CHLOROPHYLL SYNTHESIS AND PROTOCHLOROPHYLLIDE REDUCTION IN THE BARLEY MUTANT *albina-f*¹⁷

by

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Plastids isolated from dark-grown leaves of the barley chlorophyll mutant *alb-f*¹⁷ contain only 40% of the protochlorophyllide reductase enzyme present in the wild-type. The low level of enzyme is functionally linked to the similarly low level of protochlorophyllide in whole leaves. The chlorophyllide in illuminated leaves fails to undergo the Shibata shift. However, when dark-grown shoots are fed δ -aminolaevulinate, resulting in accumulation of non-photoconvertible protochlorophyllide, the newly-formed chlorophyllide undergoes a Shibata shift (18). The rate of the Shibata shift is proportional to the amount of accumulated non-photoconvertible protochlorophyllide.

It has been suggested that *alb-f*¹⁷ is blocked in the synthesis of esterified protochlorophyll and chlorophyll. It is shown that prolonged incubation of illuminated mutant leaves, whether or not fed with δ -aminolaevulinate, results in a significant accumulation of chlorophyll. The data support the view that the primary lesion is in the control of δ -aminolaevulinate synthesis.

1. INTRODUCTION

Higher plants grown in the dark accumulate the chlorophyll precursors protochlorophyllide (pchlide) and protochlorophyll (pchl). Illumination results in the photoconversion of pchlide to chlorophyllide (chlide) which subsequently becomes esterified to chlorophyll (chl) (25). The understanding of this process has been significantly advanced by the use of cell-free preparations of etioplasts (1, 5, 7, 8, 19, 20, 21). Photoconversion of pchlide is catalysed by NADPH:protochlorophyllide oxidoreductase (E.C. 1.4.99) (abbreviated reductase) (8). The protein has an apparent molecular weight of

38,000 in barley (1, 20). NADPH is the specific hydrogen donor. Pchl is not reduced by the enzyme (7, 8).

In leaves and extracts pchlide exists as two functionally distinct molecular forms characterised by red absorption maxima at 630nm and at 650 + 638nm (14). Illumination of pchlide_{650/638} results in the immediate formation of chlide absorbing at 678nm, chlide₆₇₈. In leaves or in extracts which have been supplemented with NADPH, chlide₆₇₈ shifts within a few seconds to a form absorbing at 684nm, chlide₆₈₄ (3, 21, 24). Addition of NADP⁺ or glycerol to extracts immediately after illumination stabilises the

Abbreviations: ALA = δ -aminolaevulinate; chl = chlorophyll; chlide = chlorophyllide; chlide₆₇₈ = pigment complex having red absorption maxima at indicated wavelength; pchl = protochlorophyll; pchlide = protochlorophyllide; reductase = NADPH:protochlorophyllide oxidoreductase.

Scheme 1. Proposed mechanism of the inter-conversions of the spectral forms of pchl_a and chl_a seen in etiolated and illuminated leaves.

678nm form (5, 21). In leaves and in unsupplemented extracts, chl_{ide}⁶⁸⁴ undergoes a hypochromic shift forming chl_{ide}⁶⁷²: in leaves this is referred to as the Shibata shift (21, 23). On the basis of these and other observations using semi-purified protochlorophyllide reductase preparations, Scheme 1 was proposed to account for the observed spectral forms of pchl_{ide} and chl_{ide} (5, 21). In this scheme pchl_{ide}^{638/650} is depicted as a ternary complex of the reductase, pchl_{ide} and NADPH; chl_{ide}⁶⁷⁸ as a complex of the reductase, chl_{ide} and NADP; chl_{ide}⁶⁸⁴ as a complex of the reductase, chl_{ide} and NADPH. Pchl_{ide}⁶³⁰ and chl_{ide}⁶⁷² are depicted as free or non-specifically bound pigment. The pchl_{ide} complexes are more stable than the chl_{ide} complexes and free pchl_{ide} can displace chl_{ide} from the reductase. This displacement is thought to be the driving force causing the Shibata shift (18). It ensures the availability of the reductase for continued chlorophyll synthesis in the light.

Pchlide reductase is the most abundant membrane-bound polypeptide found in etioplasts (19). The concentration of the enzyme undergoes a dramatic decline soon after the onset of illumination (17). A molecular mechanism to account for this decline in both enzymic activity

and protein quantity has been proposed (15). It has been found that NADPH, pchlide and, to a lesser extent, NADP protect the enzyme from proteolytic breakdown. The level of the enzyme, therefore, reflects the concentrations of its substrates. The steep decline in the concentration of pchlide in illuminated plants is therefore the primary cause of the illumination-induced decline in the concentration of the reductase.

The barley nuclear gene mutant *albina-f¹⁷* (28) lacks the Shibata shift and is unable to disperse the prolamellar bodies of the etioplasts into primary lamellar layers (10, 27, 29). The mutant accumulates considerably less pchl_{ide} than normal (30 – 40%) and no pchl. There is no discernable pchl_{ide}₆₃₀ and virtually all the pchl_{ide} is converted by a single flash (11, 18). NIELSEN (18) investigated the effect of ALA-feeding on the spectral forms in the mutant. Non-photoconvertible pchl_{ide}₆₃₀ was formed and the spectra of fed, illuminated leaves revealed – like in wild type – an absorption of newly formed chl_{ide} at 675 nm which was subsequently shifted to 672 nm. Unlike in the wild type esterification of chl_{ide} was not detectable in the mutant after 10 minutes (18) or up to 5 hours at 20 °C in darkness (11). The lack of the Shibata shift is apparently not due to a defect in the pchl_{ide} reductase as chl_{ide} absorption after illumination of partially purified reductase of the mutant undergoes the blue shift from 678 to 672 nm which is characteristic for the corresponding wild type preparations (11, 18).

In view of these differences between mutant and wild-type, the mutant provides an ideal system for checking the validity of the hypothesis outlined in Scheme 1. In this study we have investigated the spectral forms of pchl_{ide} and chl_{ide} in *albina-1*⁷ with reference to the nature of the Shibata shift and the factors controlling the level of the reductase protein (15). It is shown that the level of the reductase protein in the mutant leaves is low in accordance with the reduced level of pchl_{ide}. The absence of the Shibata shift in whole leaves has been confirmed, as has the synthesis of not photo-convertible pchl_{ide} after feeding of ALA to leaves. The chl_{ide} in illuminated, fed leaves underwent a Shibata shift and the rate of the shift was found to correlate with the amount of

unphotoconverted pchl_{ide}. Incubation of photoconverted, ALA-fed leaves led to the accumulation of significant quantities of esterified chl.

The characteristics of *albina-f'* are consistent with the hypothesis that the primary mutation is in the control of ALA synthesis. In the dark, low amounts of ALA are synthesised. Illumination of dark-grown leaves does not activate ALA synthesis.

3. MATERIALS AND METHODS

3.1. Plant Material

The nuclear gene mutant *alb-f'* and the mother type, *Hordeum vulgare* L. Svalöf's Bonus (28), were gifts from Professor D. VON WETTSTEIN. The seeds were surface sterilised in 1% (v/w) sodium hypochlorite for 10 min and then washed in tap water for 4 hours. The seeds were planted in trays of sterilised Levingtons' compost and grown for 7 days in darkness (7). Homozygous mutant leaves were selected by removing the apical 1 cm for examination in dim blue light. The remainder of the leaves were shielded from the light by black cloths. The leaves were homogenised using the Polytron homogeniser (Northern Scientific, Preston, UK) and total etioplast membranes were isolated by differential centrifugation (7).

3.2. Spectrophotometry

Spectra of etioplast membranes, resuspended in isolation buffer, and of intact leaves were recorded using the split-beam instrument already described (13). Interconversion of the spectral forms of chl_{ide} was followed using a dual-wavelength spectrophotometer set to measure absorbance differences at 690nm minus 710nm ($\Delta A_{690-710nm}$). The chl_{ide} forms absorbing at 678nm and 684nm have a maximum absorbance difference at 690nm. 710nm is the closest isosbestic point. Also the shift from 684 nm to 672 nm can be efficiently monitored by the absorbance difference at these two wavelengths. Actinic illumination was provided by a 10 sec pulse from a 55W quartz-halogen lamp fitted with a red cut-off filter (Wratten) giving a luminous intensity of $0.2W \cdot cm^{-2}$.

3.3. Analytical techniques

Membrane samples were analysed for polypeptide composition using 12% polyacrylamide sodium dodecylsulphate gel electrophoresis (16). Pigments were assayed by homogenising leaves in acetone containing some MgCO₃ using the Polytron homogeniser. Spectra were recorded after centrifugation. Absorption coefficients quoted by JONES (12) were used. Esterified and non-esterified pigments were separated according to TREFFRY (26). The efficiency of the phase separation was checked using paper chromatography developed in ethanol-free redistilled chloroform. Pchl_{ide} reductase activity was assayed spectrophotometrically as described before (8). The unit of activity is defined as the formation of 1 nmole chl_{ide} $\cdot min^{-1}$. Protein was assayed according to BRAMHALL et al. (4).

3.4. Other materials and methods

NADPH was added as a regenerating system consisting of (final concentrations) NADP (0.5 mM), glucose-6-phosphate (4 mM) and glucose-6-phosphate dehydrogenase (0.1 U). For ALA feeding experiments, cut ends of shoots were placed in 10 mM-ALA, pH 6.8 for 1-9 hours in darkness. All chemicals were of analytical grade and obtained from sources described earlier (7).

4. RESULTS

4.1. In vitro experiments

Spectra of the membrane samples, isolated from wild-type and mutant leaves are shown in Figure 1. Before photoconversion both membrane samples have a broad absorption band with a peak at about 633nm and a pronounced shoulder at 650nm (curves A and C). Illumination of both samples initially produced chl_{ide}₆₇₈ (data not shown) which shifted over the next 30 min to produce chl_{ide}₆₇₂ (curves B and D). The spectra also reveal a large residue of unphotoconverted pchl_{ide}₆₃₀. The shift of the initial chl_{ide}₆₇₈ to chl_{ide}₆₇₂ was not prevented by the addition of NADPH (data not shown).

The pchl_{ide} reductase specific activity of the wild-type membranes was 2.61 units $\cdot mg^{-1}$ protein. The reductase activity of the mutant was much lower, 1.05 units $\cdot mg^{-1}$ protein.

Figure 1. Spectra of mutant and wild-type etioplast membranes. Curves A and B, wild type; curves C and D, *alb-f¹⁷*. Curves A and C, before illumination; curves B and D, after 10 sec illumination and 30 min incubation with 0.5 mM-NADPH. Both samples contain 2.4 mg protein in 2.5ml isolation buffer.

The polypeptide composition of the membrane samples is shown in Figure 2. Prominent bands of 53,000 and 38,000 are much reduced in the mutant. The 38,000 band probably corresponds to the reductase protein (1, 20).

4.2. In vivo experiments

The interconversion of the chl *a* spectral forms (678, 684 and 672 nm) following illumination of etiolated leaves can be monitored using the dual-wavelength spectrophotometer set to measure $\Delta A_{690-710\text{nm}}$ (21). The large initial increase in absorbance brought about by photoconversion of pchl *a* to chl *a*₆₇₈ necessitates resetting of the instrument. Thereafter, increases in absorbance are due to conversion of chl *a*₆₇₈

Figure 2. Polypeptide composition of *alb-f¹⁷* and wild-type etioplast membranes. Samples containing 0.1 mg protein were analysed by SDS polyacrylamide gel electrophoresis.

to chl *a*₆₈₄. Subsequent decreases in absorbance are due to the conversion of chl *a*₆₈₄ to chl *a*₆₇₂.

Figure 3 shows a typical trace obtained using wild-type leaves. After photoconversion there was a rapid rise in absorbance, taking 15-20 sec, followed by a slower fall, taking 30-35 min. This trace is qualitatively identical to those obtained earlier using oat leaves (21).

As expected, a quite different trace was ob-

Figure 3. The 690-710nm absorbance changes of illuminated etiolated wild-type barley leaves. The trace was recorded using the dual wavelength spectrophotometer. R indicates resetting the chart recorder which is necessitated by the large initial increase in absorbance elicited by the initial photoconversion of pchl_{ide}. Note the break in the time scale. Arrow indicates 10 sec illumination.

Figure 4. The 690-710nm absorbance changes of illuminated etiolated *alb-f'* leaves.

tained using mutant leaves (Figure 4). After illumination and the rapid 20 sec rise there followed only a small decrease in absorbance, taking 4-6 min. The absorbance spectra of these leaves, recorded 30 min after illumination show a peak at 684nm (Figure 5). This illustrates the stability of chl_{ide}₆₈₄ in the mutant leaves. The small peak at 628 nm is due to the β -peak of chl_{ide}. The absorbance of pchl_{ide} at this wavelength is very small.

ALA feeding induces the accumulation of non-photoconvertible pchl_{ide} (6). The effect of this pigment-form on the spectral forms of

Figure 5. Spectrum of *alb-f''* leaves 30 min after illumination.

chl_a in *alb-f''* was next investigated. Mutant and wild-type leaves were fed ALA for 1 to 9 hours. The dual-wavelength trace obtained with the sample of mutant leaves fed ALA for 2 hours is shown in Figure 6. After illumination there was, as expected, the rapid, 20 sec increase in absorbance. In contrast to the unfed leaves, this was followed, over the next 15 min, by a slow fall in absorbance (Figure 6). This is reminiscent of the behaviour of wild-type leaves. The spectrum of these ALA-fed, *alb-f''* leaves, taken 20 min after illumination (Figure 7), shows peaks at 672 nm, due to chl_a, and at 633 nm due to accumulated unphotoconverted pchl_a. A pronounced shoulder at 650 nm, in the spectrum, indicates the formation of new photoconvertible pchl_a.

In an attempt to quantify the relationship between the rate of the Shibata shift and content of unphotoconverted pchl_a, a number of traces such as Figure 6 were recorded using mutant and wild-type leaves fed with ALA for various times. The extent of the slow absorbance decrease varied widely, presumably because of a varying apparent optical path-length through the different samples of leaves. As estimate of the

Figure 6. The 690-710 nm absorbance changes of etiolated *alb-f''* fed ALA for the previous 2 hours.

rate of the Shibata shift we chose to measure the reciprocal of the time (in min) taken to complete half the slow absorbance change. Subsequently the pchl_a contents of the leaf samples were measured after extraction of the pigments into acetone. Figure 8 shows the relationship between the rate of the Shibata shift and the content of non-photoconverted pchl_a. A direct correlation between the content of residual, non-photoconverted pchl_a and the rate of the

Figure 7. Spectrum of *alb-f''* leaves, fed with ALA for 2 hours, 30 min after illumination.

Figure 8. Effect of Pchl content on the rate of the Shibata shift. Samples of *alb-f¹⁷* and wild-type leaves were fed ALA for 0-9 hours. The rate of the slow decline in 690-710nm absorbance was measured using the dual-wavelength spectrophotometer. The rate $(t_{1/2})^{-1}$ was calculated as the time (min) taken to undergo half the absorbance change. The pchl contents ($\text{pmol} \cdot \text{shoot}^{-1}$) of the leaves were subsequently measured after acetone extraction.

Shibata shift is immediately apparent.

The fate of chl₆₇₂ produced by ALA-fed mutant leaves was next investigated. Samples of mutant and wild-type leaves were excised and placed in beakers containing ALA or water and

incubated overnight. Then the leaves (maintained at 10 °C) were flash-illuminated for 2 min (1 flash/10 sec) with a xenon lamp fitted with a red-filter followed by 2 hours at a flash rate of 1 flash/80 sec. After incubation for a further 3 hours, the plants were homogenised in acetone. After separation of esterified and non-esterified pigments the contents of pchl_{ide}, chl_{ide}, pchl and chl were determined. The results shown in Table I indicate, as expected, that pchl_{ide} was accumulated by the ALA-fed samples. Chl was detected in all samples. Surprisingly, the major pigment found in the water-incubated *alb-f¹⁷* leaves was chl. The chl was positively identified on the basis of its solubility and absorption spectrum in light petroleum. In agreement with earlier data (18) no pchl was detected in any of the samples.

5. DISCUSSION

5.1. Activity of pchl_{ide} reductase in *alb-f¹⁷* leaves

KAY and GRIFFITHS (15) have shown that illumination of etioplast membranes results in the specific breakdown of the reductase polypeptide(s). This is presumably catalysed by a membrane-bound protease. The available evidence strongly suggests that it is only the naked enzyme (apo-enzyme) which is susceptible to attack. The substrates, NADPH and pchl_{ide}, afford protection against breakdown. Thus it may be predicted that the level of the reductase will vary with the concentration of its substrates. In agreement with this hypothesis, it has recently been demon-

Table I

The pchl_{ide}, chl_{ide} and chlorophyll contents of illuminated wild-type and *alb-f¹⁷* leaves

	<i>alb-f¹⁷</i>		wild-type	
	on ALA	Control	on ALA	Control
	$\text{pmol} \cdot \text{shoot}^{-1}$			
Pchl _{ide}	2400	8	2730	100
Chl _{ide}	50	3	125	86
Chlorophyll a	40	34	140	160

Samples of 7 day old *alb-f¹⁷* and wild-type barley leaves were incubated with 10 mM-ALA, pH 6.8 for 16 hours. The leaves were illuminated and incubated for 5 hours as described in the text. Subsequently, the leaves were homogenised in ammoniacal acetone and the pigments were assayed as described in the methods section.

strated that etiolated oat plants which have accumulated pchl_{ide} by prior ALA-feeding, do not undergo the characteristic light-induced loss of reductase activity (9). Thus the substrates can also protect the enzyme against breakdown *in vivo*.

The nature of the primary lesion affecting chl biosynthesis in *alb-f'* has not been directly established. However, the evidence presented herein supports NIELSEN'S suggestion that the mutation does not cause a lesion in the reductase but affects the control of ALA biosynthesis. This results in the accumulation of only about 30 – 40% of the wild-type content of pchl_{ide} during dark growth. As a consequence of this there is only approximately 40% of the wild-type level of the reductase. Indeed, the prominent 38,000 mol. wt. protein found in wild-type etioplast membranes previously identified as the reductase protein (1, 20) (Figure 2) is much reduced in the mutant. The similar reductions in pchl_{ide} level, reductase activity and reductase protein in the mutant strongly support the contention that the level of the reductase is limited by the concentration of pchl_{ide}.

In vivo spectra of wild-type dark-grown leaves typically show a prominent shoulder at 630nm in addition to the major peak at 650nm (14). This 630nm absorbance has been attributed to free, or non-specifically bound and therefore non-photoconvertible pchl_{ide}. Photoconvertible pchl_{ide} which absorbs at 650nm (plus a shoulder at 638nm) is believed to be a ternary complex of reductase, NADPH and pchl_{ide} (5, 7). In contrast, the mutant leaves show only a very slight shoulder at 630nm (18). Furthermore, illumination of the mutant leaves results in the almost 100% photoconversion of the pchl_{ide}, while in the wild-type, only 60-70% of the pchl_{ide} is photoconverted by a saturating flash. These data suggest that all of the pchl_{ide} in the mutant is bound in substrate sites of the reductase enzyme. In the wild-type the presence of excess pchl_{ide} implies that the accumulation of the reductase during growth in the dark has reached a peak value and there is pchl_{ide} which could stabilise some more reductase. It would be interesting to investigate the turnover of the reductase protein in *alb-f'*. It may be that feeding small amounts of ALA to the mutant

might result in the stabilisation of wild-type levels of the reductase.

5.2. The spectral forms of chl_{ide} in *alb-f'*

alb-f' was initially characterised as a chlorophyll mutant in barley which lacked the Shibata shift and was blocked in the dispersal of the prolamellar body (10, 27, 29). Subsequent investigation showed that it contained no non-photoconvertible pchl_{ide} and that the chl_{ide} formed on illumination failed to become esterified in the dark (11, 18). In the interpretation of the various spectral forms of chl_{ide} (5, 18, 21) it was suggested that the Shibata shift is caused by free pchl_{ide} displacing enzyme-bound chl_{ide}₆₈₄. Therefore, in the illuminated, etiolated leaves of *alb-f'*, the absence of free pchl_{ide} would lead to the absence of the Shibata shift (Figures 4 and 5). Furthermore, if the etiolated leaves of the mutant are induced to accumulate free pchl_{ide} by feeding ALA, the chl_{ide} formed on illumination undergoes a Shibata shift (18) (Figures 6 and 7).

An attempt to investigate the quantitative relationship between the rate of the Shibata shift and free pchl_{ide} content was carried out. The data (Figure 8) show a high degree of scatter which may be inevitable when measuring absorbances of collections of relatively inhomogeneous leaves, especially those which have accumulated large quantities of pchl_{ide}. In spite of this, the data (Figure 8) indicate a direct relationship between the rate of the Shibata shift and free pchl_{ide} contents as predicted by Scheme 1.

Unlike whole leaves, spectra of the plastid membranes isolated from the wild-type and *alb-f'* display rather similar properties. Both show a relatively high proportion of non-photoconvertible pchl_{ide}₆₃₀ (Figure 1), compared to the spectra of whole leaves (18). Illumination of the isolated membranes in both cases produced chl_{ide}₆₇₈ which over a period of 30 min changed to chl_{ide}₆₇₂. It is apparent that the large excess of free pchl_{ide} present in both membrane preparations, prevented the stabilisation of any enzyme-bound chl_{ide} complexes. The free pchl_{ide} arises during the procedure of purifying the membranes and is considered an artefact. De-

spite the failure to prepare the membranes in a native condition, the experiment supports our interpretation that free pchl_{ide} displaces chl_{ide} from the enzyme.

5.3. Esterification of chl_{ide} and pchl_{ide}

The earlier studies of *alb-f*¹⁷ (11, 18) failed to detect any traces of esterified pchl or chl, which lead to the suggestion that the primary lesion in *alb-f*¹⁷ affected the esterifying enzyme, chlorophyll synthetase (2, 22). The absence of the Shibata shift was correlated with the absence of esterification. This implied that esterification of chl_{ide} caused the Shibata shift. NIELSEN showed that in ALA-fed leaves the Shibata shift occurred very rapidly, well before the accumulation of esterified chl (18). Thus esterification could not be the cause of the Shibata shift. This left open the question of whether the mutant could esterify chl_{ide}. In NIELSEN'S experiments the leaves of buffer- and ALA-fed mutant and wild-type leaves were incubated for only 10 min after illumination before extraction of the pigments. In the present experiments, where considerable quantities of chl were detected in both control and ALA-fed, illuminated *alb-f*¹⁷ leaves were incubated for 5 hours with additional intermittent illumination after photoconversion (Table I). This suggests, contrary to earlier conclusions (11) that active chl synthetase can be formed in the mutant plastids.

In our scheme for the action of pchl_{ide} reductase it is apparent that before chl_{ide} can become esterified, it must dissociate from the reductase – a process now demonstrated to be firmly correlated with the Shibata shift. However, no Shibata shift occurred in the mutant under conditions in which it is readily observed in the wild-type (Figure 4). Nevertheless, during the prolonged incubation period used to detect the accumulation of chl, it is likely that slow, pchl_{ide}-independent dissociation of the chl_{ide} from the reductase occurs. This would allow the synthetase access to the chl_{ide}. Therefore, during these prolonged incubation a slow hypochromic shift of the chl_{ide}₆₈₄ may well occur in the *alb-f*¹⁷ leaves.

The nature of the primary lesion affecting chl biosynthesis in the mutant remains unknown.

ALA synthesis is depressed in the dark resulting in a lower content of pchl_{ide} which limits the amount of pchl_{ide} reductase. This study has indicated that the Shibata shift can be elicited by providing additional pchl_{ide} substrate in the leaf during the isolation of membranes and esterification of chl_{ide} by prolonged intermittent illumination of the leaf. Furthermore, the hypothesis regarding the nature of the spectral forms of chl_{ide} and pchl_{ide} has enabled the reconciliation of the differences between the wild-type and the mutant on the basis of a reduced pchl_{ide} level in the mutant.

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