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PROTEIN-PROTEIN INTERACTIONS OF LIGHT-HARVESTING PIGMENT PROTEIN FROM SPINACH CHLOROPLASTS

I. Ca^{2+} BINDING AND ITS RELATION TO PROTEIN ASSOCIATION

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SUMMARY

The role of divalent cations in the regulation of the distribution of excitation energy between the two photosystems involved in green plant photosynthesis has led us to search for a better understanding of how such phenomena might occur at the molecular level. Since small changes in orientation of and distance between pigment molecules could greatly affect the distribution of excitation energy, we have decided to study the effects of ions on the light-harvesting pigment protein from spinach chloroplasts. The light-harvesting pigment protein is shown to have two types of binding sites for Ca^{2+}. Binding studies and analytical ultracentrifugation indicate that site I ($K_d = 2.5\ \mu M$, $n = 1.5$–$4.0\ \mu mol\ Ca^{2+}$ bound/mg chlorophyll) is lost as the protein associates. Site II ($K_d = 32\ \mu M$, $n = 9.5\ \mu mol\ Ca^{2+}$/mg chlorophyll) is not affected by the association of the protein. This site is responsible, however, for a further divalent cation-dependent association of the protein. The possible role of this protein in grana stacking and control of spillover is discussed.

INTRODUCTION

The control of the distribution of excitation energy between the two photosystems of higher plants has received considerable attention in recent years. In order for the 'Z' scheme of electron transport to function efficiently, equal excitation of the two photosystems is required. Thus one would expect quantum yields for reactions involving both photosystems to be maximal in spectral regions where both photosystems absorb an equal number of quanta and to drop significantly at wavelengths where only one photosystem absorbs. However, this is not found to be the case. Quantum yields do drop at far red wavelengths (> 670 nm) where only Photosystem I absorbs [1]. Yet they are remarkably constant and maximal throughout the remainder of the visible spectrum, including regions where only Photosystems II absorbs [2, 3]. To account for this, a spillover mechanism has been proposed whereby excess excitation energy from the Photosystem II light-harvesting appa-

ratus may spill over into the Photosystem I apparatus, thus enabling both photosystems to operate [4–6].

Considerable evidence has accumulated implicating both mono- and divalent cations as regulators of the spillover process. Several authors have shown that divalent cations inhibit spillover [7–15]. This is shown by an increase in steady-state fluorescence levels in the presence of 3(2,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and increase in the quantum yield of dichlorophenolindophenol (DCIP) reduction caused by the addition of divalent cations [7, 14]. Such effects have been correltaed with divalent cation-induced structural changes in the chloroplast membrane [9, 11.] In attempting to duplicate the work of Murata and coworkers [7–9], Gross and Hess [16] found that low levels of monovalent cations were required in addition to divalent cations before inhibition of spillover could be observed. The divalent cation inhibition of spillover could be correlated with the binding of divalent cations to the chloroplast membrane [17]. More recently, these effects have been related to the presence or absence of grana stacking [18]. In a low ionic strength medium, grana are stacked in the presence or absence of Ca^{2+} and spillover is inhibited. In the presence of low levels of Na^+, grana were found to be unstacked and spillover promoted. Ca^{2+} was able to reverse the Na^+-induced unstacking.

Due to the apparent relationship between divalent cation binding, grana stacking, and control of spillover, we felt it important to attempt to establish a molecular basis for these effects. A recent computer model by Seely [19] has demonstrated that the change in orientation of only a few chlorophyll molecules could greatly effect the distribution of excitation energy between the two photosystems. Since energy transfer depends on the orientation of and distance between pigment molecules [20, 21] one might expect spillover to be the result of short range pigment-pigment or protein-protein interactions. The system we have chosen to study is the light- harvesting pigment protein of spinach chloroplasts [22–25]. In this paper, we present evidence concerning the divalent cation binding properties of this protein and their relation to its state of association.

MATERIALS AND METHODS

Preparation of light-harvesting pigment protein

Washed chloroplast lamellae were isolated according to the method of Gross [26]. The washed lamellae prepared in this manner were centrifuged out of solution and resuspended in 50 mM Tris · HCl 1.0 % sodium dodecyl sulfate, pH 8.2. The suspension was stirred for 1 h after which any unsolubilized material was removed by centrifugation. A portion of the dodecyl sulfate extract was subjected to fractionation on hydroxyapatite as described by Kung and Thornber [24]. The light-harvesting pigment protein was eluted from the hydroxyapatite with 0.4 M phosphate, 1 mM $MgCl_2$, 0.05 % dodecyl sulfate, pH 7.0. Alterations on their procedure included substitution of hydroxyapatite slurry for their column and final resuspension and extensive dialysis with 50 mM Tris · HCl, pH 8.2, instead of their Mg^{2+}-containing buffer. Samples were stored in this buffer at 4 °C and dialyzed to 10 mM Tris · HCl, pH 8.2, prior to use.

Chlorophyll was determined according to the method of Arnon [27]. Protein

concentration was expressed in terms of chlorophyll concentration due to ease of measurement and low protein concentrations. Sodium dodecylsulfate content was determined by using ^{35}S-labeled sodium dodecyl sulfate (0.025–0.040 Ci/μmol dodecyl sulfate) throughout the isolation procedure and determining the amount of ^{35}S-labeled dodecyl sulfate bound to the pigment protein using a Nuclear Chicago Gas Flow Planchet Counter.

Ca^{2+} binding to light-harvesting pigment protein

Ca^{2+} binding was determined by equilibrium dialysis. 1-ml protein samples containing 1.0–4.0 μg chlorophyll/ml were placed in dialysis bags and equilibrated for 18 h against 20 ml of solutions containing 5 mM Tris · HCl, pH 8.2, and 2–250 μM $CaCl_2$. A constant amount, between 0.05 and 0.075 μCi, of $^{45}Ca^{2+}$ was placed in each tube and various amounts of unlabeled $CaCl_2$ added to obtain the desired concentration. After equilibration, the contents of the dialysis bags and samples from the equilibration medium were plated and counted using a Nuclear Chicago Gas Flow Planchet Counter. The difference between the inside and outside of the dialysis bags was taken as a measure of the amount of Ca^{2+} bound. Inhibition studies were done in the same manner with the addition of constant amounts of $MgCl_2$ or NaCl to each tube.

Ultracentrifugation studies of light-harvesting pigment protein

Sedimentation coefficients were determined using a Beckman Model E ultracentrifuge utilizing absorption optics. All runs were made at 48 000 rev./min using a 30-mm single sector cell.

Chemicals

Hydroxyapatite was obtained from Bio-Rad Laboratories and Trizma Base and dodecyl sulfate from Sigma Chemical Co. $^{35}Ca^{2+}$ and ^{45}S-labeled dodecyl sulfate were obtained from New England Nuclear. All other chemicals were of reagent grade.

RESULTS

Ca^{2+} binding to light-harvesting pigment protein

Ca^{2+} binding data was plotted according to the double reciprocal form in Eqn 1

$$\frac{1}{Ca^{2+}_{bound}} = \frac{1}{n} + \frac{K_d}{n}\frac{1}{[Ca^{2+}_{free}]} \quad (1)$$

where n equals the number of binding sites and K_d, the dissociation constant for binding to these sites. Fig. 1 shows such a plot covering the concentration range 2–250 μM $CaCl_2$. Two classes of binding sites are apparent, as represented by the two straight line segments in Fig. 1. Site I, the high-affinity site, has a dissociation constant, K_d, of 2.5 μM $CaCl_2$ and bound between 1.5 and 4.0 μmol Ca^{2+}/mg chlorophyll. Site II has a lower affinity, $K_d = 32$ μM $CaCl_2$, and bound 9.5 μmol Ca^{2+}/mg chlorophyll. Both site I and site II are inhibited in a competitive manner by Mg^{2+}. Site I is inhibited in a competitive manner by Na^+ but site II is apparently inhibited in a non-competitive manner (see Fig. 2 and 3). Inhibition constants for

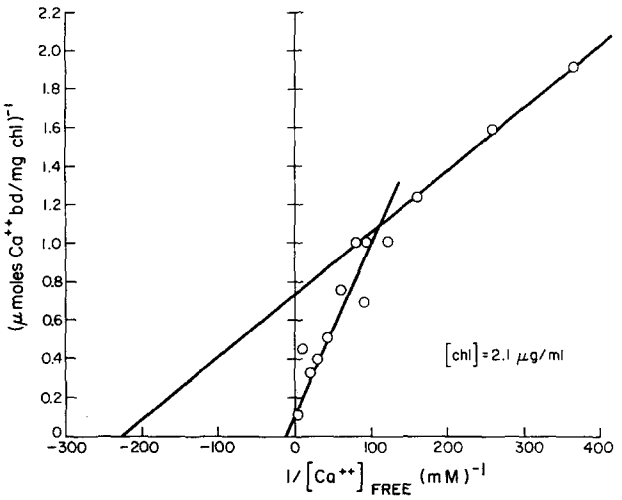

Fig. 1. Ca^{2+} binding to light-harvesting pigment protein. Ca^{2+} binding was done as described in Materials and Methods and data plotted according to Eqn 1. Chlorophyll concentration was 2.1 μg/ml. All binding experiments were done in 5 mM Tris · HCl, pH 8.2.

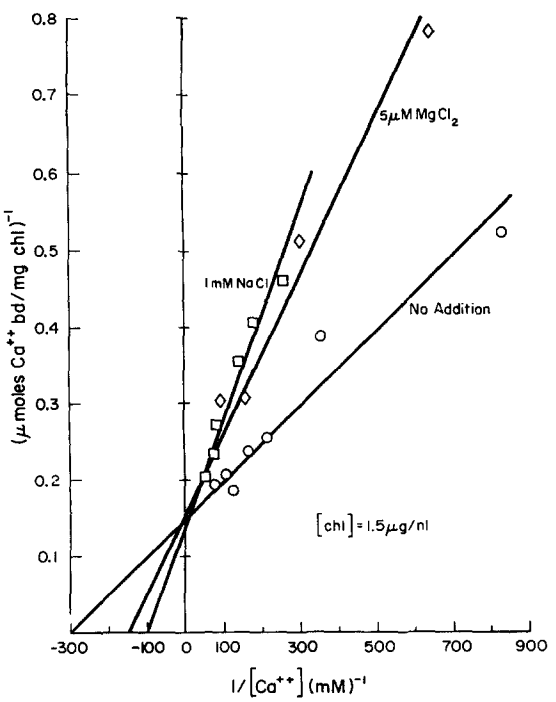

Fig. 2 Inhibition of Ca^{2+} binding to light-harvesting pigment protein site I. Ca^{2+} binding was done as described in Materials and Methods except that 1 mM NaCl or 5 μM $MgCl_2$ was present in addition to $CaCl_2$. The inhibition studies were done in 5 mM Tris · HCl, pH 8.2. Chlorophyll concentration was 1.5 μg/ml. Inhibition constants for Na^+ and Mg^{2+} were determined from Eqn 2.

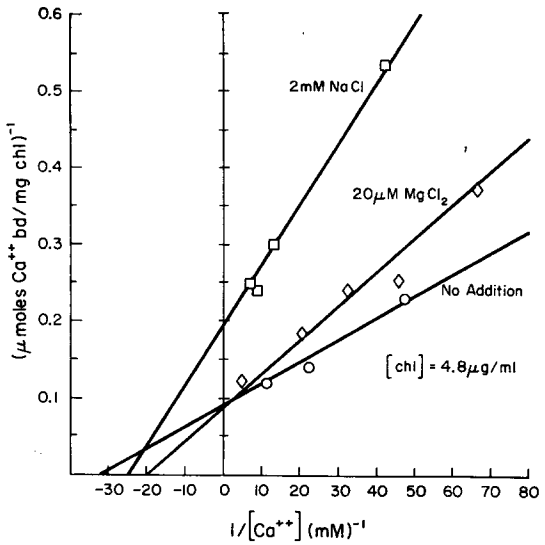

Fig. 3. Inhibition of Ca^{2+} binding to light-harvesting pigment protein site II. Inhibition studies were done as described in Fig. 2 except that the concentrations of NaCl and $MgCl_2$ were 2 mM and 20 μM, respectively. Chlorophyll concentration was 4.8 μg/ml. The advantages of working at higher protein concentrations when dealing with site II is discussed in Results presented below. Inhibition constants for Mg^{2+} and Na^+ were determined from Eqns 2 and 3, respectively.

competitive and non-competitive inhibition were determined from Eqns 2 and 3, respectively.

$$\frac{1}{Ca^{2+}_{bound}} = \frac{1}{n} + \frac{K_d}{n}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[Ca^{2+}_{free}]} \tag{2}$$

$$\frac{1}{Ca^{2+}_{bound}} = \frac{1}{n}\left(1 + \frac{[I]}{K_I}\right) + \frac{K_d}{n}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[Ca^{2+}_{free}]} \tag{3}$$

where K_I is the dissociation constant for the inhibitor and $[I]$ the concentration of inhibitor added. These values, along with a summary of the binding parameters, are presented in Table I.

TABLE I

Ca^{2+} BINDING PARAMETERS FOR LIGHT-HARVESTING PIGMENT PROTEIN

Ca^{2+} binding and dodecyl sulfate content were done as described in Materials and Methods. Data were fitted to Eqn 1 and values for n and K_d determined from the intercepts. Competitive and non-competitive inhibition constants were determined from Eqns 2 and 3, respectively. Letters in parentheses refer to competitive (c) and non-competitive (nc) inhibition. The K_d values for sites I and II are extrapolated to zero protein concentration. Dodecyl sulfate content: 0.3–0.8 μmol dodecyl sulfate/mg chlorophyll.

	Site I	Site II
K_d	2.5 μM	32 μM
n(μmol Ca^{2+} bound/mg chlorophyll)	1.5–4.0	9.5
$K_{I,\ Mg^{2+}}$	5.1 μM (c)	31 μM (c)
$K_{I,\ Na^+}$	1.0 mM (c)	1.4 mM (nc)

Sodium dodecyl sulfate content

One matter which concerned us was the possibility that the observed Ca^{2+} binding might be due to the presence of the anionic detergent, dodecyl sulfate, which is required to isolate this protein. Dodecyl sulfate is introduced at two points in the isolation procedure. The first is in the preparation of the dodecyl sulfate extract which is applied to the hydroxyapatite and the second is in the final buffer for washing the hydroxyapatite slurry (0.4 M P_i/1 mM $MgCl_2$/0.05 % dodecyl sulfate, pH 7.0 [24]). By adding ^{35}S-labeled dodecyl sulfate (specific activity 0.02–0.04 Ci/μmol dodecyl sulfate) in each of these places in independent experiments and recognizing that the maximal amount of bound detergent would be the sum of the amount of detergent bound in each experiment, we have determined that less than 1.0 μmol of dodecyl sulfate is bound per mg chlorophyll.

Although the number of counts/min obtained at the end of our isolation (dialysis included) was low, the number of counts/min used for these determinations were twice background and all data was corrected for background. Due to the low specific activity used in these experiments and the low number of counts obtained we felt that bound dodecyl sulfate cannot be responsible for the observed Ca^{2+} binding properties of Site II on this protein although residual dodecyl sulfate could not be unambiguously excluded as the source of site I binding.

We have since repeated these experiments with a 10-fold higher specific activity (0.22–0.25 Ci/μmol dodecyl sulfate) which gives us sufficient counts to state whether or not site I binding is due to bound detergent. With specific activities in this range we find a maximal detergent content of 0.2 μmol dodecyl sulfate per mg chlorophyl indicating that with the lower specific activity we may have been overestimating rather than underestimating our detergent content due to the error inherent in using a low number of counts. The detergent content determined using the higher specific activity is sufficiently less than the number of binding sites for either Site I or Site II indicating that the binding of divalent cations to these sites is not due to the presence of bound detergent.

Concentration dependence of site I

Occasionally, a Ca^{2+} binding experiment would show the presence of only one type of binding site (Fig. 4) which showed binding parameters indicating that only site II was present. Re-evaluation of existing data showed that all experiments where only one site was observed were done at the upper limit of our protein concentration range; i.e. between 2.5 and 4.0 μg chlorophyll/ml. We thus felt that a study of Ca^{2+} binding as a function of protein concentration might be informative. The results of such a study is shown in Fig. 5. The dissociation constant for site II remains constant over the entire concentration range. Site I, however, shows a striking dependence on protein concentration. At low protein concentrations the dissociation constant for Ca^{2+} binding can be extrapolated to 2.5 μM $CaCl_2$. The dissociation constant increases as protein is increased, being about equal to the dissociation constant for site II at 3.0 μg chlorophyll/ml, the concentration above which site I could not be observed. No change in the amount of Ca^{2+} bound to this site was noted over the same concentration range. We are unable to assign any functional significance to this site at this time.

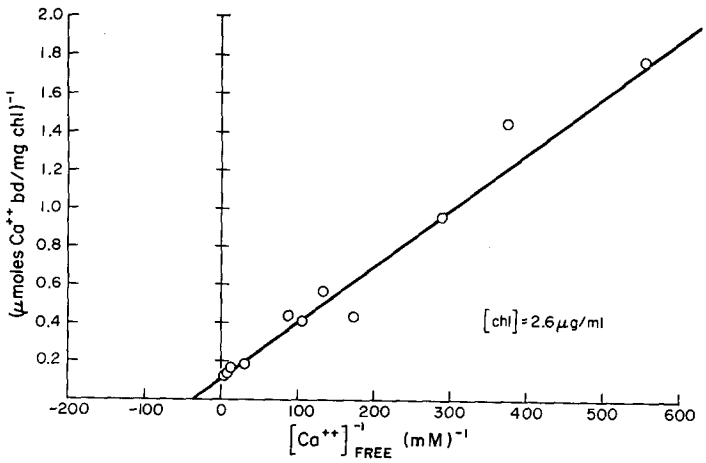

Fig. 4. Ca^{2+} binding to light-harvesting pigment protein. Ca^{2+} binding was determined as in Fig. 1. The only alteration was in the chlorophyll concentration which was 2.6 μg/ml.

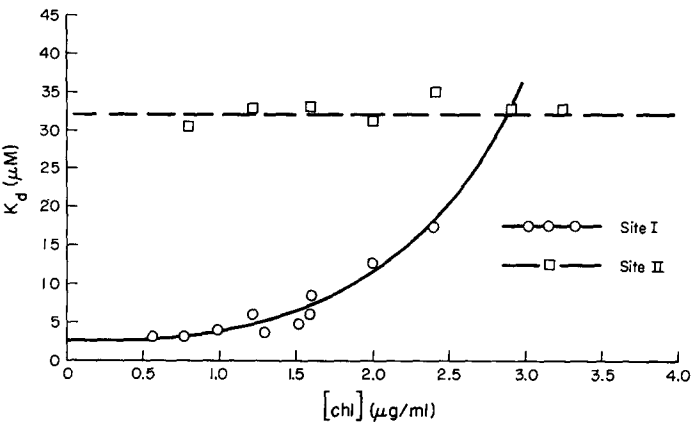

Fig. 5. Dependence of K_d for Ca^{2+} binding on light-harvesting pigment protein concentration. Ca^{2+} binding was done as described in Fig. 1. Chlorophyll concentrations were varied from 0.5 to 4.0 μg/ml. K_d values for sites I and II were determined using Eqn 1.

Ultracentrifugation studies of light-harvesting pigment protein

Cation-independent association. Such a dependence of the dissociation constant for Ca^{2+} binding to site I on protein concentration is most reasonably explained by an association of the protein which alters the environment of the groups responsible for binding. Such behavior has been observed and quantitated in the case of hemerythrin where the affinity for thiocyanate was found to vary with protein concentration [28]. To verify that association could be responsible for the observed change in K_d, we resorted to analytical ultracentrifugation. The dependence of the sedimentation coefficient on protein concentration is shown in Fig. 6. Extrapolated to zero protein, the sedimentation coefficient is 2.7 S. This agrees with the value reported by Thornber et al. [22]. In the absence of any protein-protein interaction,

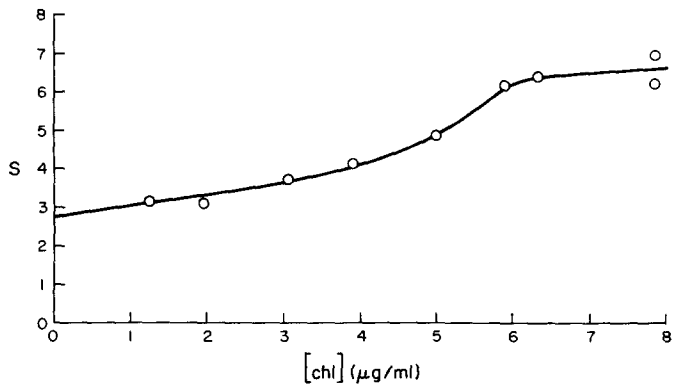

Fig. 6. Dependence of s on light-harvesting pigment protein concentration. Sedimentation coefficients were determined as a function of light-harvesting pigment protein concentration using a Beckman Model E analytical ultracentrifuge as described in Materials and Methods. All runs were made in 10 mM Tris · HCl, pH 8.2.

one would expect a plot such as that in Fig. 6 to be linear with a negative slope. This is clearly not the case. The sedimentation coefficient increases with increasing protein concentration, finally, plateauing at a value of 6.2–6.3 S at concentrations above 6.0 μg chlorophyll/ml. We believe that such an association could be responsible for the protein concentration dependence of Ca^{2+} binding to site I. The shape of the curve in Fig. 6 suggests that the association may proceed in two stages; one stage being represented by the slow, nearly linear rise which occurs below 5.0 μg chlorophyll/ml and the other by the steeper rise between 5.0 and 6.0 μg chlorophyll/ml. Further studies are under way to see if this is the case.

Cation-dependent association. The effect of the presence of ions on the sedimentation coefficient was also studied. These experiments were done on the plateau region in Fig. 6. This was done for two reasons. First, the sedimentation coefficient is no longer changing with protein concentration in this region. Any interference due to cation-independent association is therefore negated. Second, the protein concentration on the plateau is more than twice the highest concentration at which site I can be observed. Thus, by working in this region, we can observe the effects of binding to site II alone without interference from site I.

Ca^{2+} increased the sedimentation coefficient from 6.2 to 9.3 S with the half-maximal effect at 45 μM $CaCl_2$ (Fig. 7). We feel that this value provides a reasonable correlation with the dissociation constant observed for Ca^{2+} binding to site II. The difference between the two numbers could be attributed to the fact that in plotting the Ca^{2+} binding data, the free Ca^{2+} concentration is used whereas the total Ca^{2+} concentration is used in plotting curves for cation-induced changes in the sedimentation coefficient.

In the presence of NaCl, Ca^{2+} was found to increase the sedimentation from 6.2 to 12.3 S (Fig. 7). The half-maximal effect was observed at 75 μM $CaCl_2$. NaCl alone produced no changes in the sedimentation coefficient. It is difficult to rationalize this change in half-maximal concentration with the non-competitive nature of

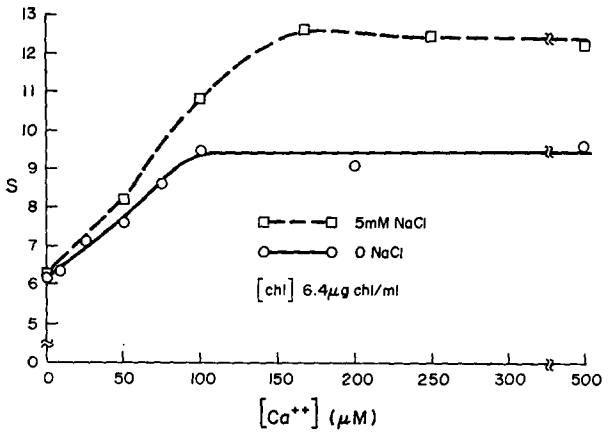

Fig. 7. Effects of Ca^{2+} on sedimentation coefficient of light-harvesting pigment protein. Sedimentation coefficients were determined as in Fig. 6. Runs were made in 10 mM Tris · HCl, pH 8.2, plus appropriate amounts of $CaCl_2$ or in 10 mM Tris · HCl, pH 8.2, minus 5 mM NaCl plus appropriate amounts of Ca^{2+}. Chlorophyll concentration was held constant at 6.4 μg/ml. No difference was observed between adding the $CaCl_2$ immediately before the centrifuge run on dialyzing the protein solution overnight indicating that a rapid equilibrium is established.

Na^+ inhibition of Ca^{2+} binding. If the inhibition were strictly non-competitive, one would expect a change in the extent of the association with no change in the half-maximal concentration. It is our feeling that the apparent non-competitive nature of inhibition by Na^+ may be due in part to intersection of the line for competitive inhibition at site I with the abcissa (compare Fig. 2 and 3). The increase in the magnitude of the Ca^{2+}-induced association in the presence of Na^+ is also surprising. Two possible explanations can be offered. Site II may represent a combination of specific and non-specific sites which possess similar binding parameters. Binding to the specific sifes might result in association while binding to the non-specific sites would be unproductive. The presence of Na^+ could alter the distribution of Ca^{2+} between specific and non-specific binding sites. Alternatively, a number of binding sites responsible for the Ca^{2+}-induced association could be buried due to the cation-independent association of the protein. Na^+ could then be supposed to loosen the association or bring about a conformational change exposing these sites. At the present time, we are unable to choose between these two possibilities.

DISCUSSION

In this paper, we have shown that the light-harvesting pigment protein of spinach chloroplasts is capable of binding considerable amounts of Ca^{2+} and that this binding brings about a cation-induced association of the protein. The presence of two types of binding sites has been demonstrated but only site II is responsible for the cation-induced association. The dissociation constant for site II was constant over the protein concentration range studied but that for site I showed a dramatic dependence on protein concentration, apparently due to cation-independent association of the protein. The binding was not due to the presence of anionic detergent used in the isolation of the protein.

We chose to study the light-harvesting pigment protein for a number of reasons. First, this protein constitutes a major portion of the chloroplast membrane, representing about 50 % of the total lamellar protein [22]. Due to the large number of binding sites Ca^{2+} on the chloroplast membrane [17], a protein present in such large amounts is a likely candidate for Ca^{2+} binding sites. (Unpublished data from this laboratory indicates that the major portion of the Ca^{2+} bound to the membrane is bound to protein.) Second, this protein is thought to be associated with the light-harvesting apparatus [25], where control of the flow of excitation energy could be most easily manipulated. Seely's [19] model predicts that a change of orientation in as few as 6 chlorophylls in a light-harvesting apparatus of 344 chlorophylls could greatly alter the distribution of excitation energy between the two photosystems [14]. Third, the light-harvesting pigment protein is the smallest unit isolated from the photosynthetic membranes of higher plants which still has attached chlorophyll present. The protein has a molecular weight of 33 000 and has one molecule each of tightly-bound chlorophyll *a* and *b* [22, 24, 25]. Recent evidence has indicated that under certain growth conditions and in a variety of mutants where chlorophyll *b* is absent, the entire protein moiety and all of the components of this pigment protein complex are also missing [25, 29].

While we have yet to show any direct evidence that the light-harvesting pigment protein is involved in control of the spillover process, indirect evidence from a number of laboratories point in this direction. If spillover and its inhibition is indeed related to the presence or absence of grana stacking, as has been suggested by Gross and Prasher [18], then the proteins which are responsible for the stacking interaction may also be involved in the control of spillover. Levine and Duram [30] have shown that when the ac-5 mutant of *Chlamydomonas reinhardi* is grown mixtotrophically a group of proteins of mol. wt 20 000–30 000 are absent and the membranes do not form grana stacks. If this mutant is grown phototrophically, this group of proteins is present and grana stacking is observed. Since Photosystem II activity was observed under both growth conditions, Levine and Duram [30] concluded that the missing proteins were not necessary for Photosystem II activity and suggested that they might be responsible for grana stacking [30]. Further work showed that this group of proteins is also absent in mutants of barley and peas [31] which show less grana stacking than is observed in the wild-types [32, 33]. Using the same barley mutant which lacks chlorophyll *b*, Thornber and Highkin [25] have shown that the light-harvesting pigment protein and all of its constituents are absent. If stacking does prove to be intimately related to spillover processes, the implication of the light-harvesting pigment protein in the stacking process may also imply its role in the regulation of spillover.

It is not hard to imagine that the stacking of grana could result in changes in spillover since the efficiency of such energy transfer depends on the distance between and orientation of pigment molecules [20, 21]. The interactions of light harvesting pigment protein in the presence and absence of ions reported in this paper could result in changes of both orientation and distance. Work is currently underway to see if such changes can be observed and related to spillover. It is hoped that further studies on this protein will better elucidate the molecular basis for grana stacking and spillover. Even if the light-harvesting pigment protein does not prove to be related to the spillover process, a study of the interactions involved in the association of this protein may lead to a better understanding of the interactions involved in maintaining membrane structure.

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