Plastid Development in Primary Leaves of *Phaseolus vulgaris*

DEVELOPMENT OF PLASTID ADENOSINE TRIPHOSPHATASE ACTIVITY DURING GREENING

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1. The etioplasts of dark-grown bean leaves showed ATPase (adenosine triphosphatase) activity which had a pH optimum of 8.5, was stimulated by dithiothreitol and unaffected by light-triggering. 2. Bean chloroplasts showed a low activity of dark-induced ATPase with a pH optimum of 8.5 and a substantial amount of light-triggered activity with a pH optimum of 8.0. The light-triggered activity depended on dithiothreitol and Mg^{2+} and was promoted by phenazine methosulphate. 3. Light-triggered ATPase activity was completely inhibited by $20\mu M$ -dicyclohexylcarbodi-imide. 4. Etioplasts developed light-triggered ATPase activity in response to 30 min illumination of the etiolated leaves. 5. During the 48 h of light-induced greening of dark-grown leaves there was a 70% increase of the chloroplast ATPase activity found after light-triggering and a 30% fall in the dark-induced activity, both expressed on a per leaf basis. As the larger part of these changes occurred during the first 30 min of illumination, it is concluded that most or all of the chloroplast ATPase was present in the etioplast, a conclusion identical with that of Lockshin *et al.* (1971) for maize. 6. During 48 h of greening there was a tenfold increase in the amount of thylakoid membrane in the leaf together with an 83% fall in the ATPase activity per m^2 of thylakoid membrane, measured after light-triggering.

The plastids (etioplasts) of dark-grown bean leaves show no photophosphorylation at a degree of illumination, $1.5mW\cdot cm^{-2}$, which is sufficient for the determination of the photophosphorylation activity of chloroplasts (Gyldenholm & Whatley, 1968). During the light-induced greening of dark-grown bean leaves photophosphorylation activity develops (Gyldenholm & Whatley, 1968), together with changes in plastid fine structure (Bradbeer *et al.*, 1974b), and the formation of certain chloroplast components that are not found in etioplasts, such as chlorophyll (Koski, 1950), the chloroplast pigment-protein complexes (Alberte *et al.*, 1972) and cytochrome *b*-559_{HP} (Gregory & Bradbeer, 1973; Plesnicar & Bendall, 1973). During greening substantial increases also occur in a number of components that are already present in the etioplasts, such as thylakoid membrane (Bradbeer *et al.*, 1974b), cytochromes *f*, *b*-559_{LP} and *b*-563 (Gregory & Bradbeer, 1973; Plesnicar & Bendall, 1973) and ferredoxin (Haslett *et al.*, 1973). In the present paper the development and properties of the ATPase† of the plastids of greening bean leaves is reported.

Coupling factor 1 is a component of the photo-

phosphorylation system of spinach chloroplasts and it has been found to possess latent ATPase activity and to stimulate phosphorylation activity in partially deficient chloroplast residues (Vambutas & Racker, 1965). The presence of a similar component has been reported in extracts of etioplasts of maize (Lockshin *et al.*, 1971) and bean (Horak & Hill, 1971, 1972) and some of the properties of these preparations have been reported. These etioplast extracts contained a Ca^{2+} -dependent dithiothreitol-activated ATPase with properties similar to those of the spinach chloroplast ATPase described by McCarty & Racker (1968). The present investigation concerns the properties and development of ATPase activity, which was examined *in situ* in the plastids of greening bean leaves.

Experimental

Materials

Seeds of *Phaseolus vulgaris* cv. Canadian Wonder were surface-sterilized in sodium hypochlorite (1% available chlorine) for 20 min, rinsed in water and sown in moist vermiculite in plastic bowls. These were placed in a darkroom at 23°C for 14 days, after which the etiolated seedlings were extracted immediately or illuminated with continuous white fluorescent

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† Abbreviation: ATPase, adenosine triphosphatase.

light ($1.6\text{mW}\cdot\text{cm}^{-2}$), in a Controlled Environments Ltd. growth cabinet maintained at 25°C . For comparison, plastids were extracted from 14-day-old greenhouse-grown plants.

Methods

Preparation of plastids. Plastids were obtained by homogenizing approx. 10g of primary leaves in an M.S.E. top-drive homogenizer, run at top speed for $3\times 5\text{s}$ intervals, in 40ml of a 0.5M-sucrose solution which also contained 0.2% bovine serum albumin and 0.1M-Tris-HCl buffer, pH8.0. The homogenate was filtered through four layers of muslin and the plastids were collected by centrifugation at $2000g$ for 10min. The pellet was resuspended in a small volume of extraction medium and immediately used in the ATPase assays. The preparation was carried out at $0-2^{\circ}\text{C}$. All steps of the etioplast preparation from dark-grown beans were carried out either in total darkness or under the illumination of a dim green safelight (a 25 W incandescent bulb behind an Ilford Spectrum Green filter).

ATPase assay. A method similar to that of Marchant (1969) was used. Approximately $3\times 10^7-1\times 10^8$ plastids in $50\mu\text{l}$ were added to a reaction mixture containing $50\mu\text{mol}$ of Tris-HCl buffer, pH8.0, $5\mu\text{mol}$ of MgCl_2 , $10\mu\text{mol}$ of dithiothreitol, $0.05\mu\text{mol}$ of phenazine methosulphate and $100\mu\text{mol}$ of sucrose in a total volume of 0.8ml. A 5min exposure to $56\text{mW}\cdot\text{cm}^{-2}$ illumination by mercury iodide lamps was used to trigger the ATPase reaction. The reaction was started by the addition of $5\mu\text{mol}$ of ATP, in 0.1ml, to each reaction mixture immediately after the end of the light treatment. During the 5min light treatment the temperature of the reaction mixtures rose from 24.0° to 25.0°C and the latter temperature was maintained for the duration of the

succeeding dark treatment. The reaction was terminated by the addition of 0.1ml of a 10% (w/v) solution of trichloroacetic acid, which also contained $3\mu\text{mol}$ of H_2O_2 to oxidize any dithiothreitol remaining in the reaction mixture. The precipitated protein was removed by centrifugation at full speed in an Eppendorf 3200 bench centrifuge and the P_i content of 0.5ml of the supernatant was determined exactly as described by Lindberg & Ernster (1956).

Plastid number. The number of plastids/ml in each preparation was determined by a haemocytometer count.

Results

Requirements for the ATPase activity of etioplasts, 30min illuminated plastids and chloroplasts

Table 1 gives a comparison of the requirements for the demonstration of ATPase activity by three distinct stages of chloroplast development. The etioplasts were obtained from 14-day-old dark-grown beans and they were not exposed to developmentally or photochemically effective illumination until they received the light-triggering treatment of the ATPase assay. The '30min plastids' were prepared from 14-day-old dark-grown beans that had been subjected to 30min of illumination with $1.6\text{mW}\cdot\text{cm}^{-2}$ white fluorescent light immediately before extraction of the plastids. This illumination was sufficient to cause transformation of the etioplast prolamellar body into an irregular structure and to trigger the formation of chlorophyll *a* from protochlorophyllide *a* (Treffry, 1970; Bradbeer *et al.*, 1974b). These 30min-illuminated plastids did not show any photophosphorylation activity in an illumination of $1.5\text{mW}\cdot\text{cm}^{-2}$ (Gyldenholm & Whatley, 1968). The chloroplasts were obtained from 14-day-old green-

Table 1. *Requirements for the demonstration of ATPase activity by bean plastids obtained at different developmental stage*

Plastid preparations consisting of $3\times 10^7-1\times 10^8$ plastids were added to the reaction mixture to give in 0.8ml $50\mu\text{mol}$ of Tris-HCl buffer, pH8.0, $5\mu\text{mol}$ of MgCl_2 , $10\mu\text{mol}$ of dithiothreitol, $0.05\mu\text{mol}$ of phenazine methosulphate and $100\mu\text{mol}$ of sucrose. A 5min exposure to $56\text{mW}\cdot\text{cm}^{-2}$ illumination was used for light-triggering. The reaction was started immediately after darkening by the addition of $5\mu\text{mol}$ of ATP in 0.1ml. It was terminated after 15min at 25°C by the addition of 0.1ml of 10% (w/v) trichloroacetic acid containing $3\mu\text{mol}$ of H_2O_2 . Etioplasts were obtained from the primary leaves of 14-day-old dark-grown beans. The '30min plastids' were obtained from similar bean plants that had been subjected to 30min illumination of $1.6\text{mW}\cdot\text{cm}^{-2}$ at 25°C immediately before plastid preparation. Chloroplasts were obtained from the primary leaves of 14-day-old greenhouse-grown beans. For further details see the text.

Reaction mixture	ATPase activity (μmol of P_i released/h per 1×10^8 plastids)					
	Light-triggered			Dark-induced		
	Etioplasts	'30min plastids'	Chloroplasts	Etioplasts	'30min plastids'	Chloroplasts
Complete	4.3	16.0	6.3	4.6	7.8	0.5
-Dithiothreitol	1.1	4.2	0.5	—	6.1	—
-Phenazine methosulphate	—	10.0	2.1	—	7.9	—
- MgCl_2	4.4	7.5	0.5	—	4.3	—

house-grown beans that had developed full photosynthetic activity.

In Table 1 it can be seen that chloroplast ATPase activity was dependent on light-triggering and the presence of dithiothreitol and MgCl_2 . Omission of phenazine methosulphate gave only one-third of the activity of the complete system. These requirements are similar to those reported by McCarty & Racker (1968) for the ATPase activity of spinach chloroplasts *in situ*.

In contrast the ATPase activity of etioplasts was not dependent on light-triggering or the presence of MgCl_2 , but there was a substantial stimulation by dithiothreitol, the activity in its absence being only 25% of that in its presence. Lockshin *et al.* (1971) reported that dithiothreitol increased the ATPase activity of maize etioplasts *in situ* by about 30%. The properties of etioplast ATPase have been reported for EDTA extracts of etioplasts, which, in the case of bean, required dithiothreitol and Mg^{2+} , or Ca^{2+} whereas light-triggering had no effect (Horak & Hill, 1971). A similar preparation from maize etioplasts was activated by dithiothreitol, Mg^{2+} , Ca^{2+} and incubation with trypsin, but the effects of illumination were not reported (Lockshin *et al.*, 1971). In both cases Ca^{2+} gave a greater activation than Mg^{2+} .

For 30 min-illuminated plastids light-triggering approximately doubled the amount of ATPase activity over that shown in the dark. The increase in activity due to light-triggering was dependent on the presence of dithiothreitol, phenazine methosulphate and Mg^{2+} . In contrast, the dark-induced ATPase activity of '30-min plastids' was not affected by the absence of phenazine methosulphate and was only slightly decreased by the absence of dithiothreitol. Both the light-triggered and the dark-induced activities were decreased by about 50% in the absence of Mg^{2+} , however. Thus the 30 min illumination of the leaves resulted in a substantial change in the properties of the ATPase activity.

Effect of pH on ATPase activity

Fig. 1 shows the effect of the pH value of the reaction mixture on the light-triggered and dark-induced ATPase activities of etioplasts, '30 min plastids' and chloroplasts. Each curve represents a typical result of an experiment that was repeated at least three times. In the case of the etioplasts the light and the dark experiments gave the same results, i.e. light-triggering had no effect and there was a pH optimum of 8.5. Fully developed chloroplasts had an optimum pH of 8.0 for light-triggered ATPase activity and the dark-induced activity had an optimum pH of 8.5. The '30 min plastids' showed maximum dark-induced ATPase activity at pH 8.5 and 7.0, but, after light-triggering, peak activities were found at pH 8.0 and 8.5. The latter result was obtained in five replications of this experiment, during which

some measurements at pH values different from those shown in Fig. 1 established that the peak values were at pH 8.0 and 8.5. Thus for all three types of plastid a pH optimum of 8.5 was found for dark-induced ATPase activity. After only 30 min of illumination of the leaves of dark-grown plants the plastids had developed some light-triggered ATPase activity with the same pH optimum as that of the light-triggered ATPase of normal chloroplasts. The double peaks of activity shown in Fig. 1 for the light-triggered ATPase of '30 min plastids' are considered to represent the newly developed light-triggered ATPase activity superimposed on a substantial amount of residual dark-induced ATPase activity.

Effect of dicyclohexylcarbodi-imide on ATPase activity

McCarty & Racker (1967) reported that dicyclohexylcarbodi-imide was a potent inhibitor of spinach chloroplast ATPase. Table 2 shows that a 5 min

Fig. 1. Effect of pH on ATPase activity of bean plastids

Activities were measured as for Table 1. (a) Etioplasts; (b) '30 min plastids'; (c) chloroplasts. ●, Dark-induced activity; ○, activity after light-triggering.

Table 2. *Effects of dicyclohexylcarbodi-imide on the ATPase activities of bean plastids obtained at different stages of development*

The plastids were incubated in the reaction mixture for 5 min before light-triggering. For details see Table 1 and the text.

Reaction mixture	Light-triggering	ATPase activity (μmol of P_i released/h per 1×10^8 plastid)		
		Etioplasts	'30-min plastids'	Chloroplasts
Complete	+	14.0	7.2	12.0
Complete	—	14.0	3.6	1.5
Complete+20 μM -dicyclohexylcarbodi-imide	+	7.0	3.3	1.6
Complete+20 μM -dicyclohexylcarbodi-imide	—	7.0	—	—

Table 3. *ATPase activity of the plastids of bean leaves at different stages of development*

Etioplasts were obtained from 14-day-old dark-grown leaves. The 14-day-old dark-grown leaves gave '30 min plastids' after 30 min of illumination, '20 h plastids' after 20 h of illumination and '48 h plastids' after 48 h of illumination. Chloroplasts were obtained from 14-day-old greenhouse-grown leaves. ATPase activity is expressed as μmol of P_i released/h. For further details see Table 1 and the text.

	Etioplasts	'30 min plastids'	'20 h plastids'	'48 h plastids'	Chloroplasts
Thylakoid membrane area/leaf (cm^2)	218	218	331	2220	5660
Plastid number/leaf	2.84×10^8	2.84×10^8	3.35×10^8	3.35×10^8	7.4×10^8
ATPase activity/ 1×10^8 plastids					
Light-triggered	11.0	16.0	15.9	16.0	12.0
Dark-induced	11.0	7.8	6.1	6.8	1.5
Ratio light-triggered/dark-induced	1.0	2.05	2.60	2.35	8.0
ATPase activity/leaf					
Light-triggered	31.2	45.4	53.2	53.6	88.8
Dark-induced	31.2	22.1	20.4	22.8	11.1
ATPase activity/ m^2 of thylakoid membrane					
Light-triggered	1434	2072	1605	241	157
Dark-induced	1434	1009	615	102	20

preincubation with 20 nmol of dicyclohexylcarbodi-imide immediately before light-triggering decreased both the chloroplast and the '30 min plastid' ATPase activities to the values of the dark-grown controls, inhibitions of 87 and 54% respectively. The etioplast ATPase activity, which was not affected by light-triggering, was inhibited by 50%.

Effect of NH_4Cl on ATPase activity

When NH_4Cl was added to the ATPase assay of spinach chloroplasts immediately after light-triggering there was a substantial increase in activity, the optimum promotion being found with 1 mM- NH_4Cl (McCarty & Racker, 1968; Marchant, 1969). In the present work bean chloroplast ATPase was similarly stimulated by NH_4Cl , the optimum concentration of the NH_4Cl being 0.4 mM. The '30 min plastids' also showed a stimulation of ATPase activity by NH_4Cl , but in this case the effect increased over the whole range of NH_4Cl concentrations tested up to 1.6 mM- NH_4Cl . Dark-induced ATPase activity was not affected by NH_4Cl at any stage of plastid development. Marchant (1969) has reviewed

the range of variation of the reported effects of NH_4Cl on ATPase activity.

Development of plastid ATPase activity during greening

Table 3 shows the results of an experiment in which 14-day-old dark-grown beans were greened under continuous illumination, plastids were prepared from the primary leaves at certain stages and their ATPase activity was determined. Plastid number per leaf and the area of thylakoid membrane per leaf were determined in similar experiments by the methods of Bradbeer *et al.* (1974a). When the prolamellar body was present (etioplasts and '30 min plastids') the area of its membrane was included in the value representing thylakoid area.

During the first 30 min of illumination there was a rise in the light-triggered ATPase activity and a fall in the dark-induced ATPase activity for all three ways of expressing the results in Table 3. On a per plastid basis the light-triggered activity then remained steady up to 48 h, but the dark-induced activity fell slightly. Greenhouse-grown leaves gave plastids with a slightly lower light-triggered ATPase activity than

Table 4. *Light-triggered and dark-induced ATPase activities of developing plastids*

Light-triggered ATPase was determined at pH8.0 and dark-induced activity at pH8.5. For further details see Tables 1 and 3 and the text.

	ATPase activity (μmol of P_i released/h per 1×10^8 plastid)		
	Light-triggered (pH8.0)	Dark-induced (pH8.5)	Light-triggered/ dark-induced ATPase
Etioplasts	11.0	16.0	0.7
'30-min plastids'	16.0	8.6	1.9
'20-h plastids'	15.9	7.4	2.1

'48-h plastids' and a much lower dark-induced ATPase activity. During greening the ratio light-triggered/dark-induced ATPase activities increased, but did not reach the high ratio of greenhouse-grown leaves.

On a per leaf basis the ATPase activity in the light-triggered assay increased by 71% during the first 20h of illumination whereas the dark-induced ATPase activity fell by 35%. When ATPase activity was expressed per area of thylakoid membrane there was a 45% increase in the activity in the light-triggered assay during the first 30min of illumination, but further illumination resulted in a fall in activity. However, dark-induced ATPase activity fell continually during greening.

Table 4 shows that for the first 30min of illumination the increase in the light-triggered ATPase activity at pH8.0 coincided with an approximately equal fall in the dark-induced ATPase activity at pH8.5. Subsequently little change occurred in the greening experiment. The changes during the first 30min of illumination are consistent with the light-triggered ATPase being a modified version of the dark-induced ATPase as the rise in the former activity corresponds with the fall in the activity of the presumed precursor.

Discussion

To establish the occurrence of a change in the amount of a constituent of greening bean leaves it is necessary to express the data on a per leaf basis. Over a 48h period of greening the bean leaves showed a 72% increase of ATPase activity in the light-triggered assay, whereas the dark-induced activity fell by 30%. The increase in activity is low in comparison with the simultaneous increases of other membrane components during 48h greening, such as a ninefold increase in thylakoid area (Table 3), five-nine-fold increases in plastid cytochromes (Gregory & Bradbeer, 1973), a 12-fold increase in

ferredoxin (Haslett *et al.*, 1973) and the formation of about 130nmol of chlorophyll/leaf when the dark-grown leaf, before the commencement of illumination, contains no chlorophyll and less than 1nmol of protochlorophyllide/leaf. The effects of illumination on ATPase activity were to bring about a response to light-triggering, which was further stimulated by NH_4^+ , and to develop a requirement for Mg^{2+} . This activity thus resembles the light-triggered ATPase activity reported by McCarty & Racker (1967, 1968). Since the effects of illumination on the development of ATPase activity seems to involve modification of the enzyme properties it is possible that the small increase in light-triggered activity may be accounted for by activation rather than by synthesis *de novo*. This suggestion is supported by the finding that the main changes in ATPase activity per leaf occurred during the first 30min of illumination (Tables 3 and 4), and thus would seem more likely to result from a light-induced enzyme modification. Lockshin *et al.* (1971), who studied the development of chloroplast ATPase activity in greening maize leaves without the use of a light-triggering treatment in their assays, came to the conclusion that most or all of the coupling factor (measured as ATPase) activity of a chloroplast may be present in the etioplast from which it develops. Our results support this conclusion and extend it to apply also to the development of light-triggered chloroplast ATPase activity.

Horak & Hill (1972) found that the EDTA-extractable ATPase activity of bean plastids increased in specific activity (μmoles of P_i released/h per mg of protein) during greening and that this increase in specific activity was inhibited by both chloramphenicol and cycloheximide. The increase in the specific activity of an enzyme in such a leaf fraction does not show whether or not the ATPase activity increases on a whole-leaf basis. An increase in the specific activity of an enzyme in one leaf-protein fraction merely shows that the activity has increased in relation to the protein content of the fraction, a change that might result from an increase in the activity of the enzyme concerned, or a fall in the relative concentration of the other proteins. Thus the work of Horak & Hill (1972) is inconclusive with respect to the possible synthesis *de novo* of chloroplast ATPase in greening leaves.

The lack of an apparent synthesis of ATPase *de novo* in greening leaves contrasts with the light-induced synthesis of most other chloroplast components (Bradbeer, 1973). It must be inferred that the bean leaf synthesizes its full complement of chloroplast ATPase irrespective of illumination. In dark-grown leaves most of the other chloroplast components are only synthesized to about one-tenth of the amount they will reach when the leaves are allowed to green. Thus for example the greening of

dark-grown bean leaves results in a substantial fall in ATPase activity expressed on the basis of unit area of thylakoid membrane as a consequence of the lack of further ATPase formation, while a tenfold increase in thylakoid membrane occurs.

Despite the rapid development of light-triggered ATPase activity during greening there was a lag of 4 or 5 h before the developing chloroplasts showed detectable photophosphorylation (Gyldenholm & Whatley, 1968; Howes & Stern, 1973). This lag implies that at least one component of the photophosphorylation system is lacking during the first few hours in greening bean leaves. Since Plesnicar & Bendall (1973) observed no lag in the development of cyclic photophosphorylation in greening barley it is possible that the deficiency is peculiar to *Phaseolus vulgaris*. In this laboratory it is intended to continue to study the development of the individual components of the thylakoid electron-transport system and of the subunits of the ATPase.

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