Covalent labelling of the NADPH: protochlorophyllide oxidoreductase from etioplast membranes with [³H]*N*-phenylmaleimide

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[³H]*N*-Phenylmaleimide has been used to covalently label in a specific manner the substrate-protected thiol groups of the enzyme protochlorophyllide reductase. In membrane preparations from oat (*Avena sativa*) and runner-bean (*Phaseolus vulgaris*) seedlings, two related peptides of mol.wts. 35 000/37 000 and 34 000/35 000 respectively and showing properties expected of the reductase have been identified, whereas the same technique with barley (*Hordeum vulgare*) extracts resulted in labelling a single peptide of mol.wt. 38 000. Evidence is presented that both NADPH and protochlorophyllide are required for protection of the essential thiol groups on the reductase in oat extracts, a situation favouring a ternary complex as the structure for the photoactive enzyme–substrates intermediate.

Since detection of the activity of the enzyme NADPH:protochlorophyllide oxidoreductase (Griffiths, 1978) the mystery surrounding the function of the protochlorophyllide holochrome in bringing about the photoconversion of protochlorophyllide to chlorophyllide has been largely rationalized. This enzyme, universally distributed in etiolated higher plants, has been shown to catalyse the formation of a ternary complex with its substrates, NADPH and protochlorophyllide, and, on absorption of light, hydrogen transfer occurs on a time scale of less than 10 μ s, giving the products NADP⁺ and chlorophyllide, which dissociate from the enzyme.

Despite the recognition of the activity of protochlorophyllide reductase, identification of the enzyme as a molecular species has yet to be achieved. Studies with this aim in mind, but directed towards the apoprotein of protochlorophyllide holochrome, have been largely hindered by the fact that the molecule is an insoluble component of the etioplast membrane. Such attempts have normally involved purification and identification of protein bound to protochlorophyllide. However, owing to solubility problems, detergents have had to be employed both to facilitate solution of the protein and also to ensure complete disaggregation before any attempts at molecular-weight determination. Unfortunately, detergents invariably lead to dissociation of the pigment from the protein, rendering the latter no

longer identifiable. Detergents can also, under certain conditions, produce artificial pigment–protein complexes giving further complications in identification attempts (Thomas *et al.*, 1976).

These difficulties are well illustrated by the large numbers of protochlorophyllide protein complexes that have been described in the literature, all of which are apparently different (Schopfer & Siegelman, 1968; Guignery *et al.*, 1974; Canaani & Sauer, 1977; Redlinger & Apel, 1980). In the present paper, the enzyme protochlorophyllide reductase is identified by a technique that is completely free of these problems. By using this procedure we have identified thiol-containing polypeptides that can be protected by NADPH and protochlorophyllide from *N*-phenylmaleimide, but that become exposed to this reagent specifically on illumination. The technique identifies protochlorophyllide reductase of oat (*Avena sativa*) membranes as a pair of closely related peptides of mol.wts. 35 000 and 37 000. These same peptides have already been found to co-purify with the enzymic activity of protochlorophyllide reductase (Beer & Griffiths, 1981). In the present paper, also, the same substrate-protection technique is used to gain information on the nature of substrate binding to the enzyme. A brief report of some of these data has already been published (Oliver & Griffiths, 1980).

Experimental

Biological materials

Etiolated barley (*Hordeum vulgare* L., cultivar

Abbreviations used: SDS, sodium dodecyl sulphate; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Proctor) and oats (*Avena sativa* L., cultivar Pennal) were cultivated for 6 days, and runner bean (*Phaseolus vulgaris* L., cultivar Streamline) were cultivated for 13 days, in the dark as described previously (Griffiths, 1975). Etioplasts were isolated from the plant tissue by differential centrifugation of homogenates as previously described (Griffiths, 1975).

Etioplast membranes were obtained by resuspending pelleted etioplasts in buffer II (20 mM-Tes/20 mM-Hepes/20 mM-sucrose/5 mM-cysteine/2 mM-MgCl₂, pH 7.5) at 0°C and centrifuging at 9000 *g* for 30 min. In some experiments a membrane fraction purified by centrifugation on sucrose gradients was used. Oat etioplast membranes, resuspended in buffer II at a protein concentration of 6–10 mg/ml, were sonicated three times for 5 s in an MSE probe sonicator by using low power and setting no. 5. During this treatment the sample was kept cold by immersion of the sonication tube in a freezing mixture of ice and NH₄Cl. A portion (2–3 ml) of the resulting suspension was layered on to a discontinuous step gradient consisting of 13 ml of 40% (w/v) sucrose and 20 ml of 20% (w/v) sucrose, made up in buffer II. After centrifugation at 75 000 *g* for 2 h, the material at the interface of the layers was removed, diluted with 4 vol. of buffer II and re-centrifuged at 38 000 *g* for 30 min. This final pellet was resuspended in isolation buffer (Griffiths, 1975) at a concentration of 2–3 mg of protein/ml and stored at –20°C until required.

Preparation of substrate-protected N-phenylmaleimide-reacted etioplast membranes

Oat etioplast membranes or samples purified on sucrose density gradients were incubated for 10 min at room temperature in darkness with 4.7 μ M-protoclorophyllide and 1 mM-NADPH. Then 8.5 mM-*N*-phenylmaleimide was added and the mixture incubated for a further 10 min in darkness in order to cause all the membrane thiol groups not associated with protoclorophyllide reductase activity to react. Finally, the membranes were passed through a loosely packed column (0.5 cm $\times$ 5 cm) of medium-grade Sephadex G-50 equilibrated in buffer II minus cysteine, all the operations being performed under dim green illumination. For barley membranes, incubation with protoclorophyllide and NADPH was performed for 15 min and with *N*-phenylmaleimide for 5 min. Also, both incubations and chromatography were performed in the cold-room at 4°C. These extra precautions were required in order to retain photoconvertibility in the more labile barley membranes. These substrate-protected *N*-phenylmaleimide-reacted membrane samples were used in the various experiments described in the Results section.

Chemicals

[³H]*N*-Phenylmaleimide was synthesized by a scaled-down version of the procedure described by Cava *et al.* (1961). Briefly, 9 μ l of aniline [5 mCi of [*ring*-G-³H]aniline, 150 mCi $\cdot$ mmol^{–1} (The Radiochemical Centre, Amersham, Bucks., U.K.)] was dissolved in 100 μ l of freshly distilled dry diethyl ether at 0°C in an Eppendorf polypropylene microcentrifuge tube. To this was added 80 μ l of a solution of maleic anhydride in diethyl ether (120 mg $\cdot$ ml^{–1}). The resulting yellow slurry was mixed for 30 min at 20°C in a Hillsonic sonicating bath and the precipitate was collected by centrifugation and washed once with 100 μ l of diethyl ether. After the addition of 4 mg of dry sodium acetate and 40 μ l of acetic anhydride, the mixture was heated in a boiling-water bath for 30 min. The brown liquid so formed was cooled on ice, diluted with 70 μ l of ice-cold distilled water and stirred in the sonicator bath at 4°C for 30 min. The resultant precipitate was collected by centrifugation as described above, and washed twice with 30 μ l of ice-cold water and once with 30 μ l of light petroleum (b.p. 40–60°C). The preparation was purified by dissolving the precipitate in 80 μ l of aq. 95% (v/v) ethanol at 60°C, cooling to 0°C, centrifuging and removing the liquor. The yellow precipitate was dried in a vacuum desiccator, dissolved in 300 μ l of acetone and then portions equivalent to 135 μ Ci were dispensed into plastic tubes, which were dried out and kept at –20°C until required.

Protochlorophyllide was isolated from etiolated barley and oat tissue and dispensed in cholate solution as described previously (Griffiths, 1978). Other chemicals were from the same sources as described previously (Griffiths, 1978).

SDS/polyacrylamide-gel electrophoresis

Membranes were analysed with polyacrylamide gels cast and run as described by Laemmli (1970). The separating gel consisted of 12% (w/v) acrylamide, 0.3% bisacrylamide, 0.1% SDS and the stacking gel consisted of 6% acrylamide, 0.16% bisacrylamide and 0.1% SDS. Gels were electrophoresed at a constant voltage of 60 V overnight, stained with 0.25% (v/v) Coomassie Brilliant Blue R, destained in methanol/acetic acid/water (5:7:88, by vol.) and photographed to produce a permanent record. Standard proteins of known molecular weights (bovine serum albumin, 65 000; ovalbumin, 43 000; glyceraldehyde 3-phosphate dehydrogenase, 35 000 and carbonic anhydrase, 29 000) were simultaneously run to enable relative molecular weights to be estimated.

Fluorography and densitometric scanning

Stained gels were fluorographed as described by Laskey & Mills (1975). Conditions were arranged

such that the extent of fogging of the photographic plate was proportional to radioactivity. Densitometric scans of the fluorographs were obtained with a computer-linked Unicam SP.500 spectrophotometer.

Protochlorophyllide reductase assay

Protochlorophyllide reductase was assayed spectrophotometrically as described previously (Griffiths, 1978).

Protein assay

Protein was assayed as described by Bramhall *et al.* (1969), with bovine serum albumin as standard.

Results

It has previously been shown that when substrates become bound to form the protochlorophyllide reductase-substrates complex, they mask some essential thiol groups on the enzyme, preventing them from reacting with thiol reagents. On illumination, however, products are formed that dissociate from the enzyme, thereby exposing the previously substrate-protected groups to attack by thiol reagents. This basis of the conclusion, arrived at by using the hydrophobic thiol-group-specific reagent *N*-phenylmaleimide, is demonstrated in Fig. 1. Protochlorophyllide reductase-enriched membrane fractions prepared by sonication of oat etioplasts followed by purification on a stepwise sucrose density gradient (Beer & Griffiths, 1981) still

retain their reductase as the photoactive enzyme-substrates complex. This becomes photoconverted to give chlorophyllide on illumination, as can be seen from the flash-induced increase in absorbance at 672 minus 710 nm [Fig. 1(i)]. This photoconversion is complete after seven or eight flashes, as indicated by the absence of any further flash-induced absorbance increase. Addition of NADPH (as a regenerating system) and protochlorophyllide at this stage results in restoration of the flash-induced chlorophyllide formation, indicating the activity of protochlorophyllide reductase. On repeating this procedure with the exception that after photoconversion of the endogenous photoactive complex, 350 μ M-*N*-phenylmaleimide is added [Fig. 1(ii)], followed 1 min later by protochlorophyllide and NADPH, no chlorophyllide synthesis occurs after resumption of flash-illumination, indicating complete inhibition of the reductase by *N*-phenylmaleimide. Another repeat of the original procedure, with a fresh membrane sample that had been preincubated for 1 min, prior to any illumination, with the same concentration of *N*-phenylmaleimide (350 μ M) shows that initial photoconversion of the endogenous photoactive complex is unaffected by this reagent, whereas, as before, re-formation of the complex from added substrates is completely inhibited [Fig. 1(iii)].

By using the experimental conditions indicated in Fig 1(ii), but with various amounts of phenylmaleimide added after the initial photoconversion, the inhibitor-concentration-dependence of the reac-

Fig. 1. Inhibition of protochlorophyllide reductase by *N*-phenylmaleimide

Purified oat etioplast membranes (56 μ g of protein) were incubated in a buffer containing 0.3 mM-Triton X-100 in a cuvette in a dual-wavelength spectrophotometer and absorbance changes at 672 minus 710 nm monitored while flash-illuminating the sample as described in the text. Additions to, and treatment of, the various samples were then performed as indicated in the Figure. (i), (ii) and (iii) are referred to in the text.

Fig. 2. Concentration-dependence of the inhibition of protochlorophyllide reductase by *N*-phenylmaleimide. Purified oat etioplast membranes were flash-illuminated to photoconvert the endogenous photoactive pigment followed by incubation for 60 s with various concentrations of *N*-phenylmaleimide. After this, substrates were added and the activity of protochlorophyllide reductase assayed by the standard procedure.

tion rate with exogenous substrates was determined. From the data obtained, Fig. 2 reveals that half-maximum inhibition of protochlorophyllide reductase activity occurs on addition of $2 \mu\text{M}$ *N*-phenylmaleimide under these experimental conditions. Investigation of the more hydrophilic thiol-group-specific reagent *N*-ethylmaleimide, under similar conditions, gave a half-maximum inhibition of the enzyme by this reagent at the slightly higher concentration of $10 \mu\text{M}$ (R. P. Oliver & W. T. Griffiths, unpublished work).

The amount of enzyme occurring in the form of the photoactive enzyme-substrates complex in the sucrose-gradient-purified membranes was maximized by incubation of the preparation with protochlorophyllide and NADPH, followed by treatment of the mixture with unlabelled *N*-phenylmaleimide, after which excess of the substrates and inhibitor were removed by gel filtration. This substrate-protected thiol-reacted preparation was then exposed to $[^3\text{H}]\text{N}$ -phenylmaleimide and flash-illuminated in the dual-wavelength spectrophotometer (conditions that should lead to the specific labelling with ^3H of the protochlorophyllide reductase substrate-protected thiol groups). Fluorographic analysis after SDS/polyacrylamide-gel electrophoresis of samples taken during the flash-illumination gave the peptide labelling pattern seen in Fig. 3.

Fig. 3. Illumination-dependent labelling of peptides of purified etioplast membranes by $[^3\text{H}]\text{N}$ -phenylmaleimide. NADPH- and protochlorophyllide-protected *N*-phenylmaleimide-reacted purified oat etioplast membranes were flash-illuminated at one flash/80 s, chlorophyllide formation being monitored by our usual technique. The process was performed in the presence of $[^3\text{H}]\text{N}$ -phenylmaleimide. After each flash, portions were removed and after a further 10 min were quenched with 0.23 M-mercaptoethanol. Equal portions of the differently photoconverted samples were then electrophoresed and finally stained, photographed and fluorographed as described in the text. The Figure shows a photograph of the original Coomassie Blue-stained gel and the final fluorograph. Tracks 1–13 correspond to the fluorographed samples that have been exposed to one to thirteen flashes respectively.

Included and superimposed on the fluorograph is a photograph of a track from the stained gel before fluorography. This shows the usual characteristic two groups of peptides of mol.wts. approx. 36 000 and 60 000 that are normally found in these purified membrane samples (Beer & Griffiths, 1981). The fluorograph shows that successive illumination results in an increasing incorporation of ^3H specifically into the peptides of mol.wts. 35 000 and 37 000, an observation confirming previous data from similar experiments performed on whole etioplast membranes (Oliver & Griffiths, 1980).

The fluorograph from the total-etioplast-membrane experiment published previously (Oliver & Griffiths, 1980) was now densitometrically scanned, providing graphic presentation of the ^3H incorporation by the total membranes as a function of illumination time. The resulting scans for the samples taken after illumination periods resulting in photo-

Fig. 4. Densitometric scans of a fluorograph from electrophoresed oat etioplast membrane peptides labelled with [^3H]N-phenylmaleimide for different periods of illumination

Substrate-protected thiol-reacted oat etioplast membranes incubated with [^3H]N-phenylmaleimide were flash-illuminated and the progress of photoconversion was monitored spectrophotometrically as described in Fig. 3. Samples were removed at various intervals, quenched with mercaptoethanol and electrophoresed, and finally fluorographed as described in Fig. 3. The Figure shows the densitometric scans of the fluorographed samples that had been illuminated so as to effect 21% (—) and 52% (---) photoconversion of the endogenous photoactive pigment respectively.

conversion of the endogenous photoactive complex to the extent of 21 and 52% respectively are shown in Fig. 4. This demonstrates the more-or-less specific incorporation of ^3H into an asymmetric unresolved peak of mol.wt. approx. 36 000 corresponding to the 35 000/37 000-mol.wt. labelled doublet clearly seen in the original fluorograph (Oliver & Griffiths, 1980). Furthermore, it is obvious from Fig. 4 that the extent of ^3H incorporation into the doublet increases with length of illumination or extent of photoconversion. This is further illustrated by the data in Fig. 5, where the extent of ^3H incorporation into the 35 000- and 37 000-mol.wt. peptides, calculated from densitometric scans similar to those in Fig. 4, is plotted as a function of the inhibition of the reductase activity consequential of illumination of the sample in the presence of the [^3H]N-phenylmaleimide. Fig. 5 shows the linear relationship between ^3H incorporation and illumination-induced inhibition of protochlorophyllide reductase. Bearing in mind the already documented correlation under these conditions between inhibition of the reductase and extent of photoconversion, (Oliver & Griffiths, 1980), then it is obvious that incorporation of ^3H into these two peptides is directly related, not only to the inhibition of the reductase, but also to the extent of photoconversion of the endogenous photoactive complex. Since increased photoconversion in the presence of [^3H]N-phenylmaleimide is accompanied by the parallel inhibition of the reductase activity and the specific incorporation of radioactivity into the 35 000- and 37 000-mol.wt. peptides, then these two peptides must identify with the reductase.

Fig. 5. Correlation between the inhibition of protochlorophyllide reductase of oat etioplast membranes by [^3H]N-phenylmaleimide and the incorporation of ^3H into peptides of mol.wts. 35 000 and 37 000

Substrate-protected thiol-reacted oat etioplast membranes were flash-illuminated in the presence of [^3H]N-phenylmaleimide as shown in Fig. 3. Samples removed after each flash were quenched with mercaptoethanol and portions were assayed for protochlorophyllide reductase activity and also electrophoresed and fluorographed as described in the text. The fluorographs were densitometrically scanned as in Fig. 4, and the labelling of the 35 000- and 37 000-mol.wt. peptides calculated as peak areas. These are plotted against the corresponding percentage inhibitions of the reductase activity.

Fig. 6. *Light-dependent labelling of barley etioplast membranes*

The labelling of barley etioplast membranes with [^3H]N-phenylmaleimide and subsequent electrophoresis and fluorography was performed as described for oat membranes in Fig. 3. Track 1 is the fluorograph of the membrane sample exposed to the radioactive inhibitor for an extended period of time (30 min) under flash illumination. Tracks 2–8 are fluorographs of the samples removed after one to seven flashes respectively in the presence of labelled inhibitor, and track 10 is the fluorograph of a portion of the labelled oat membrane sample from Fig. 3 simultaneously re-analysed here for comparison. Track 9 is a photograph of a track from the original stained gel before fluorography, superimposed on the fluorograph and shows the peptides associated with unfractionated barley membranes.

It would be expected, of course, that if this conclusion were correct, then these peptides would be found to display the same properties in etioplasts from all protochlorophyllide reductase-containing plants. To check whether this is so, the same experimental approach for labelling protochlorophyllide reductase was repeated with total membrane preparations from barley and bean etioplasts. The fluorograph resulting from the barley experiment, together with a photograph of the original stained gel taken before fluorographic treatment to illustrate the peptide pattern of the membrane, is shown in Fig. 6. This shows that the barley etioplast membranes have a very similar peptide composition to oat membranes, with very pronounced clusters of peptides in regions corresponding to mol.wts. of approx. 10000, 38000 and 60000. However, of these peptides, the only one to become specifically labelled [^3H]phenylmaleimide under the present conditions is a single band corresponding to mol.wt. 38000, in contrast with the labelled doublet of oats (Fig. 3).

Fig. 7. *Light-dependent labelling of bean etioplast membranes*

Substrate-protected thiol-reacted bean etioplast membranes, prepared as described for oat membranes in Fig. 3, were flash-illuminated (one flash/80s) in the presence of [^3H]N-phenylmaleimide for 14 min and then quenched with mercaptoethanol. A control sample of membranes was incubated with the radioactive inhibitor in darkness for 14 min and then similarly quenched. Finally both samples were electrophoresed, stained, fluorographed and densitometrically scanned as already described. The Figure shows the Coomassie Blue-stained-peptide pattern (a) together with the densitometer tracings (b) of the fluorographs from the dark (---) and illuminated (—) samples respectively.

A somewhat similar picture is obtained when the procedure is repeated with bean membranes (Fig. 7). The stained peptides of bean etioplast membranes appear electrophoretically very similar to those seen in both oat and barley (cf. Figs. 3 and 6). Again the enzyme ^3H -labelling technique results in labelling two bean peptides corresponding to mol.wts. approx. 34000 and 35000 (Fig. 7). Although this conclusion is obvious from the original fluorograph (results not shown), it is also quite apparent from the densitometric scan of the fluorograph reproduced in Fig. 7 (—).

The substrate-protection experiments were extended in an attempt to gain information of the specificity or mechanism of substrate binding to protochlorophyllide reductase. In this experiment, substrate-protected thiol-reacted membranes purified on sucrose gradients were used. Portions of the membranes were illuminated to photoconvert endogenous substrates and then incubated with no additions, with NADPH and/or protochlorophyll-

Table 1. *Effect of NADPH, NADH and protochlorophyllide on the labelling of the 35 000- and 37 000-mol.wt polypeptides of oat etioplast membranes by [³H]N-phenylmaleimide*

Substrate-protected thiol-reacted oat etioplast membranes prepared as described in Fig. 3 were illuminated for 30 s at 15 cm from a 30 W tungsten-filament lamp to effect complete photoconversion of endogenous photoactive pigment. To equal portions of these were added the reagents indicated in the Table (NADH at 1.35 mM, NADPH at 0.135 mM and protochlorophyllide at 2 μ M). After 10 min incubation in darkness, 30 μ Ci of [³H]N-phenylmaleimide was added and incubation continued for a further 10 min, followed by quenching with 0.7 M-mercaptoethanol. Fluorographs of the electrophoresed samples prepared as described in Fig. 3 were densitometrically scanned and the incorporation of ³H into the 35 000- and 37 000-mol.wt. peptides was estimated as described in Fig. 5.

Membranes incubated with [³ H]N-phenylmaleimide in the presence of:	Relative labelling of the 35 000- and 37 000-mol.wt. doublet (%)
Protochlorophyllide and NADPH	11.5
NADPH	21.8
Protochlorophyllide	55.2
None	100
NADH	100

ide or with NADH. Incubations were performed for 10 min, after which [³H]N-phenylmaleimide was added and incubation continued for a further 10 min in darkness. Finally, the samples were quenched with mercaptoethanol and subjected to electrophoresis and fluorography. The degree of protection offered by the various compounds is, of course, related to the extent of ³H incorporation. Estimates of this incorporation were obtained as peak areas from densitometric scans of the final fluorograph and are presented in Table 1. If the incorporation of ³H into the enzyme in the absence of any additions is given the arbitrary value of 100, then, on the same scale, incorporation of ³H in the membrane protected with protochlorophyllide and NADPH is 11.5 (Table 1). Protochlorophyllide alone gave an incorporation of 55.2, whereas protection by NADPH alone led to an incorporation of 21.8. NADH gave no protection whatsoever, as this sample incorporated ³H to the same extent as the unsupplemented control.

Discussion

Attempts at identification or characterization of the protein moiety of protochlorophyllide holochrome, i.e. the enzyme protochlorophyllide reductase, have to date met with only limited success. This is undoubtedly due, we think, to the fact that the methods used in the past have all relied on detecting

bound protochlorophyllide as the means of identifying the protein. The drawback of this approach is the well-known labile nature of the native protochlorophyllide-protein association (Manetas & Akoyunoglou, 1976; Redlinger & Apel, 1980), which has necessitated using only the mildest of detergents in isolation attempts to avoid dissociating the complex. However, such mild conditions we now know (Beer & Griffiths, 1981) lead to incomplete solubilization of the protochlorophyllide-protein complex and produces macromolecular aggregates with erroneously high molecular weights (Schopfer & Siegelman, 1968; Henningsen & Kahn, 1971; Rotfarb *et al.*, 1974). Studies involving harsher solubilizing conditions, however, have suffered from the possibility of creating artificial pigment-protein complexes in strong detergents (Thomas *et al.*, 1976).

In recent work in our laboratory, both these problems have been avoided. Beer & Griffiths (1981) monitored protochlorophyllide reductase enzymic activity during the development of a procedure for purifying the enzyme. This work led to the copurification of the enzyme from oat extracts with two closely related peptides with mol.wts. 35 000 and 37 000. In the present paper, identification of these peptides as protochlorophyllide reductase has been strengthened. It is shown that photoconversion, with total or purified oat etioplast membranes, leads to the exposure of thiol groups on two peptides with mol.wts. 35 000 and 37 000 that before photoconversion must have been protected by the substrates of protochlorophyllide reductase (Figs. 3 and 4). These peptides have been unambiguously identified by covalently labelling them with [³H]N-phenylmaleimide. The extent of this labelling (Fig. 5) has been shown to be directly related to the illumination-induced inhibition of the enzyme by the thiol reagent. Therefore the peptides must correspond to the reductase.

Similar conclusions can be drawn from the results obtained from application of the same technique to membranes from barley and bean etioplasts (Figs. 6 and 7). Despite the presence of many proteins in extracts from these membranes, the only ones to become labelled under our experimental conditions, which are designed to specifically identify protochlorophyllide reductase, are the pair of peptides with mol.wts. 34 000 and 35 000 in bean and a single peptide of mol.wt. 38 000 for barley. Why a single peptide should become labelled in barley when two peptides become labelled in oat and bean remains unclear at this stage. Extracts from both these plants have been extensively studied for protochlorophyllide-protein complexes in the past (Schopfer & Siegelman, 1968; Canaani & Sauer, 1977; Henningsen & Kahn, 1971; Rotfarb *et al.*, 1974), but the results have failed to realize a consistent model of

general application. In contrast, we consider that the results presented here can be used as a basis for a generally applicable model for protochlorophyllide photoreduction by protochlorophyllide reductase.

It is noteworthy that these peptides that we have identified as protochlorophyllide reductase have been described as major components of etioplast membranes several times in the past (Hoyer-Hansen *et al.*, 1976; Apel & Kloppstech, 1978; Grebanier *et al.*, 1979). All these reports were concerned with changes in membrane proteins accompanying greening and they all reported that although the doublet with molecular weight centred around 36000 was a major feature of peptide patterns from etioplast membranes, the amount of both peptides decreased during greening. We have repeated this observation (N. S. Beer & W. T. Griffiths, unpublished work) and it takes on renewed significance when the identification of the doublet with protochlorophyllide reductase in the current paper is borne in mind, coupled with our previous observation of the dramatic decrease in activity of this enzyme that accompanies the illumination of dark-grown plants (Maplestone & Griffiths, 1977, 1980). It therefore appears that the decrease in activity of protochlorophyllide reductase on illumination may reflect some coarse control mechanism for modifying the activity of the enzyme.

Although we felt confident from our evidence that we have identified the protochlorophyllide reductase peptides, some precautions about the interpretations of the results from our techniques should nevertheless be pointed out. Thus the method we have used here specifically and unambiguously identified substrate-protected thiol groups that become exposed to thiol-alkylating reagents after illumination. However, if photoconversion were to be accompanied by dissociation of the enzyme into subunits, some of which did not possess any substrate-protected thiol groups, perhaps by a mechanism analogous to the proposal of Canaani & Sauer (1977), then such subunits would escape detection in the present work. It is considered, however, in the case of protochlorophyllide reductase, that such events are unlikely in view of the fact that the enzyme in a very active and therefore presumably native form co-purifies with a protein fraction of mol.wt. approx. 40000 only and containing solely the two closely related peptides identified here (Beer & Griffiths, 1981).

Again, it is feasible that light could expose hidden thiol groups on peptides other than protochlorophyllide reductase by, for example, an illumination-induced conformational change, even in these substrate-protected membranes. In fact, a protein, present in etioplast membranes, known to behave in such a manner is the γ -subunit of the chloroplast ATPase (Bogorad *et al.*, 1971; McCarty & Fagan,

1973) and this also has a mol.wt. of 35000. However, in view of the absolute requirement for both NADPH and protochlorophyllide to afford protection from thiol reagents of the two peptides labelled in our experiments (Table 1), it is considered unlikely that any of the peptides we have identified corresponds to the ATPase subunit. What is perplexing from our data is why there are two closely related peptides that both qualify for identification as protochlorophyllide reductase in bean and oat membranes, whereas, in contrast, in barley only a single polypeptide becomes labelled. In the previous work, referred to above, on the etioplast-membrane peptides similar to those we identify here as protochlorophyllide reductase, the suggestion was made that the doublet of mol.wt. 36000 arose as an artefact of the electrophoretic procedure (Hoyer-Hansen *et al.*, 1976). Although this possibility cannot be overlooked at this stage, we have considered the additional possibility of the two peptides being isoenzymes of the reductase. In view of the polyploidy of the plant material we have been investigating to date, the possibility of the existence of isoenzymes must be considered a real one. We have at this stage, however, no explanation for the labelling of only a single peptide in barley under conditions when the doublet becomes labelled in bean and oat preparations.

Besides providing confirmation of the identity of the protochlorophyllide reductase peptides, the technique used here has also provided some information on substrate binding at the enzyme's active thiol group. The protection experiment (Table 1) can be interpreted as the thiol site binding the NADPH rather than the protochlorophyllide, but that the presence of the latter enhances the NADPH binding, i.e. as a ternary complex. The failure of NADPH to protect the thiol site suggests the involvement of the 2'-phosphate group of NADPH in the binding.

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