Phytochemistry, 1980, Vol. 19, pp. 2541–2545 © Pergamon Press Ltd Printed in England. 0031 9422/80 1201 2541 $02.00/0

δ-AMINOLEVULINATE DEHYDRATASE FROM *RAPHANUS SATIVUS* COTYLEDONS

A. P. BALANGE and C. LAMBERT

Laboratoire de Photobiologie (Laboratoire associé au C.N.R.S. L.A 203), Centre de Recherche de Biochimie et Physiologie Cellulaires, Faculté des Sciences, B.P. 67, 76130 Mont Saint Aignan, France

(*Received* 31 *January* 1980)

Key Word Index—*Raphanus sativa*; Cruciferae; δ-aminolevulinate dehydratase; phytochrome; cycloheximide; etioplasts; erythromycin.

Abstract—Phytochrome induces δ-aminolevulinate dehydratase (ALAD) activity in radish seedling cotyledons under continuous far red light. Analysis of the enzymatic activity in etioplasts vs total activity shows a constant ALAD level in these organelles (10%) in etiolated seedlings. In far red irradiated seedlings, the percentage of enzyme detected into etioplasts increases up to 45% of the total. Comparative kinetic studies of ALAD activity detected in the cytoplasm and the etioplasts indicate an increase in both compartments with a maximum value reached respectively at 96 and 120 hr from sowing. Treatment with cycloheximide shows a very fast abolition of cytoplasmic ALAD activity which is always correlated to an etioplast decrease with a time shift of *ca* 24 hr. Erythromycin acts only on the cytoplasmic level of ALAD, and only for far red irradiated seedlings, with an increase of activity twice the level detected in untreated ones. This unexpected effect is discussed.

INTRODUCTION

In higher plants a considerable number of enzymes are under phytochrome control and much attention has been paid to the possible mechanisms involved [1].

In tetrapyrrol biosynthesis in animals, ALA synthetase plays a key role, but there is no evidence for its existence in higher plants: ALA must be synthesized by different pathways depending essentially on environmental conditions [2–4]. On the other hand, ALAD is present and detectable in most plants and hence is the first enzyme readily available for studies of phytochrome action on tetrapyrrol biosynthesis.

Previously [5] we have shown that ALAD activity in radish seedlings is enhanced by continuous far red (FR) light treatment, via the phytochrome system. The clear increase of ALAD activity, detected at 40 hr from sowing, takes place in two waves with maxima at 68 and 88 hr. The first phase required a light treatment given at least between 24 and 48 hr from sowing. The second can be induced by light given prior to 24 hr from sowing. In etiolated seedlings we have also noted two phases, which are correlated in time with those observed under FR light conditions, but the level of ALAD is lower.

The use of different light treatments, protein synthesis and of inhibitors allowed us to examine original (detected in the dark) and inducible (light promoted) ALAD activities. These latter are sensitive to cycloheximide (CHI) and presumably correspond to a newly synthesized enzyme.

Since ALAD is considered a specific enzyme of etioplasts and chloroplasts [6–8] we have investigated the distribution of its activity in seedlings with time. Under our experimental conditions, we were unable to detect ALAD activity in an enriched mitochondria fraction prepared according to Douce [9]. We have thus restricted our study to plastids and the cytoplasm. At the physiological development of the seedlings studied, plastids are rapidly transformed by any light treatment, so we do not distinguish between the various forms and will call the organelles 'etioplasts'.

In the present paper, we report investigations on the modification of ALAD distribution in cells of radish cotyledons grown in FR light. We have made no estimate of the intactness of organelles we have examined. We cannot exclude that some of them may be disrupted but nevertheless collected in the pellet during purification procedures. However, after disruption of organelles in hypotonic medium, we have never observed any ALAD activity in the pellet following centrifugation (unpublished results). Thus we assume, along with other authors [8, 10] that ALAD is a stromal enzyme.

As carotenoids form the reference point of our calculations (see Experimental), organelles which pellet without their stroma can only lead to an underestimate of the ALAD related by calculation to the etioplasts. We are thus not able to state absolutely that variations in ALAD distribution are correlated with modifications of adsorption on etioplast membranes.

RESULTS

ALAD activity, related to etioplasts, expressed as a percentage of total activity shows a nearly constant value of about 10% during 144 hr from sowing, in radish seedlings

Abbreviations: δ-ALA—δ-aminolevulinic acid; ALAD—δ-aminolevulinic acid dehydratase (EC.4.2.1.24); FR—standard far red light ($\lambda \simeq 720$ nm); PBG—porphobilinogen; CHI—cycloheximide; ERT—erythromycin.

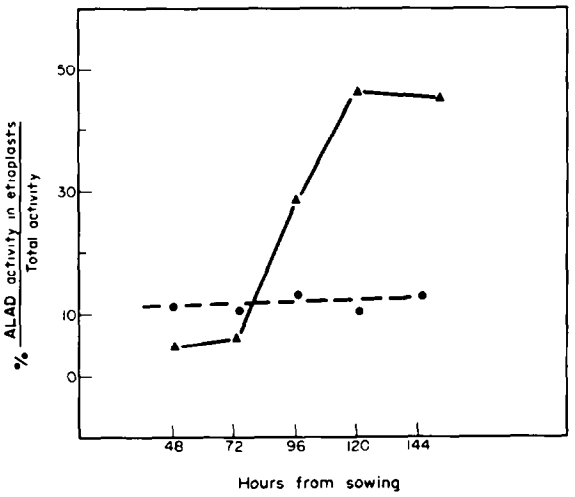

Fig. 1. Distribution of ALAD expressed as percentage of enzymatic activity in etioplasts vs total cytoplasmic activity. ●---●, seedlings maintained in total darkness, ▲---▲, far red irradiated seedlings.

kept in total darkness (Fig. 1). In FR irradiated seedlings, however, we observe that at 72 hr after sowing, a marked increase of etioplastic ALAD vs total activity, which rises to a value of 45% at 120 hr (Fig. 1).

The kinetics of ALAD activity in the etioplast and cytoplasmic fraction are studied in cotyledons kept in total darkness or under continuous FR light (Fig. 2a, 2b). In total darkness etioplast ALAD activity presents a constant and very low level, but in the cytoplasmic fraction, the activity increased from 48 to 72 hr (Fig. 2b).

Results obtained in FR light conditions indicate that both cytoplasmic and etioplast ALAD activities are greatly increased from 48 to 96 hr and 72 to 120 hr respectively after sowing (Fig. 2a). In this case, variations of ALAD activity in these two fractions show similar kinetics, with a time shift of 24 hr for etioplasts.

The use of ribosomal inhibitors give some extra information on the origin of these variations. Cycloheximide (CHI) at 28 μg/ml and erythromycin (ERT) at 250 μg/ml were applied to seedlings as described elsewhere [5]. These act respectively either on cytoplasmic ribosomes or on ribosomes in organelles (plastids, mitochondria).

We observed in previous work [5] that ERT, given at 48 hr from sowing, increases ALAD activity only in seedlings kept in continuous FR light. This was confirmed (Fig. 1a) and there is no real difference between treated and untreated seedlings kept in total darkness (Fig. 2b). For FR irradiated seedlings we note an important increase of ALAD activity only in the cytoplasmic fraction, with a maximum at 96 hr from imbibition, and then a decrease and stabilization at twice the level found in the untreated control (Fig. 2a).

The action CHI is shown in Fig. 3 and Table 1. The use of a 28 μg/ml concentration leads to a 75% inhibition of ALAD activity instead of a complete disappearance as happens to phenylalanine ammonia-lyase under the same conditions [11]. Furthermore, it was impossible to give a continuous treatment with CHI to seedlings either in total darkness or in FR light. After more than a 24 hr treatment the physiological state of cotyledons does not allow ready isolation of etioplasts, according to our experimental procedure. Thus seedlings were treated only during the first 24 hr, and ALAD activity immediatly estimated. Under

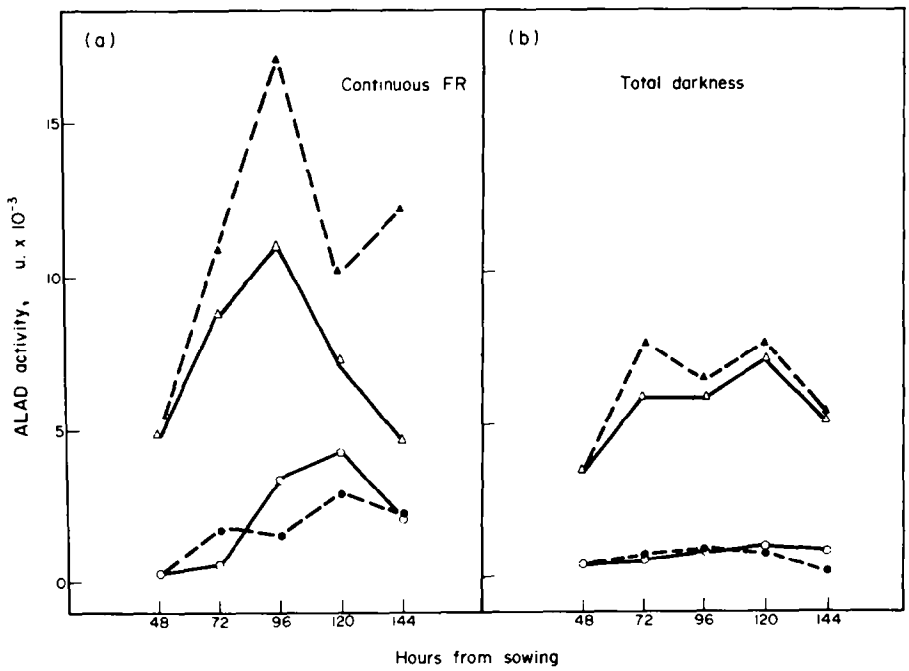

Fig. 2. (a) Appearance of ALAD activity of seedlings kept on water under continuous far red light. △ △, cytoplasmic fraction; ○——○, etioplasts; or after erythromycin treatment (250 μg/ml) 48 hr from sowing. ▲- --▲, cytoplasmic fraction; ●----●, etioplast. (b) The same symbols are employed for results obtained from seedlings kept in total darkness.

Table 1. ALAD activity detected in cytoplasmic fraction before and after 24 hr cycloheximide treatment. For etioplast activity see Fig. 3

	Cytoplasmic ALAD activity in U × 10^{-4} for 200 cotyledons			
	At start		After 24 hr CHI treatment	
Hr from sowing	FR irradiated seedlings	Dark control	FR irradiated seedlings	Dark control
48	483	352	–	–
72	860	587	250	98
96	1099	581	210	133
120	636	714	123	153
144	457	514	222	109

these conditions, the effect of CHI is very rapid: enzymatic activity decreases within 4 hr and a constant level is established until the end of the treatment (24 hr) in the etioplast but not in the cytoplasmic fraction (unpublished results).

We observed (Table 1) that cytoplasmic activity is severely depressed in etiolated and FR irradiated seedlings after 24 hr CHI treatment. The most important response to application of antibiotic was observed from 72 to 120 hr from sowing in irradiated seedlings, the time at which phytochrome promotes ALAD activity in the control. CHI lowers enzymatic activity in a constant manner in etiolated radishes.

The results observed in etioplasts are shown in Fig. 3. In complete darkness CHI reduces ALAD activity to a constant level (Fig. 3b). In FR irradiated seedlings, CHI slightly inhibits the enzymatic increase observed at 72 hr, but inhibits mainly at 96 and 120 hr from sowing. The levels reached after treatment are similar to those observed under dark conditions (Fig. 3a).

DISCUSSION

We had previously observed [5] along with other authors [12] that ALAD from radish cotyledons is a phytochrome-dependent enzyme. The comparison between the results obtained with etiolated or FR irradiated seedlings shows that phytochrome induces an important increase in ALAD activity at 48 hr in the cytoplasm with a maximum at 96 hr from sowing. This increase is detectable only at 72 hr and is maximum at 120 hr from sowing in the etioplasts.

CHI inhibits these two increasing levels of ALAD, but the cytoplasmic one is affected within 48 hr from

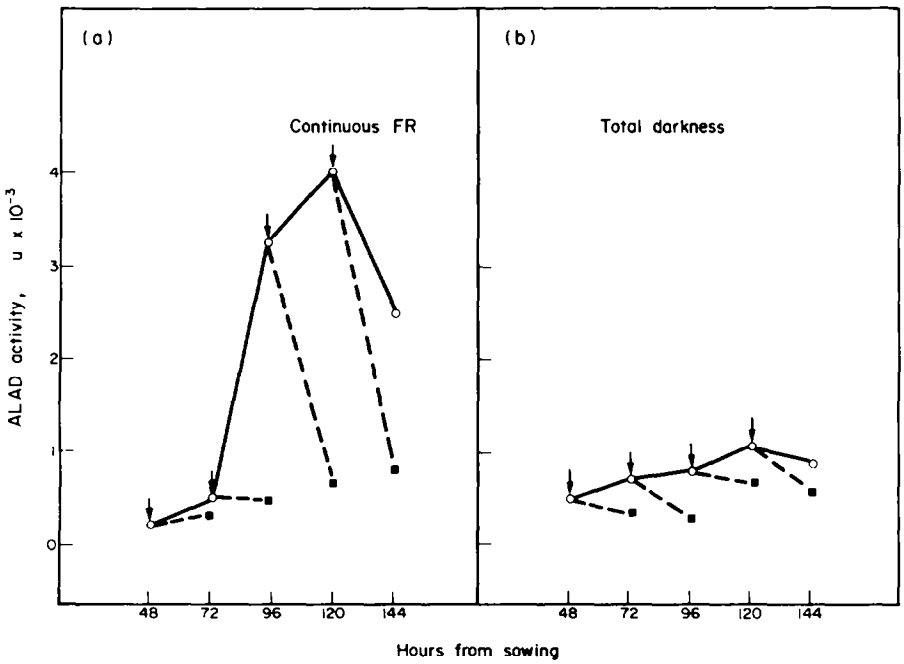

Fig. 3. ALAD activity detected after cycloheximide treatment (28 μg/ml) in etioplasts; ○ —○, seedlings kept on water; ■---■, results obtained after 24 hr of antibiotic treatment. (a) Far red irradiated seedlings, (b) etiolated seedlings. Arrows show time of treatment.

imbibition, whereas the etioplast takes 96 hr. The action of CHI treatment on cytoplasmic ALAD may correspond to the inhibition of its synthesis which is under the control of phytochrome. The antibiotic also induces a decrease of ALAD activity detected in etioplast with a time shift of *ca* 24 hr.

In etiolated seedlings, a slow rate of synthesis is maintained in the dark (Fig. 3b). CHI treatment prevents this slow synthesis and we can observe a decrease in ALAD as soon as 48 hr both in cytoplasmic and the etioplast fraction. In FR irradiated seedlings, phytochrome promotes the cytoplasmic synthesis (Fig. 3a). At 48 and 72 hr from sowing, there is only a 75% inhibition by CHI of cytoplasmic synthesis allowing a partial compensation of the turnover of molecules in organelles and we observe a constant level of ALAD after a 24 hr treatment.

At 96 and 120 hr from imbibition of the seeds the cytoplasmic synthesis is lowered in the untreated control and CHI treatment prevents any remaining synthesis. The enzyme is apparently no longer available for transport and does not compensate for the turnover in organelles. Then at 96 and 120 hr from sowing we detect a constant level of ALAD activity in the organelles instead of clear increases.

These results agree with those obtained by an autoradiographic experiment with bean leaves [13] which showed labelling of proteins and their transport into organelles with a similar lag period. They are also in accord with other results showing the translocation of at least a part of ALAD from cytoplasm to the organelles [6, 10, 14]. Thus we interpret our results as the appearance (synthesis or another mechanism) of ALAD in the cytoplasm and its transport into etioplasts with a lag phase of *ca* 24 hr. The results from the CHI experiments allow us to believe that this may be due to an inhibition of cytoplasmic synthesis, but are not sufficiently precise to determine whether 'promotion' or 'increase' of ALAD activity are only the results of *de novo* synthesis.

An analysis of the results from ERT treatment is more confusing. The results presented here show that the antibiotic induces an increase in ALAD activity to twice that detected in the control, only in the cytoplasmic fraction of FR irradiated seedlings with a much lower, decrease in the organelles (Fig. 2a).

Different hypotheses may be advanced to explain such an unexpected effect. The inhibition of etioplast protein synthesis by ERT may mean that amino acids, are more available in the cytoplasm [15]. This explanation supposes that ERT treatment increases various cellular pathways: phenylalanine ammonia-lyase, measured under the same consitions as ALAD is also increased (unpublished results). We may also explain such an increase of enzymatic activity as due to the inhibition by ERT of an etioplast regulator which is able to modulate cytoplasmic enzymes via a feedback process, specific or not. In these two hypotheses, the lower activity observed in organelles after ERT treatment would depend on a modification of ALAD transfer. ERT, by its action as an inhibitor of protein synthesis, may have such an effect [16].

Another possibility may be advanced according to the scheme of the synthesis of the light subunit of RuDP carboxylase. This fraction, which is synthesized in cytoplasm, is transferred into chloroplasts [17]. It has been shown that the cytoplasmic molecule is different in size from those observed in the chloroplasts. We can postulate an identical process for ALAD. The cytoplasmic molecule may have a different structure and be more active; its structure and activity being modified during transfer into etioplasts. After ERT inhibition the more active molecule stays in the cytoplasm where a high activity is detected, whereas a decrease into etioplasts related to lack of transfer.

We shall in future work focus our experiments on the regulation of ALAD into etioplasts and on the mechanisms involved in the increase of ALAD in cytoplasm firstly through the phytochrome system, secondly through ERT action.

EXPERIMENTAL

Raddish seeds (*Raphanus sativus* L. cv Longue Rave saumonée) were sown either in the dark or under continuous FR light. Enzymatic measurements, chemical products and light conditions are described elsewhere [5].

Etioplast isolation. The different manipulations were carried out under a dim green safe-light, and at 0°. 350–450 cotyledon pairs, according to seedling differentiation (10 g fr. wt) were collected and ground in 30 ml buffer by using a Waring Blendor adapted with a special razor blade bucket, according to Kannangara [18]. The buffer used was: Tris–HCl 100 mM pH 8.5, containing 10 mM $MgCl_2$, bovine serum albumin 0.2%, β-mercapto-ethanol 1 mM, D-mannitol 600 mM. The homogenate was filtered on cheesecloth, and centrifuged 100 × *g*, 15 mn (Sorvall, rotor SM 24). Etioplasts were collected from the supernatant centrifuged at 1000 *g*, 15 min. Pellets were carefully resuspended in 10 ml of extraction buffer and purified through centrifugation on discontinuous sucrose gradients (2 M, 1.3 M, 1 M, 0.7 M, 4000 *g* Beckman SW 27). 1 ml fractions were collected for measurements. ALAD activity is recovered at 1.3/2 M and 1/1.3 M interfaces.

Measurements and expression of results. To prevent artefacts with highly concentrated buffers on ALAD activity, all fractions are made 100 mM in sucrose by dilution with Tris–HCl buffer 0.05 M pH 8.5, containing 10 mM $MgCl_2$ and 1 mM β-mercaptoethanol.

Total carotenoids are estimated according to Kirk [19] after dilution v/v with 80% acetone. As carotenoids are only located in the plastids, a pigment present in the supernatant comes from broken organelles. They can thus serve as reference for an estimation of ALAD activity previously present in etioplasts before grinding: the measurement of carotenoids from the supernatant and etioplast fractions, after grinding, gives a determination of ALAD activity level present in etioplasts before grinding. In such a procedure, if broken organelles are collected with the etioplast fractions, it has no ALAD activity but has carotenoids. By this calculation our results on extrapolated ALAD activity may be understated.

Results are expressed in nM of δ-ALA transformed in PBG ml/sec [20]. Values are corrected to 200 pairs of cotyledons. Each result is the mean value of 4–6 independent experiments.

Acknowledgements – We are grateful to Professor P. Rollin for skillful discussions and Mrs. M. Troly for technical assistance. This work was supported in part by the Centre National de la Recherche Scientifique (LA 203).

REFERENCES

1. Schopfer, P. (1977) *Annu. Rev. Plant. Physiol.* **28**, 223.
2. Rebeiz, C. A. and Castelfranco, P. A. (1973) *Annu. Rev. Plant. Physiol.* **24**, 129.
3. Beale, S. I. (1970) *Plant Physiol.* **45**, 504.
4. Beale, S. I. (1978) *Annu. Rev. Plant. Physiol* **29**, 95.
5. Balangé, A. P. and Rollin, P. (1979) *Physiol. Veg.* **17**, 153.

6. Sluiters-Scholten, C. M. Th., Van den Berg, F. M. and Stegwee, S. (1973) *Z. Pflanzenphysiol.* **69**, 217.
7. Hampp, C. and Ziegler, H. (1975) *Planta* **124**, 255.
8. Schneider, Hj. A. W. (1976) *Z. Naturforsch.* **31**, 55.
9. Douce, R., Christensen, E. L. and Bonner, W. D. (1972) *Biochim. Biophys. Acta* **275**, 148.
10. Hovenkamp-Obbema, Moorman, A. and Stegwee, D. (1974) *Z. Pflanzenphysiol.* **72**, 277.
11. Klein-Eude, D., Huault, C. and Rollin, P. (1971) *C. R. Acad. Sci.* **273**, 1276.
12. Kasemir, H. and Masoner, M. (1975) *Planta* **129**, 119.
13. Strzalka, K. and Kwiatkowska, M. (1979) *Planta* **146**, 393.
14. Beisenherz, W. W. Hj. and Schneider, A. W. (1974) *Ber. Dtsch. Bot. Ges.* **87**, 161.
15. Averina, N. G., Polikarpova, N. N. and Shlyk, A. A. (1977) *Biochemistry* **42**, 2064.
16. Asselineau, J. and Zalta, J. P. (1973) in *Les Antibiotiques: Structure et Exemples de Modes d'Action* (Herman, ed.). Paris.
17. Ellis, R. J. and Barraclough, R. (1978) in *Chloroplast Development*, p. 185 (G. Akoyunoglou ed).
18. Kannangara, C., Gough, S. P., Hansen, B., Rasmussen, J. N. and Simpson, D. J. (1977) *Carlsberg Res. Commun.* **42**, 431.
19. Kirk, J. T. O. and Allen, R. L. (1965) *Biochem. Biophys. Res. Commun.* **21**, 523.
20. Shemin, D. (1962) in *Methods in Enzymology* (Colowick, S. C. and Kaplan, N. O., eds) Vol. 5, pp. 883–884. Academic Press, New York.