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NADPH-Protochlorophyllide Oxidoreductase: Reciprocal Regulation in Mono- and Dicotyledonean Plants

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Abstract

The influence of light on the expression of NADPH: protochlorophyllide oxidoreductase has been studied in different plant species. The presumptive precursors to this enzyme have been characterized by in vitro translation of poly(A)RNA and immunoprecipitation. Two bands of apparent molecular weights of about 42 000 and 44 000 have been found in light- and dark grown monocotyledonean species, whereas a single band has been observed preferentially in light grown species of dicotyledonean plants. Membrane proteins reacting with the antibody to protochlorophyllide oxidoreductase have been identified by the method of immune blotting. On the basis of these findings it is concluded that protochlorophyllide oxidoreductase proteins are present in the membranes of all illuminated plants for at least several days. The mode of regulation, however, has been found different in mono- and dicotyledonean plants.

Introduction

In angiosperm plants the formation of chlorophylls is controlled by the external factor of light at several levels. The essential light dependent reaction consists of the photoreduction of protochlorophyllide to chlorophyllide (see Boardman et al. 1978).
This reaction can be mimiced in vitro in the presence of a 36 000 protein of the etioplast membranes of oats or barley with protochlorophyllide and NADPH as substrate and cofactor, respectively (Beer and Griffiths 1981, Apel et al. 1980).

In vivo the enzyme itself, NADPH: protochlorophyllide oxidoreductase (EC 1.6.99.-), is controlled in a twofold manner via the factor of light at least in barley and oats, where the measurable enzyme activity declines upon illumination of etiolated plants (Mapleston and Griffiths 1980). Furthermore, in barley the enzyme protein of 36 000 has been described to vanish quite rapidly from the membranes with illumination (Santel and Apel 1981). Finally, the mRNA for this enzyme has been found to disappear from the poly(A)RNA fraction in a phytochrome controlled step (Apel 1981). These findings are remarkable because the synthesis of chlorophyll is by no means completed at this time.

Two working hypotheses have been developed in order to explain this apparent paradoxon. While Mapleston and Griffiths (1980) calculate a residual activity of the enzyme in illuminated plants which is sufficient to account for the observed rate of chlorophyll synthesis in light grown plants, Santel and Apel (1981) postulate an alternative mechanism of chlorophyll synthesis which substitutes the function of the etioplast protochlorophyllide oxidoreductase upon illumination, the latter enzyme exerting only a limited but essential function.

Both groups worked primarily with monocotyledonean species. We found considerable species specific differences in the light dependent regulation of the chloroplast specific glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) (Cerff and Kloppstech 1982) as well as of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.9) (Cerff and Kloppstech, unpublished) especially between mono- and dicotyledonean species. It therefore seemed promising to investigate the regulation of protochlorophyllide oxidoreductase in different species on both the level of proteins and of translatable mRNAs in order to gain additional access to the understanding of the mechanisms of regulation of this particular protein. An interpretation of the obtained results leads to the conclusion that both mentioned working hypotheses seem to be realized to some extent in cereals but that a different mode of regulation exists in dicotyledonean species.

Methods

Bean (*Phaseolus vulgaris* cv. Sotexa), pea (*Pisum sativum* cv. Rosakrone), and tomato (*Lycopersicum esculentum* cv. Lukullus) were grown on moist vermiculite at 25° C for 9 days in a 12:12 h light-dark cycle at 7500 lx. Wheat (*Triticum aestivum* cv. Reso), oats (*Avena sativa* cv. 7770 v. Lochow-Petkus), and rye (*Seccale cereale* cv. Halo) were cultivated under the same conditions but for 5 and 7 days, respectively.

Monocotyledonean plants were cut about 2 cm above the ground. From the green dicotyledonean plants merely the leaves were harvested, whereas from etiolated pea and bean the upper 3 cm of the plants were collected after removal of the cotyledons (bean).

The isolation of poly(A)RNA, its translation and the analysis of the products have been performed as described (Apel and Kloppstech 1978). The method of immunoprecipitation has been modified as follows. In one set of experiments the translation mixture has been diluted fivefold with PBST-buffer (0.01 M Tris/HCl, pH 7.1, 0.15 M NaCl, 0.1% sodium dodecylsulfate, and 1% Triton X-100) prior to addition of 2.5 µl anti-

body to ribulose-1,5-bisphosphate carboxylase per 160 µl of translation supernatant, incubation for 30' at 4° C and removal of the immunoglobulins by adsorbtion to 50 µl of a 30% suspension of Staphylococcus aureus membranes (Cullen and Schwartz 1976). This step has been repeated with the antibody against the light-harvesting chlorophyll a/b protein and 60 µl Staphylococcus membranes before the final addition of 2.5 µl antibody against protochlorophyllide oxidoreductase and proceeding according to the published procedure (Cullen and Schwartz 1976). In the second procedure the precursors from 160 µl translation supernatant were precipitated with the antibody against protochlorophyllide oxidoreductase, the complex removed from the Staphylococcus membranes in PBST-buffer containing 2% sodium dodecylsulfate at 50° C. The supernatant was diluted twentyfold with PBST-buffer without sodium dodecylsulfate and the immunoprecipitation step repeated by addition of another 2.5 µl antibody. The immunoglobulins were thereafter adsorbed to 100 µl of Staphylococcus membranes and eluted at 100° C as described by Neville (1971).

The plastid membranes were prepared following the method of Apel et al. (1980), dissolved and analyzed according to Neville (1971).

The transfer of proteins was done as published (Burnette 1981) at 6V/cm (48 V total) but for 16 h at 10° C in a stirred buffer compartment to which 0.1% sodium dodecylsulfate and 10 mM $MgCl_2$ had been added (Pharmacia application note). The detection of proteins was performed as described (Towbin et al. 1979) with the following modifications. Binding of antibodies (1:50 diluted in 5% bovine serum albumine) and peroxidase conjugated antiserum to antibodies (1:250 in serum albumine) as well as all washing steps were done in PBST-buffer except for the last washing step and the peroxidase reaction itself in order to reduce the background staining.

Results

Poly(A)RNA from different light- and dark grown plants has been translated and treated with the antibody raised against protochlorophyllide oxidoreductase of barley in order to isolate the precursors to this protein. In contrast to the products from etiolated plants where the immunoprecipitation is comparatively easy to handle, it turned out rather difficult to obtain clear precipitation pattern especially from light grown plants despite the pretreatment with antisera to prevalent chloroplast proteins (Figs. 1 and 2). The precursor bands of ribulose-1,5-bisphosphate carboxylase small subunit and light-harvesting chlorophyll a/b protein coprecipitate with the immunospecific bands. That indeed the bands marked by the crosses in figures 1 and 2 represent specific immunoreactive bands can be concluded from Figure 3, where the products of the first immunoprecipitation have been dissolved and precipitated a second time with the antibody against the reductase. The following results have been obtained. From the RNA of all investigated light grown plants translation products have been precipitated that react with the antibody and most probably represent protochlorophyllide oxidoreductase proteins both on the basis of immunoreaction and of their apparent molecular weights. The data can be arranged in two groups. On the one hand all investigated monocotyledonean species like wheat and oats (Figs. 1-3) but also rye and barley (not shown) possess two immunoreactive bands of about equal intensity and of 42 000 and 44 000 apparent molecular weight. Whereas on the other hand in the second group, to which all investigated dicotyledonean species belong, only one major species has been found. In addition to bean, pea, and tomato (Figs. 1-3) the same result has been obtained for spinach, cucumber, and pumpkin (not shown).

The differences in the translation products obtained from the RNA of etiolated plants are somewhat more complex. The monocotyledonean species again show two immunoreactive bands; from these the lower molecular weight bands are likewise heavily overrepresented. This band which fades considerably during illumination apparently corresponds to the precursor band described by Apel (1981).

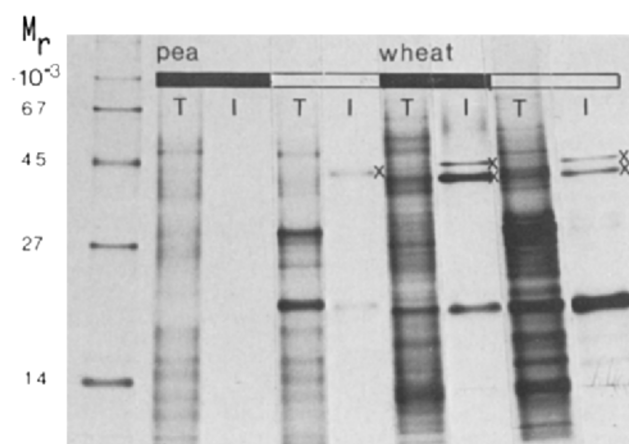


Fig. 1 In vitro translation products obtained from poly(A)RNA of light- and dark grown pea and wheat.

Poly(A)RNA was translated at rate limiting concentrations in a wheat germ system. One tenth volume (160 ul total) was precipitated with trichloroacetic acid (T) and the remainder with the antibody to protochlorophyllide oxidoreductase according to the first procedure described in methods (I). Electrophoresis in a 10-15% polyacrylamide gel according to Neville (1971). Lane 1: reference proteins. Black and white bars: dark and light, respectively.

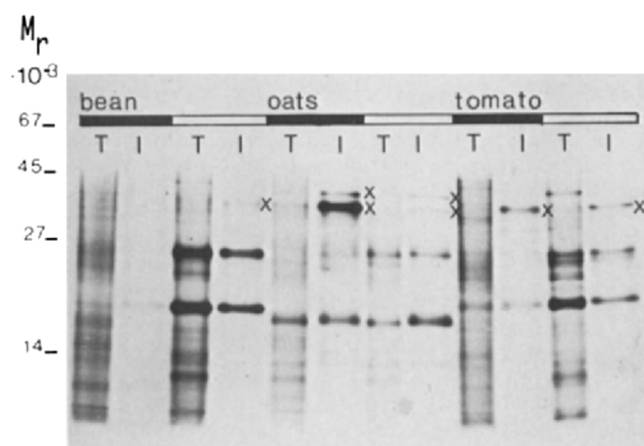
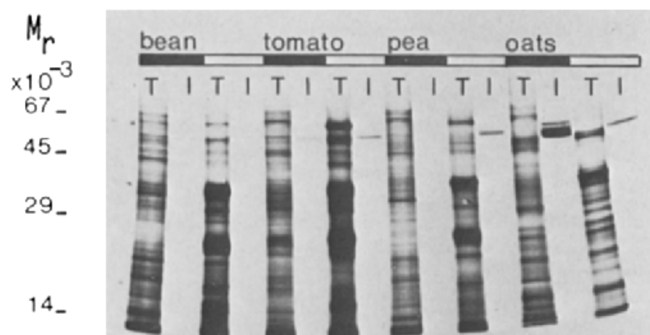


Fig. 2 In vitro translation products from poly(A)RNA of light- and dark grown bean, oats, and tomato

For explanation see legend to Figure 1.



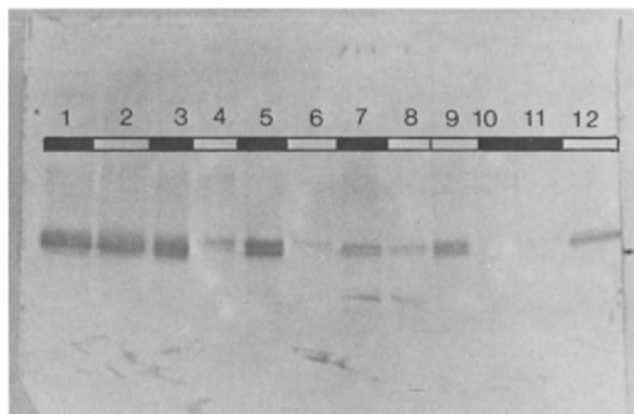


Fig. 3 Translation products of poly(A)RNA from light- and dark grown bean, tomato, pea, and oats.

Immunoprecipitates (I) prepared according to the second procedure of the methods section and trichloroacetic acid precipitates (T) were separated on a 10 to 15% polyacrylamide gel. The figure is a composite of two different exposures of the same gel.

Fig. 4 Immune reaction of plastid membrane proteins with the antibody to protochlorophyllide oxidoreductase.

Membrane proteins (50 µg) were separated in a 10 to 15% polyacrylamide gel, transfered to nitrocellulose, treated according to Burnette (1981), and the immunoreactive proteins were detected with peroxidase-conjugated anti-immunoglobulins according to Towbin et al. (1979) with the modifications outlined in the methods section. Lanes: 1: barley etioplast, 2: barley illuminated for 4 h, 3,4: wheat, 5,6: oats, 7,8: tomato, 10,9: bean, and 11,12: pea of dark- and light grown plants, respectively. Black and white bars indicate dark and light treatment of plants.

It has been reported that parallel to the decrease of protochlorophyllide oxidoreductase mRNA also the enzyme protein disappears rather extensively from the membranes of barley plastids (Santel and Apel 1981), These results had been obtained by comparison of the membrane protein composition of etioplasts and of plastids during the greening period. We used the method of immune blotting to investigate this aspect and found that there is a considerable reduction of immunoreactive protein in monocotyledonean species upon illumination, thereby confirming the previous work, but by no means a complete disappearance (Fig. 4). In detail, it seems that as in vitro there are also two bands in vivo but of 36 000 and 38 000 apparent molecular weight at least in wheat and oats. The presence of two bands in oats is well known from the work of Oliver and Griffiths (1981). From these two bands, as in the in vitro translation products, it is the lower band that disappears with illumination, whereas, even after seven days of light-dark regime, the higher molecular weight band is still detectable in the membranes. Again, as in the translation assays, we find correspondent results for the immunoreactive protein in the membranes of tomato, bean, and pea. In tomato the protein is present in the membranes of dark as well as of light grown plants, whilst in dark grown pea and bean the reaction with the antibody is quite weak but an intensive reaction can be obtained in light-dark grown plants.

Discussion

The data presented in this paper point out that the results obtained for the protochlorophyllide oxidoreductase of oats (Mapleston and Griffiths 1980) and barley (Santel and Apel 1981) cannot be generalized for higher plants, there are considerable differences between the investigated species in the expression of the mRNA, at least within the poly(A)RNA fraction, as well as in the expression of the protein itself. The used technique of immune blotting, however, leaves some questions unanswered and so far gives semiquantitative results at the most. This is preferentially the result of the limitation that only the antibody against the 36 000 protein of barley could be used. The fact that we obtain massive immunoprecipitation in mono- but not in dicotyledonean species of in vitro translation products might be the result of a different extent of cross-reaction between different species; this point awaits further clarification in the future.

The same argument of different extents of cross-reactivity might be valid for the second band of higher molecular weight which seems absent from dicotyledonean plants. An alternative explanation, namely that a contaminant protein of 38 000 had been present in the antigen preparation, can be excluded both by the method of preparation of the antigen as well as by the fact that analytical gels gave no indications of a higher molecular weight contaminant in the antigen preparations. Furthermore, our results are in agreement with earlier observations of Griffiths that there are two bands of molecular weights rather close to those described in this paper in oats and bean (Mapleston and Griffiths 1980). On the basis of his and our evidence we favor the idea that there are two isoenzymes present in mono- and probably also in dicotyledonean plants, which in analogy to the results obtained for the light-harvesting chlorophyll a/b protein, may not be separated electrophoretically in all species. In this connexion it is of interest that upon illumination the higher molecular weight band prevails over the lower molecular weight band in wheat and oats (Fig. 4). This observation might indicate that the 36 000 protein becomes at least partially replaced by a 38 000 protein in monocotyledonean plants. This idea needs confirmation by an enzyme assay that works with membranes as well as with the isolated protein from greened plants.

The main finding presented in this paper is the observation that the antagonistic regulation of the light-harvesting chlorophyll a/b protein and of protochlorophyllide oxidoreductase described for barley (Apel

1981) is not valid for all higher plant species or at least not to this high extent. In dicotyledonean species there are at least two different modes of regulation. In one group to which so far only tomato belongs we find the RNA for the precursor as well as the immunoreactive protein both in light- and dark grown plants. In the other group, of which pea and bean are representatives, both the protein and its mRNA are present in light grown plants even after 12 days under a 12:12 h light-dark regime but only to a very low extent in dark grown plants. This might indicate that eventually all monocotyledonean species but surely the cereals have evolved a specific adaptation that enables them to react very efficiently upon access to the environmental factor of light in order to provide the reactioncenter proteins very rapidly with chlorophyll. Since it has been found that the disappearance of the mRNA for protochlorophyllide oxidoreductase is under control of phytochrome in barley (Apel 1981), we have to conclude that the coupling of the expression of this particular protein to the phytochrome system remains very flexible even between rather closely related species and therefore should be a rather recent invention in evolution. It remains to be proven whether in pea the induction of protochlorophyllide oxidoreductase is under positive control of phytochrome.

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Abbreviations: poly(A), polyadenylated; PBST, buffer as described under Methods.