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Chloroplast Alkaline Fructose 1,6-Bisphosphatase Exists in a Membrane-Bound Form

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ABSTRACT

An alkaline fructose 1,6-bisphosphatase activity associated with soybean (*Glycine max* cv Beeson) chloroplasts appears to be membrane-bound. The pH optimum of the membrane-associated activity corresponds to that found for activity associated with the stroma. Illumination of washed thylakoids results in an increase in alkaline fructose 1,6-bisphosphatase activity in the absence of any added stromal factors. Exposure to pH 8.0 results in a partial release of enzyme activity from the membrane. The activation status of the enzyme does not appear to alter its association with the membrane.

Alkaline FBPase[1] (EC 3.1.3.11) is exclusively a chloroplast enzyme (27). Work in several laboratories showed that alkaline FBPase plays a key role in the regulation of carbon fixation in the chloroplast (9, 17). Its sensitivity to changes in pH and in Mg^{2+} levels are two means by which this regulation is effected.

Light causes alkalization of the chloroplast stroma (18) and also a change in stromal Mg^{2+} (20). Conditions which exist in illuminated and photosynthetically functional chloroplasts are optimal for FBPase function, whereas in the dark it is estimated that *in vivo* the enzyme should have very little activity.

Champigny and Bismuth (13) and Anderson and Avron (3) independently demonstrated that a light-generated reductant was involved in activation of the enzyme in isolated chloroplasts. The activities of several key enzymes of the reductive phosphate cycle are now known to be modulated by light and the operation of the photosynthetic electron transport chain in a similar fashion (2, 9).

The work of Buchanan and his colleagues has demonstrated the role of soluble Fd and of thioredoxin and Fd-thioredoxin reductase (9) in the regulation of chloroplast FBPase. The work of Anderson's group (5, 3) implicated membrane-bound reductants in the LEM of chloroplast enzyme activity. Ashton, Brennan and Anderson (8) subsequently reported that chloroplast membranes possess a firmly bound thioredoxin activity. The differences between the results of the two groups could perhaps be in part experimental as suggested by Buchanan (9). Scheibe and Beck (26), Fisher and Latzko (14), and Anderson and Ben-Bassat (4) each found evidence for membrane-bound carbon metabolism enzymes in isolated chloroplasts. Anderson and Ben-Bassat (4) found that illumination of these membranes resulted in a release of a part of the enzyme activity from them.

The data presented here constitute evidence for the existence of another membrane-bound carbon metabolism enzyme-alkaline FBPase. This form of the enzyme is light-activatable and can be partially released from the membrane by exposure to pH 8.0. The possible significance of this finding is discussed in the context of *in vivo* light activation.

[1] Abbreviations: FBPase, fructose 1,6-bisphosphatase; LEM, light effect mediator.

MATERIALS AND METHODS

Experimental Plants. Soybean (*Glycine max* [cv Beeson]) were used for this study. Plants were grown from seed in 13 cm pots in a controlled temperature greenhouse (26.7°C day, 21°C night). Natural daylight was supplemented with illumination from metal halide lamps during the period October to May. At approximately the 5th trifoliate stage, they were transferred to controlled environment chambers where they were maintained on a 16-h photoperiod under subdued light conditions (100 μE m^{-2} s^{-1}) for 5 d. Relative humidity in the chambers was about 50%. Mature leaves from these plants were used for chloroplast isolation as described below.

Chloroplast Isolation. All manipulations described in this section were performed under a green safelight. Chloroplast isolation and preparation of washed thylakoids was a combination of the methods of Ashton and Anderson (7) Alscher-Herman (1) and Nakatani and Barber (24). Grinding and resuspension buffer compositions and centrifugation times used are those described by Nakatani and Barber. Chopped soybean leaves (15 g) were ground in 150 ml of partially frozen 330 mM sorbitol, 200 mM $MgCl_2$, 20 mM Mes (adjusted to pH 6.5 with 1 M Tris), using setting No. 9 on a Polytron homogenizer. The brei was filtered through a pad of 10 layers cheesecloth, one layer Miracloth, and one layer nonabsorbent cotton and then through one layer of 15 μm precision woven nylon mesh to remove the whole cells which commonly contaminate preparations of chloroplasts from soybean. The filtrate was centrifuged at 4,500*g*. for 30 s at speed, with a 90 s total centrifugation time.

The resulting pellet was resuspended in 10 ml 330 mM sorbitol adjusted to pH 7.5 with Tris-base, again filtered through 15 μm nylon mesh and centrifuged at 4,500*g*. for 20 s at speed, with a total centrifugation time of 60 s.

Broken Chloroplasts and Washed Thylakoids. The pellet obtained was resuspended in a minimum volume of 50 mM Hepes (pH 6.8) containing 1 mM EDTA, 10 mM KCl, and 5 mM $MgCl_2$. Aliquots of the suspensions were removed, extracted with 80% acetone and Chl concentrations determined by the method of Arnon (7). The Chl concentration of the suspension was adjusted to 1 mg Chl ml^{-1} and membranes were recovered from the suspension by centrifugation at 23,500*g* for 20 min. The supernatant (stromal extract) obtained was stored at 4°C for further use. The membranes were resuspended in 27 ml ice-cold distilled H_2O, to which 3 ml of 500 mM Hepes (pH 6.8) containing 10 mM EDTA, 100 mM KCl, and 5 mM $MgCl_2$ were subsequently added. The suspension was re-centrifuged as before. This step was repeated twice. The membrane pellet obtained after the final washing was resuspended in 50 mM Hepes buffer (pH 6.8) containing 1 mM EDTA, 10 ml KCl, 5 mM $MgCl_2$. This procedure is described in Figure 1.

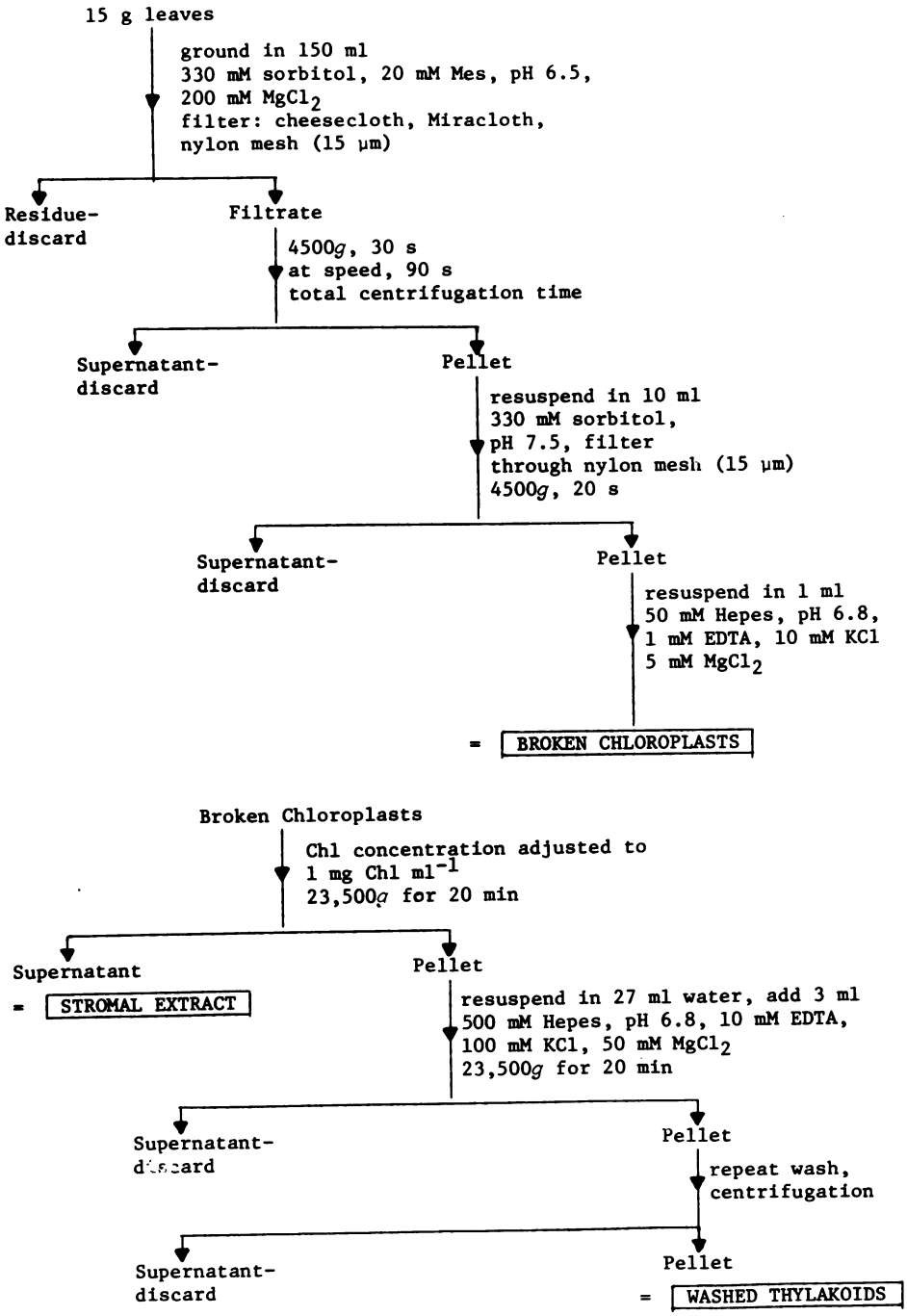

FIG. 1. Procedure for the isolation of soybean chloroplasts from leaves.

Intact Chloroplasts. In the case of experiments where intact chloroplasts were prepared, the pellet obtained after the second centrifugation was resuspended in 330 mM sorbitol (pH 7.5) (adjusted with Tris-base) instead of in the Hepes buffer indicated in Figure 1.

Light Activation. The washed thylakoids obtained as described above were resuspended at 100 μg Chl ml^{-1} in the Hepes buffer described above and illuminated for 5 m at 25°C at 900 μE m^{-2} s^{-1}. Activation was fully attained within the 5-min illumination period (preliminary data not shown here). The suspensions were stored in liquid N_2. The extensive manipulations carried out on the membranes after illumination were all carried out at 4°C. At this temperature and within this time period, no inactivation of the enzyme was detected (data not shown). Aliquots were subsequently used in determinations of FBPase activity. (Light activation ability does not appear to survive freezing and thawing, whereas FBPase activity did not appear to be affected by storage in liquid N_2, preliminary data, not shown here.) In the case of the stroma, stromal extracts were combined with washed membranes at a final Chl concentration of 100 mg Chl ml^{-1}, and illuminated for 5 min as described above.

Assay of FBPase Activity. Assays of FBPase activity were carried out either by the method of Preiss and Greenberg (25) or by measuring the release of Pi, using the phosphate reagent described by Taussky and Schorr (28).

Measurement of FBPase Activity through Release of Pi. Stromal extract, or reaction mixtures from the light activation step were incubated for 30 min at 30°C ± 8 mM FBPase. Reaction

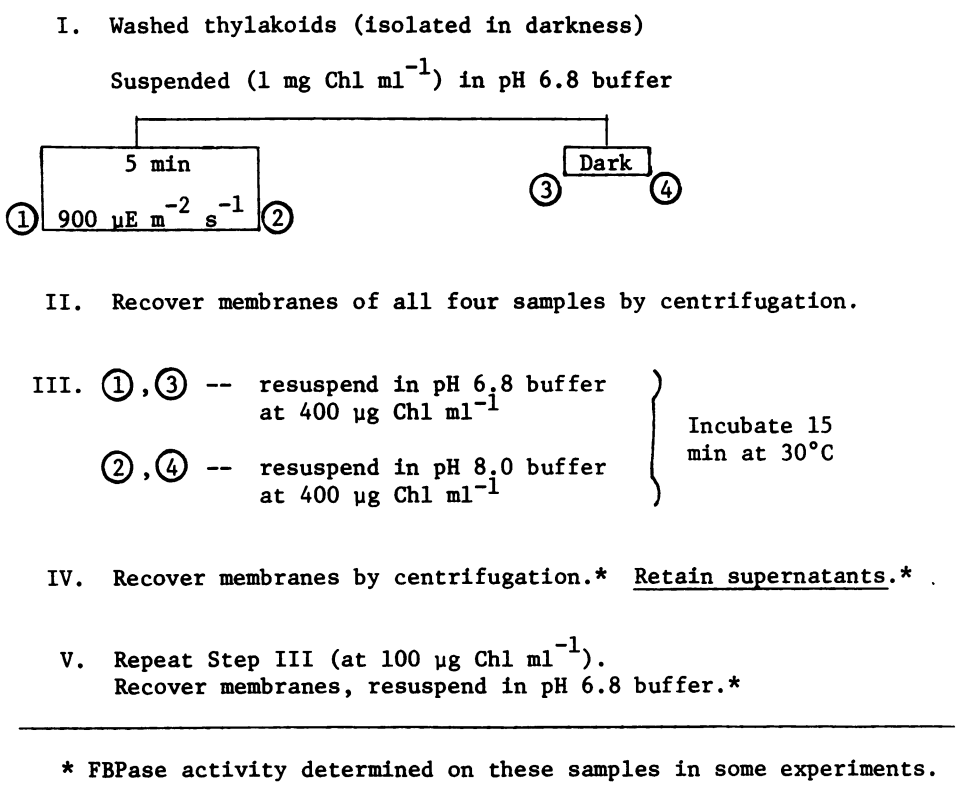

FIG. 2. Summary of procedures followed to investigate thylakoid-FBPase association in soybean.

mixtures were 100 mM Tris-HCl (pH 7.0, 7.5, 8.1, 8.5, or 9.0) 18 mM $MgCl_2$. After the incubation, the reaction was stopped by making the mixture 2% with respect to TCA. When broken chloroplast mixtures were used, 525 µl water was added, and the precipitated protein was removed by centrifugation. In the case of stromal extract, 525 µl water were also added, but the centrifugation step was omitted. To these mixtures, 600 µl phosphate reagent was added, and A_{740} was read after exactly 5 min. The amount of Pi released was determined from a standard curve for phosphate after correction for substrate breakdown and nonspecific phosphatase activity.

Coupled Assay for FBPase Activity. Reaction mixtures contained 100 mM Tris-HCl (pH 8.5) 10 mM $MgCl_2$, 3.2 mM EDTA (disodium salt), 4.9 mM FBPase, glucose-6-P dehydrogenase and phosphohexose isomerase (10 µg ml^{-1}), and 0.5 mM NADP. Either 50 or 100 µl aliquots of chloroplast samples were used, the mixture brought to a final volume of 1 ml with distilled H_2O and changes in A_{340} followed using a linear recorder attached to a Gilford model 250-1 spectrophotometer.

Chemicals and Supplies. All chemicals, including enzymes, were obtained from Sigma. Precision woven nylon mesh was obtained from Tetko, Inc., Elmsford, NY.

Determination of the Effect of pH on Membrane-Bound FBPase. A summary of the procedures followed to investigate thylakoid-FBPase association in soybean is shown in Figure 2. Thylakoids prepared as described above were the starting material for all experiments described here. The first incubation (step III) was carried out at a relatively high Chl concentration (400 µg Chl ml^{-1}) so as to allow for detection of released enzyme activity in the wash medium.

RESULTS

A Method for Obtaining Chloroplasts from Soybean Leaves. Figure 3, A and B demonstrate the effectiveness of filtering through precision woven nylon mesh as a method for removing whole cells from soybean leaf homogenates. The chloroplasts obtained from the leaves of plants which had been subjected to a subdued light regime appear to be 40% to 50% intact as judged by their appearance under the phase contrast microscope. Attempts to determine per cent intactness by conventional biochemical means (ferricyanide assay of Heber and Santarius [16] purification with Percoll by Mills and Joy [23] were not successful. The intact chloroplasts did, however, exhibit some carbon fixation ability as assayed by incorporation of $^{14}CO_2$ into acid-insoluble products (data not shown). Washed thylakoids did not exhibit this capacity. This constitutes further evidence of the absence of whole cells from the preparations.

pH Optimum of Stromal and Membrane-Associated Enzymes. The pH optimum of FBPase activity present in stromal extracts of chloroplasts obtained from soybean leaves as described above is shown in Figure 4B. No activity was detected below pH 8.0, a finding which is in good agreement with the long-established alkaline pH optimum of chloroplast FBPase (27). FBPase activity associated with the washed thylakoid fraction had a similar pH optimum (Fig. 4A). This peak of enzyme activity in the alkaline range gives further indication that whole cells are effectively absent from these samples, inasmuch as a contribution of activity in the neutral pH range might be expected from the cytoplasmic enzyme, were it present and active in the assay mixture.

Thylakoid-Associated Alkaline FBPase Can Be Light-Activated. Washed soybean thylakoids isolated under a green safelight as described in "Materials and Methods" and illuminated at pH 6.8 show increased FBPase activity, as is demonstrated by the data of Table I, with a L:D ratio of 1.62. This increase is in good agreement with the 1.8-fold light activation of alkaline FBPase reported by Anderson (2). This stimulatory effect of light on thylakoid-associated enzyme activity was obtained in the absence of any added stromal factors.

Similar results were obtained using washed thylakoids obtained

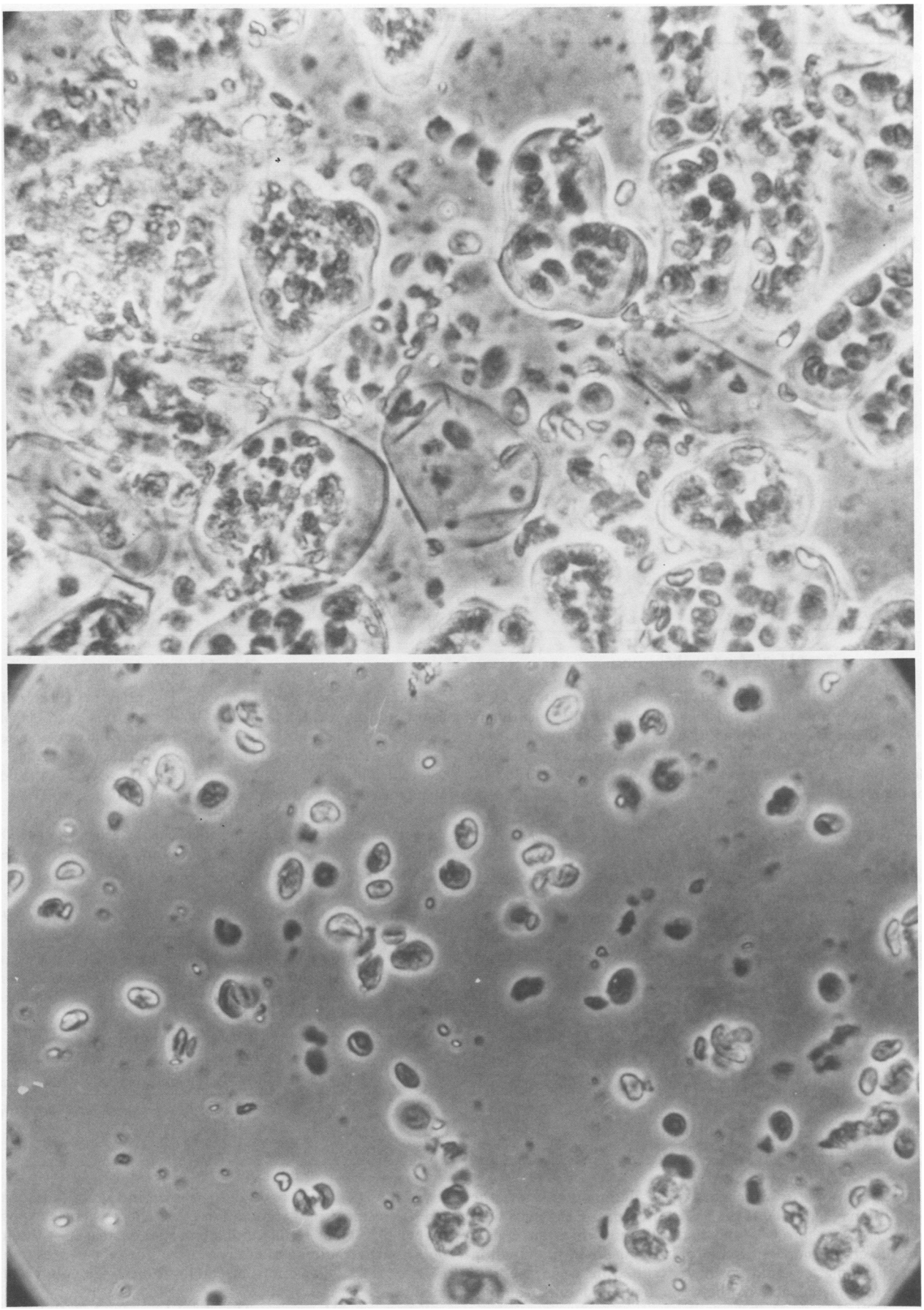

FIG. 3. Light micrographs (Zeiss) showing effect of filtering through 15 μm precision woven nylon mesh on pellet obtained by the chloroplast isolation procedure described in "Materials and Methods." A, Pellet obtained, omitting the filtration step. B, Pellet obtained when filtration step was included.

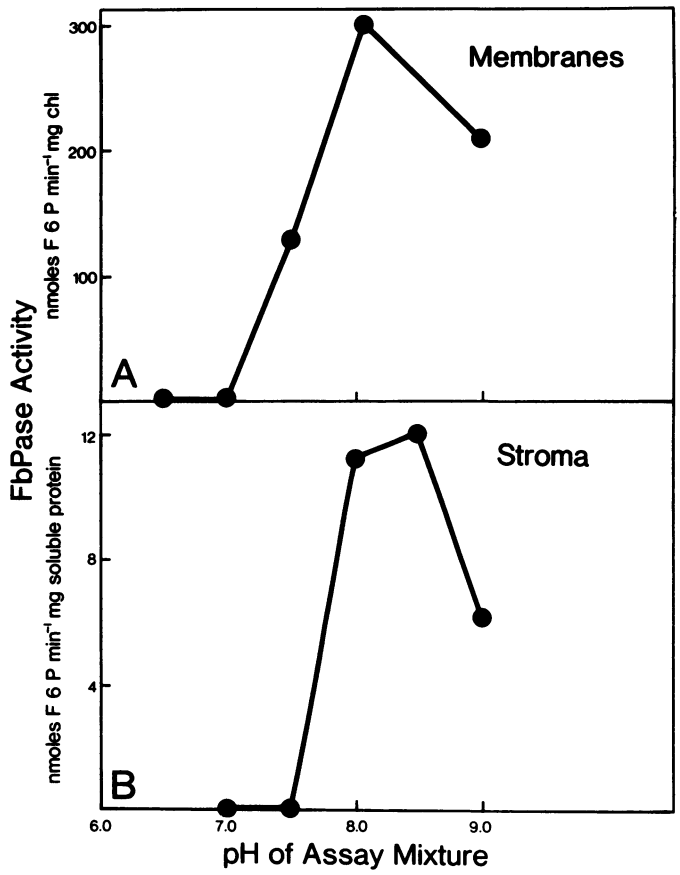

FIG. 4. Effect of pH of the assay mixture on FBPase activity. A, Effect of pH of the assay mixture on thylakoid-associated FBPase activity. B, Effect of pH of the assay mixture on FBPase activity of the stromal extract. Stroma and washed thylakoids were obtained as described in "Materials and Methods." Tris-HCl (100 mM) was used at pH 7.0 to 9.0 and 100 mM Mes for the pH 6.5.

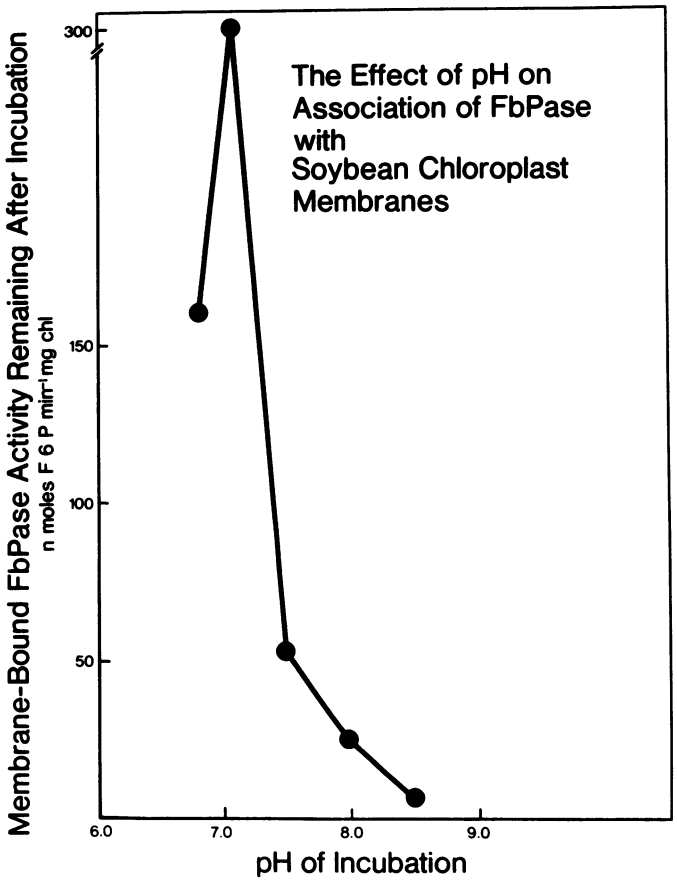

FIG. 5. The effect of incubation at varying pH on the activity of FBPase associated with soybean thylakoids. Washed thylakoids were prepared as described in "Materials and Methods," including the filtration step. Aliquots were suspended in 50 mM Tris-HCl, adjusted to pH 7.2, 8.0, 8.5, or 9.0, containing 1 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$. An additional aliquot was suspended in 50 mM Hepes (pH 6.8), 1 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$. All suspensions were incubated at 30°C for 15 min, membranes recovered by centrifugation, and thylakoid-associated FBPase activities determined at pH 8.1 by the Preiss and Greenberg assay described in "Materials and Methods."

Table I. *Effect of Illumination of FBPase Activity Associated with Soybean Thylakoids*

Intact chloroplasts (1) or washed thylakoids (2) were isolated in darkness from mature soybean leaves. The effect of illumination (900 μE m^{-2} s^{-1} for 5 min at 25°C at pH 6.8 of (1) and (2) on FBPase activity (at pH 8.1) associated with washed thylakoids obtained from (1) after illumination and with (2) was determined. All enzyme assays were carried out at pH 8.1.

Sample	Membrane-Associated FBPase Activity		L:D
	Light	Dark	
	nmol FBP produced min^{-1} mg^{-1} Chl		*ratio*
Intact chloroplasts (1)	90	61	1.48
Washed thylakoids (2)	128	79	1.62

Table II. *Effect of exposure to Alkaline pH on Membrane-Bound FBPase*

Washed, nonilluminated thylakoids were incubated at 30°C for 15 min at pH 8.0 or 6.8 at 400 μg Chl ml^{-1} and recovered from the suspension by centrifugation. Aliquots of the supernatants obtained were assayed for FBPase activity, as were aliquots of the resuspended membranes. All enzyme assays were carried out at pH 8.1

pH of Incubation	Total FBPase Activity	
	Activity released	Membrane
	nmol FBP produced min^{-1} mg^{-1} Chl	
6.8	14	172
8.0	112	91

from intact chloroplasts which had been illuminated under the same conditions (Table I). If the association of the enzymes with the thylakoid were purely an adventitious one which arose as a result of the circumstances of chloroplast lysis, then illumination of intact chloroplasts would not have resulted in the measurable difference in enzyme activity between light and dark samples shown in Table I.

Effect of Incubation at Varying pH on Thylakoid-Associated FBPase Activity. The effect of incubation at varying pH of washed thylakoids at 100 μg Chl ml^{-1} is shown in Figure 5. Exposure to pH 7.2 resulted in a stimulation of activity, whereas exposure to higher pH values resulted in substantial decreases, with virtually no activity remaining after exposure of the membranes to pH 8.5. FBPase activity was not detectable in the large volume of wash medium.

Effect on pH on the Thylakoid-Enzyme Association. When washed thylakoids which had not been exposed to light were incubated at 30°C for 15 min in a pH 8.0 buffer at 400 μg Chl

Table III. *Effect of Exposure to Light on the Binding of FBPase to Thylakoid Membranes*

The procedure described in Figure 2 was followed in order to determine if light-activated membrane-bound enzyme differed from nonactivated enzyme with regard to its association with the membrane. Thylakoids were subjected to steps I to V of Figure 2. FBPase activities remaining with the membranes after two cycles of incubation (Inc. I and Inc. II) at pH 6.8 or 8.0 were determined (Fig. 2, step V). All enzyme assays were carried out at pH 8.1.

Treatment	Membrane-Associated FBPase Activity (nmol FBP min^{-1} mg^{-1} Chl)											
	Experiment I				Experiment II				Experiment III			
	After Inc. I	L:D	After Inc. II	L:D	After Inc. I	L:D	After Inc. II	L:D	After Inc. I	L:D	After Inc. II	L:D
		ratio		*ratio*		*ratio*		*ratio*		*ratio*		*ratio*
Dark, pH 6.8	170		178		270		270		803		760	
		1.9		1.8		1.9		1.4		1.4		1.3
Illuminated, then exposed to pH 6.8	320		318		502		370		1,101		1,005	
Dark, pH 8.0	90		ND[a]		160		150		680		ND	
		2.4				1.6		1.5		1.5		
Illuminated, then exposed to pH 8.0	220		230		250		230		803			

[a] Not detectable.

ml^{-1}, thylakoid-associated FBPase activity decreased by approximately 50% (Table II). The higher Chl concentration was chosen in order to try and detect released enzyme activity in the wash medium. Considerable activity was released into the medium as a result of incubation at pH 8.0, whereas an incubation carried out under the same conditions, except at pH 6.8, resulted in release of only a small amount (about 8%) of the total membrane-associated FBPase activity.

Effect of Light on the FBPase-Thylakoid Association. Anderson and Ben-Bassat (4) found that light brought about partial release of other thylakoid-bound carbon metabolism enzymes to the stroma in pea chloroplasts. The results of experiments designed to determine whether this was also the case for soybean thylakoids and alkaline FBPase are shown in Table III. The effect of illumination on the amount of enzyme activity associated with the membrane was determined, as was the effect of activation status on the ability of pH 8.0 conditions to remove the enzyme from the thylakoids. It can be seen from the results of Table III that light does not cause the release of enzyme from the membrane. In addition, the portion which remains associated with the membrane after its exposure to pH 8.0 is activated by light to an extent (L:D, 1.56), which is entirely comparable to the activation obtained for membrane-associated enzyme exposed to the control pH of 6.8 (L:D, 1.85). Enzyme remaining associated after a second incubation at 100 μg Chl ml^{-1} was activated by light as well. As in the case of the results of Figure 4, FBPase activity could not be detected in the large volume of wash medium.

DISCUSSION

The method described here represents a means of obtaining chloroplasts from soybean leaves. Studies of photosynthetic function in isolated soybean chloroplasts have been rare and reports of levels of activities of many chloroplast enzymes do not exist due to the technical difficulties presented by the leaf material. The filtering step which I have included in the isolation procedure effectively removes contaminating whole cells from the leaf homogenate and the prior 5-d exposure of the whole plants to low light conditions makes possible the isolation of chloroplast pellets which contain some stromal material. The thylakoid-associated FBPase reported here has an alkaline pH optimum similar to that obtained for activity associated with the stromal fraction. Inasmuch as the pH of the assay corresponds to the pH range which causes release of the enzyme, it is possible that some release from the membrane may have taken place during the assay procedure. The coupled assay system for FBPase involves an immediate determination of the rate of enzyme activity. If release did take place, it did not appear to alter the kinetics of enzyme action, because both stromal and membrane-bound fractions gave linear rates of FBPase activity for up to 15 min.

The evidence of Table I shows that the membrane-associated FBPase can be light-activated in the absence of any added soluble factors. Although an adventitious binding of enzyme to membrane during the initial homogenization step cannot be entirely ruled out, it does appear that the results shown in Table I do not arise as a consequence of any nonspecific binding which might occur during the chloroplast lysis step, inasmuch as light would not influence such a process, and thus the amount of membrane-associated activity obtained in the case of samples, which had been illuminated before lysis, should not be influenced by light. Attempts to bring about binding of stromal FBPase to washed thylakoids were unsuccessful (data not shown). This result, although disappointing, makes the possibility of an adventitious binding of stromal enzyme to the thylakoids less likely.

The chloroplast preparations which I obtained were approximately 40% intact as based on the observations made in the phase contrast microscope. Hence, the values obtained for the membrane-associated FBPase activity contain a large contribution from naked membranes present in its mixed population. However, inasmuch as there is no large difference in the values for membrane-associated activity between "intact" and lysed preparations, it would seem that the membranes of the "intact" samples do have enzyme associated with them.

The removal of a part of the enzyme from the membrane by a simple exposure to pH 8.0 suggests a possible physiologic significance for the enzyme-membrane association. As the stroma reaches its more alkaline pH in the light, alkaline FBPase may be released from a site of activation on the membrane. This immediately raises the intriguing question of the nature of this putative site of activation. As Scheibe and Beck (26) point out in their discussion of the mechanism of light activation of the NADP-dependent malate dehydrogenase of spinach chloroplasts, the activation process is rapid and must involve close contact between activating system and thylakoid at some point. An association such as the one described here for soybean thylakoids would constitute such a close contact. Fischer and Latzko (14) described a loose association of ribulose-5-P kinase with spinach thylakoids. They raise the possibility that a direct interaction between thyla-

koid sulfhydryl groups and enzyme occurs after illumination. This recalls Anderson and Avron's (3) membrane-bound reductants (LEM). Although at first it was proposed that no soluble factors were required for light activation in the LEM model, Ashton and Anderson (7) subsequently found that a soluble protein named Protein Modulase was required for light activation in pea chloroplasts. Lara *et al.* (21) described a new protein factor which functions as a Fd-independent mechanism for the modulation of FBPase by light. Scheibe and Beck (26) interpreted their results to indicate that enzyme plus activator were membrane-bound with the activator being more loosely bound than the enzyme. The membrane-bound soybean enzyme described here may also be associated with its activator which may be the protein factor described by Lara *et al.* (21). Activator as well as enzyme may be released from the membrane by exposure to pH 8.0, inasmuch as membranes which had been depleted of enzyme by this treatment could not mediate light activation of added stroma (data not shown). Previous work in this laboratory, on the other hand, has demonstrated *in vitro* light activation of stromal FBPase in broken soybean chloroplast preparations (1). Thus, soybean thylakoids do have the ability to bring about *in vitro* light activation of FBPase.

Inasmuch as no reliable physiologic method for determination of intactness was possible, I was not able to determine the distribution of enzyme between stroma and thylakoids with any degree of accuracy. However, rates of alkaline FBPase activity in soluble extracts of soybean leaves were found to be in the range of 200 μmol FBP min^{-1} mg^{-1} Chl (Alscher-Herman and Jeske, submitted for publication). This is roughly equivalent to the rates reported here for soybean thylakoids and would suggest a major contribution by bound enzyme (under the appropriate illumination conditions) to total chloroplast FBPase activity.

The chloroplast preparations used for this study were obtained from plants which had been in the dark for 16 h before harvesting and the isolation procedure itself was carried out under green light. These precautions were taken in the hopes of obtaining a distribution of enzyme between membranes and stroma which would accurately reflect *in vivo* conditions in the dark. Inasmuch as the transition from pH 6.8 to 8.0 removes some of the membrane-associated enzyme, it is possible that at the lower pH (about 7.0) which obtains in the stroma in the dark a large proportion of FBPase is loosely bound to the thylakoids. On illumination, the enzyme is activated by photochemically generated reductant through an activator(s), the stromal pH rises, and dissociation from the membrane takes place. This hypothesis carries with it the implication of a cyclic association and dissociation of the enzyme and the membrane. The existence of such a phenomenon remains to be proven. Its relation to the soluble light-activating system which has been so fully characterized by Buchanan and his colleagues (9–12, 21) should then become clear.

Note Added in Proof. During the period of time in which this work was carried out, the results of Charles and Halliwell 1980 Biochem J 185: 689–693; 1981 Planta 151: 242–246 appeared which demonstrated that the form of FBPase, which is inactive at the substrate concentrations which occur *in vivo* (100–400 μM) does have activity at higher substrate concentrations. Thylakoids prepared as described in this report have now been assayed for FBPase activity at lower substrate and Mg concentrations (500 μM, 5 mM Mg) and been found to have enzyme activity (about 90 nmol FBP mg^{-1} Chl min^{-1}) which can be light-activated (about 1.5-fold activation). In addition, the values reported by the Halliwell group for FBPase activity in spinach chloroplasts are higher (spinach, about 2,800 nmol FBP min^{-1} mg^{-1} Chl at 4 mM FBP; soybean thylakoids, 200–500 nmol FBP min^{-1} mg^{-1} Chl). This may reflect a species difference and/or the relative distribution of enzyme between stroma and thylakoids.

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