Identification of the polypeptides of NADPH–protochlorophyllide oxidoreductase

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The peptides of protochlorophyllide reductase have been identified by a covalent-radioactive labelling technique. This produced two labelled peptides of mol.wts. 35 000 and 37 000.

Dark-grown leaves of angiosperms accumulate the pigment protochlorophyllide, which is reduced to chlorophyllide on illumination. A photoactive protochlorophyllide–protein complex, designated protochlorophyllide holochrome, was first isolated from etiolated leaves as long ago as 1952 (Smith, 1952). Progress on the chemical characterization of this complex, and also on defining the mechanism of the unique light-requiring reduction, has, however, been slow.

With regard to the former, many studies aimed at defining the size of the ‘holochrome’ and its subunits have been performed, based largely on identification of protein fractions (Smith & Kupke, 1956; Boardman, 1962; Schopfer & Siegelman, 1968) or peptides (Guignery *et al.*, 1974; Canaani & Sauer, 1977; Redlinger & Apel, 1980) associated with pigment. However, a uniformly consistent picture for the holochrome or its subunits has yet to emerge from such studies. Recently, mechanism for the light-dependent reduction of protochlorophyllide has been proposed (Griffiths, 1978). The reaction has been shown to be catalysed by a membrane-bound enzyme. NADPH–protochlorophyllide oxidoreductase, which forms a ternary enzyme–protochlorophyllide–NADPH complex in darkness. Illumination of such a complex results in hydrogen transfer from the NADPH to the protochlorophyllide, followed by release of the products, thereby restoring the enzyme to its original state. It must be emphasized that, in this interpretation of the photo-conversion, the ‘protochlorophyllide holochrome’ of earlier literature is identified as the enzyme–substrate ternary complex of protochlorophyllide reductase. It is therefore of some considerable importance to identify the protochlorophyllide reductase protein. Such an identification has been

achieved here by covalently labelling the enzyme with a radioactive inhibitor.

The technique employed involved labelling of the substrate-binding thiol groups, exposed on illumination of the photoactive enzyme–substrates complex, with ^3H -labelled *N*-phenylmaleimide, followed by identification of the labelled peptide by SDS/polyacrylamide-gel electrophoresis and fluorography. Added substrates, protochlorophyllide and NADPH, were used initially to protect the enzyme’s essential thiol groups, whereas others were made to react with unlabelled *N*-phenylmaleimide. Excess of the reagent and substrates were then removed by gel filtration, [^3H]*N*-phenylmaleimide added and the active-site thiol group exposed to the reagent by illumination, resulting in specific covalent labelling of the enzyme.

Experimental

Materials

Biological material. Etioplasts were prepared from 7-day-old dark-grown oat (*Avena sativa* L., var. Pennal) leaves by homogenization in isolation buffer, followed by differential centrifugation as previously described (Griffiths, 1975). Etioplast membranes were obtained by lysis of the etioplasts in buffer II (20 mM-sucrose, 20 mM-Hepes, 20 mM-Tes, 2 mM-MgCl₂, 5 mM-cysteine, pH 7.5) and centrifuging at 14 000*g* for 20 min, followed by resuspension of the pellet in isolation buffer to a concentration of 6–12 mg of protein · ml⁻¹.

Chemicals. Protochlorophyllide and NADPH were obtained and used as before (Griffiths, 1978). *N*-Phenylmaleimide was synthesized from aniline, maleic anhydride and acetic anhydride as described by Cava *et al.*, (1961). [^3H]*N*-phenylmaleimide was similarly prepared by using [*ring-G*- ^3H]aniline (sp. radioactivity 150 mCi/mmol) supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

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

Methods

Enzyme assay. Protochlorophyllide reductase activity was routinely assayed by using the spectrophotometric technique previously described (Griffiths, 1978).

Light-dependent ^3H -labelling of etioplast membranes. The rationale underlying this procedure has been outlined above. In detail, etioplast membranes (say 0.75 ml containing 8.25 mg of protein) were incubated with protochlorophyllide ($4.7\ \mu\text{M}$) and NADPH (1.4 mM) for 10 min at room temperature in darkness to maximize formation of the enzyme (protochlorophyllide reductase)-substrates complex. A solution of *N*-phenylmaleimide was then added to a concentration of 7.2 mM and allowed to react with the substrate-protected membrane for 10 min, after which time the excess was removed by passage of the mixture through a loosely-packed column ($0.5\ \text{cm} \times 3\ \text{cm}$) of Sephadex (Medium grade) G-25 previously equilibrated with Buffer II minus cysteine.

$[^3\text{H}]\text{N}$ -Phenylmaleimide ($135\ \mu\text{Ci}$, 9 mM) was added to the column eluate and the mixture transferred into a cuvette in a dual-wavelength spectrophotometer set to assay absorbance changes at 672 nm relative to 710 nm. The samples were flashed at $1\ \text{flash} \cdot 80\ \text{s}^{-1}$ to effect photoconversion as described previously (Griffiths, 1978). After addition of the radioactive reagent and after each flash, portions were removed and 2 mins later were each quenched by addition of mercaptoethanol to 0.6 M. Again after each flash the extent of photoconversion was monitored by the spectrophotometer. Conditions, i.e. concentration of membranes in the cuvette and intensity of the flash, were prearranged such that about eight or so flashes were required to effect complete photoconversion of the photoactive complex. Illumination of the residual material remaining after photoconversion was continued for 30 mins to effect maximal incorporation of radioactivity. Samples treated exactly as above but not exposed to illumination were also taken and quenched with mercaptoethanol after 7 and 14 min incubation with the radioactive reagent and served as controls.

Electrophoretic separation and identification of radioactively labelled proteins. Portions of the ^3H -labelled samples from above were electrophoresed for 16 h at 4°C and 60 V on a separating gel composed of polyacrylamide (12%, w/v) bisacrylamide (0.32%, w/v) and a stacking gel of polyacrylamide (5%, w/v) bisacrylamide (0.13%, w/v) and SDS (0.1%, w/v), with the discontinuous buffer system described by Laemmli (1970). After electrophoresis, gels were stained, destained and photographed. Finally the gels were processed for fluorography as described by Laskey and co-workers (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Protein assays. Protein was assayed by the method of Bramhall *et al.* (1969), with bovine serum albumin as standard.

Results and discussion

Considerable controversy surrounds identification of the apoprotein of 'protochlorophyllide-holochrome' a protein that has recently been ascribed the function of protochlorophyllide reductase. This probably arises from the fact that identification of the protein was invariably only been indirectly attempted by monitoring the chromophore as a marker or probe for the protein. The distribution of protochlorophyllide-binding proteins has been investigated in plant extracts under both non-denaturing (Schopfer & Siegelman, 1968; Henningsen & Kahn, 1971; Canaani & Sauer, 1977) and completely denaturing conditions (Guignery *et al.*, 1974; Canaani & Sauer, 1977; Redlinger & Apel, 1980). Under non-denaturing conditions the well-known hydrophobic nature of such complexes leads to aggregation in aqueous buffers, giving largely anomalous values of molecular weights calculated from pigment-distribution data. Again, under denaturing conditions (e.g. during SDS/polyacrylamide-gel electrophoresis), in view of the weak nature of the bonding between protochlorophyllide and the protein (Manetas & Akoyunoglou, 1976; Redlinger & Apel, 1980), it is rather surprising to find that such a complex should survive even the slightest trace of detergent, especially during the course of electrophoretic analysis. Indeed, in contrast with the reports mentioned above, we have repeatedly failed to detect any protein-bound protochlorophyllide during SDS/polyacrylamide-gel electrophoresis of etioplast extracts under the mildest of conditions. Another potential drawback of this type of approach is that detergent might remove pigment from its apoprotein, associate with other protein, thereby creating artefacts capable of surviving solubilization in low concentrations of SDS and again leading to anomalous molecular-weight values of the apoprotein from pigment-distribution studies. Conditions favouring such artefacts might include high pigment concentrations coupled with low concentrations of detergent. It is possible that the recent report by Redlinger & Apel (1980) claiming at least four protochlorophyllide-binding proteins in etioplasts could well be interpreted in this manner. In the present work these shortcomings have been largely overcome by covalently labelling protochlorophyllide reductase with $[^3\text{H}]\text{N}$ -phenylmaleimide, followed by identification of the resulting radioactive peptides.

The effects of thiol-specific reagents such as *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid) on the activity of protochlorophyllide reductase have already been described (Griffiths,

1975). These inhibit the regeneration of the enzyme-substrates complex but have no effect on the photoconversion of the preformed complex. These results imply that essential thiol group(s) in the reductase molecule are protected from thiol-specific reagents by the substrates. However, on illumination and release of the products, these essential thiol groups become exposed to thiol-specific reagents. This suggested to us that if these thiol groups exposed by illumination could be covalently labelled with a radioactive compound, a specific stable marker for the enzyme would be provided. Again, these earlier results imply that, in the substrate-protected enzyme, the degree of inhibition of the enzyme by thiol-specific reagents should be proportional to the extent of photoconversion of the enzyme-substrate complex.

The validity of this latter prediction is illustrated in Fig. 1. This experiment was carried out with etioplast membranes pretreated with substrates and the excess removed by gel filtration. The hydrophobic inhibitor *N*-phenylmaleimide was then added and the mixture exposed to a flash of light every 80 s, while the progress of photoconversion was monitored spectrophotometrically. During the

Fig. 1. Relationship between photoconversion and inhibition of protochlorophyllide reductase. Substrate-protected oat etioplast membranes were photoconverted in the presence of *N*-phenylmaleimide. During the photoconversion, samples were removed and their reductase activity assayed as described in the text.

Fig. 2. Light-dependent labelling of etioplast membranes by [^3H]*N*-phenylmaleimide. Substrate-protected *N*-phenylmaleimide-treated oat etioplast membranes were photoconverted in the presence of [^3H]*N*-phenylmaleimide and samples were removed after various time intervals for electrophoresis and fluorography. Control samples incubated for prolonged periods in the dark and light were also analysed.

experiment, samples were removed, excess reagent quenched with mercaptoethanol and finally the activity of the reductase remaining assayed as usual.

The results in Fig. 1 show, as expected, a complete correlation between inhibition of reductase activity and the degree of photoconversion in the presence of the inhibitor. These data offer confirmation of the feasibility of our proposed procedure for the specific radioactive labelling of the reductase outlined above. Fig. 2 shows the results of such a labelling experiment.

SDS/polyacrylamide-gel electrophoresis of the various protein samples resulting from the experiment gave, on staining, the expected identical peptide pattern for all the samples. The developed gel at this stage was photographed, providing a permanent record of this pattern. The fluorograph resulting from exposing the scintillant-impregnated gel to photographic film for 4 days at -70°C is reproduced in Fig. 2. On either side of the fluorograph in the Figure we have included, for comparison, the peptide pattern of the samples taken from the original photograph. Also in the Figure are included the percentage-photoconversion data of the various samples, together with details of incubation times and conditions, i.e. illumination or darkness.

In considering this data it must be emphasized that equal quantities ($123\mu\text{g}$ of protein) of each sample were electrophoresed, so that a direct comparison of incorporation of radioactivity into each sample is possible from the fluorograph. The exception is the sample that received a prolonged period of illumination ($>30\text{ min}$) in the presence of the labelled inhibitor. Owing to the high ^3H incorporation in this sample, two relatively small portions [corresponding to approx. 70 and $35\mu\text{g}$ of protein (tracks 13 and 12 respectively)] were electrophoresed in order that these tracks on the final gel would contain roughly the same amounts of radioactivity as the others.

The developed fluorograph (Fig. 2) shows (i) incubation of the membranes in darkness with the radioactive inhibitor for up to 14 min results in only slight incorporation of ^3H into peptides (tracks 1 and 2). It is significant (see below) that the radioactivity was confined to a closely running pair of peptides of approx. mol.wts. 35000 and 37000. (ii) By comparison, flash-illumination of the incubated membranes gives a rapid incorporation of ^3H into the same doublet. Furthermore, the extent of incorporation appears qualitatively to correlate with the percentage photoconversion resulting from the illumination, e.g. both appear to saturate after 10 min of flash-illumination. Bearing in mind the rationale behind this experiment, it is apparent that these two peptides possess thiol groups that can be

protected from *N*-phenylmaleimide by NADPH and protochlorophyllide and, as such, we believe, qualify as the peptides of the enzyme protochlorophyllide reductase. The small amount of ^3H incorporated into these peptides in the samples that should have been protected by the substrates during incubation in the dark (see above) is probably accounted for by a slow diffusion by the substrates off the enzyme in darkness, as already noted previously for protochlorophyllide by Manetas & Akoyunoglou (1975) and Redlinger & Apel (1980). The general labelling of the peptides in the membranes seen in the long-term-illuminated samples (Fig. 2, tracks 12 and 13) probably reflect non-specific events such as, e.g., the light-induced breakdown of NADPH or porphyrin attached to proteins other than the reductase during the substrate-protection step, this breakdown being accompanied by non-specific exposure of thiol groups.

In conclusion, the present paper announces the first unequivocal identification of the peptide(s) of the enzyme protochlorophyllide reductase. Whether the two peptides are subunits of a polymeric reductase or, as appears more likely, represent two isoenzymes of the reductase, remains to be established.

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