SOURCE OF REDUCING EQUIVALENTS FOR THE IN VITRO SYNTHESIS OF CHLOROPHYLL FROM PROTOCHLOROPHYLL

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1. Introduction

The terminal reactions of chlorophyll(ide) synthesis in higher plants involve the photoreduction of protein bound protochlorophyll(ide) by an unidentified hydrogen donor. Etiolated leaves of angiosperms accumulate both photoconvertible and non-photoconvertible PChl* species which can be identified by their absorption maxima in vivo in the red region of the spectrum [1]. The major phototransformable form is P_{652} – the PChl holochrome. A second phototransformable species P_{637}, occurs to a lesser extent in etiolated leaves. Non-phototransformable P_{630} varies in amount in different species and with age of plant but its level increases with δ-aminolevulinic acid (ALA) feeding [2].

Various explanations have been suggested to acount for the existence of the different forms of PChl ranging from whether the molecule exists free or bound to protein [3], whether it is phytylated or not [4] or whether aggregation occurs to give monomeric or dimeric PChl complexes [5,6]. Further, attempts to physically separate the different absorbing forms have also been made [7–9].

The studies reported here provide evidence suggesting NADPH as the hydrogen donor for PChl photoreduction. Again, some suggestions regarding the relationship between non-photoconvertible P_{630} and photoconvertible P_{652} can also be offered from the results of this study.

* Abbreviations: ALA: δ-aminolevulinic acid; PChl: protochlorophyll(ide) *a*; P_{630}; P_{652}: forms of PChl occuring in vivo absorbing light at the wavelengths indicated. Chl: chlorophyll(ide).

2. Materials and methods

Etioplasts were prepared from 7 day-old dark grown barley seedlings by a modification of the procedure of Horton and Leech [10]. This involved the use of a slightly different isolation medium which comprised 0.5 M sucrose, 0.2% bovine serum albumin, 5 mM cysteine, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM TES [*N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid] adjusted with KOH to pH 7.2. The etioplast pellet was resuspended in isolation medium supplemented with 1.5 mM ATP either in the presence or absence of 5 mM cysteine and was used without further purification. The protein concentration of the final suspension, as assayed by the Folin method [11] was arranged by dilution to be between 6–8 mg ml^{-1}. Chlorophyll levels in the etioplasts were estimated from the intensity of the red chlorophyll absorption band assuming linearity between absorption and chlorophyll concentration (see Boardman [12]).

Water lysing of the etioplasts was achieved by a tenfold dilution with water at 0°C. After 5 min, the lysed membranes were sedimented by centrifuging at 30 000 *g* for 15 min and finally resuspended to their original concentrations in buffer appropriate to the study. Absorption spectra of the preparations were recorded on a split beam spectrophotometer described by Jones and Saunders [13], using a dilute solution of milk as a blank to compensate for the scattering effect of the preparation. P_{630} enriched etioplasts were isolated from seedlings which had been fed on ALA for 6–8 hr by standard procedures [14]. Illumination of the etioplasts was achieved by one of two methods. For a single illumination to estimate photoconvertibility of the protochlorophyll(ide) in preparations, the

sample, usually in the spectrophotometer was illuminated for 90 sec by a 100 W tungsten filament lamp held at a distance of 10 cm away. For flash illumination the samples were exposed to 1msec flashes from a photographic Xenon lamp (Mecablitz) supported 10 cm from the sample. Detailed experimental procedures are to be described separately [15].

2.1. *Chemicals and enzymes*

Glucose 6-phosphate, L-malate, ATP, $NADP^+$ NAD^+ were purchased from Boehringer, as also were the enzymes glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and L-malate: NAD oxidoreductase (EC 1.1.1.37), ALA was a product of Sigma Chemical Co.

3. Results

The absorption spectrum of an etioplast suspension isolated from seven day old etiolated barley is shown in fig. 1 (curve a). The main red absorption band at

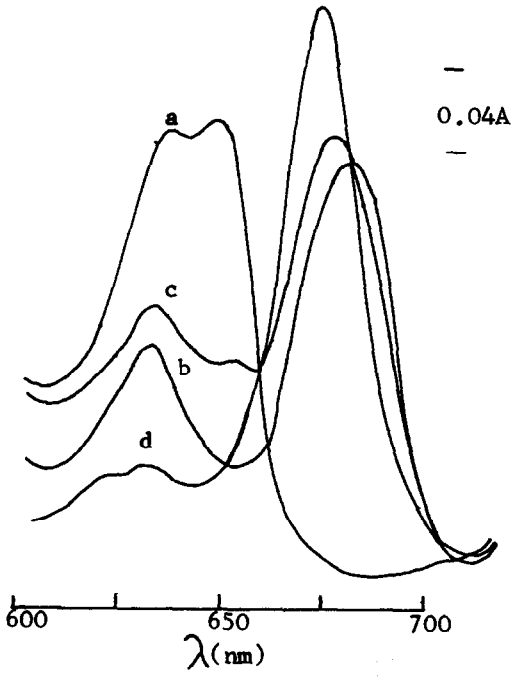

Fig. 1. Absorption spectra of etioplasts isolated from 7 day-old barley. Curve a: sample before illumination, b: after illumination for 90 sec with a 60 W tungsten lamp, c: after 25 min in darkness following illumination, d: sample given a 1 msec Xenon flash every 15 min overnight (12 hr).

Table 1
Chlorophyll synthesis in etioplasts

System	Chlorophyll level
	(% of control)[1]
No additions	132
+ Cysteine	138
[2] NADH regenerating	163
[3] NADPH regenerating	158
H_2O lysed, no additions	16

[1] Control level taken as that attained by illuminating the freshly isolated etioplasts for 90 sec as in fig. 1.
[2] 1.6 mM NAD, 6 units malate dehydrogenase and 5 mM malate.
[3] 0.5 mM NADP, 5 units glucose 6-phosphate dehydrogenase and 5 mM glucose-6-P.
Etioplasts were isolated as described in Materials and methods and resuspended in the absence of cysteine. Chlorophyll synthesis was then induced in the etioplasts by overnight flashing under different incubation conditions.

652 nm is due to P_{652} whilst the shoulder at 630–635 nm is attributed to P_{630}. On illumination (curve b) the P_{652} absorption disappears, being replaced by Chl absorption at 682 nm and now revealing the non-photoconvertible P_{630} absorption as a peak at 633 nm. As already described [15] if the illuminated sample is left in darkness for 20 min and its spectrum redetermined an increased absorption at 652 nm results (curve c) indicating the regeneration of P_{652} which can again the photoconverted to increase the Chl level. If instead of a single flash of light etioplasts are given a series of 1 msec flashes every 15 min overnight (12 hr) the spectrum given in curve (1d) is obtained. In this case there is seen a greatly increased absorption at 675 nm whilst the absorption at 633 nm has almost completely disappeared, indicating the formation of Chl from P_{630} as previously described [15].

Table 1 gives the effects of various pretreatments and additives on the relative amounts of Chl produced in etioplasts by flash illuminating overnight. No great differences in the Chl levels as a result of these treatments are seen with the exception of the water lysed preparation incubated in the absence of cysteine in which the level is dramatically reduced.

Fig. 2 records the absorption spectra of overnight flashed, water lysed etioplasts (curve a). As already indicated in table 1 very little Chl appears in the water lysed preparation on flashing overnight – note low absorbancy at 675 nm with a corresponding high

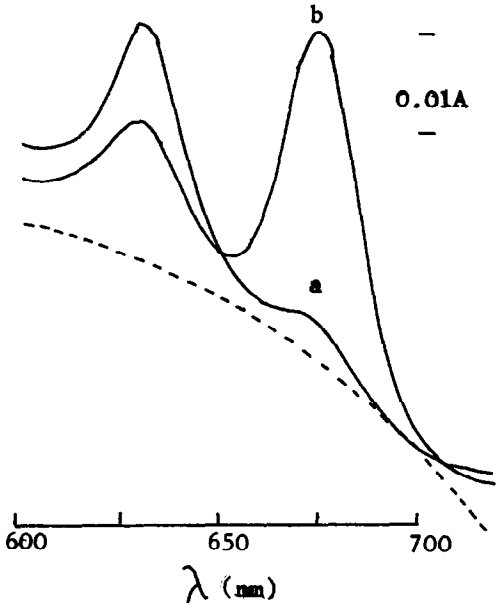

Fig. 2. Absorption spectra of flashed lysed etioplasts. Water lysed etioplasts resuspended in the absence of cysteine were flashed overnight as in fig. 1. Curve a: with no additions, b: in the presence of an NADPH regenerating system.

absorbancy at 633 nm. A similar spectrum is seen if, to the water lysed sample, the water washings are added at twice the original concentration. However, in the sample incubated in the presence of an NADPH regenerating system (curve 2b) a greatly increased absorption due to chlorophyll at 675 nm is seen, accompanied by a decrease in the absorption due to PChl at 633 nm.

Table 2 records the Chl levels obtained by flashing depleted etioplasts overnight in the presence of various reducing systems. The inclusion of ATP during the incubation failed to stimulate Chl synthesis, cysteine or a supply of NADH produced a slight stimulation, whereas a supply of NADPH either in the presence or absence of ATP again produced a dramatic increase in the Chl level.

Fig. 3 represents the difference in absorption of lysed etioplasts incubated in the presence of an NADPH regenerating system *minus* a similar sample incubated in the absence of such an NADPH supply. The series of curves represent the difference spectra obtained after various incubation times given in minutes. With increasing incubation time there is an increase in the

Table 2
Specificity for hydrogen donors and ATP of the reconstituted chlorophyll synthesising system

System	Chlorophyll
	(%)
No additions	36
1.5 mM ATP	38
5mM cysteine	98
ATP + NADH generating	95
ATP + NADPH generating	274
NADPH generating, no ATP	269

Water lysed etioplasts prepared from ALA fed plants were resuspended in isolation medium devoid of cysteine and ATP and incubated in the presence of various hydrogen donating systems overnight under flash illumination. Chlorophyll levels were estimated from the absorption spectra of the samples and are expressed as a % of the chlorophyll formed by illuminating the freshly isolated etioplasts. The basic incubating solution comprised the resuspending medium devoid of ATP and cysteine. Other details as for table 1.

absorption at 652 nm coupled with a decrease in the absorption at 633 nm suggesting a time and NADPH dependent conversion of P_{633} to P_{652}, cf. curve obtained after 10 min incubation with curve obtained after 95 min incubation.

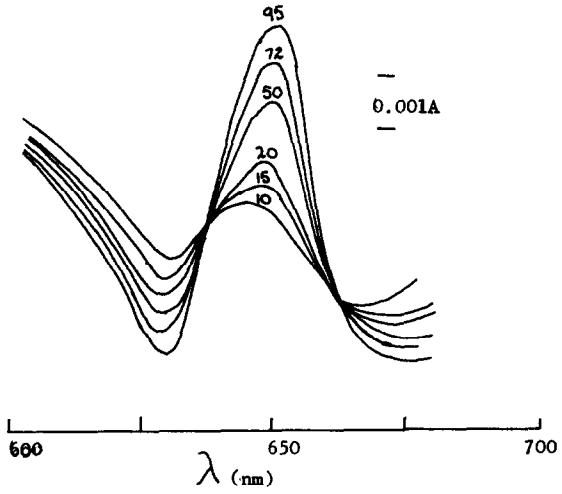

Fig. 3. NADPH induced absorbancy changes in etioplasts. Identical samples of water lysed etioplasts in measuring and reference beams (——)NADPH regenerating system added to sample in measuring beam and difference spectra recorded after the time intervals indicated in minutes.

4. Discussion

Etioplasts isolated from seven day old dark grown barley have already been shown to be capable of converting normally inactive P_{630}, whether of endogenous origin or derived from exogenous ALA, into chlorophyll by a flashing light technique (fig. 1) [15]. It is shown in the present studies that this process is inhibited by water lysing the etioplasts (table 1) but can effectively be reconstituted by incubating the depleted preparation with an NADPH regenerating system in the form of glucose 6-phosphate, NADP and glucose 6-phosphate dehydrogenase (fig. 2b). Other reductants failed to produce such a reactivation (table 2). It is worth noting that ATP also proved to be ineffective in this respect (table 2). This is in contrast to the specific effect of ATP on the conversion demonstrated in aged etioplasts by Horton and Leech [10]. An explanation for this discrepancy is currently being sought.

In the inactive preparation, PChl is present as P_{630} whereas in the NADPH reconstituted preparation the PChl is present as the active form, P_{652}, (fig. 3). This implies that reconstitution involves the NADPH dependent conversion of P_{630} to P_{652}. A direct demonstration of this conclusion is given by the difference spectra recorded in fig. 3. The series of time dependent difference spectra for the conversion reveal an isosbestic point for the change at 639 nm contrary to the results obtained with whole leaves [4]. Failure to detect an isosbestic point for the P_{630} to P_{652} reaction in whole leaves can probably be accounted for by interference from a combination of ultrastructural changes and complex Chl absorbancy changes, both of which are known to occur under the conditions where the transition has been studies in vivo [16].

These experiments identify for the first time NADPH as a likely reductant for Chl synthesis from PChl and also describe the relationship between P_{630} and P_{652}. The mechanism by which NADPH converts P_{630} into P_{652} is at present under study. Experiments are in progress designed to distinguish between the direct reduction of the P_{630} protein complex by NADPH to give P_{652} or, alternatively, the formation of a ternary NADPH–protein–PChl complex as the active PChl holochrome.

One possible site for the regulation of Chl synthesis in higher plants may be at the P_{630} to P_{652} reaction which may well be controlled by the NADPH/NADP ratios existing within the etioplast. Evidence for such a regulation in Chl synthesis is also currently being sought.

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