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5-AMINOLAEVULINATE DEHYDRATASE OF WHEAT LEAVES: DISTRIBUTION AND RESPONSE TO LIGHT

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; enzymology; 5-aminolaevulinate dehydratase; localization; phytochrome.

Abstract—5-Aminolaevulinate dehydratase occurs almost exclusively in the stroma of etioplasts and chloroplasts of young wheat leaves and is absent from mitochondria or cytosol fractions. Etiolated plants show little or no change in activity of the enzyme during the first 30 hr of illumination except for a small transient increase in the first hour which may be under phytochrome control.

INTRODUCTION

Previous data on the localization within green tissue of 5-aminolaevulinate dehydratase [ALAD; 5-aminolaevulinate hydrolase (adding 5-amino-laevulinate and cyclizing) EC 4.2.1.24] have been obtained by grinding the tissue (with blendors, mortar and pestle, etc.) and fractionating. Such treatments bring about breakage of the outer membrane system of etio- and chloroplasts with release of stromal contents. Even the least damaging of these grinding methods, e.g. the modified Braun blendor of Kannangara and Gough [1], results in 15–18% rupture of chloroplasts. Thus, while the previous data [2–7] indicate ALAD to be found within etioplasts (except possibly in bean leaves [8]), it has not been possible to decide on its presence or absence in other subcellular fractions. There are also conflicting reports as to the intraplastidic site of ALAD. In tobacco [7], bean [6] and probably cucumber [9], ALAD is a stromal enzyme; in *Kalanchoe* [4] and *Euglena* [5], it appears to be membrane-bound. In this report we have used a method of cell fractionation, stemming from mild rupture of enzymically isolated protoplasts, to examine the question of ALAD subcellular localization in wheat leaves.

The possible response of ALAD levels to illumination of the plant has been examined previously in a variety of ways. The greening of etiolated plants is usually associated with an increase in ALAD activity but at no time did the enzyme appear to be rate-limiting in the overall biosynthesis of chlorophyll [2, 4, 6, 8, 10]. Thus changes in ALAD may simply be reflecting the growth and transformation of plastids which follow illumination. Nevertheless, there does appear to be in mustard seedlings a minor involvement of phytochrome in ALAD activity [11]. This paper describes a transient increase of ALAD during the greening of etiolated wheat seedlings which is related to phytochrome status.

RESULTS AND DISCUSSION

Preliminary measurements showed that in the crude homogenate ALAD activity was constant for at least 120 min incubation at 37° and no pre-incubation was necessary. At pH 7.7, the K_m for ALA was 10^{-3} M, in good agreement with Nandi and Waygood [3] who used a partially purified enzyme preparation from wheat leaves. Routinely, 7.5 mM magnesium chloride was added to our incubations although no loss of activity was seen when magnesium chloride was not added. However, as in ref. [3], addition of EDTA (1–3 mM) to incubation mixtures (without added magnesium chloride) reduced ALAD activity, which was overcome with added magnesium chloride.

A typical set of data showing subcellular distribution of ALAD activity is given in Table 1. Clearly ALAD is present almost exclusively in the etioplast fraction (part a). The partial reconstitution experiment (part b) demonstrates that lack of ALAD activity in the mitochondria and supernatant could not be attributed to the presence of possible ALAD inhibitors in the fractions. Taking into account the limits of sensitivity of the ALAD assay in our hands, it was calculated that, at most, the extra-etioplast ALAD activity would be less than 8% of the total activity of the protoplast but in view of the ALAD activity recoveries observed (etioplast compared to protoplast) the actual figure was near 4–5%. Similar subcellular distribution was observed in protoplasts prepared from leaves of plants grown in daylight for 7 days. The results demonstrate the usefulness for subcellular distribution studies of preparations stemming from protoplasts gently ruptured through fine nylon mesh.

Rupture of the plastids with hypotonic medium demonstrates that ALAD activity was almost entirely within the stroma (Table 2). However, there was always a residual amount of ALAD activity found in the membrane fraction which was less than 1% in etioplasts and between 5 and 8% in chloroplasts. More vigorous treatments, e.g. ultrasonic, were not resorted to in order to decide whether this residual activity was bound or trapped in the membrane. There may be species differences in the nature of the association of ALAD within the plastid. Wheat is different in this respect from bean [8], *Euglena* [5] and *Kalanchoe* [4] but is similar in this respect to some other species, e.g. oats [2] and cucumber [9].

Table 1. Subcellular localization of ALAD activity*

Fraction	Total protein (mg)	Sp. act. (nmol/hr per mg protein)	Total activity 100%
(a) Protoplasts	9.9	17.6	100
Etioplasts	3.4	52.5	97
Mitochondria	0.55	—	N.D.†
Supernatant	6.3	—	N.D.
(b) Etioplasts			100
Etioplasts + mitochondria			106
Etioplasts + supernatant			107

*Protoplasts were prepared from 7-day-old etiolated leaf samples and fractionated. Protoplasts, etioplasts and mitochondria were lysed and assayed in hypotonic medium, i.e. isolation medium minus the osmoticum sorbitol. In (b), mixtures were as in proportions in protoplasts.

†N.D. = Not detected.

Table 2. Extraction of ALAD activity from etioplasts and chloroplasts*

Fraction	Protein (mg)	ALAD sp. act. (nmol/hr per mg protein)	Total activity (%)
Lysed etioplasts	37.8	40.4	100
S_1	9.7	142.8	91.4
S_2	1.9	45.6	6.9
S_3	0.8	N.D.†	N.D.
Membranes	25.1	1.6	0.4
Lysed chloroplasts	30.2	61.2	100
S_1	9.0	184.8	90
S_2	1.8	N.D.	N.D.
S_3	N.D.	N.D.	N.D.
Membranes	19.1	8.0	8

*Etioplasts were isolated from leaves of 7-day-old etiolated and 10-day-old green plants and lysed in hypotonic medium. Membranes were removed from supernatant S_1 by centrifuging at 16000 *g* for 10 min. Membranes were then extracted twice to produce S_2, S_3 and residual membranes.

†N.D. = Not detected.

Figure 1 shows a typical set of data relating to the response of 6½-day-old etiolated wheat plants to illumination by white light. The results are expressed as activity per 50 plants (sample size) rather than in terms of weight, protein, or specific activity of the leaf in order to provide a measure of the response of the whole plant to illumination. The total amount of ALAD activity (on a whole plant basis) was very little altered by 24–30 hr illumination even though during this period there was chlorophyll biosynthesis and an increase in total protein. The rate of chlorophyll biosynthesis over 5–20 hr of illumination was *ca* 0.1 μmol chlorophyll per 50 plants and was approximately that which would be expected during the maximum rate during the greening of wheat [12]. The capacity of ALAD was around 1 μmol/hr per 50 plants and thus probably at no stage limiting for chlorophyll synthesis and not causally related to the lag phase of chlorophyll biosynthesis. The data of Fig. 1 show that the specific activity of ALAD fell during illumination. However, Fig. 2a with individual results from two separate experiments indicates that ALAD activity during the first 4 hr of continuous illumination could show a marked transient increase which is evident after 30 min, maximal at 1–2 hr and decreasing to dark levels by *ca* 4 hr. The two examples indicate the variation in response. Figure 2b shows that a 2 min exposure to red light followed by darkness induces a transient increase in ALAD activity which is smaller in magnitude and always with a shorter time course than that seen in continuous white light. When the red light treatment is followed immediately by far-red light (Fig. 2c), the transient increase in ALAD activity is largely prevented. The far-red light treatment does not in itself cause a discernible change in ALAD activity. The protein content of the samples did not show any significant changes which relate to the changes in ALAD activity of Fig. 2. The rate of the

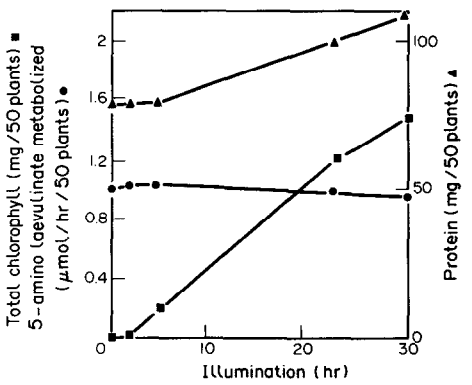

Fig. 1. Response of etiolated wheat to white light. 6½-day-old etiolated wheat plants were placed under white tungsten light (65 W/m²) at 25°. A sample (50 whole plants) was taken and homogenized. Chlorophyll was determined on the homogenate and ALAD activity and protein on the 'crude homogenate'. Chlorophyll at the commencement of illumination was determined as protochlorophyll [12].

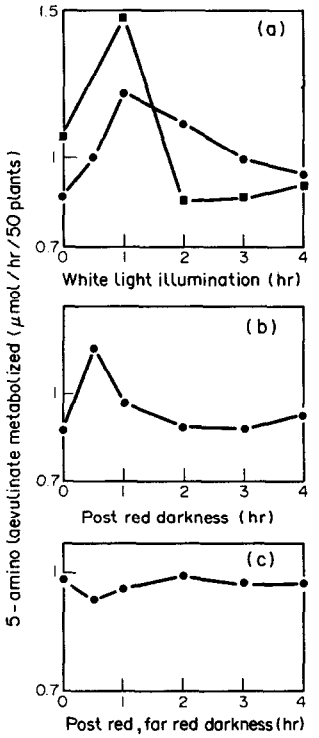

Fig. 2. Effect of white, red and far-red light on ALAD activity in crude homogenate. 6½-day-old etiolated wheat plants were treated as follows. (a) Placed under white tungsten light (● and ■, two separate experiments). (b) Placed under red light for 2 min and then returned to darkness. (c) As for (b) given 5 min far-red light immediately following red light, then returned to darkness. ALAD and proteins were determined on the 'crude homogenate'. (a), (b) and (c) were performed on different batches of plants.

reaction was constant over the 90 min incubation in samples showing the light induction, i.e. the increase in activity was stable in the crude homogenate. The ALAD assay measures the sum of porphobilinogen and porphyrins formed during the metabolism of ALA. In the crude homogenates, both aspects of the assay manifested the light-induced transient increases. Also the rate of disappearance of added porphobilinogen (as a substrate) in a preliminary experiment with a crude homogenate underwent a similar transient increase during continuous white light illumination.

The transient increase in activity of ALAD would, from the responses to red and far-red light, be related to phytochrome activity. One other report [13] shows that the synthesis of 5-aminolaevulinate in plastids from greening maize leaves shows a similar but more pronounced transient. This reaction was followed by incorporation of radioactivities from [^{14}C]glutamate in 5-aminolaevulinate and would have been sensitive to transient changes in concentrations of endogenous metabolites as well as in the activity of the enzymes concerned. The former possibility was probably not significant in relation to the results with ALAD in which mM concentrations of substrate and known activators (Mg^{2+} and –SH) were used to assay activity. The mechanism of the transient effect is unknown but may represent the synthesis or activation of a small amount of ALAD which is rapidly degraded or inactivated *in vivo*. The small stimulation of ALAD activity in cotyledons and hypocotyls of mustard [11] attributed to phytochrome would seem to be a different phenomenon to the transient effect seen in wheat.

Thus, in wheat, ALAD activity whilst present almost entirely within the etioplast or chloroplast is relatively unaffected by light which stimulates many transformations within plastids, including overall chlorophyll biosynthesis (of which ALAD is an integral part). This behaviour of wheat ALAD is in contrast to that of bean [6, 8], oats [2] and cultured tobacco [10].

EXPERIMENTAL

Wheat (*Triticum aestivum* var. Olympic) was germinated and grown under natural light or in complete darkness at 26–27° for 6–7 days on vermiculite moistened with Hoagland's soln. Whole seedlings, i.e. growth above vermiculite surface, were used to prepare 'crude homogenate' [8] and ALAD assays were commenced within 5 min. Protoplasts were isolated from laminae (top 6–8 cm) by an enzymatic digestion procedure [14], washed once in protein-free medium, and finally suspended in 0.5 M sorbitol containing 25 mM K_2HPO_4, 10 mM ascorbate, 5 mM $MgCl_2$, 3 mM *o*-phenanthroline and 10 mM 2-mercaptoethanol, pH 7.6. Protoplasts were ruptured by gently forcing them 3 × through 20 μm nylon mesh, and the plastid, mitochondria and supernatant fractions were obtained by differential centrifugation [15]. ALAD activity was determined by assaying for porphobilinogen formation [8], plus a correction for ongoing porphyrin formation [16]. Porphyrin assays from fully green samples were given an extraction with Me_2CO–C_6H_{12} prior to HCl treatment to remove chlorophyll. Also it was found necessary to dissolve the ALA immediately prior to each assay as ALA appeared to be unstable at the pH of this assay (pH 7.6). Protein determination [17] was by a microbiuret assay on TCA precipitable material (extracted with Et_2O in the case of fully green samples). Chlorophyll was determined by the method of ref. [18]. Pre-illumination with red or far-red light was per-

formed in a set-up like that described in ref. [19] which was modified to include an extra filter (Schott RG 780) in the far-red box.

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