EFFECTS OF ILLUMINATION OF ETIOLATED LEAVES ON THE REDOX STATE OF NADP IN THE PLASTIDS

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Received 8 June 1978

1. Introduction

Higher plants are unable to develop chloroplasts in the absence of light, and in darkness they accumulate the chloroplast precursors designated etioplasts [1]. These are largely undifferentiated organelles containing most of the components found in normal chloroplasts, but at a greatly reduced level. The notable exception however is chlorophyll, which is completely absent from etioplasts, being replaced here by a trace amount of its immediate precursor, protochlorophyllide. Exposure of dark grown plants to light results in the synchronous conversion of the etioplasts into functional chloroplasts.

Details of the mechanism by which illumination of etiolated leaves mediates the various reactions resulting in the etioplast to chloroplast transformation remain largely uncharacterized. However, in this context, one well-defined light-requiring reaction is the phototransformation of protochlorophyllide to chlorophyllide. Whether the light requirement for chloroplast development can be accounted for solely by this reaction or whether other photomorphogenetic reaction(s) (e.g., involving phytochrome) are involved in chloroplast formation is still a matter of considerable controversy (discussed [2]). Thus, the fact that certain plants such as gymnosperms [3] or various algae [4], which can make chlorphyll in darkness, can develop structures that appear like normal chloroplasts, without any illumination, would support the former contention. However, the extensive literature on the photomorphogenetic effects mediated by the pigment phytochrome would support the idea that photomorphogenic light is also needed for other reactions during chloroplast development [2]. Irrespective of this however, no mechanism, supported by direct experimental evidence, has been offered to explain how the light reaction(s) affects the phototransformation of etioplasts to chloroplasts. In its simplest form the photocontrol of chloroplast development might be expected to involve a primary photoreaction, e.g., protochlorophyllide or phytochrome transformation producing an effector capable of 'switching on' the various pathways, the concerted operation of which results in chloroplast development. In this respect it is worth recalling that the products of protochlorophyllide phototransformation, as described [5], are $NADP^+$ and chlorophyllide.

We report here on a remarkable effect of illumination on the redox state of the NADP pool within the plastids of etiolated barley plants. Possible regulatory implications of this effect to the general process of chloroplast development are discussed. Evidence of parallel changes occuring in the activity of at least one enzyme of chlorophyll biosynthesis is also presented.

2. Materials and methods

Etioplasts were isolated by differential centrifugation of 7 day old etiolated barley *(Hordeum vulgare* L., cultivar Proctor) leaf homogenates as in [6] except that for nucleotide analyses, cysteine was omitted from the isolation buffer and the plastids were resuspended in 0.5 M sucrose, 100 mM Tris adjusted with KOH to pH 7.6.

Activity of the enzyme protochlorophyllide

 Elsevier/North-Holland Biomedical Press

reductase in etioplasts was measured as the rate of flash-induced chlorophyllide formation in the presence of excess substrates, protochlorophyllide and NADPH, as in [5]. Triton X-100 was routinely added to the assay medium, at a pre-determined optimum level, to overcome problems of impermeability.

Pyridine nucleotide levels were measured using the enzyme cycling method in [7]. For the NADP estimations, plastids equivalent to ~ 10 mg protein were found, on extraction, to produce reliable dichlorophenolindophenol reduction rates, whereas this amount had to be increased ~ 3-fold for the assay of NAD. It was also found necessary in the NAD assays to use large amounts (45 units) of alcohol dehydrogenase to overcome inhibition of the enzyme by some endogenous material present in the extract.

Protein was measured by the Lowry method [8] using bovine serum albumin as standard.

Glucose 6-phosphate, ATP and $NADP^+$ were purchased from Boehringer as also were the enzymes yeast glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and yeast alcohol dehydrogenase (EC 1.1.1.1.)

3. Results

The regeneration of photoactive protochlorophyllide during chlorophyll biosynthesis by higher plants is known to have an obligate requirement for NADPH [5]. The fact that isolated barley etioplasts, as routinely prepared in this laboratory, have already been shown to be capable of extensive synthesis of photoactive protochlorophyllide without any supplementation [6] indicates that etioplasts largely retain their nucleotide components during isolation in aqueous buffer. This is confirmed by the data in table 1, which records our estimations of the redox levels of the pyridine nucleotides in 3 different etioplast preparations. Whereas some variation in the total recovery of nucleotides was noted in the different samples, reflecting varying degrees of intactness of the preparations, the estimated $NAD(P)H/NAD^+(P)$ values show a high degree of consistency.

Table 1 shows that in etioplasts from dark grown plants the $NADPH/NADP^+$ ratio is very high (~ 3.6) compared with the corresponding $NADH/NAD^+$ value (~ 0.23). Illumination of the plants for 35 min results in considerable oxidation of the plastid NADP whereas the redox state of the $NAD^+/NADH$ couple remains largely unaffected by this treatment.

The effect of 30 min illumination followed by varying periods in darkness on the NADP redox level in plastids is shown in table 2. Thus, as before, 30 min illumination results in the plastid NADP occurring chiefly as the oxidised form ($NADPH/NADP^+ = 0.76$). However, after returning the plants to darkness the plastid NADP is seen to become progressively more reduced again, until after 3 h in darkness the ratio ($NADPH/NADP^+$) has returned to 2.2 (table 2). This data indicates that it is possible to largely reverse the illumination-induced oxidation of etioplast NADP merely by following the illumination with a period in darkness.

In this experiment activity of the enzyme protochlorophyllide reductase was also assayed in the various plastid preparations and the results, expressed in units of nmol chlorophyllide formed/mg protein/h, are included in table 2. Thus, the reductase in plastids

Table 1
Redox states of pyridine nucleotides in plastids from dark and briefly illuminated barley plants

	$NADPH/NADP^+$			$NADH/NAD^+$		
Exp. no.	1	2	3	1	2	3
Dark plastids	3.56	4.02	3.40	0.223	0.242	0.226
Illuminated 35 min. plastids	0.53	0.38	0.63	0.172	0.196	0.215

Plastids were isolated from 7 day old etiolated barley plants before and after a 35 min illumination from a bank of 60 W tungsten lamps 50 cm away and redox rates of the nucleotides in extracts of the plastids assayed as described in the text. The data from 3 separate experiments are included

Table 2
Effect of illumination of barley plants followed by a dark period on the redox state of NADP and activity of protochlorophyllide reductase in plastids

	NADPH/NADP$^+$	Protochlorophyllide reductase (nmoles/min/mg protein)
Dark control	4.83	1.82
Light 30 min	0.76	0.479
Light 30 min + dark 1 h	0.995	0.610
Light 30 min + dark 2 h	1.65	0.934
Light 30 min + dark 3 h	2.22	1.250

Plastids were isolated from 7 day old dark grown plants, after 30 min illumination and after further 1 h, 2 h and 3 h in darkness and the redox state of NADP and activity of protochlorophyllide reductase assayed as described in the text

from the unilluminated plants had spec. act. 1.82. This decreased to 0.479 in plastids from the plants illuminated for 30 min whereas a further 3 h in darkness led to a rise in the specific activity of the enzyme in the plastids to 1.25 (table 2). Recalling the NADPH/NADP$^+$ results, this data indicates a correlation in plastids between the redox state of NADP and the specific activity of the enzyme protochlorophyllide reductase.

Table 3 further bears out this correlation and provides additional characterization of the illumination induced change in NADP levels. Thus, it is apparent that continuous light is not required to produce the NADPH oxidation since 2 min illumination of etiolated plants results in considerable oxidation of the plastid NADPH. However, this oxidation continues in the absence of light for a further 1 h before the nucleotide becomes re-reduced as before, on prolonging the time in darkness to 4 h (table 3).

Regarding the correlation between NADPH levels and activity of the enzyme protochlorophyllide reductase table 3 shows that 2 min illumination of etiolated plants triggers a decrease in the specific activity of the reductase, a decline which continues for a further 1 h without light. Prolonged (4 h) darkness however leads to a marked restoration of the enzymic activity in a manner exactly analogous with the changes already seen in the NADP levels (table 3).

2. Discussion

The role of light in regulating the various metabolic activities, oxidative and reductive, associated with the mature chloroplasts is currently attracting much interest. Thus, it is becoming increasingly apparent that photosynthetically produced reductants, e.g., reduced dithiols, ferredoxin or NADPH can activate chloroplast enzymes associated with anabolic activities, e.g., carboxydismutase [9], NADPH-linked

Table 3
Effect of light triggering followed by a dark treatment of etiolated barley plants on the plastid NADPH/NADP$^+$ ratios and activity of protochlorophyllide reductase

	NADPH/NADP$^+$	Protochlorophyllide reductase (nmol/min/mg protein)
Dark control	3.62	3.35
Light 2 min	1.76	2.03
Light 2 min + dark 1 h	0.50	0.78
Light 2 min + dark 4 h	2.78	2.01

The methods employed were as in table 2 except that only a brief 2 min illumination was given to the plants

glyceraldehyde-3-phosphate dehydrogenase [10] and sedoheptulose [11] and fructose diphosphate phosphatases [12] while serving to depress the activities of catabolic, oxidative enzymes, e.g., glucose 6-phosphate dehydrogenase [13,14]. In darkness, due to the cessation of photosynthesis the reverse situation exists with conditions now favouring the oxidative enzymes and enzymes of the reductive pathway being largely inhibited.

Illumination of dark grown plants activates various metabolic processes which co-operatively results in chloroplast development. The question is, does illumination influence this development by a mechanism similar to that operational in the light control of normal chloroplast metabolism mentioned earlier? Thus, is it possible to ascribe the influence of light on chloroplast development to a product resulting directly from the illumination? The findings reported here of illumination-induced changes in the redox state of NADP within barley plastids might well be worth considering in such a context. Further, the fact that oxidation of the plastids' NADPH by illumination is accompanied by a decrease in the activity of the enzyme protochlorophyllide reductase might point to a general relationship between the redox state of the plastids' NADP and activity of certain important chloroplast biosynthetic enzymes. Thus, modification of the former by illumination might lead to regulation of the activities of the latter.

The different effects of light on NADPH/$NADP^+$ ratios in etioplasts and chloroplasts is striking. Thus, whereas illumination of dark-grown leaves results in an oxidation of the etioplast NADPH, similar treatment of green leaves or chloroplasts [7,13] leads to the reduction of $NADPH^+$. Whereas the chloroplast and green tissue results are readily accounted for in terms of photosynthesis it is possible that the high NADPH level in etioplasts is due to the activity of the plastid glucose 6-phosphate dehydrogenase – an enzyme which is active in the dark [15]. Light-induced oxidation of the NADPH in the etioplasts can possibly be the result of the activity of the protochlorophyllide reductase, a unique light requiring reaction which also oxidises NADPH [5]. The fact that a brief (2 min) period of illumination is sufficient to effect the change in the redox state of the NADP (table 3) supports this possibility.

What is not clear in the data presented in this paper is what is the significance to the plant of the decrease in activity of protochlorophyllide reductase seen on illumination (table 3) since it is known that illumination of leaves results in considerable synthesis of chlorophyll? A possible explanation may be that this decrease in activity is only apparent, and a consequence of the fact that the reductase activity in the etioplast is abnormally high as some result of prolonged etiolation. Illumination then merely restores it to an activity more compatible with other enzymes of chlorophyll biosynthesis. Thus, the reductase activity, even at the reduced, illuminated level, is more than adequate to account for the overall rate of chlorophyll synthesis achieved by the whole plants [5].

Before ascribing too much regulatory significance to the illumination effects noted here, and as a cautionary note, it should be pointed out that in these experiments we have not made the distinction between free and bound nucleotides in the plastids. Thus, the thermodynamically active nucleotide pools may in fact show a different response to illumination from those described here. However it will still be of great interest to investigate the effect of the reported illumination-induced change in plastid NADP levels on key chloroplast biosynthetic enzymes in an effort to explain the effect of light on chloroplast development.

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