Light Modulation of Enzyme Activity

ACTIVATION OF THE LIGHT EFFECT MEDIATORS BY REDUCTION AND MODULATION OF ENZYME ACTIVITY BY THIOL-DISULFIDE EXCHANGE?[1]

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ABSTRACT

Light and dark modulation experiments with pea (*Pisum sativum* L.) chloroplast stromal fractions pretreated with dithiothreitol (to reduce protein disulfide bonds) or with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (to block sulfhydryl groups) suggest that light modulation involves thiol-disulfide exchange on the modulatable stromal enzyme protein. Light-dependent reduction of DTNB involves a photosynthetic electron transport chain component located on the reducing side of photosystem I prior to ferredoxin; DTNB may be acting as a light effect mediator substitute. The thylakoid-bound light effect mediator system, then, in its light-activated reduced form probably catalyzes thiol-disulfide exchange reactions on stromal enzymes.

Light modulates the activity of several chloroplastic enzymes by a process involving vicinal-dithiol, thylakoid-bound light effect mediators (LEMs).[3] There are at least two different LEMs. Both apparently accept electrons from the photosynthetic electron transport chain on the reducing side of PSI, but one (LEM_I) interacts with a component of the electron transport system before ferredoxin, while the other (LEM_{II}) interacts at the level of, or beyond, ferredoxin (3).

When crude pea leaf stromal extracts are treated with DTT the five known light-activatable chloroplastic enzymes are activated and glucose-6-P dehydrogenase is inactivated. This observation led us to speculate that light modulation involves reduction of disulfide bonds in the light-modulatable enzymes; but there is still no definitive evidence for reduction, and, in fact, compounds which catalyze disulfide interchange sometimes affect the modulatable enzymes (1). The first purpose of the present experiments was to determine what effect treating stromal extracts with compounds which react with free sulfhydryl groups, thereby blocking thiol-disulfide exchange, might have on modulation. The results lead us to conclude that light modulation probably involves intramolecular thiol-disulfide exchange, at least in the case of the two chloroplast enzymes studied in the present experiments, NADP-linked malic dehydrogenase and glucose-6-P dehydrogenase.

Since the LEMs are sensitive to sulfite, disulfide bonds are apparently essential for LEM structure and/or function (3). The

popular reagent for the detection of thiol groups, DTNB, first shown to be reduced photochemically in bacterial chromatophore preparations by Newton (15), is included in a recent listing of Hill reagents (21). Because DTNB, like the LEMs, is a disulfide, it seemed of interest to determine the point at which DTNB interacts with the chloroplast electron transport chain. Our results indicate that DTNB is photoreduced at, or close to, the site involved in photoreduction of LEM_I and is, therefore, potentially useful as an analogue of LEM_I.

The LEMs, then, are probably reductively activated thylakoid-bound proteins which catalyze thiol-disulfide group exchange in certain stromal enzymes.

MATERIALS AND METHODS

Pea (*Pisum sativum* L., var. Little Marvel) plants were grown in soil or Vermiculite in a greenhouse. Leaves were harvested when the plants were between 10 and 14 days old. Chloroplast membrane fragments and stromal extracts were prepared essentially as described previously (3) except that: (*a*) no attempt was made to shield plants or preparations from light; and (*b*) $MgCl_2$ was omitted from the buffer used for suspending the particulate fraction.

Effector Experiments. Stromal fractions were made 1 mM with respect to DTNB, or 10 or 50 mM with respect to DTT, as indicated, and allowed to stand for 1 hr on ice. The effector compound was then removed by gel filtration (100- to 300-μm G-25 Sephadex, column size 0.7 × 4.7 cm, 1-ml aliquot applied to column, first 11 drops discarded, next 12 collected for use in reconstituted chloroplast system). Control samples were treated in exactly the same manner except that the effector compound was omitted. The reconstituted chloroplast system contained: (*a*) particulate material to give a final Chl concentration of 60 μg ml^{-1}, and (*b*) stromal fraction having, within each individual experiment, equivalent units of triose-P isomerase activity. (Triose-P isomerase was used as a standard enzyme for "normalizing" stromal protein levels because it was not affected by any of the effectors used here. We could not use protein values to normalize fractions because of the limited amounts of material available.) Stromal protein concentration in reaction mixtures was estimated from isomerase levels and the protein concentration in stromal fractions before gel filtration. Samples were preincubated in the dark at room temperature for 5 min, and then exposed to white light (see above) for 5 min (light-treated). Modulation was stopped by 10-fold dilution of the modulation assay components with ice-cold deionized H_2O. Particulate material was removed by centrifugation and enzyme activity was assayed immediately.

Enzyme Assays. Enzyme activity was measured using methods previously employed in this laboratory (11) except that the change in reduced NADP was sometimes followed by change in fluorescence (340 + 360 nm exciting light) using an Eppendorf photometer 1100 adapted for fluorimetry. All assays were run at room temperature (about 20 C).

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[3] Abbreviations: diquat: 1,1'-ethylene-2,2'-dipyridylium dibromide; DPIP: 2,6-dichlorophenolindophenol; DSPD: disalicylidenepropanediamine; DTE: dithioerythritol; DTNB: 5,5'-dithiobis(2-nitrobenzoic acid); LEM: light effect mediator.

Gel Electrophoresis Experiments. These experiments were conducted essentially as described previously (5) except that after electrophoresis the gels were frozen on dry ice, sliced into 1- or 2-mm segments (Bio-Rad model 190 gel slicer) and the segment was either incubated overnight in a small volume of the assay mixture or was extracted overnight, and activity of solubilized enzyme was assayed on the following day. Activity was proportional to disappearance of substrate (NADP-linked malic dehydrogenase) or appearance of product (ribulose-5-P kinase, sedoheptulose-1,7-diP phosphatase). Assay mixtures used were those used previously in this laboratory (11). All absorbancy readings were made with a Gilford 2400 spectrophotometer.

Assay for Hill Reaction. Resuspended membrane fragments were preincubated for 10 min in the dark in cuvettes in 1 ml (final volume) of solution containing 1 μmol of DTNB, 25 μmol of pH 7.4 potassium HEPES buffer, 5 μmol of KCl and 0.5 μmol of EDTA. Cuvettes and contents were then irradiated with white light from two Westinghouse 125- to 130-v, 75-w flood lamps, separated from the cuvettes by a water bath. Light intensity was 3,500 ft-c, which is the lower limit for maximal activity. High light intensity (over about 6,000 ft-c) inactivates. At 4-min intervals A_{412} was measured against a neutral density filter (frosted side of a glass cuvette) in a Gilford 2400 spectrophotometer. Four readings were taken on each sample. Total light exposure was 12 min. Dark controls were run in all cases. There was no change in the dark control and no change in the irradiated samples when they were removed from the light. We detected no photoactivity in boiled thylaloid preparations. The reaction was linear with respect to time for at least 12 min. It was not apparently linear with respect to Chl concentration because of the turbidity of the membrane suspension. Since our aim was to compare the systems for the photoreduction of the LEMs and for DTNB and because experimental material was limited, we did not determine exact equivalence of A_{412} units and nmol of monothionitrobenzoate formed in each experiment. Results are expressed as change (increase) A_{412} min^{-1} ml^{-1}. One A_{412} unit is equivalent to the formation of approximately 50 nmol of 2-nitro-5-thiobenzoate with our experimental set-up.

All assays were run at room temperature (about 20 C).

Determination of Optimal pH. pH of the assay mixture was measured with a Radiometer pH meter 26 following irradiation.

Protein and Chl Estimation. Protein was estimated by the biuret method after precipitation with acetone (2) and Chl from A_{665} and A_{649} readings in 80% acetone (18).

Chemicals. Biochemicals and coupling enzymes were products of Sigma Chemical Co. DSPD was the product of Fluka; diquat, of Plant Production Ltd., Jeolott's Hill Research Station, Yalding, England. DCMU used in these experiments was a product of Pfaltz and Bauer, Inc. and was recrystallized from ethanol prior to use. It contained 50% CMU which was corrected for in making up DCMU solutions. Other chemicals were analytical reagent grade. Pea seeds were obtained from Northrup and King Seed Company, Chicago.
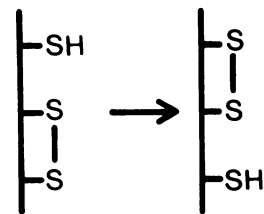
RESULTS AND DISCUSSION

What Is the Nature of the Change in the Light-modulatable Enzymes? Our reasoning with regard to this question was as follows. If enzyme disulfide or sulfhydryl groups are involved in light modulation then the three simplest possible changes in the enzyme molecule are:
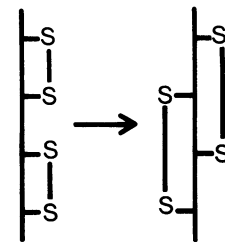
reduction of a disulfide bond
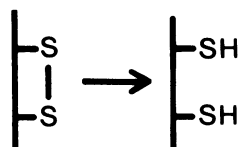
(Model I)

thiol, disulfide exchange

(Model II)

disulfide exchange

(Model III)

If DTT acts as a reducing agent and reduces disulfide bonds and DTNB acts by blocking free sulfhydryl groups, then DTT should reduce the disulfide bond(s) in each of the models whereas DTNB should only react with the free sulfhydryl group in model II.

We first treated the stromal fraction with DTT, and then used this fraction as substrate for the LEM system. We observed that the effect of DTT on the activity of the dehydrogenase in the stromal fraction was similar to the effect of light mediated by the LEM in the reconstituted chloroplast system (Table I). This is consistent with previous work in this laboratory (1).

Let us examine each model with respect to the effect of DTT. In the case of model I DTT and light will be equivalent. Light, *in vivo*, and DTT, *in vitro*, do inactivate glucose-6-P dehydrogenase and activate malic dehydrogenase (Table I). On the basis of the experimental data model I is possible.

In the case of model II DTT *in vitro* and light *in vivo* will be equivalent if a new sulfhydryl group is required for the alteration in enzyme activity, but not if a new disulfide bond is required. On the basis of the data (Table I) model II is still possible.

In the case of model III DTT and light cannot be equivalent. However, we observed (Table I) that DTT treatment and light modulation mediated by the LEM system produce similar changes in the activity of the two dehydrogenases. Therefore, model III is impossible and can be eliminated.

DTT treatment should appear to inhibit light modulation in either case I or II, since reduced enzyme will not react with the LEM system. This is indeed observed (Table I).

DTNB should not react with the disulfide in model I, but should react with the free thiol group in model II blocking modulation of enzyme activity by the LEM system. DTNB does block modulation (Table I). Therefore, model I is eliminated and only model II remains. We conclude that light modulation probably involves thiol-disulfide exchange of enzyme cysteine and cystine groups, and not reduction of a disulfide bond.

Although DTT and DTNB could affect soluble mediators as well as the modulatable stromal enzymes, we have not been able to find any evidence for the participation of such mediators in the LEM system (see later).

We have done a series of gel electrophoresis experiments to compare the effects of DTT and light treatment. In the first of these experiments we found that NADP-linked glyceraldehyde-3-P dehydrogenase was apparently shifted from one conformer to another by light or DTT treatment (5). In the case of NADP-linked malic dehydrogenase only the enzyme from the DTT-

Table I. Effect of pretreatment on stromal fraction with DTNB or DTT on light and dark modulation in reconstituted chloroplast system

Experimental conditions as outlined in Materials and Methods. Light modulation here is expressed as percent activity remaining after light treatment, i.e. in control, first line, there is a 9.4-fold stimulation of malic dehydrogenase activity, in control, line 3, there is 58% inhibition of glucose-6-P dehydrogenase activity.

| Effector	Enzyme	Activity (nmoles) min^{-1} ml^{-1}		Effect of Effector on Enzyme	Light modulation (%)		Inhibition of Modulation	Stromal protein concentration in assay (mg ml^{-1})
		Control	Treated		Treated	Control		
1 mM DTNB	Malic Dehydrogenase	0.3	0.3	none	180	940	80	3.25
		0.4	0.4	none	none	4450	100	4
	Glucose-6-P Dehydrogenase	1.1	0.63	43% inhib.	13	58	77	3.25
		0.525	0.2	62% inhib.	29	50	42	3.9
10 mM DTT	Malic Dehydrogenase	0.12	6.65	55x stim.	160	4450	96	4.0
50 mM DTT		0.12	8.9	74x stim.	170	4450	96	4.0
		0.16	19.7	123x stim.	158	2900	95	3.9
10 mM DTT	Glucose-6-P Dehydrogenase	2.2	0.7	68% inhib.	33	45	26	7.0
		1.6	0.65	59% inhib.	25	48	48	6.7
50 mM DTT		1.6	0.3	81% inhib.	14	48	71	6.7
		1.5	0.3	80% inhib.	21	63	66	4.0
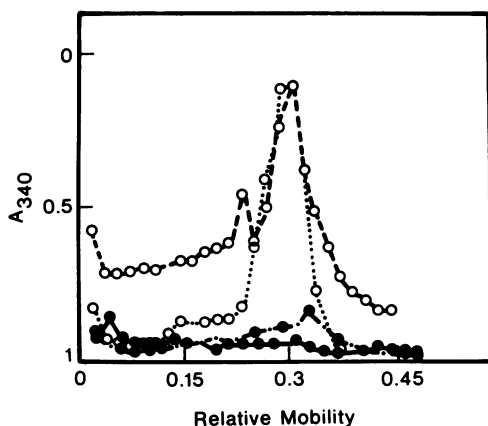
FIG. 1. Activity of NADP-linked malic dehydrogenase after light and dark treatment of whole plants or dithiol treatment of extract or gel slice *versus* relative electrophoretic mobility. Plants were held in the dark overnight, then irradiated. Extracts were prepared by grinding leaves in gel electrophoresis buffer. DTT treatment before electrophoresis consisted of incubating the extract from nonirradiated leaves in 50 mM DTT for 15 min. After electrophoresis gels were sliced into 1-mm segments. The discs were incubated in assay mixture overnight and A_{340} was measured on the following day. For dithiol treatment after electrophoresis the assay mixture was made 10 mM in DTE; gel used was duplicate of gel from dark-treated sample. Amount of protein applied to gels was 180 μg in case of extract from light-treated leaves, 370 μg in other cases. ●——●: light-treated; ●— · —●: dark-treated; O— - -O: DTT before electrophoresis; O · · · · O: DTT after electrophoresis.

behavior between DTT- and light-activated enzymes is predicted, while in the case of model II variation is possible.

Reduction of disulfide groups (as in model I) must result in production, however transient, of vicinal dithiol groups. If light modulation is reductive, then after light modulation the modulatable enzymes should be arsenite-sensitive. However, when we stopped the light reaction by diluting the reconstituted chloroplast system with 100 μM arsenite instead of water, there was no effect on the activity of NADP-linked malic dehydrogenase or of glucose-6-P dehydrogenase (data not shown). Gibbs and Calo (12)
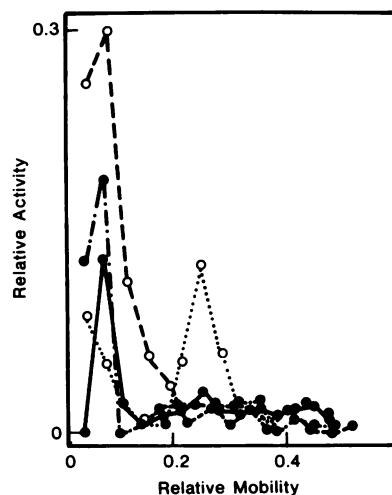

FIG. 2. Activity of ribulose-5-P kinase after light and dark treatment of whole shoots or DTT treatment of extract or gel slice *versus* relative electrophoretic mobility. Plants were held in dark overnight, then irradiated. Extracts were prepared by grinding leaves in gel electrophoresis buffer. DTT treatment before electrophoresis consisted of incubating the extract from nonirradiated leaves in 50 mM DTT for 15 min. After electrophoresis gels were sliced into 2-mm segments and incubated overnight in 0.5 ml of 0.1 M (pH 7.8) Tris-HCl buffer at 4 C. For DTT treatment after electrophoresis the buffer solution was made 50 mM in DTT; gel used was duplicate of gel from dark-treated sample. Ribulose-5-P kinase activity which leached out into buffer was assayed. Amount of protein applied to gels was 250 μg in case of extract from light-treated shoots, 360 μg in other cases. Activity values for light-treated extract gel have been normalized to correspond to protein applied to the other gels. ●——●: light-treated; ●— · —●: dark-treated; O— - -O: DTT before electrophoresis; O · · · · O: DTT after electrophoresis.

treated extract remained fully active during subsequent electrophoresis (Fig. 1). Clearly the light effect is not the same as the DTT effect in this experiment. Likewise, the effects of light and of DTT treatment are not the same in the case of ribulose-5-P kinase (Fig. 2). Light activation does not persist through the electrophoretic process and, after DTT activation, the enzyme has been changed to a faster moving form. The slower moving form of the enzyme can be activated by DTT treatment after electrophoresis. Light, but not DTE, activates the slower moving form of sedoheptulose-1,7-diP phosphatase, while light and DTE have similar effects on the activity of the faster moving form (Fig. 3). Here, DTE and light are not equivalent for one form, but similar effects are found with the other form. These experiments are consistent with the thiol-disulfide exchange mechanism (model II): in the case of model I (disulfide reduction) no difference in mobility or
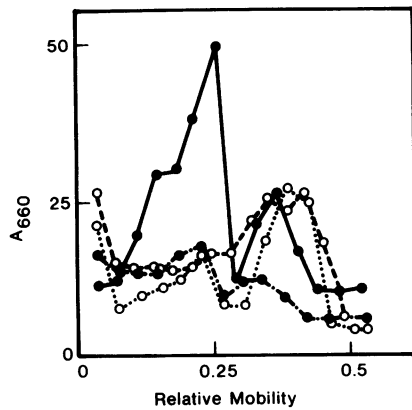
Fig. 3. Activity of sedoheptulose-1,7-diP phosphatase after light and dark treatment of whole shoots of DTE treatment of extract, or gel slice, versus relative electrophoretic mobility. Plants were held in dark overnight, then irradiated. Extracts were prepared by grinding leaves in gel electrophoresis buffer. DTE treatment before electrophoresis consisted of incubating extract from nonirradiated leaves in 50 mM DTE for 15 min. After electrophoresis gels were sliced into 2-mm segments and incubated overnight in sedoheptulose-1,7-diP assay mixture at room temperature. For DTE treatment after electrophoresis the assay mixture was made 10 mM in DTE. Reagents for inorganic phosphate determination (which terminate enzyme reaction) were added directly to the incubation mixture and A_{660} was measured spectrophotometrically. Amount of protein applied to gels was 350 µg in case of extract from light-treated shoots, 900 µg in other cases. Activity is expressed as A_{660} units/g of protein applied to gel. ●——●: light-treated; ●— · —●: dark-treated; O— - -O: DTT before electrophoresis; O · · · ·O: DTT after electrophoresis.

also found no effect of arsenite on several Calvin cycle enzymes. Although transient vicinal dithiol formation is not ruled out by this experiment, the light-modulated forms of these enzymes do not, apparently, contain essential, arsenite-sensitive, vicinal dithiol groups.

Of our three hypothetical models only the thiol-disulfide exchange model is consistent with all of these experiments.

Is the Disulfide DTNB a Potentially Useful Analogue of the Disulfide LEMs? The data in Table II indicate that DTNB is reduced by a component of the electron transport system located on the reducing side of PSI prior to ferredoxin. Inhibition by DCMU indicates that photosynthetic electron transport is involved (13). Inhibition by diquat, a compound which short circuits electron transport by transferring electrons from the PSI electron acceptor to O_2 (23) indicates involvement of PSI. Reduction of DTNB is enhanced about 10-fold when ascorbate (10 mM), DPIP (200 µM) replaces water as electron donor in presence of 2 µM DCMU (data not shown), which again indicates that PSI is involved in this light-dependent reduction (7). The electron transport component responsible for the reduction of DTNB must precede ferredoxin in the electron transport chain since: (a) ferredoxin is almost certainly washed out of the membrane fraction during preparation; (b) inclusion of 5 µM spinach ferredoxin in the reaction mixture is without effect (data not shown); and (c) the ferredoxin antagonist DSPD at levels of 1 mM has essentially no effect on DTNB reduction in this system (Table II). The light-dependent reduction of DTNB then involves an electron transport chain component located on the reducing side of PSI prior to ferredoxin. The LEM_l systems for the activation of ribulose-5-P kinase, NADP-linked glyceraldehyde-3-P dehydrogenase, and NADP-linked malic dehydrogenase and inactivation of glucose-6-P dehydrogenase, likewise, involve electron transport chain component(s) located on the reducing side of PSI prior to ferredoxin (3, 8).

The optimal pH for the photoreduction of DTNB in this system is 7.4 (Fig. 4), which is about one unit lower than the usual optimum for DTNB reaction with sulfhydryl groups (20). Above

pH 8 DTNB reacts rapidly with available thiol groups in this system (data not shown). The pH dependency curves for light inactivation of glucose-6-P dehydrogenase and for light activation of ribulose-5-P kinase are remarkably similar, being essentially superimposable on the DTNB photoreduction curve.

Treating the membrane particles with 1 mM arsenite (in light or dark) or with 100 µM sulfite does not affect light-dependent DTNB reduction, although 10-fold lower concentrations of either compound have been shown to inhibit transfer of electrons to the LEM system (3). Therefore, it seems that DTNB accepts electrons directly from the electron transport system or from some component of the LEM system lying before the arsenite- or sulfite-sensitive components.

The LEM could be a disulfide isomerase closely related to the enzyme which has been isolated from microsomal membranes by Anfinsen and co-workers (6) and which is thought to be responsible for catalyzing disulfide interchange in mammalian systems. However, we have not been able to renature ribonuclease or soybean trypsin inhibitor with the LEM system from the green leaf: both are renatured in the microsomal system. Nor have we been able to detect light or dark modulation of mammalian fructose-6-P kinase or glyceraldehyde-3-P dehydrogenase, yeast glucose-6-P dehydrogenase, or native soybean trypsin inhibitor with the LEM system. If the LEM does catalyze thiol-disulfide exchange, it exhibits a high degree of specificity for substrate.

Recently there have been reports of soluble protein factors or enzymes which are involved in DTT-dependent activation of three of the light-activatable chloroplastic enzymes (10, 14, 16, 19). Two

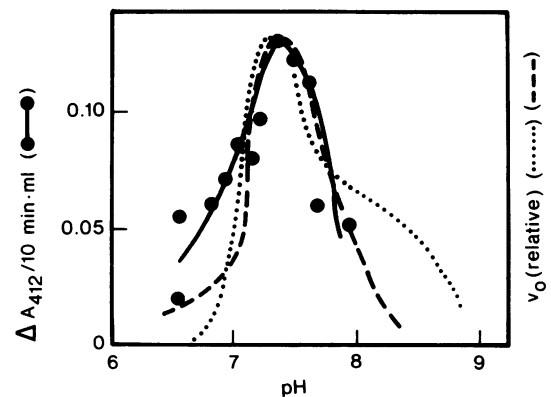| Inhibitor	Concentration µM	Inhibition %	Control rate ΔA_{412} $min^{-1}ml^{-1}$	Chl ug/ml
DCMU	2	80	0.01	22.8
		100	0.0125	22.8
Diquat	200	50	0.0076	29.5
		50	0.0088	17.7
DSPD	1000	0	0.007	29.5
		10	0.007	25.0

Fig. 4. Comparison of pH dependence of thylakoid LEM-catalyzed inactivation of glucose-6-P dehydrogenase (-----), thylakoid LEM-catalyzed activation of ribulose-5-P kinase (· · · · ·), and light-dependent DTNB reduction (———). Curve for LEM-catalyzed inactivation of the dehydrogenase is from reference 4; curve for LEM-catalyzed activation of the kinase is unpublished work of J. X. Duggan. Data points are for light-dependent DTNB reduction. In this experiment each cuvette contained 50 µmol of potassium HEPES buffer, 1 µmol of DTNB and chloroplast particulate fraction (18 µg of Chl) in a total volume of 1 ml. pH was determined after activity was measured. The experiment was repeated three times with consistent results. The pH optimum for light-dependent DTNB reduction is 7.4.
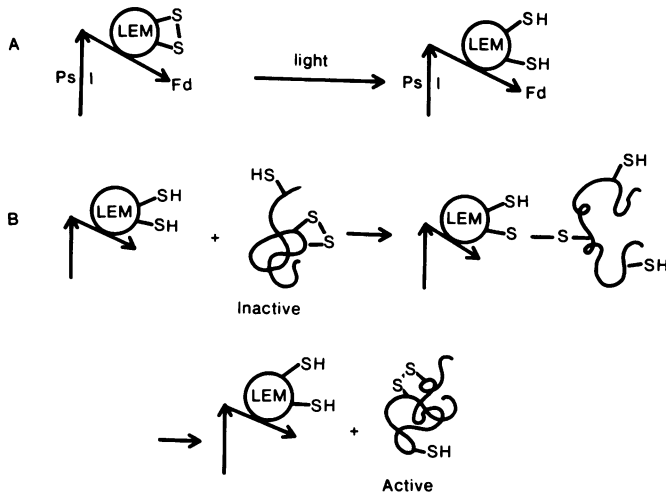
LIGHT MODULATION OF ENZYMES

FIG. 5. Simplest hypothetical scheme for light modulation of enzyme activity. A: reductive activation of LEM; B: activation of stromal enzyme by thiol-disulfide exchange. (For glucose-6-P dehydrogenase read "inactive," "active" and "active," "inactive.")

of these, ferrodoxin, thioredoxin reductase and thioredoxin, have been implicated in the ferredoxin-dependent light activation system which is currently being studied by Buchanan and co-workers (9, 22). If the LEM is involved in reducing the light-modulatable enzymes one might expect the LEM (or some part of the LEM system) to be identical with one or more of these soluble protein factors. We have not been able to obtain any evidence for the participation of soluble factors in light modulation and all of our experiments to date, albeit preliminary, indicate that the LEM system is very tightly bound to, or an integral part of, the thylakoid membrane. There is no loss of light modulatability when stroma containing modulatable enzyme is subjected to gel filtration on Sephadex G-25 or to chromatography on Shaltiel's (17) hydrocarbon-coated agaroses (L. Anderson, unpublished). Chloroplast thylakoid membranes retain light modulation activity after rather strenuous treatment with some chaotrophic reagents (K. Manabe, unpublished). Although positive experimental results would prove the occurrence of soluble factors, negative results do not disprove their existence. Nevertheless, we have as yet no evidence for participation of soluble protein factors in light modulation of enzyme activity in pea leaves.

Our current working hypothesis is that light reductively activates the LEM which then catalyzes thiol-disulfide exchange on the light-modulatable enzyme (Fig. 5). Resolution of the exact nature of the change mediated by the LEM system awaits purifi-

cation of stable active and inactive forms of the light-modulatable enzymes.

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