Biochem. J. (1986) 236, 741–748 (Printed in Great Britain)

Photoreduction of protochlorophyllide and its relationship to δ-aminolaevulinic acid synthesis in the leaves of dark-grown barley (*Hordeum vulgare*) seedlings

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The photoreduction of protochlorophyllide (Pchl) in dark-grown leaves of barley (*Hordeum vulgare*) brings about the synthesis of δ-aminolaevulinic acid (AmLev). Manipulation of the Pchl level in the leaves by incubation in AmLev indicated that the production of AmLev was intimately related to the state of the Pchl reductase ternary complex. Free Pchl reductase that is unassociated with substrate/product appeared at first to be essential for the photoinduction of AmLev synthesis. Experiments on the photoreduction of Pchl in dark-grown leaves exposed to low-energy red-light, however, showed that photoreduction and AmLev synthesis would occur when the Pchl reductase, together with substrate, was maintained at relatively high endogenous concentration. Under such conditions the availability of free reductase protein would be negligible. An alternative scheme is presented, therefore, that can explain many, if not all, of the observations on AmLev synthesis and its close relationship to Pchl reduction, and which is based on a common supply of NADPH for the reduction of glutamate to AmLev and the synthesis of chorophyll(-ide).

INTRODUCTION

The first, relatively stable, characteristic precursor of chlorophyll in the leaves of higher plants is the aminoketone δ-aminolaevulinic acid (AmLev). The etiolated leaves of dark-grown cereals, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), contain appreciable protochlorophyllide (Pchl) that is largely present in a photoactive form which, in the presence of light, is photoreduced to chlorophyll(-ide) (Klein *et al.*, 1977). The phototransformable species of Pchl is present as a ternary complex together with the Pchl reductase protein and the co-substrate, NADPH (Brodersen, 1976; Griffiths *et al.*, 1976). If dark-grown leaves are exposed to light, the photoreduction of the Pchl occurs, and within a short time new Pchl is synthesized (the so-called 'PChl regeneration'; Virgin, 1955). The protein-bound Pchl rapidly builds back up to almost the same level as that present in the leaves before the light treatment and then ceases. The re-formation of the Pchl after the light treatment is due to a stimulation in AmLev synthesis (Augustinussen & Madsen, 1965; Stobart *et al.*, 1972; Virgin, 1984). The reaction steps between AmLev and Pchl appear to be non-limiting (Nadler & Granick, 1970; Stobart *et al.*, 1972), and it is considered that a major site for regulation by light is at the level of AmLev formation (Fluhr *et al.*, 1975; Klein *et al.*, 1977). The most logical candidate to exert control over AmLev synthesis is Pchl (Bogorad, 1976; Klein *et al.*, 1977; Gough, 1978; Ford & Kasemir, 1980) and we have suggested that, in chlorophyll synthesis in etiolated leaves given brief light-treatments, the primary involvement of light may only be in the photoreduction of the bound Pchl and that the stimulation in AmLev formation is a consequence of this (Stobart & Ameen-Bukhari, 1984). In the present paper we report further evidence suggesting an intimate relationship between the state of the Pchl–protein complex and AmLev synthesis in etiolated barley leaves and suggest a model that can explain many of the observations on AmLev formation and the photoreduction of bound Pchl.

MATERIALS AND METHODS

Plant material

Barley (*Hordeum vulgare* L.) seeds were soaked in water overnight and, after sowing in moist Perlite, allowed to germinate in the dark at 25 °C. The top 3 cm of the primary leaf at the desired age was removed and usually used in groups of 5 (approx. 0.2 g) per treatment. All manipulations were carried out in a darkroom under a dim green safelight.

AmLev extraction and estimation

Laevulinic acid (Lev), a competitive inhibitor of AmLev dehydratase, was used to block the utilization of AmLev in chlorophyll synthesis in the leaf segments (Harel & Klein, 1972). Segments were placed with their cut ends in the test solution or Lev (80 mM) in glass vials in the dark. After the desired treatment the AmLev was extracted from the segments by boiling for 15 min in 4% (w/v) trichloroacetic acid and determined as the pyrrole–Ehrlich's reagent complex after condensation with ethyl acetoacetate (Stobart & Ameen-Bukhari, 1984).

Pchl extraction and determination

Leaf segments (0.2–0.4 g. fresh wt.) were extracted in cold acetone/water (4:1, v/v) in the dark with the aid of

Abbreviations used: AmLev, δ-aminolaevulinic acid; Pchl, protochlorophyllide; Lev, laevulinic acid.

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a dim green safelight. Well over 95% of the pigment was recovered in one extraction. A_{626}, A_{645} and A_{663} were used to calculate total Pchl (free and bound Pchl are estimated as total Pchl after extraction in acetone) in equations derived from those of Anderson & Boardman (1964).

Spectroscopy of the leaves *in vivo*

Leaf segments were inserted in a special glass cuvette, in the dark, to give a light path of one leaf thick. The loaded cuvette was placed in a double-beam spectrophotometer and the absorption recorded from 600 to 700 nm against a filter-paper blank (Stobart *et al.*, 1972).

Light treatment

The photoreduction of all the endogenous bound and photoactive Pchl was achieved by exposing the leaves to white fluorescent light ($370\ \mu E \cdot m^{-2} \cdot s^{-1}$) for 30 s. Low-energy red-light (about $1\ \mu E \cdot m^{-2} \cdot s^{-1}$) was provided from a modified Hilger and Watts monochromator by passing the light beam through a neutral-density filter.

RESULTS

Utilization of AmLev in dark-grown leaves

Many of the anticipated experiments required the formation of bound Pchl directly from a pool of free Pchl rather than from endogenously synthesized AmLev. This would permit the precise manipulation of the bound Pchl and free reductase protein content in the leaves and enable one to study the effect of this on AmLev accumulation in the presence of Lev. The increase in free Pchl could only be achieved, however, by incubating the leaves in AmLev. It was therefore essential to establish whether conditions existed under which it was possible to measure the endogenous formation of AmLev after exogenously supplied AmLev has entered the leaf.

The cut ends of the top 3 cm of 7-day-old dark-grown leaves were stood in AmLev (1 mM) for 2 h in the dark and, after rinsing, they were transferred to distilled water for a further 2 h. At regular intervals throughout the incubation the AmLev in the leaves was determined. The results (Fig. 1) show that the AmLev rapidly entered the cut leaves at an almost linear rate and reached a maximum of 100 nmol/g fresh wt. above that of the AmLev present in control tissue treated with distilled water only (< 2 nmol/g fresh wt.). Within 20 min of transferring the leaf segments from AmLev to water, the AmLev content decreased rapidly and was some 50% of the observed maximum concentration in the tissue. After 2 h in water the AmLev was only slightly greater than that in the leaves which were given no prior treatment in AmLev. Leaves incubated in AmLev for 4 h, however, maintained the high level of Amlev.

The results indicate that any Amlev which is made available to, or even synthesized in, the leaf is probably utilized at a relatively rapid rate. Dark-grown leaves incubated in AmLev normally accumulate excessive quantities of free Pchl with an absorption maximum *in vivo* at 630 nm (Stobart *et al.*, 1972), and other workers report other metabolic fates for AmLev (Gassman *et al.*, 1978; Duggan *et al.*, 1982). Further experiments were therefore conducted to assess the efficiency of free Pchl formation from exogenously supplied AmLev.

Leaf tips (top 3 cm) from the dark-grown leaves were pre-incubated for 2 h in the dark in either water or Lev (80 mM) and then transferred after washing to AmLev (1 mM). The Amlev and the total Pchl in the leaf segments were determined at regular intervals. The results (Fig. 2) show that, in tissue that was treated with water before transfer to AmLev, the AmLev accumulated to about 80 nmol/g fresh wt. after 40 min and remained at this level with further time. The Pchl, on the other hand, increased at a linear rate throughout the incubation in AmLev, and after 3 h its level was nearly 3-fold greater than that in the leaves before transfer to AmLev. In leaf segments first treated with Lev before incubation in AmLev there was a rapid accumulation of AmLev, which continued throughout the experiment with little change in the Pchl content. The results show that Lev efficiently blocked the utilization of supplied AmLev and, under these conditions, AmLev accumulated. It is also evident that the quantity of Pchl in the leaves treated with water before transfer to AmLev was equivalent to the AmLev which accumulated in the Lev-treated leaves.

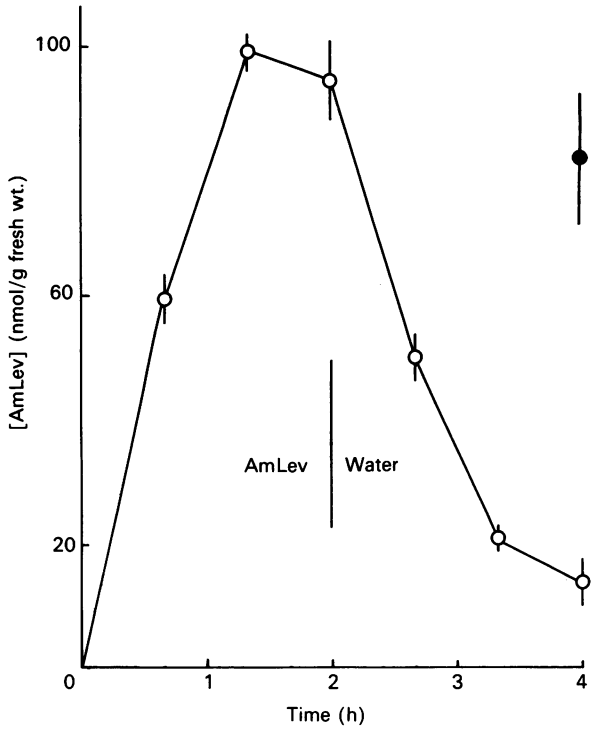


Fig. 1. AmLev uptake and utilization

Leaf segments of 7-day-old dark-grown seedlings were stood in AmLev (1 mM) for 2 h and then transferred to water. The AmLev in the leaves was determined at regular intervals (○). Control segments were stood in AmLev for 4 h and their AmLev content determined (●). The results are means (±S.D.) for three replicates.

Effect of Pchl on AmLev production

The above experiments demonstrate that it is possible to manipulate quite precisely the free Pchl in the leaves by incubation in AmLev and to investigate the effect of this on the biosynthesis of endogenous AmLev. The rationale behind such experiments is to produce in the leaves amounts of free Pchl that will, after a light treatment to reduce the photoactive species of Pchl (i.e. Pchl bound to the Pchl reductase protein and which has

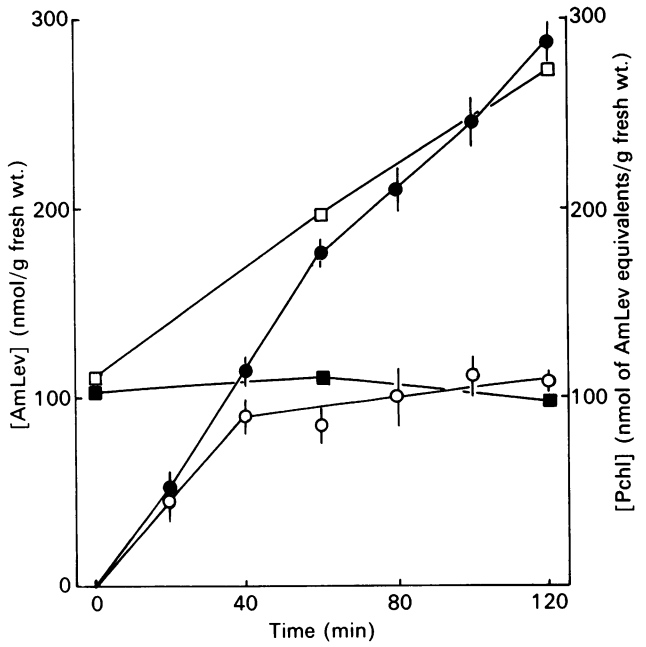


Fig. 2. Effect of Lev on the accumulation of exogenously supplied AmLev and the production of Pchl

Leaf segments of 7-day-old dark-grown seedlings were pre-incubated for 2 h in the dark in either water or Lev (80 mM) and then transferred to AmLev (1 mM). At regular intervals the [AmLev] and total [Pchl] in the tissues were determined. Symbols used: ○ and □, AmLev and Pchl in the water controls respectively; ● and ■, AmLev and Pchl in the Lev-treated segments respectively. The results for AmLev are means (±S.D.) for three replicates.

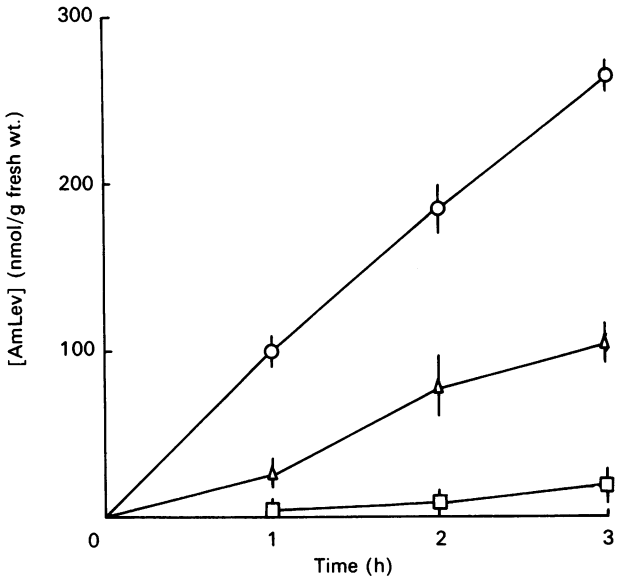


Fig. 3. Effect of Pchl on AmLev production

Leaf segments of 7-day-old dark-grown seedlings were incubated in the dark in AmLev (1 mM) for 1 and 2 h and transferred to water for 2 and 1 h respectively, followed by a further 2 h in Lev (80 mM). After a brief light treatment the [AmLev] in the leaves was determined at regular intervals. Control leaf segments were incubated in water for 3 h followed by 2 h in Lev. Symbols used: ○, water controls; △, 1 h in AmLev; □, 2 h in AmLev. The results are means (±S.D.) for three replicates.

an absorption maximum at 652 nm *in vivo*), rapidly recharge the reductase enzyme.

Leaf segments were incubated in the dark in AmLev for 1 and 2 h and transferred to water for 2 and 1 h respectively, followed by a further 2 h in 80 mM-Lev. At the end of the incubation sequence the leaves were given a brief light treatment of just sufficient duration to photoreduce almost all the endogenous photoactive Pchl. At intervals thereafter the AmLev in the leaves was determined. Control leaf segments were incubated in water for 3 h, followed by 2 h in Lev. The total Pchl in the leaves at the end of the Lev treatment and before exposure to light was 14, 34 and 42 nmol/g fresh wt in the water controls, the 1 h and 2 h AmLev treatments respectively. The major species of Pchl in the water-control leaves, as judged from spectroscopy *in vivo*, was the photoactive form with an absorption maximum at 650 nm, whereas the additional Pchl in the AmLev-treated leaves was the free non-photoactive species absorbing at 630 nm. At 30 min after the light treatment, 14, 33 and 86% regeneration of bound Pchl had occurred in the water, 1 and 2 h AmLev treatments respectively. Almost the same amounts of chlorophyll, as judged by spectroscopy *in vivo*, were present in the leaves treated with water and AmLev after the light-treatment. The accumulation of AmLev in the leaves after the light treatment and return to the dark is given in Fig. 3. In the control tissue, AmLev accumulated at an almost linear rate, and at the end of the experiment had reached a final concentration of some 260 nmol/g fresh wt. However, in the leaf segments that contained the highest Pchl (42 nmol/g fresh wt), the AmLev present in the leaves after the light treatment was always extremely low. On the other hand, leaf segments with the intermediate Pchl produced, after the light treatment, substantial AmLev and this accumulated at a rate (~ 42 nmol/h) that was almost half that observed in the water-control tissue.

The results show that the amount of free Pchl in the leaves at the onset of a brief light treatment appears to influence the accumulation of AmLev in the presence of Lev. The excess free Pchl in the leaf regulates, in these experiments, the availability of substrate-free Pchl reductase protein present after the light treatment. In the leaves with a large pool of free Pchl there is a rapid recharging of the reductase protein with substrate after the photoreduction, and under these conditions very little AmLev was found to accumulate.

These proposals were further investigated in experiments in which the level of free Pchl was manipulated so that there would be just sufficient substrate present to fill a limited number of binding sites on the reductase protein. Leaf segments were incubated in AmLev for 25 and 75 min and then transferred to Lev for a further 2 h. The total Pchl in the AmLev-treated leaves was 19.1 ± 0.4 and 28.3 ± 0.6 nmol/g fresh wt., and this compared with 15.0 ± 0.4 nmol/g fresh wt. in the water-treated control tissue. After a brief illumination, the Pchl present in the control and AmLev treatments was 2, 5 and 9 nmol/g fresh wt., respectively. Most of the Pchl at this stage was present as the bound, photoactive, species as evidenced by spectroscopy of the leaves *in vivo*. The AmLev that accumulated in the leaves after the light treatment is given

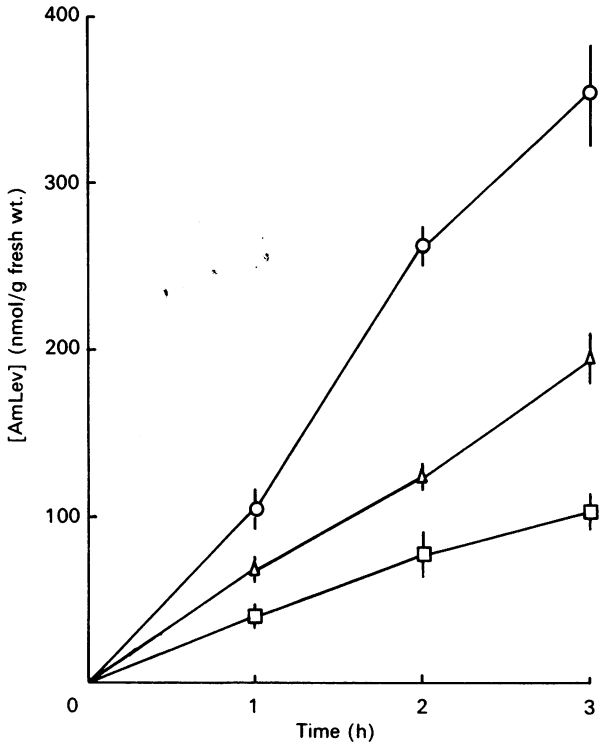


Fig. 4. Effect of relatively low free Pchl on AmLev production

Leaf segments of 7-day-old dark-grown seedlings were incubated in AmLev (1 mM) for 25 and 75 min and then transferred to Lev (80 mM) for 2 h. After a brief light treatment the AmLev in the leaves was determined at regular intervals. Control leaf segments were incubated in water for 75 min before transfer to Lev. Symbols used: ○, water controls; △, 25 min in AmLev; □, 75 min in AmLev. The results are means (±S.D.) for three replicates.

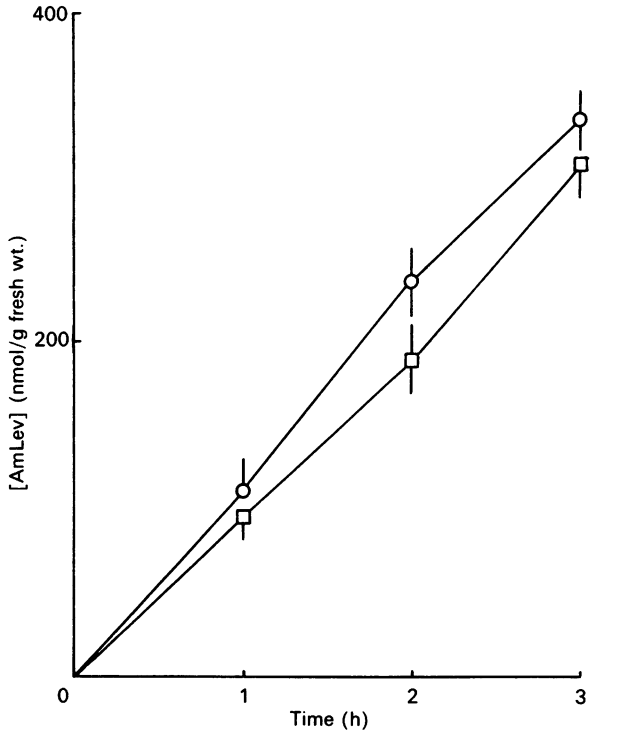


Fig. 5. AmLev production in high-Pchl-containing leaves given sufficient light to photoreduce all the Pchl

Leaf segments of 7-day-old dark-grown seedlings were incubated in AmLev (1 mM) for 3 h and then transferred to Lev (80 mM) for a further 2 h. After a light treatment of sufficient duration to photoreduce all the Pchl, the leaves were returned to the dark and the AmLev determined at regular intervals thereafter. Control leaf segments were incubated in water for 3 h before transfer to Lev. Symbols used: ○, water controls; □, 3 h in AmLev. The results are means (±S.D.) for three replicates.

in Fig. 4. The rates of AmLev accumulation in the water controls and the leaves which contained 5 and 9 nmol/g fresh wt. of bound Pchl were 125, 66 and 28 nmol/h per g fresh wt. respectively. The results show that the synthesis of AmLev in dark-grown leaves given a light treatment appears to be governed by the photoreduction of Pchl and perhaps the state of the Pchl–reductase protein complex.

This was further substantiated in experiments in which dark-grown leaf segments were incubated in AmLev to elevate substantially the free Pchl. Segments given AmLev for 3 h and transferred to Lev for a further 2 h contained 40 nmol/g fresh wt., and this compared with 15 nmol/g fresh wt. in control leaves. The leaves were given a 5 min light treatment of sufficient intensity to photoreduce the bound Pchl and bring about the further binding of the free Pchl to the reductase protein and its subsequent reduction. Absorption spectra of the leaves *in vivo* indicated that the AmLev-treated tissues contained over twice as much chlorophyll(-ide) as the water controls. Little difference, however, was observed between the accumulation of AmLev after the light treatment in leaves that had contained the high free Pchl content and that in the water-control tissue (Fig. 5). The explanation for these observations is that in this experiment all the bound and free Pchl had been utilized in chlorophyll(-ide) synthesis, leaving similar amounts of substrate-free reductase protein. Again, it seems that the production of AmLev is dependent upon conditions that favour the appearance of free reductase protein in the leaves.

Effect of glycerol on the 'Shibata shift' and AmLev production

Characteristic absorption changes *in vivo* are associated with the photoreduction of Pchl and the synthesis of chlorophyll(-ide) in dark-grown leaves given a light flash. The absorption at 652 nm due to the photoactive species of Pchl disappears on photoreduction and is replaced by a major band at ~ 680 nm. In the dark the absorption moves from 680 to 670 nm. The movement in absorption towards the blue end of the spectrum is referred to as the 'Shibata shift' (Shibata, 1957) and is associated with the release of photoproducts from the reductase protein (Broderson, 1976; Griffiths, 1975). The Shibata shift, therefore, results in the appearance of free reductase protein that is unassociated with substrate and/or product. Broderson (1976) showed that the shift and the release of chlorophyllide from the reductase could be slowed down considerably in etioplast preparations by increasing the viscosity of the medium with glycerol. Similar experiments were performed with dark-grown leaves. The segments were incubated in glycerol (1.5 M)

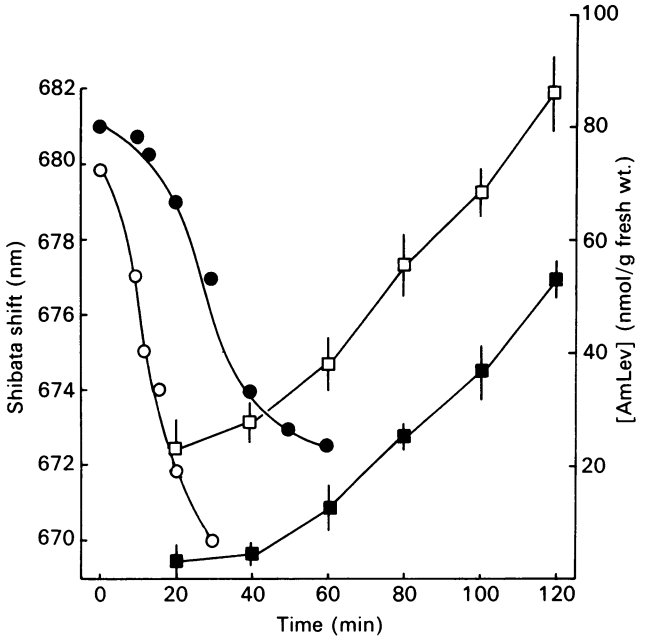


Fig. 6. Effect of glycerol on the Shibata shift and the accumulation of AmLev

Leaf segments of 7-day-old dark-grown seedlings were incubated in glycerol (1.5 M) containing Lev (80 mM) for 4 h in the dark. The segments were given a brief light treatment to photoreduce all the Pchl and the Shibata shift (movement towards the blue end of the spectrum) monitored at regular intervals. Similarly treated segments were assayed for AmLev. Control leaf segments were incubated in Lev only for 4 h before exposure to light. Symbols used: ○ and □, Shibata shift and AmLev production in water controls respectively; ● and ■, Shibata shift and AmLev production in the glycerol treatments. The results for AmLev are means (±S.D.) for three replicates.

containing Lev (80 mM) for 4 h and then given a brief light treatment. The results of a typical experiment show (Fig. 6) that the glycerol slows down the Shibata shift *in vivo*. In the glycerol-treated leaves the shift took some 30 min longer to complete than it did in the water-control tissue. The accumulation of AmLev in the glycerol treatments was also slower than observed in the water controls. These observations show that the photostimulation in AmLev synthesis is not in itself due to the reduction of Pchl, since a similar photoreduction had occurred in both the glycerol- and water-treated leaves. Rather the dissociation of the complex between reductase protein and its substrates/products appears to be the factor that is intimately involved in regulating AmLev synthesis.

Effect of low-energy light on Pchl reduction and AmLev synthesis

In the above experiments the most characteristic feature related to the stimulation in AmLev synthesis appeared to be the presence of free Pchl reductase protein. A further plausible candidate as an effector of the AmLev synthetase system, however, must be considered. The photoreduction of Pchl utilizes NADPH, which is bound together with Pchl in a ternary complex with the reductase protein (Griffiths *et al.*, 1976). After a light treatment to photoreduce the Pchl, the resulting $NADP^+$ is also released from the reductase and is re-reduced by a glucose-6-phosphate dehydrogenase which is associated with the prelamellar body (Griffiths, 1976). The NADPH/$NADP^+$ ratio, which changes after the photoreduction of Pchl in etioplastids (Griffiths & Mapleston, 1978) could be involved, therefore, in regulating the activities of the enzymes that synthesize AmLev. Experiments were designed to see if it was possible to photoinduce AmLev production in the leaves without the appearance of free reductase protein. It may be possible, under the correct conditions of illumination, to photoreduce the Pchl at a similar rate to its synthesis. Under such conditions the Pchl–reductase complex would remain in a steady state and at the same high endogenous concentration found in the etiolated leaf. Leaf segments from 7-day-old dark-grown seedlings were exposed to red light of a low intensity and at regular intervals the absorption spectrum *in vivo* between 600 and 700 nm was recorded. The results (Fig. 7) show that, in low-energy light, the photoreduction of Pchl will occur at a slow rate and that the photoactive species of Pchl remains at a high and relatively constant level. These observations indicate that sufficient Pchl is being synthesized at the low light intensity to be equivalent to the rate of photoreduction and the release of the photoproducts from the reductase complex. Under such conditions no free reductase protein would be present, since the synthesized free Pchl directly displaces the photoproduct from the active site of the enzyme.

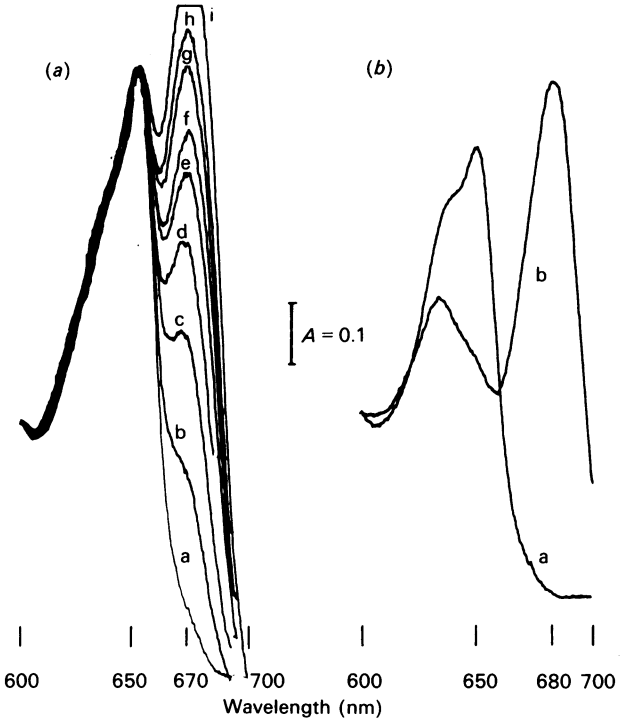


Fig. 7. Effect of low-energy red light on Pchl reduction

Leaf segments of 7-day-old dark-grown seedlings were exposed to low light intensity and at 15 min intervals (a–i) the absorption spectrum *in vivo* between 600 and 700 nm was recorded (*a*). For comparison the spectrum of etiolated leaves before (a) and immediately after a high-light intensity flash (b) is presented (*b*).

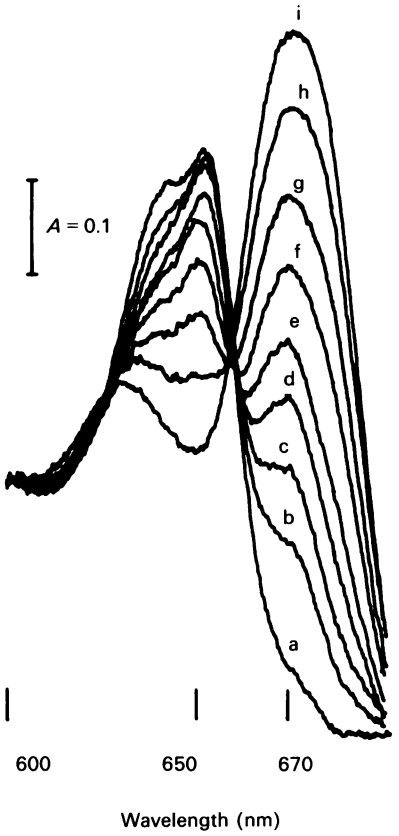


Fig. 8. Effect of low-energy red light on the photoreduction of Pchl in Lev-treated leaf segments

Leaf segments of 7-day-old dark-grown seedlings were incubated in the dark in AmLev (80 mM) for 2 h and then exposed to low-intensity light. At 15 min intervals (a–i) the absorption spectrum *in vivo* between 600 and 700 nm was recorded.

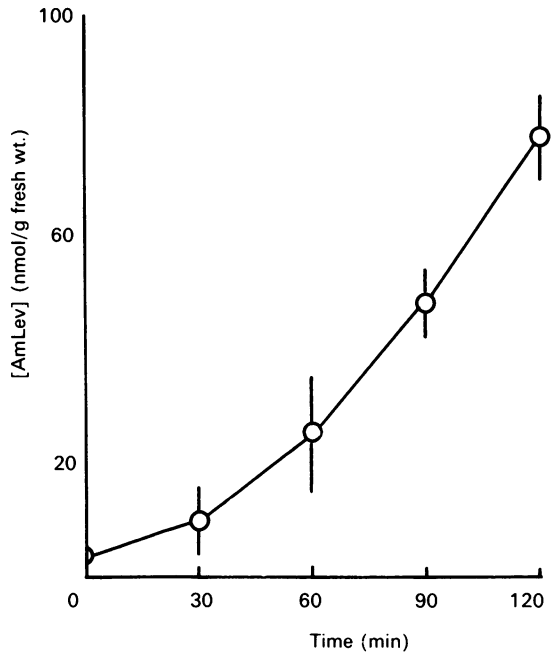


Fig. 9. AmLev production in Lev-treated leaves under low-energy red light

Leaf segments of 7-day-old dark-grown seedlings were incubated in Lev (80 mM) in the dark for 2 h and then exposed to low-intensity light for periods up to 2 h. Segments which were exposed to light for less than 2 h were returned to the dark for a period of time so that time in light + time in dark = 2 h. At the end of the 2 h the AmLev in the leaves was determined. The results are means (±S.D.) for three replicates.

To satisfy the high and steady-state level of photoactive Pchl under low light, there must be some stimulation in the synthesis of AmLev. This is illustrated in the absorption spectra *in vivo* of 8-day-old dark-grown leaves which had been incubated in Lev (80 mM) for 2 h in the dark before being subjected to low-intensity red light. The spectra show (Fig. 8) that, in red light, the photoactive species of Pchl slowly declines, since Lev has inhibited AmLev synthesis and there is no Pchl formation to recharge the Pchl reductase protein. In a separate experiment, leaf segments were incubated in Lev (80 mM) in the dark for 2 h and then exposed to low-energy red light for various periods up to 2 h. After a total period of 2 h in the light and dark (i.e. time in light + time in dark = 2 h) the AmLev that had accumulated in the leaves was determined. The results (Fig. 9) show that as more and more photoreduction of Pchl occurs (see Fig. 8), there is a greater accumulation of Amlev in the tissues.

DISCUSSION

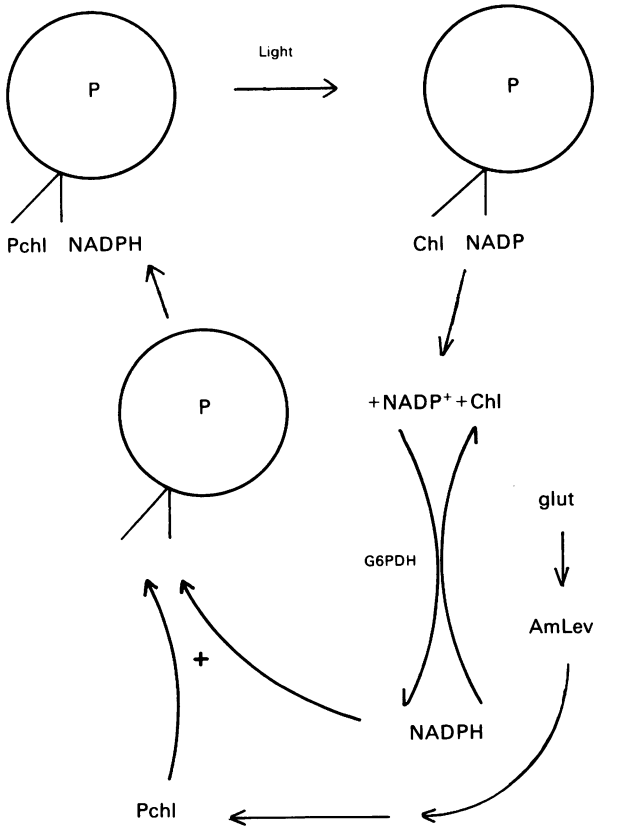
The rapidity of Pchl synthesis from exogenously supplied AmLev (Fig. 2) makes it possible to manipulate the pool of free Pchl in the leaf and to study the effect of this on the photostimulation of AmLev biosynthesis. The results with relatively high and low concentrations of free Pchl in the leaf (Figs. 3 and 4) strongly indicate a close relationship between the photoreduction of bound Pchl and the 'switch-on' in AmLev synthesis. In many of these experiments it at first appeared that the ability to synthesize AmLev requires conditions that would produce free reductase protein. This was also indicated in experiments designed to modify the rate of the Shibata shift. Glycerol slowed down the dissociation of the photoproducts from the reductase protein, and associated with this was a concomitant decrease in the appearance of AmLev (Fig. 6).

We have previously considered (Stobart & Ameen-Bukhari, 1984) the possibility that the free reductase protein that appears after a photoreduction event in the dark-grown leaves could be involved directly in AmLev synthesis. The effect of low-intensity light on Pchl reduction and the high and unchanged level of the Pchl reductase complex that is still maintained in the leaves under these conditions, however, makes this hypothesis rather unlikely. The photoreduction of the bound Pchl requires NADPH, and Griffiths *et al.* (1976) have shown that the photoactive species of Pchl exists as a ternary complex of substrate and enzyme. After photoreduction the products are released from the reductase protein. Besides the sudden appearance of free reductase, therefore, $NADP^+$ also is made available. It is considered likely that the $NADP^+$ is reduced by a glucose-6-phosphate dehydrogenase associated with the prolamellar body and that NADPH is recycled for binding with Pchl to the

reductase protein and further photoreduction (Griffiths, 1975).

The regulation of the NADPH/$NADP^+$ ratio by the photoreduction of Pchl offers, therefore, a possible mechanism to explain the close and intimate relationship observed between the state of the Pchl reductase complex and the ability of the leaf to synthesize AmLev. As an experimental model we propose the following sequence of events (see Scheme 1).

1. After a light treatment the oxidized $NADP^+$ is released from the reductase complex to be reduced by a glucose-6-phosphate dehydrogenase.
2. The reformed NADPH is used to reduce glutamate in AmLev synthesis (Kannangara *et al.*, 1984).
3. The coupling of the glucose-6-phosphate dehydrogenase and the enzyme(s) synthesizing AmLev results in sufficient AmLev for Pchl production.
4. NADPH is removed from the active pool and bound to the reductase protein only when Pchl becomes available.



Scheme 1. Proposed model for the control of AmLev production mediated through the photoreduction of Pchl

Pchl reductase (P) catalyses the photoreduction of Pchl to chlorophyll(-ide) (Chl). The oxidized $NADP^+$ is released from the Pchl–reductase–NADPH ternary complex and is re-reduced by glucose-6-phosphate dehydrogenase (G6PDH). The NADPH is used initially to reduce glutamate (glut) to the level of AmLev. AmLev is required for the synthesis of Pchl. The Pchl that is formed binds to the reductase protein together with NADPH. The formation of Pchl and its binding to the reductase will remove NADPH from the active pool, and under these conditions AmLev synthesis will cease.

Thus the formation of the ternary substrate–enzyme–NADPH complex will require the coupling of NADPH production with the synthesis of Pchl. This would of course necessitate the presence of a limited and common pool of NADPH for photoreduction and AmLev synthesis.

The competition between the binding of NADPH to the reductase protein in the presence of Pchl and the reaction(s) in Amlev synthesis which require(s) NADPH could control quite precisely the switching on and off of the production of AmLev for Pchl formation. With the formation of Pchl the NADPH co-substrate is removed from the active pool as they both become associated in the reductase complex, and under these conditions the synthesis of AmLev will cease. The competition between the enzymes that require NADPH need not necessarily depend solely on the affinity of the different proteins for the co-substrate. The relative quantities of the enzymes that are available in the dark-grown leaf exposed to flash-illumination could be important. The Pchl reductase is one of the most abundant proteins in the etioplast, and this alone could create the bias required for regulating the availability of reductant for AmLev synthesis and the rapid 'switching off' of AmLev formation after Pchl regeneration in dark-grown leaves (Stobart & Ameen-Bukhari, 1984). Regulation through a common pool of NADPH/$NADP^+$ can explain many, if not all, of the observations on photoactive Pchl synthesis and the formation of AmLev. The uncontrolled production of AmLev that occurs after the photoreduction of Pchl in the presence of Lev (Stobart & Ameen-Bukhari, 1984), for instance, would result from the fact that no Pchl is synthesized and hence none of the NADPH will be removed from the pool by its binding to the reductase protein. Under such conditions the NADPH/$NADP^+$ would be in continuous re-formation through the glucose-6-phosphate dehydrogenase and glutamate/AmLev systems and an overproduction of AmLev would result.

I.A.-B. was in receipt of a postgraduate scholarship from King Saud University, Riyadh, Saudi Arabia, A.K.S. thanks Dr W. T. Griffiths, Department of Biochemistry, University of Bristol, for a critical appraisal of the manuscript and Mr. B. Perham for technical assistance.

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Received 25 November 1985/27 January 1986; accepted 12 February 1986