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Light-Induced Breakdown of NADPH-Protochlorophyllide Oxidoreductase *In Vitro*[1]

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ABSTRACT

Light-induced loss of the enzyme protochlorophyllide reductase (EC 1.6.99.1.), already described as a characteristic of whole plants, has now been demonstrated *in vitro* using etioplast membrane preparations of *Avena Sativa* L. var Peniarth and *Secale cereale* L. var Rheidol. Some evidence is presented, based upon temperature, pH, and inhibitor sensitivity of the process, that loss of enzyme may be the result of proteolysis. The light-induced process can, *in vitro*, be largely prevented by addition of the substrates of the reductase, protochlorophyllide and NADPH. It is concluded that light causes the breakdown of the reductase *in vivo* and *in vitro* by producing ligand-free enzyme as a consequence of the photoconversion reaction.

It has previously been demonstrated (17, 18) that illumination of etiolated plants leads to a dramatic decrease in the activity of the plastid-localized enzyme Pchlide reductase. It has also been observed that such *in vivo* illumination simultaneously caused oxidation of the plastid NADPH and a decrease in Pchlide level as a result of photoconversion. Redarkening of briefly illuminated plants produced an exact reversal of the situation with re-reduction of the plastid NADP and resynthesis of Pchlide accompanying recovery of Pchlide reductase activity (17–19). After identification of the enzyme protein (1, 20), it became apparent that the observed changes in enzyme activity results from a degradation and resynthesis of the actual enzyme protein under conditions of illumination and redarkening. However, the mechanism whereby light produced this change is unexplained. Again, the significance, if any, of the accompanying changes in Pchlide and NADPH, in regulation of the level of Pchlide reductase, also remained obscure.

In the present paper, the light-induced loss of Pchlide reductase has been mimicked by illumination of cell-free preparations *in vitro*. Further, and more significantly, the process is shown to be largely inhibited by addition of Pchlide and NADPH prior to illumination. From the data, a simple model to account for the light-induced loss of reductase activity is proposed. An account of this work has already been presented briefly at the Nato Advanced Study Institution on "Molecular models of photoresponsiveness", Pisa, Italy, September 1982.

MATERIALS AND METHODS

Plant Material. Oats (*Avena sativa* L. var Peniarth) were grown for 6 or 7 d in complete darkness as previously described (9). Rye (*Secale cereale* L. var Rheidol) was germinated and grown for 7 d at 22°C or 33°C in the dark, exactly as reported earlier (2).

Preparation of Etioplasts and Etioplast Membrane Fractions. Shoots of oats and rye were homogenized in an Atomix blender and etioplasts isolated by differential centrifugation of the homogenates using a modification of the previously reported method (10). Etioplast pellets were gently resuspended using a cotton wool-covered glass rod and then filtered through glass wool to produce a homogeneous suspension.

Oat and rye etioplast membranes were prepared by lysing whole, intact etioplasts in hypotonic buffer (20 mM sucrose, 20 mM Tes, 20 mM Hepes, 2 mM $MgCl_2$, 5 mM cysteine, pH 7.5) followed by centrifugation (21). In some experiments (see text), a further purification step was carried out by centrifugation on a 20% to 40% (w/w) discontinuous sucrose gradient as before (21). Samples were finally resuspended in a buffer containing 0.5 M sucrose, 20 mM Tes, 20 mM Hepes, 2 mM $MgCl_2$, 5 mM cysteine, pH 7.5. All steps were performed under a dim green safelight at 4°C.

Enzyme Assay. Pchlide reductase (EC 1.6.99.1) was assayed as described by Griffiths (10). A unit of enzyme activity was taken as that causing the conversion of 1 nmol Pchlide to Chlide/min under standard assay conditions (10).

***In Vitro* Incubation Conditions.** Samples of etioplasts and etioplast membranes (typically, 200 μl of material containing approximately 1 mg of protein) were illuminated on ice for 2 min by a 50-w focussed quartz halogen lamp at a distance of 5 cm, followed by 3 min red light (Kodak Wratten filter No. 25) of an intensity of 15 w m^{-2}. Incubation on ice ensured that no significant rise in temperature occurred during the illumination. The illumination conditions were chosen as those producing maximal photoconversion of Pchlide in etioplasts with the minimum of photooxidative damage. The plastids or plastid membranes were then incubated at room temperature in darkness and aliquots (approximately 15 μl) removed at various time intervals for either enzyme assay or analysis by SDS-PAGE[2]. In experiments where compounds were added to membrane preparations, additions were made 5 min prior to illumination at a final concentration of 2 mM, except for Pchlide and oxidized GSH which were added at 80 and 50 μM, respectively. In the proteolytic inhibitor studies, inhibitors were added to membrane samples 10 min prior to illumination, at concentrations of 0 to 100 μg ml^{-1}.

For the determination of the pH profile of reductase breakdown, samples of etioplast membranes were suspended to a concentration of 5 mg protein ml^{-1} in a buffer consisting of 10 mM phthalate, 10 mM Mes, 10 mM Hepes, and 10 mM Tricine adjusted to the various pH values. The samples at different pH values were then illuminated with 2 min white light, followed by 3 min red light, to effect complete photoconversion. After incubation at room temperature for a further 30 min in darkness, aliquots were taken for enzyme assay.

[1] Supported by grant GR/B/71992 from the Science and Engineering Research Council.

[2] Abbreviations: PAGE, polyacrylamide gel electrophoresis; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase.

Controls appropriate to each particular experiment were carried out under the conditions specified in the text.

Electrophoretic Analysis of Peptides. Aliquots of membranes or plastids were solubilized in a buffer containing 1% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8. Approximately 70 μg of protein was loaded on each track and electrophoresed on 12% or 15% polyacrylamide gels according to Laemmli (16). Mol wt marker proteins were always run and consisted of BSA (66 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), and lysozyme (12 kD). Gels were fixed and then stained with Coomassie Brilliant Blue R.

Protein Assay. Protein was assayed by the method of Bramhall *et al.* (5) using BSA as standard.

Spectral Measurements. Absorbance spectra of samples suspended in buffer were recorded using a split-beam spectrophotometer as previously described (10).

Chemicals. Pchlide was purified from acetone extracts of etiolated oats as described previously (10). NADPH was produced from a regenerating system containing G-6-P (4 mM), NADP (0.5 mM), and G-6-PDH (0.1 units). The protease inhibitors were obtained from Sigma Chemical Co. Ltd. (London, U.K.). All other chemicals were A.R. grade where possible from sources already described (10).

RESULTS

Etioplasts prepared from 7-d-old dark-grown oats contain a very active Pchlide reductase with, typically, a specific activity of about 3.2 units/mg protein. The enzyme in such preparations remains stable for prolonged periods if stored at −20°C in darkness. At room temperature (22°C) in darkness, the enzyme is again relatively stable with activity readily detectable, albeit at a reduced level, even after 3 h under such conditions (Fig. 1), and after only 50 min as much as 90% of the original enzymic activity

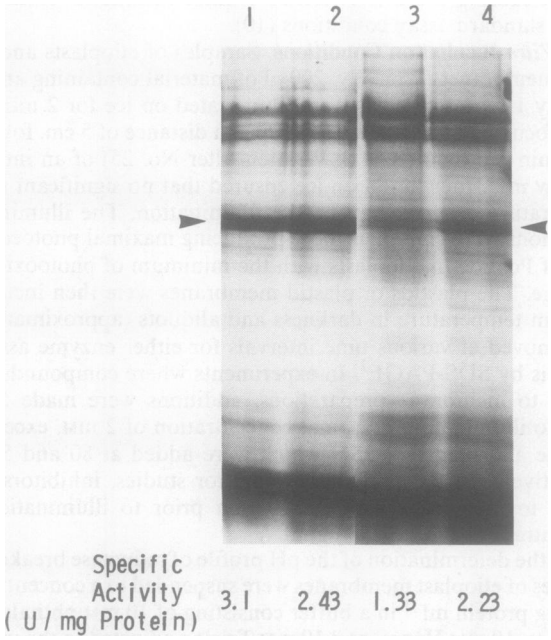

FIG. 1. Light-induced decrease in Pchlide reductase activity in isolated etioplasts. Isolated oat etioplasts resuspended in buffer at a protein concentration of 5.4 mg ml^{-1}, as described in the text, were illuminated for 2 min with white light, followed by red light for 3 min, and then incubated in darkness at 22°C. Aliquots were removed at intervals and reductase activity assayed (●). Simultaneously, a nonilluminated sample was also similarly incubated and the enzyme activity again assayed at intervals (○). The results are expressed as percentages of the activity present prior to incubation.

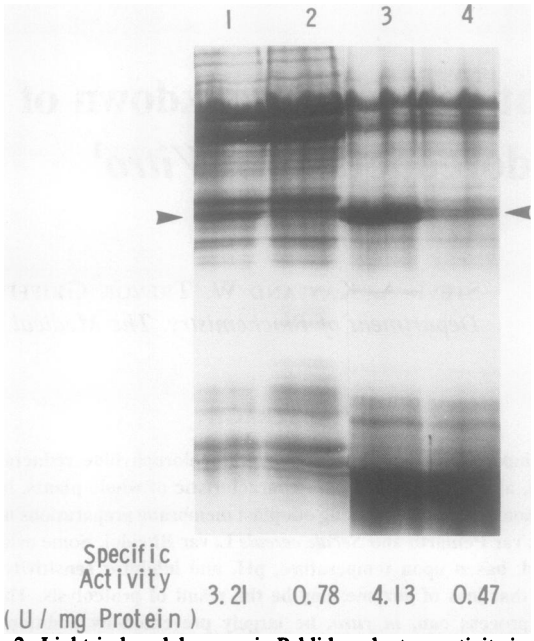

FIG. 2. Light-induced decrease in Pchlide reductase activity in etioplast membranes. Etioplast membranes prepared by osmotic lysis of oat etioplasts were incubated at a protein concentration of 7.0 mg ml^{-1} with (●) and without (○) prior incubation as described in Figure 1. Aliquots were removed at intervals and assayed for residual reductase activity. Activities are expressed as percentages of the activity of the original membranes.

is present. In marked contrast, however, if the etioplasts are illuminated prior to incubation at room temperature, then a dramatic and rapid decrease in the activity of the enzyme occurs with approximately 40% lost within 15 min of illumination (Fig. 1), and after 2 h, only about 25% of the activity remains. This observation is very reminiscent of the behavior of the enzyme in whole leaves under similar conditions (17–19).

A similar behavior was observed on illumination of isolated etioplast membranes. Before illumination, such membranes had a reductase specific activity of 4.13 units/mg protein. Over a 3-h incubation in darkness, this declined to about 65% of its original level. In contrast, however, illumination of the membranes prior to such an incubation in darkness led to a 50% drop in reductase activity within 20 min, and after 3 h, less than 20% of the original activity was still detectable (Fig. 2).

Figure 3 shows the peptide profiles of etioplasts (tracks 1 and 2) and membranes (tracks 3 and 4) sampled before (tracks 1 and 3) and 3 h after (tracks 2 and 4) illumination, respectively. The specific activity of Pchlide reductase in each sample is also included. In the etioplasts prior to illumination (track 1), the presence of the reductase is quite discernable (arrowed bands of mol wt 36 and 38 kD) despite the large number of other etioplast proteins in such preparations. After illumination followed by 3 h incubation in darkness, however, the intensity of the reductase bands have greatly diminished (Fig. 3, track 2). In the unilluminated membranes, the reductase bands are a very prominent feature of the peptide profile (track 3). In the illuminated 3-h incubated sample, however, a treatment which leads to loss of nearly 90% of the reductase activity, a dramatic decrease in amount of the enzyme protein is apparent (track 4). While minor changes in other peptides can also be discerned from the figure, these become insignificant when compared with the gross changes in the level of the reductase. It is interesting that no additional bands corresponding to possible breakdown products of the reductase are apparent among the peptides in the incubated samples (Fig. 3, tracks 3 and 4).

The effect of addition of Pchlide and NADPH, together and

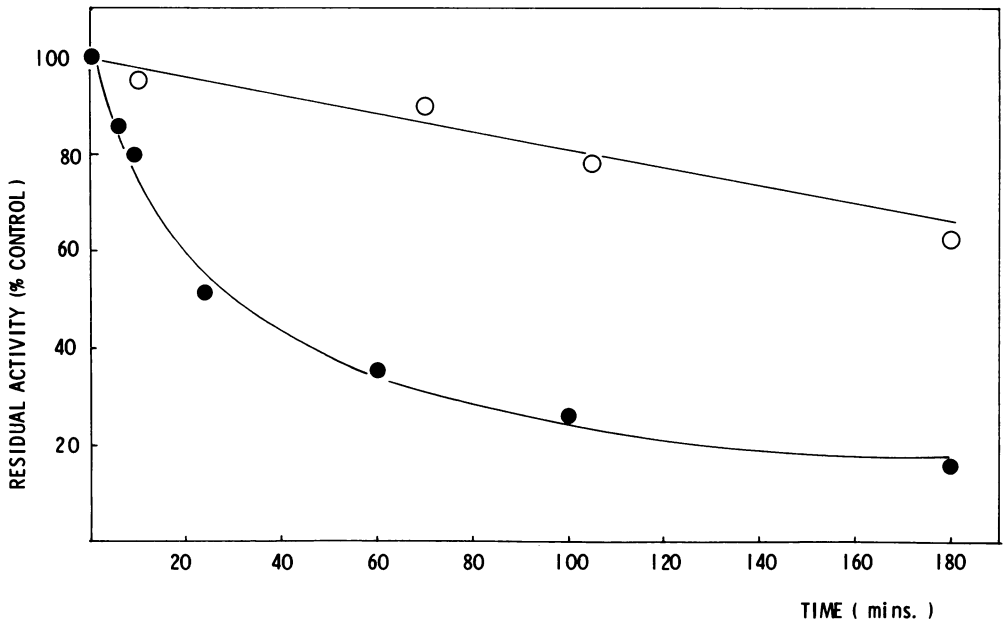

FIG. 3. Light-induced decrease in the Pchlide reductase protein *in vitro*. Isolated etioplasts and etioplast membranes were illuminated as described in Figures 1 and 2, with samples taken immediately and after a further 3-h incubation in darkness, and polypeptides were analyzed by SDS-PAGE. Tracks 1 and 3 correspond to the unincubated etioplasts and membrane samples, respectively, while tracks 2 and 4 are from the corresponding samples after 3 h incubation postillumination. The arrow indicates the 36 to 38 kD polypeptides of Pchlide reductase. The specific activity of the reductase corresponding to each sample is also included.

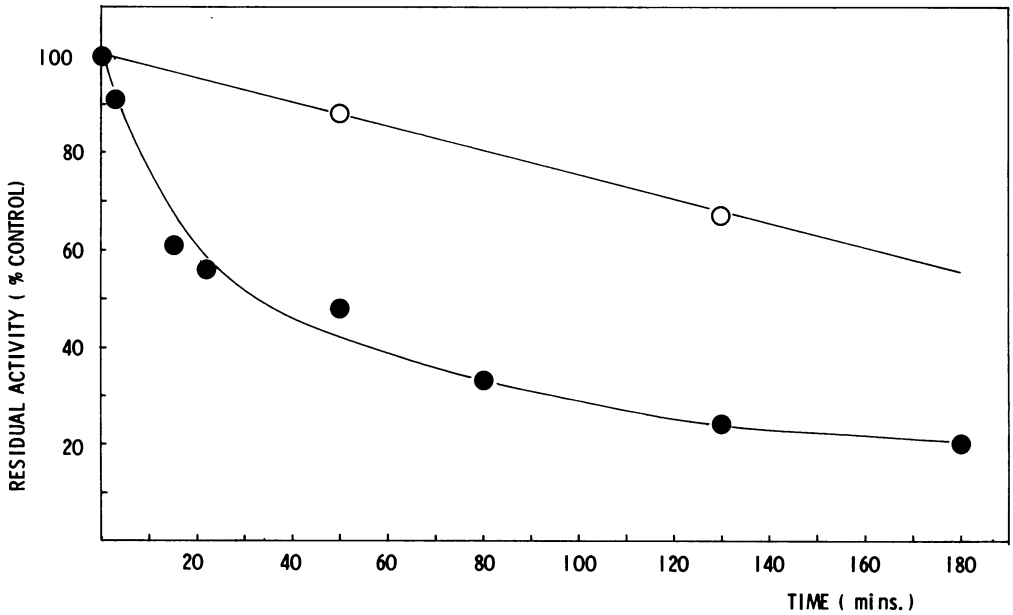

FIG. 4. Substrate protection of Pchlide reductase from light-induced breakdown *in vitro*. Isolated etioplast membranes were illuminated as in Figure 1 in the presence of Pchlide and NADPH (80 μM and 2 mM, respectively, track 2), 80 μM Pchlide alone (track 3), or 2 mM NADPH (track 4) and incubated in darkness at room temperature for 3 h, followed by SDS-PAGE. An aliquot of the membranes was also analyzed before illumination (track 1). The figure also includes data on the reductase specific activity of the different samples.

separately, on the light-induced breakdown of the reductase *in vitro* are shown in Figure 4. The reagents were added prior to illumination of isolated membranes and the figure records details of the peptide profiles of the various samples, together with the corresponding Pchlide reductase specific activity values. Isolated membranes supplemented with substrates (2 mM NADPH and 80 μM Pchlide), prior to any illumination or incubation, have a specific reductase activity of 3.1 units/mg protein. After illumination and subsequent incubation at 22°C for 3 h, the activity had declined a little to 2.43 units/mg protein, but without any significant change visible in the amount of stained peptides corresponding to the enzyme (tracks 1 and 2). Supplementation of the illuminated membranes with Pchlide alone produced a final sample with reductase specific activity 1.33 units/mg protein, whereas in the NADPH-incubated sample, the reductase specific activity was 2.23 units/mg protein. The greater retention of activity by the supplemented membranes is somewhat reflected in the elevated amount of the enzyme protein in these preparations (Fig. 4, tracks 3 and 4) compared with the amount present in the unsupplemented membranes noted earlier (*cf.* Fig. 3, track 4). The difference becomes particularly apparent when comparing the proteins of the NADH-supplemented (Fig. 4, track 4) and the

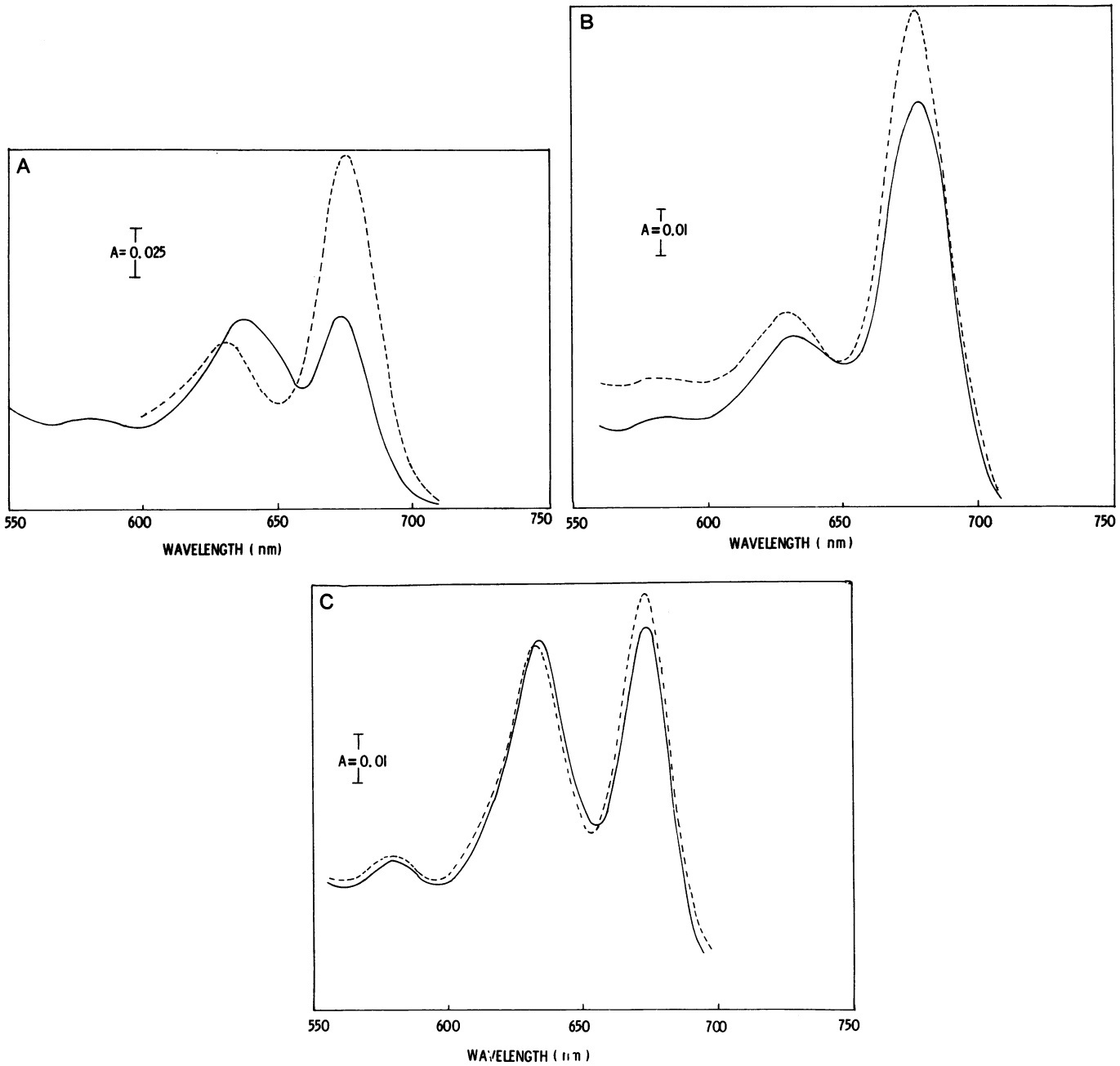

FIG. 5. Absorption spectra of illuminated oat membrane samples after incubation for 3 h in the presence of: A, NADPH + Pchlide; B, NADPH; C, Pchlide as in Figure 4. After reading the initial spectra (——), each sample was illuminated for 2 min in the spectrophotometer and the absorption spectra re-recorded (– – –) to estimate the amount of photoactive pigment present.

unsupplemented membranes (Fig. 3, track 4).

Absorption spectra of the various samples described in Figure 4, recorded at the end of the incubation period, before and after a 30-s saturating flash of light are shown in Figure 5. Illumination of every sample produced additional Chlide, indicated by the increased *A* at approximately 670 to 680 nm, following the saturating flash. This was a relatively large amount in the case of the NADPH-plus-Pchlide incubation (Fig. 5A), but with a slightly reduced amount in the plus-NADPH sample (Fig. 5B), and only a trace formed in the plus-Pchlide-incubated sample (Fig. 5C). It is worth mentioning that no Chlide was formed on illumination of the sample incubated without any additions (data not shown). Bearing in mind our earlier (10) characterization of photoactive Pchlide as the ternary complex of Pchlide reductase with its substrates, this spectral data offers a useful measure of the amount of enzyme in each preparation. This data supports the conclusions already drawn from the peptide and activity analyses (Fig. 4).

The effects of other reagents on the light-induced loss of enzyme were similarly examined by incubation of oat membranes in the presence of various additions, after preillumination *in vitro* as before. The results obtained are summarized in Figure 6. Inclusion of 2 mM NADP resulted unexpectedly in greater recovery of enzyme (activity and protein) compared with the unsupplemented control (*cf.* Fig. 6, tracks 1 and 5). NADH and NAD (both at 2 mM) and oxidized GSH at 50 μM were less effective in this respect. The presence of GSH in fact resulted in more extensive breakdown and loss of enzyme activity than the unsupplemented sample (*cf.* Fig. 6, tracks 4 and 5).

At this stage, proteolysis was considered as possibly providing the most likely explanation of the data. Experiments were there-

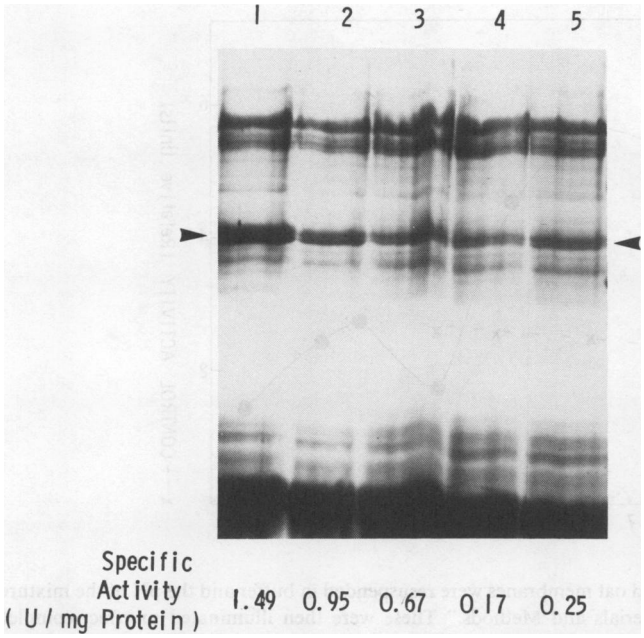

FIG. 6. Specificity of protection of Pchlide reductase from degradation by light. Oat etioplast membranes were supplemented with the various compounds as indicated followed by illumination as in Figure 1 and incubation in darkness for 100 min at room temperature. The samples were finally assayed for reductase activity and peptide profiles. Track 1, NADP; track 2, NADH; track 3, NAD; track 4, oxidized GSH; track 5, no additions.

fore carried out to obtain support for the presumed proteolytic breakdown of Pchlide reductase *in vitro*. The pH dependency of the process is illustrated in Figure 7, which shows reductase activity remaining, after incubating unsupplemented oat membranes for 30 min at various pH values. Control samples were incubated under identical conditions but received no illumination. Over the pH range studied, a gradual decrease in the activity of the control sample was seen on decreasing the pH, due to breakdown of the enzyme in a light-independent manner (Fig. 7). Values for the light-induced breakdown in the figure correspond to the activity of the incubated illuminated samples expressed as a percentage of the corresponding dark control values. It is, of course, obvious that a minimum in this plot corresponds to a maximum degradation of the enzyme. Two pH optima for enzyme degradation are apparent *viz.* a broad optimum of activity at approximately pH 6 and a sharper optimum of higher activity at pH 8 (Fig. 7). Repeating the experiment with a fresh batch of membranes produced an almost identical profile subscribing to the validity of the data. Again, analysis of the breakdown products at the various pH values by SDS-PAGE (unpublished work) indicated that, at the lower pH values, several of the proteins originally present were degraded, whereas at the high pH values breakdown was limited to the reductase.

The effect of temperature on the loss of Pchlide reductase activity was estimated by incubating illuminated membranes for 20 min at different temperatures followed by assaying the residual activity at the standard temperature of 22°C and comparing with control nonilluminated membranes similarly treated. The data (not presented) indicated only a slight loss of reductase below 15°C, the process increasing rapidly with rise in temperature above 20°C, as might be expected for enzymic breakdown of the enzyme. It was impossible to extend the range of this data beyond 33°C due to inactivity of the reductase above this temperature. Again, a similar response to temperature was observed for loss of the reductase in unilluminated control membranes, except that here the extent of the process was considerably reduced.

The properties of the reductase in enzyme-enriched, sucrose-gradient-purified membranes was next investigated by the usual procedure, with the results shown in Figure 8. The specific activity of the reductase in this particular preparation was somewhat lower than usual (3) at approximately 6 units/mg protein, but the enzyme was still by far the major protein in the sample (Fig. 8, track 2). On illumination of aliquots of the membranes in the presence or absence of NADPH and Pchlide, followed by incubation for 50 min at 22°C, the activity of the unsupplemented membrane fell to 30% of its original value, accompanied by considerable reduction in the amount of enzyme visible in the profile of the separated peptides (Fig. 8, track 3). In contrast, the substrate-supplemented preparation under identical conditions, suffered only a slight decrease in activity with no apparent change in the actual amount of enzyme protein (Fig. 8, track 4). Again, it is worth pointing out, as commented earlier, that no extra peptide bands become apparent in extracts from the sample in which enzyme loss had occurred.

The data already presented is compatible with proteolysis causing the loss of Pchlide reductase observed on illumination of etioplast membranes *in vitro*. It should therefore be possible to inhibit the process by one of the many protease inhibitors that have lately been described. Table I records the effect of some of these inhibitors on the reductase in illuminated membranes incubated for 60 min in darkness. The data are presented as the concentration of inhibitor required to abolish by 50% the reductase lost under identical conditions from unsupplemented illuminated membranes. From the table, both Antipain and leupeptin appear effective inhibitors of the breakdown with ID_{50} values of 45 and 83 μg/ml, respectively, whereas the remaining compounds tested failed to produce a significant effect at the concentrations tested.

It was considered of interest to study the phenomenon of Pchlide reductase breakdown in rye plants grown at 33°C and, as such, deficient in plastid protein synthesis (8). Table II shows the effect of illumination in the presence and absence of substrates, on the activity of the reductase in crude membranes isolated from 33°C-grown etiolated rye plants. The results in the table are compared with the corresponding data obtained for membranes prepared from rye grown at 22°C. All the activities are expressed as a percentage of the activities of identical controls assayed at zero time. The table shows that illumination of unsupplemented rye membranes, followed by incubation for 100 min at room temperature in darkness, results in a 73% loss of Pchlide reductase activity, whereas in the presence of substrates the original activity has in fact been increased (by 47%) under such conditions. An even more dramatic loss of activity (97%) is seen in the unsupplemented membranes from the 33°C-grown plants, whereas again in the presence of substrates the original activity is increased (by 70%) as a result of the 100-min incubation.

DISCUSSION

The biological role of proteases, both in the formation and breakdown of many proteins, is becoming increasingly more apparent. Proteolysis is important not only in the final processing of many protein precursors (15), but also as a coarse control mechanism for regulating the actual level of proteins (22). The light-requiring enzyme Pchlide reductase may be subjected to both proteolytic processing and regulation. The enzyme is synthesised in plants as a larger precursor on 80S ribosomes and proteolytically cleaved to its final size on entry into plastids (1). Again, light appears to regulate the activity of the enzyme by causing its almost complete disappearance on illumination of etiolated tissues (17, 19).

In the present paper, we have set out to answer the question: why does the enzyme Pchlide reductase appear to decline so dramatically on illumination of etiolated tissues? A solution to this problem may be provided by the data presented here based

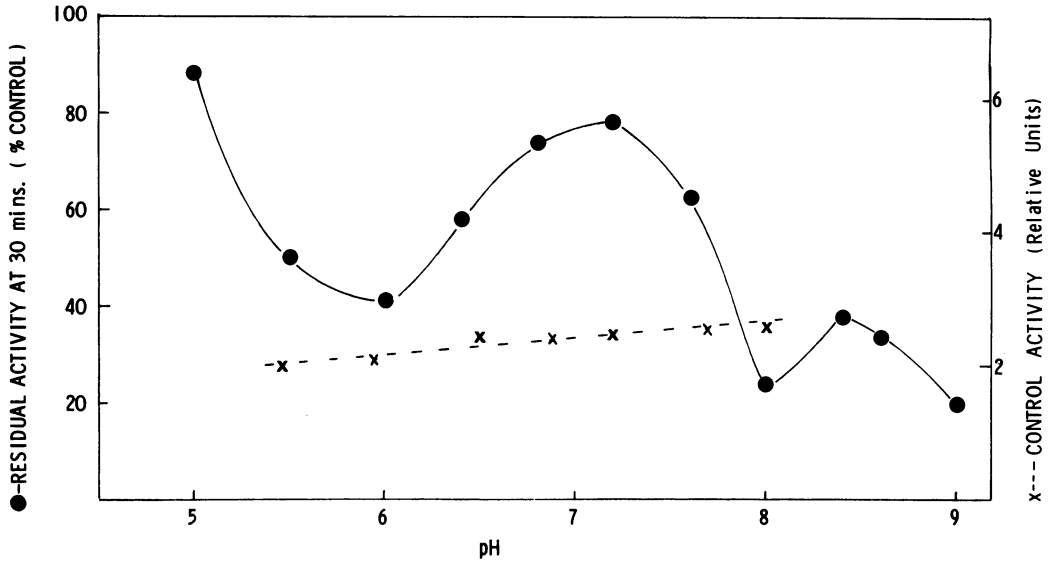

FIG. 7. pH dependency of Pchlide reductase degradation. Aliquots of isolated oat membranes were resuspended in buffer and the pH of the mixtures adjusted to the various values ranging from 5.0 to 9.0 as described in "Materials and Methods." These were then illuminated to effect complete photoconversion and incubated in darkness for 30 min. Aliquots were then immediately transferred into normal assay buffer and the residual reductase activity assayed. Control samples were similarly treated but without any illumination, and the experimental results (O) are expressed as the residual activity in the illuminated sample as a percentage of that in the corresponding dark sample. The effect of pH on the control activity is also presented (×).

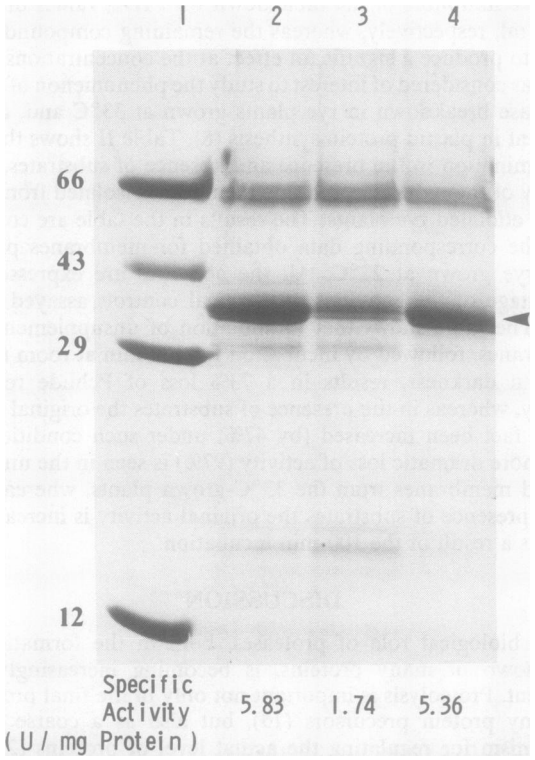

FIG. 8. *In vitro* breakdown of Pchlide reductase in purified oat etioplast membranes. Pchlide reductase-enriched membranes from oat etioplasts were isolated and illuminated in the absence (track 3) or presence (track 4) of substrates followed by incubation in darkness for 100 min. Aliquots were then assayed for reductase activity or peptide profile. An aliquot (track 2) was also analyzed before any illumination or incubation.

Table 1. *Effect of Protease Inhibitors on Pchlide Reductase Breakdown In Vitro*

Inhibitors were added to incubated membranes as described in the text. Aliquots of membranes were taken at 60 min following illumination and assayed for activity. The residual activity of samples containing inhibitors was compared with illuminated controls incubated with no additions. ID_{50} refers to the concentration of inhibitor ($\mu g \cdot ml^{-1}$) required to inhibit enzyme breakdown by 50%.

Inhibitor	ID_{50}
Antipain	45
Leupeptin	83
PMSF[a]	>100
TLCK[a]	>100
Pepstatin	>100

[a] PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone.

on our observation that the light-induced loss of the reductase can be reproduced *in vitro*. It has been shown that isolated etioplasts (Fig. 1), or etioplast membranes (Fig. 2), in the absence of substrates, respond to illumination in much the same way as whole plants with respect to Pchlide reductase disappearance. Further, purified etioplast membranes enriched in the enzyme also display a loss of reductase in the light (Fig. 8). Despite the fact that no breakdown products of the enzyme have yet been identified (*cf.* especially Fig. 8), the most likely explanation for the loss of the reductase induced by illumination must surely be the result of proteolysis. The enzymic nature of this loss is supported by the temperature sensitivity and pH dependence (Fig. 7) of the process. The two pH optima for degradation, at pH 6 and pH 8, respectively, deserve comment. The loss of reductase at pH 6 probably reflects the activity of nonspecific proteases since, at this pH, breakdown of the enzyme has been shown by SDS-PAGE analysis to be accompanied by breakdown of a large number of etioplast proteins. In contrast, loss of reductase at pH 8 is probably due to the action of a specific Pchlide reductase protease since, at this pH, specific loss of the enzyme protein alone has been demonstrated by electrophoretic analysis (S. A. Kay and W. T. Griffiths, unpublished work). Additional evidence supporting proteolytic degradation of the reductase in the light is provided by the protease inhibitor studies (Table I). Antipain, and to a lesser extent leupeptin (22), both specific protease inhibitors, largely

Table II. *In Vitro Breakdown of Pchlide Reductase in Membranes from Rye Grown at 22°C and 33°C*

Etioplast membranes were prepared from 22°C and 33°C cultivated rye plants as described in the text. These were assayed to provide a measure of control activity. Aliquots were then illuminated in the presence and absence of substrates followed by incubation at room temperature in darkness for 100 min as described previously (Fig. 1). The samples were then reassayed for Pchlide reductase activity. The table gives the residual activity of the illuminated samples expressed as a percentage of the original control activity.

Additions	Residual Activity at 100 Min Postillumination	
	22°Rye	33°C Rye
	% control	
None	27	3
+ Substrates	147	170

abolish light-induced loss of the enzyme at very low concentrations.

If our assumption regarding the proteolytic nature of the reductase disappearance is correct, then the data presented in Figure 8 implies a close physical association of the inferred protease with the reductase molecule on etioplast membranes. Further, in view of the demonstration (Table II) that light-induced loss of the reductase in rye is independent of the presence of 80S ribosomes, the putative protease or whatever else may be involved in the process may, like Pchlide reductase, be a product of cytoplasmic protein synthesis.

The inferred occurrence of proteolysis in etioplasts is not novel as there is already evidence available for turnover of some membrane-bound plastid proteins, such as the light harvesting Chl *a/b* protein (2, 4) and the 32 kD peak D protein (6, 24). Further, direct evidence for the existence of etioplast proteases, active in degrading prolamellar body membrane proteins, has recently been provided (14). Our consistent failure to detect any proteolytic breakdown products might imply that degradation of the reductase is more or less complete, producing very small peptides migrating with the ionic front during electrophoresis. Alternatively, the process might result in the formation of a large number of different fragments, none of which predominate and become discernible as specific bands during electrophoresis.

Pchlide reductase displays the remarkable property of being protected from light-induced degradation *in vitro* by the presence of its substrates, Pchlide and NADPH. Thus, in all the systems tested here, supplementation with these two substrates led to almost complete protection of the enzyme from degradation and loss of activity in the light (Figs. 3, 4, and 8). Although the data of Figure 4 (tracks 3 and 4) might imply that Pchlide or NADPH alone can afford protection, it is clear from the spectra recorded at the end of the incubations (Fig. 5) that some endogenous free Pchlide and NADPH, that had escaped the original photoconversion, must have been present in the membranes. During the incubation period, the free Pchlide and NADPH reacts with the reductase in the presence of the exogenously added second substrate, forming some photoactive ternary complex. This was detected as the additional Chlide formed on illumination of the supplemented samples at the end of the experiment (Fig. 5, b and c). It must be emphasized, however, that the concentrations of free substrates present in the original membranes must have been too low to produce any significant amount of the photoactive ternary complex without any additions, since no Chlide was formed at the end of the incubation in the unsupplemented samples.

It is feasible that the substrates afforded protection of the reductase by binding at the enzymes' active site. The fact that NADP as well as NADPH and Pchlide resulted in protection would support this conclusion since these three compounds have already been shown to prevent reaction of an -SH group at the enzymes' active site with the hydrophobic thiol reagent *N*-phenyl maleimide (20; B. El Hamouri, R.P. Oliver, and W.T. Griffiths, in preparation). There is also available spectroscopic evidence supporting the binding of both NADPH and NADP to the reductase in the presence of Pchlide (7). It is of interest also that neither oxidized nor reduced NAD provided any protection, which, if the inference above is correct, implies the requirement of a 2′-phosphate group for co-enzyme binding, supporting our earlier work (11). However, another possible interpretation of the substrate protection data, which cannot be eliminated at the present, is that these compounds actually inhibit the reductase degrading system.

These *in vitro* findings offer a rational explanation of the earlier reported *in vivo* breakdown of Pchlide reductase accompanied by oxidation of plastid NADPH and loss of Pchlide (18, 19). *In vitro* enzyme loss can be prevented by supplementation with substrates which bind to the enzyme. It is also possible, by feeding 5-aminolevulinic acid to whole leaves, to supplement the *in vivo* system, at least with Pchlide. Under such conditions, loss of the reductase *in vivo* in the light is again largely inhibited (R.E. Mapleston, N.S. Beer, and W.T. Griffiths, in preparation). Normally, however, following photoconversion *in vivo*, free enzyme is eventually made available which, in the absence of Pchlide and/or NADPH resynthesis, may be degraded. Degradation of the enzyme *in vivo*, therefore, depends upon the extent of illumination relative to amounts of Pchlide and NADPH in the plastids.

This paper thus offers an explanation of why the reductase responds as it does to illumination. However, we remain unsure of the mechanism of enzyme loss. It may be that illumination effects the enzyme by causing its inactivation by aggregation and, in so doing, rendering it insoluble or unresolvable by SDS-PAGE. Alternatively, as supported in this paper, loss of reductase may be due to degradation by a protease, the existence of which, can at this stage be only inferred. Despite this, it is apparent from the data presented here that loss of reductase activity on illumination *in vitro* is a biphasic process (see Figs. 1 and 2). The initial phase probably reflects product release and relaxation of the enzyme molecule into an inactive state, this conformational change being reversed by substrates. This phase is characterized by loss of enzyme activity but without any decrease in enzyme concentration, as measured by SDS-PAGE. The existence of this initial phase probably accounts for the previously reported lack of correlation between reductase activity and protein level, observed during the early stages of illumination of leaves when inactivation precedes breakdown (23). The same explanation can be offered to explain some of the data reported here for rye membranes. Table II shows that illuminated, substrate-supplemented rye membranes after incubation at room temperature for 100 min have higher reductase activity than the original membranes, despite the fact that the enzyme peptide level in both is similar (unpublished observations). It may be that during preparation of the original membranes dissociation of substrates from some of the enzyme could have resulted in these undergoing the reversible conformational change to the inactive form as above. In the illuminated samples, this inactivation would have been reversed over the incubation period by the presence of excess added substrate, resulting in the observed increase in activity. This reversible initial phase of reductase breakdown also probably accounts for an earlier observation from this laboratory (13), in which we reported reactivation of the enzyme in plastids from briefly illuminated plants by incubation *in vitro* with Pchlide and NADPH.

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