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PROTEIN-PROTEIN INTERACTIONS OF THE LIGHT-HARVESTING CHLOROPHYLL *a*/*b* PROTEIN

II. EVIDENCE FOR TWO STAGES OF CATION INDEPENDENT ASSOCIATION

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SUMMARY

In a previous paper, we observed a two-stage cation-independent association of the light-harvesting chlorophyll *a*/*b* protein from spinach chloroplasts based on concentration-dependent changes in the sedimentation coefficient. The two stages of association occurred between (2–4) and (4–7) μg/ml chlorophyll. In this paper, we provide further evidence for this association.

This includes: (1) A decrease in the number of divalent cation binding sites in the second stage of association. (2) A corresponding decrease in the extent of the cation-dependent association. (3) A positive deviation from Beer's law for chlorophyll *b* for both stages of the cation-independent association and a positive deviation for chlorophyll *a* for the second stage of association only. (4) A change in the fluorescence emission of both chlorophyll *a* and *b*. The change for chlorophyll *b* was observed for both steps of association whereas that for chlorophyll *a* was observed for the second step of association only. Therefore, the first stage of association affects only chlorophyll *b* whereas the second stage alters the environment of both chlorophyll *a* and *b*. (5) In addition, divalent cations quenched chlorophyll fluorescence. However, the quenching which required 200–300 μM divalent cation for half-maximal effects was related neither to divalent cation binding nor to the divalent cation-induced association of the protein.

INTRODUCTION

In the previous paper of this series [1], we showed that the light-harvesting chlorophyll *a*/*b* protein of spinach chloroplasts [2–5] associates with itself as its concentration is increased. In addition, divalent cation binding to the protein caused a further association [1]. In this paper, we will show how the two stages of cation-independent association affect the environment of the chlorophylls and the extent of the cation-dependent association.

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The light-harvesting chlorophyll *a/b* protein was first isolated by Thornber and coworkers [2–5]. It has a mol. wt. of 33 000 and a chlorophyll *a/b* ratio of 1.0. It is absent in mutants with a reduced ability to form grana stacks [6–9]. We have used a modification of the Kung and Thornber procedure [5] and obtain a protein which is essentially detergent-free (It contains approx. 1 mol sodium dodecyl sulfate/mol protein) but is sparingly soluble in water (up to 15–20 μg/ml chlorophyll) [1]. This allows us to study its interactions in the ultracentrifuge.

A study of the physical properties of the light-harvesting chlorophyll *a/b* protein is important for the following reasons. First, this protein comprises 50 % of the total protein of the chloroplast membrane [4]. Therefore, it must be important in maintaining membrane structure and integrity and in determining permeability properties.

Second, it is an important component of the light-harvesting apparatus of the chloroplast since it contains all of the chlorophyll *b* and 50 % of the total chlorophyll of the chloroplast [4].

Third, it is probably the site of divalent cation regulation of the excitation energy distribution between the two photosystems of green plant photosynthesis. Divalent cations have been shown to cause structural changes [10–15] (shrinkage and restacking of unstacked lamellae) which, in turn, alter the distribution of excitation energy between the two photosystems [13–26]. These effects are due to divalent cation binding to the chloroplast membrane [27]. The following evidence indicates that the binding sites in question reside on the light-harvesting chlorophyll *a/b* protein. The protein binds divalent cations with a dissociation constant similar to that obtained for whole chloroplasts [1]. Furthermore, there is simultaneous development of grana stacking, cation-regulation of energy transfer, divalent cation binding and the light-harvesting chlorophyll *a/b* protein in a greening system [28, 29].

Fourth, this study will provide insight into the general behavior of integral membrane proteins.

MATERIALS AND METHODS

Preparation of the light-harvesting chlorophyll a/b protein. The chlorophyll *a/b* protein was prepared by a variation of the method of Kung and Thornber [5] as described previously [1]. Chlorophyll content was determined according to the equations of Arnon [30]. Protein concentrations were routinely expressed in terms of chlorophyll concentrations due to ease in measurement at the low concentrations used in these studies.

Ca^{2+} binding to the light-harvesting chlorophyll a/b protein. Ca^{2+} binding to the light-harvesting chlorophyll *a/b* protein was determined by the equilibrium dialysis method using $^{45}Ca^{2+}$ as described in an earlier paper [1].

Ultracentrifugation studies of the light-harvesting chlorophyll a/b protein. Sedimentation coefficients were determined using a Beckman Model E analytical ultracentrifuge [1]. All runs were made at 20 °C at a speed of 48 000 rev./min using a 30 mm single sector cell.

Absorption spectra studies of the light-harvesting chlorophyll a/b protein. Absorption spectra of the light-harvesting chlorophyll *a/b* protein were recorded using an Aminco-Chance spectrophotometer in the split beam spectral mode of

operation. Data was treated as described in Results. The absorbance of dilute samples was verified by rerunning the spectra on expanded scales.

Fluorescence studies on the light-harvesting chlorophyll a/b protein. Fluorescence excitation and emission spectra were recorded using an Aminco-Bowman spectrofluorimeter. All studies were done at room temperature in 10 mM Tris · Cl, pH 8.2. Where indicated, ions were added just prior to the fluorescence measurements.

RESULTS

Ultracentrifugation studies

Fig. 1. shows the two stages of cation-independent association of the light-harvesting chlorophyll *a/b* protein. The first stage of association is represented by the increase in sedimentation coefficient which occurs between 2 and 4 μg/ml chlorophyll. The line would have a negative slope in the absence of interactions due to excluded volume effects. The first stage of association is accompanied by an increase in the dissociation constant for Site I divalent cation binding to the protein [1]. The second stage of association is represented by the increase in sedimentation coefficient which occurs between 5 and 6 μg/ml chlorophyll.

The state of cation-independent association also affects the extent of the cation-dependent association. It occurs at all protein concentrations but occurs to a lesser extent above the second transition. This difference is erased by the addition of 5 mM NaCl. Although the extent of the association is affected, the concentration of $CaCl_2$ required for half-maximal increases in the sedimentation coefficient is indepen-

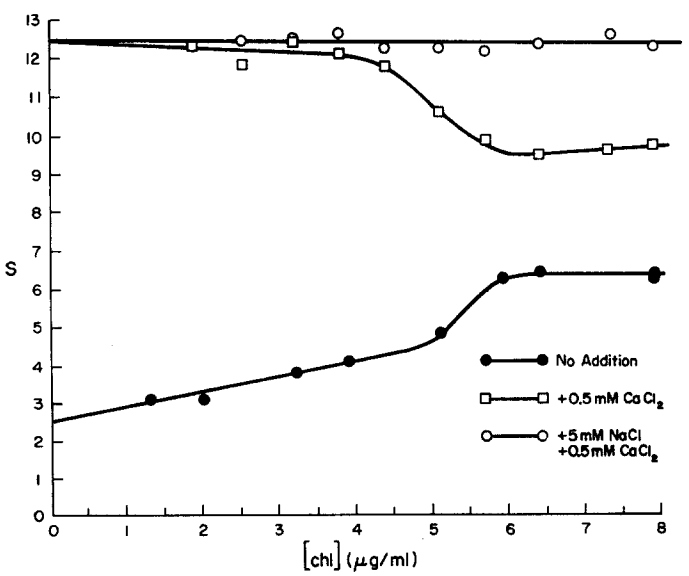

Fig. 1. Protein concentration dependence of sedimentation coefficient in the presence and absence of divalent cations. Sedimentation coefficients were determined as a function of light-harvesting chlorophyll *a/b* protein concentration in the presence and absence of 0.5 mM $CaCl_2$ as described in Materials and Methods. All runs were made in 10 mM Tris · Cl, pH 8.2.

TABLE I

HALF-MAXIMAL CONCENTRATIONS FOR Ca^{2+}-INDUCED INCREASES IN THE SEDIMENTATION COEFFICIENT OF THE LIGHT-HARVESTING CHLOROPHYLL *a/b* PROTEIN

Ultracentrifugation was carried out as described in the Materials and Methods section. The concentration of the light-harvesting pigment protein was 3.2 or 6.4 μg/ml chlorophyll. Sedimentation coefficients were determined at a number of Ca^{2+} concentrations ranging from 0 to 500 μM after which the concentration required for half-maximal effects ($C_{\frac{1}{2}max}$) was determined.

Conditions	Chlorophyll conc. (μg/ml)	$C_{\frac{1}{2}max}$
10 mM Tris · Cl pH 8.2	3.2	45
	6.4	45
10 mM Tris · Cl+5 mM NaCl	3.2	75
	6.4	70

dent of protein concentration (Table I). The increase in the amount of $CaCl_2$ required for half-maximal effects in the presence of NaCl appears to be due to a mixed effect of ionic strength and competition for the divalent cation binding sites.

The effect of the second stage of cation-independent association on divalent cation binding to the light-harvesting chlorophyll a/b protein.

In the first paper of this series [1] we showed that the first stage of cation-independent association caused an increase in the dissociation constant for divalent

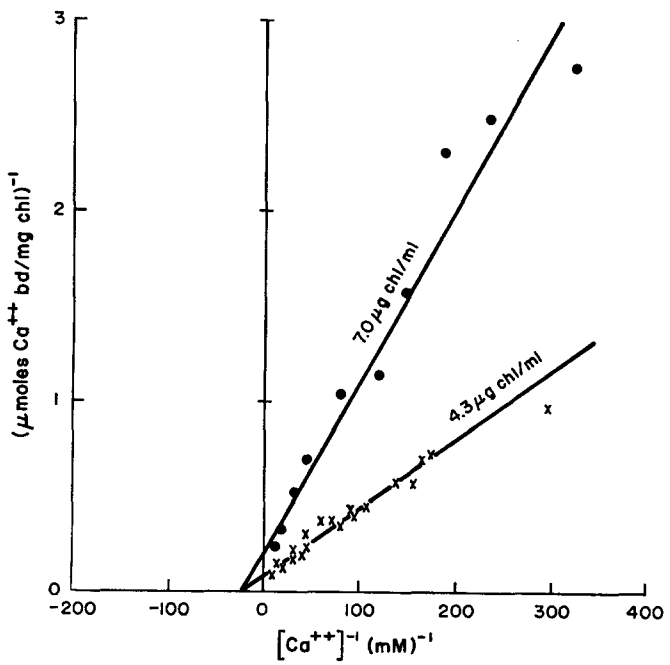

Fig. 2. Ca^{2+} binding to the light harvesting chlorophyll *a/b* protein at two concentrations. Ca^{2+} binding was determined by equilibrium dialysis using $^{45}Ca^{2+}$ as described previously [1]. Light-harvesting chlorophyll *a/b* protein concentrations were 4.3 and 7.0 μg chlorophyll/ml.

cation binding to the high-affinity sites (Site I) on the protein. However, this did not affect the extent of divalent cation-induced association of the protein (Fig. 1) since this is caused by binding to the low affinity sites (Site II). Now we want to know whether the decrease in the extent of cation-dependent association observed above the second transition is due to a change in divalent cation binding. Double reciprocal plots of calcium binding vs. Ca^{2+} concentration were made at two protein concentrations, which are below and above the transition (Fig. 2). Only one site (i.e. a single straight line segment) is observed since site I disappears above the first transition [1]. There is no change in the dissociation constant (K_d). This agrees with the observation (Table I) that there is no change in the divalent cation concentration required for half-maximal increases in the sedimentation coefficient. However, there is a decrease in the number of binding sites going from 10 μmol Ca^{2+} bound/mg chlorophyll to 4.3 μg/ml chlorophyll to 5.7 μmol/mg chlorophyll at 7.0 μg/ml chlorophyll. This suggests that some potential binding sites are buried during the second stage of association. The remaining binding sites are not sufficient to promote maximum association. Therefore, NaCl must alter the structure so that these sites can participate in aggregate formation.

The effect of cation-independent association on the environment of the chlorophylls. Changes in pigment absorption

Next, we examined the effect of protein association on the environment of the pigments as indicated by their absorption and fluorescence characteristics. The cation-independent association did not affect the position of the absorption maxima (not shown) but did alter the magnitude of the absorption (Figs. 3 and 4). A plot of chlorophyll *a* absorbance at 436 and 668 nm (Fig. 3) shows a positive deviation from

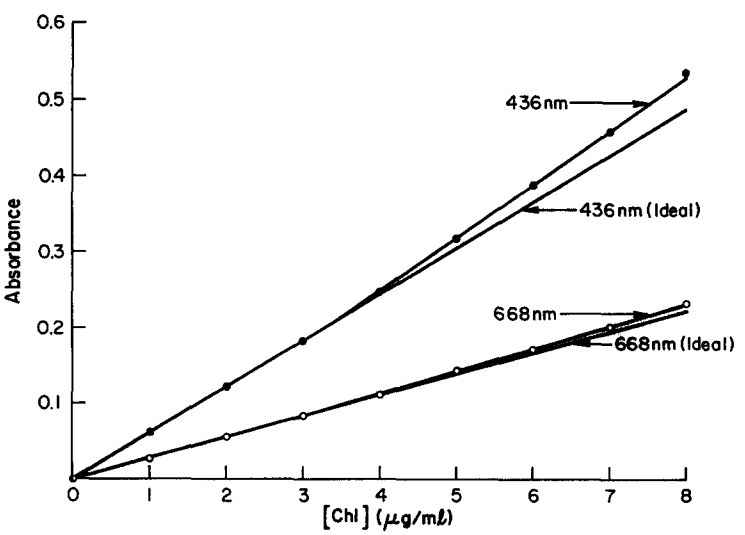

Fig. 3. Concentration dependence of chlorophyll *a* and *b* absorbance: deviation from Beer's law. The absorbance at 436 and 668 nm was determined as a function of light-harvesting chlorophyll *a*/*b* protein concentration. The ideal line was obtained by extension of the linear portion of the curves to higher concentrations.

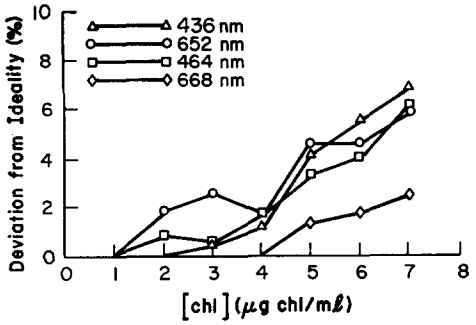

Fig. 4. Deviation from ideality of chlorophyll *a* and *b* absorbance as a function of protein concentration. The deviation from ideality was calculated as described in the text and plotted against protein concentration (expressed in terms of chlorophyll).

the expected Beer's law relationship. We can quantitate the deviation using the following equation.

$$\text{Deviation from ideality } (\%) = \frac{A_{obs} - A_{calc}}{A_{calc}} \times 100 \qquad (1)$$

where A_{obs} is the observed absorbance at the stated wavelength and protein concentration, whereas A_{calc} is the "ideal" absorbance under the same conditions. It is obtained by extrapolating the line through the data points obtained at low protein concentrations to higher concentrations. Fig. 4 shows a plot of the data for both chlorophyll *a* ($\lambda = 436$ and 668 nm) and chlorophyll *b* ($\lambda = 463$ and 652 nm) as a function of protein concentration. The chlorophyll *b* peaks show a deviation from ideality for both stages of cation-independent association whereas the chlorophyll *a* peaks show a deviation for the second stage of association only.

The effect of cation-independent association on the fluorescence properties of the light-harvesting chlorophyll a/b protein

We have also studied the effect of protein association on the fluorescence excitation and emission spectra of the light-harvesting chlorophyll *a*/*b* protein. The fluorescence emission maximum (Fig. 5) occurs between 670 and 675 nm when either chlorophyll *a* (430–435 nm) or chlorophyll *b* (464–470) is excited. However, there is a shoulder on the short wavelength side of the emission band (650 nm) when chlorophyll *b* is excited which is less pronounced when chlorophyll *a* is excited. Excitation spectra (Fig. 6) show that the emission at 650 nm is excited to a greater extent by chlorophyll *b* than by chlorophyll *a* whereas the reverse is true for the chlorophyll *a* emission at 670 nm. These results are consistent with a portion of the fluorescence arising from chlorophyll *b*. However, Brown [31] did not observe any chlorophyll *b* fluorescence in her preparation. Also chlorophyll *b* is not fluorescent in vivo [32, 33], since its excitation energy is transferred to chlorophyll *a* with 100 % efficiency. However, chlorophyll *b* fluorescence has been observed in green algae under high light conditions in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea which would effectively close the Photosystem II traps [34].

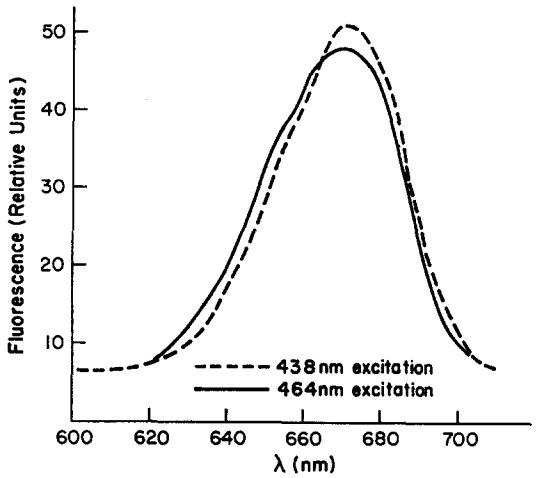

Fig. 5. Emission spectra of light-harvesting chlorophyll *a/b* protein. Emission spectra were recorded using an Aminco-Bowman spectrofluorimeter with excitation of either 438 or 464 nm. The fluorescence measurements were made in 10 mM Tris · Cl, pH 8.2. The emission maximum was at a shorter wavelength than reported previously [4, 31] due to the use of the R-136 photomultiplier. This does not affect the relative changes in chlorophyll *a* fluorescence observed in the different structural states. The chlorophyll concentration was 7 μg/ml.

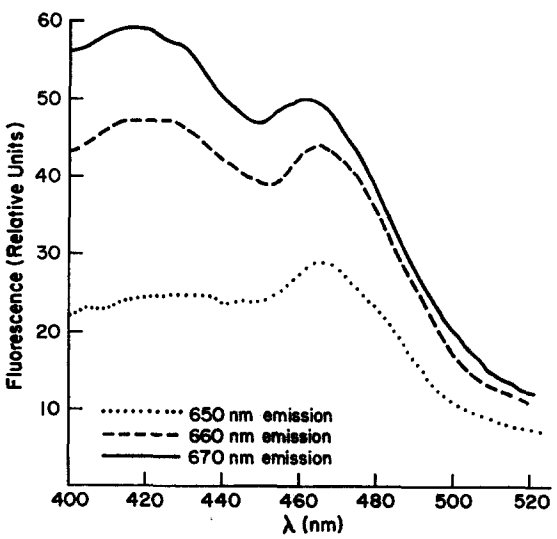

Fig. 6. Excitation spectra of the light-harvesting chlorophyll *a/b* protein. Excitation spectra were recorded by monitoring the fluorescence at a fixed wavelength (650, 660, or 670 nm) while changing the wavelength of excitation. Measurements were made in 10 mM Tris · Cl, pH 8.2. The chlorophyll concentration was 7 μg/ml.

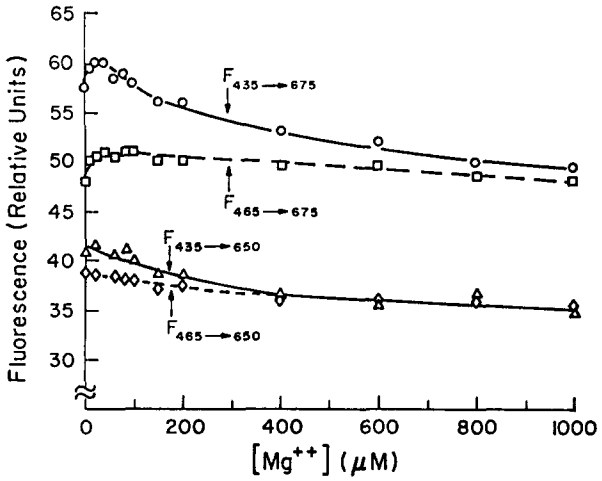

Fig. 7. Comparison of fluorescence excitation as a function of light-harvesting chlorophyll *a*/*b* protein concentration. The fluorescence at either 650 or 675 nm was determined as a function of protein concentration with excitation wavelength of 435 or 465 nm. At each protein concentration, ratios of $F_{465 \rightarrow 650}/F_{435 \rightarrow 650}$ and $F_{465 \rightarrow 675}/F_{435 \rightarrow 675}$ were calculated and plotted as a function of protein concentration expressed in terms of chlorophyll.

We then determined the chlorophyll *a* fluorescence properties as a function of protein concentration. First, we examined the relative effectiveness of chlorophyll *b* and chlorophyll *a* at exciting fluorescence from chlorophyll *a* (Fig. 7). This was expressed as the ratio $F_{465 \rightarrow 675}/F_{435 \rightarrow 675}$ where $F_{465 \rightarrow 675}$ represents fluorescence from chlorophyll *a* (at 675 nm) due to excitation of chlorophyll *b* whereas $F_{435 \rightarrow 675}$ represents chlorophyll *a* fluorescence due to the direct excitation of chlorophyll *a*. The first stage of cation-independent association does not affect this ratio. However, there is an increase in this ratio during the second stage of cation-independent association. One explanation is that there is an increase in the transfer of excitation energy from chlorophyll *b* to chlorophyll *a* during the second stage of association. There could be an intrasubunit rearrangement or a decrease in the distance between a molecule of chlorophyll *b* on one subunit and a molecule of chlorophyll *a* on an adjacent one.

Next, we examined the relative effectiveness of chlorophyll *b* and chlorophyll *a* at exciting chlorophyll *b* fluorescence. This is represented by $F_{465 \rightarrow 660}/F_{435 \rightarrow 660}$. This ratio shows an increase for both phases of cation-independent association (Fig 7).

From both absorption and fluorescence measurements, we can conclude that the first stage of cation-independent association affects only the environment of chlorophyll *b* whereas the second stage of association affects the environment of both chlorophylls.

The effect of divalent cations on the fluorescence from the light-harvesting chlorophyll a/b protein

Divalent cations decrease the fluorescence of the light-harvesting chlorophyll *a*/*b* protein (Fig. 8). This is in contrast to the divalent cation-induced increases in fluorescence usually observed in whole chloroplasts [13–25, 27]. Wydryznski et al.

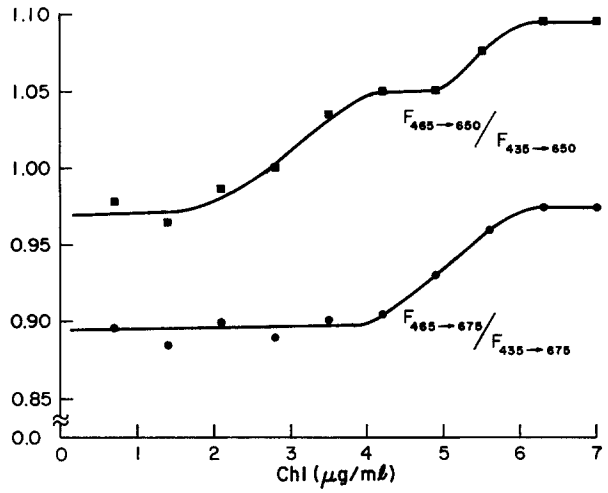

Fig. 8. Effect of $MgCl_2$ on chlorophyll fluorescence from the light-harvesting chlorophyll *a/b* protein. Fluorescence at 650 and 675 nm was measured with excitation of either 435 or 465 nm as a function of $MgCl_2$ concentration. The light-harvesting chlorophyll *a/b* protein concentration was 1.8 μg chlorophyll/ml.

TABLE II

THE EFFECT OF O_2 ON Mg^{2+}-INDUCED CHANGES IN CHLOROPHYLL FLUORESCENCE FROM THE LIGHT-HARVESTING CHLOROPHYLL *a/b* PROTEIN

Measurements of chlorophyll fluorescence were made as described in Materials and Methods. Samples were deaerated by bubbling with argon just prior to the fluorescence measurements. The Mg^{2+} concentration was 1 mM.

Condition	Chlorophyll conc. (μg/ml)	Mg^{2+}-induced decreases in fluorescence (per cent)
Aerated	1.8	24
Deaerated	1.8	20
Aerated	8.0	35
Deaerated	8.0	33

[25] did observe small decreases in chlorophyll fluorescence when divalent cations were added to chloroplasts incubated under low ionic strength conditions. These fluorescence decreases are not related either to divalent cation binding to the protein or the cation-induced association since the fluorescence decreases require 200–300 μM $CaCl_2$ for half-saturation whereas 35–45 μM is sufficient for half-maximal saturation of the binding sites and divalent cation-induced association. One possible explanation is that the divalent cations could facilitate the reaction of the chromophore with molecular oxygen resulting in fluorescence quenching. However, removal of the oxygen by bubbling with nitrogen did not alter the results (Table II).

TABLE III

EVIDENCE FOR THE TWO STAGES OF CATION INDEPENDENT ASSOCIATION OF LIGHT-HARVESTING CHLOROPHYLL *a/b* PROTEIN

Stage I
1. Concentration range: 1–4 μg chlorophyll/ml
2. Increase in K_d for divalent cation binding to Site I
3. Positive slope of plot of sedimentation coefficient against light-harvesting pigment protein concentration
4. Change in environment of chlorophyll *b*
Stage II
1. Concentration range: 4–6 μg chlorophyll/ml
2. Decrease in the number of Site II divalent cation binding sites
3. Transition in plot of sedimentation coefficient against light-harvesting pigment protein concentration
4. Difference in the extent of the divalent cation induced increase in sedimentation coefficient on opposite sides of the above mentioned transition
5. Changes in the environment of both chlorophyll *a* and chlorophyll *b*

DISCUSSION

The data presented here and in the previous paper [1] show that there are two stages of cation-independent association of the light-harvesting chlorophyll *a/b* protein. The phenomena associated with these two stages are listed in Table III.

Stage I is accompanied by an increase in the dissociation constant for Site I divalent cation binding and a change in the environment of chlorophyll *b*. Stage II is accompanied by (1) a decrease in the number of divalent cation binding sites and the extent of divalent cation-induced association and (2) changes in the environment of both chlorophyll *a* and chlorophyll *b*.

The ease with which the light-harvesting chlorophyll *a/b* protein associates with itself both in the presence and absence of added cations suggests that interactions such as these may be important in determining the state of associationof the proteins in the chloroplast membrane and the transfer of excitation energy between individual pigment protein molecules. However, more work is required to determine the state of the protein in the membrane. In particular, we need to study the interaction of this protein with the core complexes of Photosystem I and Photosystem II and chloroplast lipids before we can determine the exact role of this protein in the transfer of excitation energy in photosynthesis.

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