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Studies on δ-amino levulinic acid dehydratase in radish cotyledons during chloroplast development

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The subcellular localization and biosynthetic site of δ-amino levulinic acid dehydratase [EC 4.2.1.24, ALAD] were investigated in relation to chloroplast development in radish cotyledons.

ALAD was mainly located in the chloroplasts and cytoplasm. Most of the ALAD in the chloroplasts was readily released by hypotonic shock. The enzyme was also found in the proplastids of etiolated cotyledons.

The normal increase in the activity of ALAD in the chloroplasts as well as the cytoplasm was inhibited by cycloheximide but unaffected by D-*threo* chloramphenicol and kanamycin during the greening of radish cotyledons. We concluded that the ALAD in both the cytoplasm and chloroplasts was synthesized on the cytoplasmic 80S-ribosomes. This suggests that the ALAD formed on the 80S-ribosomes might be incorporated into chloroplasts during their development.

When etiolated radish seedlings were illuminated, ALAD in both the cytoplasm and chloroplasts increased up to the point of the full development of the chloroplasts, and thereafter it decreased.

δ-Amino levulinic acid dehydratase, the second enzyme in the biosynthetic route of chlorophylls as well as of cytochrome hemes, catalyzes the formation of one molecule of PBG from 2 molecules of ALA. During studies on the mechanism of chloroplast development in etiolated radish cotyledons (*11–14*, *18*), we were prompted to investigate the nature of ALAD.

We report the subcellular localization, the biosynthetic site and some enzymic properties of ALAD in greening radish cotyledons. The relationship between ALAD formation and chloroplast development is also discussed.

Materials and methods

Chemicals ALA hydrochloride was obtained from Nakarai Chemical Co. Ltd., Kyoto. D- and L-*threo* Chloramphenicol were the gift of Dr. M. Ishida of the Agricultural Chemical Research Laboratories, of Sankyo Co. Ltd. Cycloheximide and kanamycin were given by Dr. Y. Ohhashi of the Microbial Chemistry Research Laboratory of Tanabe Seiyaku Co. Ltd.

Abbreviations: ALAD, δ-amino levulinic acid dehydratase [EC 4.2.1.24]; ALA, δ-amino levulinic acid; PBG, porphobilinogen.

Plant materials and culture conditions Radish seeds (*Raphanus sativus* Linn.) obtained from Takii Shubyo Co. Ltd., Kyoto, were allowed to germinate on absorbent cotton in petri dishes containing 1 mM potassium phosphate, pH 7.0, in the dark at 22°C for 4 days. These etiolated radish seedlings were then illuminated with fluorescent lamps (1500 lux) for specified intervals.

Assays ALAD activity was assayed according to Shemin (*17*) with a slight modification. The reaction was performed at 30°C in a total volume of 3 ml containing 100 μmoles of Tris-HCl buffer, pH 8.0, 10 μmoles of 2-mercaptoethanol, 10 μmoles of $MgCl_2$, 10 μmoles of ALA-HCl (neutralized with Tris) and 1.5–2.5 mg of enzyme protein. After 1 hr of incubation, the reaction was terminated by adding 3 ml of 8% trichloroacetic acid containing 10 mM $HgCl_2$. The protein precipitate was removed by centrifugation. An aliquot of the supernatant was treated with an equal volume of the Ehrlich reagent (*9*), then after 10 min the absorbance at 555 nm was measured. The molar extinction coefficient of PBG, 6.2×10^4 M^{-1} cm^{-1} (*17*), was used. In the crude enzyme preparation, a part of the PBG formed is presumably converted to porphyrin compounds. To restore this loss, another aliquot of the supernatant was diluted with an equal volume of 5 N-HCl, and its absorbance at 406 nm was measured using the molar extinction coefficient of porphyrin, 53×10^4 M^{-1} cm^{-1} (*17*). The total amount of PBG was calculated by assuming that 1 mole porphyrin is equal to 4 moles of PBG. The control was run with the reaction mixture without substrate. Under the present assay conditions, the reaction proceeded linearly for at least 3 hr. One unit of ALAD is defined as the amount of enzyme producing 1 nmole of PBG per hr.

NADPH-diaphorase [EC 1.6.99.1c], was assayed by following the rate of the enzyme catalized reduction of 2,6-dichlorophenol indophenol by NADPH (*10*). The activity of cytochrome c oxidase [EC 1.9.3.1] was determined at 550 nm by following the oxidation of reduced cytochrome c (*8*). Chlorophyll was determined according to the method of Mackinney (*7*). Protochlorophyllide was extracted with acetone under dim green safe light, then estimated spectrophotometrically (*16*). Protein contents were determined according to Lowry et al. (*6*).

Fractionation of radish cotyledons Greening radish cotyledons (20 g) were excised from seedlings illuminated for 3 days, then they were homogenized in 40 ml of 0.5 M sucrose containing 0.05 M Tris-HCl buffer, pH 7.8 and 0.01 M 2-mercaptoethanol for 5 sec in a Waring blendor. The homogenate was squeezed through 8 layers of nylon cloth then centrifuged at $500 \times g$ for 2 min. The pellet (P) was resuspended with 5 ml of grinding medium and centrifuged again at $500 \times g$ for 2 min to give pellet (P_1). The supernatants of both P and P_1 were combined. This combined supernatant was then subjected to successive differential centrifugation as follows: $1500 \times g$ for 10 min, $3000 \times g$ for 20 min and $20{,}000 \times g$ for 20 min to give the pellets, P_2, P_3 and P_4, respectively. Each pellet was washed as described for P_1. The washing medium was then combined with the original supernatant. The final supernatant was designated the S fraction. Before the enzyme assays, each fraction was divided into two parts. One part was dialyzed for 6 hr against 10 mM Tris-HCl buffer, pH 7.8 containing 2 mM 2-mercaptoethanol then used for the ALAD assay. The other was dialyzed against 10 mM Tris-HCl buffer containing 10 mM NaCl then used for the diaphorase assay to avoid the chemical reduction of indophenol by 2-mercaptoethanol. The dialyzate against 10 mM

Tris-HCl buffer containing 10 mM NaCl was used for the cytochrome c oxidase assay.

For simple separation of the chloroplasts from the squeezed filtrate, the latter was centrifuged at 24,000×*g* for 20 min. The resulting precipitate was designated the 24,000×*g* fraction and the remaining solution the "Supernatant".

Isolation and purification of proplastids Etiolated radish cotyledons were homogenized in 0.5 M Tris-HCl buffer, pH 8.0, containing 0.5 M sucrose, 1 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The homogenate was squeezed through 8 layers of nylon cloth then centrifuged for 15 min at 400×*g* to collect the first crude proplastids. The sediment obtained was resuspended in the homogenizing medium and designated step 1 proplastids. These step 1 proplastids were then purified by differential centrifugation (40×*g* for 5 min to 400×*g* for 15 min). These purified proplastids were designated step 2 proplastids. The step 2 preparation was further purified by discontinuous sucrose gradient centrifugation techniques (2.0 M sucrose, 25 ml; 1.3 M sucrose, 10 ml; the step 2 suspension, 10 ml; 2500×*g* for 30 min) (*5*). The purified proplastids, which accumulated at the interface between 2.0 M and 1.3 M sucrose, were designated step 3 proplastids.

Results

Subcellular distribution of ALAD in greening radish cotyledons

The distribution of ALAD, NADPH-diaphorase and cytochrome c oxidase among the several cellular fractions separated by differential centrifugation is presented in Table 1. A considerable amount (11%) of ALAD was present in the chloroplast-enriched fractions (P_2 and P_3), as indicated by their chlorophyll contents. The P_2 fraction had a high specific activity of ALAD and retained 7.4% of the total activity. In contrast, the low specific activity and the retention of only 2.4% of the total ALAD by the mitochondrial fraction (P_4), as assumed by the high activity of cytochrome c oxidase in this fraction, suggest that ALAD may be absent from the mitochondria. Approximately 76% of the total ALAD was found in the S fraction and the high specific activity in this fraction was comparable to that in the P_2 fraction. About 20% of the NADPH-diaphorase, a marker enzyme for chloroplasts (*19*), was associated with the chloroplast fraction. The

Table 1 *Distribution of enzymes among fractions obtained by differential centrifugation of radish cotyledons*

Fraction	Total protein	Total chlorophyll	ALAD		NADPH-diaphorase		Cytochrome c oxidase
			Total activity	Specific activity	Total activity	Specific activity	Total activity
	mg fraction^{-1} (%)	mg fraction^{-1} (%)	units fraction^{-1} (%)	units mg protein^{-1} ($\times 10^{-3}$)	μmoles fraction^{-1} (%)	μmoles min^{-1} mg protein^{-1} ($\times 10^{-3}$)	μmoles fraction^{-1} (%)
P_1	72.8 (5.6)	1.62 (19.4)	1.13 (10.9)	15.5	1.7 (7.9)	32.2	9.22 (4.3)
P_2	77.6 (8.1)	3.80 (45.5)	0.77 (7.4)	9.9	2.8 (13.1)	36.1	1.67 (7.8)
P_3	67.0 (6.9)	2.20 (26.3)	0.38 (3.6)	5.6	1.7 (7.9)	38.0	1.88 (8.7)
P_4	44.7 (4.6)	0.49 (5.8)	0.25 (2.4)	5.5	1.3 (6.1)	29.1	2.98 (13.8)
S	697.1 (73.0)	0.25 (3.0)	7.91 (75.8)	11.4	13.9 (64.9)	19.9	14.07 (65.4)

Table 2 *ALAD in the purification steps of chloroplasts*

Chloroplast	ALAD activity		Protein/Chlorophyll
	(units/mg chlorophyll)	(units/mg protein)	(mg/mg)
Step 1	280	11.5	24.4
Step 2	430	22.9	18.9
Step 3	530	31.1	17.1

Radish cotyledons (20 g) which had been illuminated for 2 days were homogenized in 0.05 M Tris-HCl, pH 7.8, containing 0.4 M sucrose, 0.01 M NaCl and 0.01 M 2-mercaptoethanol for 5 sec in a Waring blendor. The homogenate was squeezed through 8 layers of nylon cloth then centrifuged at 4000×*g* for 10 min. The pellet was resuspended with the grinding medium and designated the step 1 chloroplast. The step 1 preparation was further purified according to the method cited by Yamashita et al. (*20*). The step 1 preparation was centrifuged at 300×*g* for 1 min and the pellet obtained was discarded. The supernatant was centrifuged at 600×*g* for 7 min to give a pellet, which was resuspended as described above. This suspension was designated the step 2 chloroplasts. The step 2 preparation was further centrifuged at 300×*g* for 1 min, and the resulting supernatant was centrifuged at 1500×*g* for 7 min. The pellet obtained was resuspended as described above and designated the step 3 chloroplasts.

activity appearing in the S fraction presumably results from the release of the enzyme from the chloroplasts during fractionation, since the specific activity of the S fraction was low. Although the ALAD in the S fraction is considered to contain released ALAD from the chloroplasts, the high specific activity of ALAD, in contrast to that for NADPH-diaphorase, shows that ALAD is present in the cytoplasm as well. No ALAD activity could be found in the microsomal fraction obtained by centrifugation (105,000×*g* for 90 min) of the S fraction. As shown in Table 2, ALAD activity on a chlorophyll basis as well as its specific activity increased during the purification of chloroplasts as indicated by the content of protein per chlorophyll. Thus, we inferred (see Tables 1 and 2) that ALAD is mainly located in the chloroplasts and cytoplasm of greening radish cotyledons.

Table 3 *Effect of treatments of chloroplasts on ALAD*

Treatment	(A) Chloroplast ALAD (units/mg chlorophyll)	(B) Distribution of ALAD (units/fraction)	
		Chloroplast	Supernatant
None	411.6		
0.5 M Sucrose	408.1	168.7	8.8
10 mM Tris-HCl pH 7.8	71.5	25.9	147.5
Water	12.5	4.5	140.3
10 mM Tris-HCl pH 7.8 3 times washed	43.0		

The P_2 fraction, described in the text was suspended in 0.5 M sucrose, 10 mM Tris-HCl pH 7.8 or water and kept in an ice bath for 1 hr with occasional stirring. Chloroplasts were collected by centrifugation at 10,000×*g* for 15 min. These were resuspended in, and dialyzed against 10 mM Tris-HCl buffer containing 2 mM 2-mercaptoethanol. ALAD activity was assayed and calculated on a chlorophyll basis (A). The remaining supernatant was dialyzed then used for the ALAD assay. The total units of ALAD in both the chloroplast fraction and supernatant were determined (B).

Table 4 *ALAD in the purification steps of proplastids*

Proplastid	ALAD (units/mg protein)	Protein/protochlorophyllide (g/nmole)
Step 1	13.4	6.57
Step 2	17.1	4.01
Step 3	23.3	3.43

To examine the release of ALAD from chloroplasts, the P_2 fraction described in Table 1 was treated with an isotonic 0.5 M sucrose solution or with hypotonic 0.01 M Tris-HCl buffer or water. As shown in Table 3, treatment with the isotonic solution resulted in very little release of enzyme activity from the chloroplasts on a chlorophyll basis. However, treatment with the hypotonic solution brought about an 85% release of activity from the chloroplasts, with about 10% of the activity remaining even after three washings with Tris buffer. It, thus, appears that the ALAD in the chloroplast is not tightly bound to the chloroplast components.

As shown in Table 4, the specific activity of ALAD increased during purification of the proplastids, as indicated by the protein content on a protochlorophyllide basis. It, therefore, appears that ALAD is already present in the proplastids of etiolated radish cotyledons.

Table 5 *Effect of antibiotics on ALAD formation*

Treatment	Chlorophyll (μg/g fresh cotyledon)	ALAD (units/g fresh cotyledon)		
		24,000×*g*	Supernatant	Total
K-phosphate	245.4	0.33	1.19	1.52
Cycloheximide (μg/ml)				
0.25	180.6	0.24	0.84	1.08
0.5	82.7	0.22	0.81	1.03
0.75	50.6	0.22	0.83	1.05
1.0	28.1	0.22	0.77	0.99
D-Chloramphenicol (μg/ml)				
250	116.8	0.33	1.04	1.37
750	66.0	0.28	0.92	1.20
Kanamycin (μg/ml)				
250	119.0	0.30	1.01	1.31
750	75.9	0.32	0.96	1.28
K-phosphate	359.1	0.32	1.31	1.63
L-Chloramphenicol (μg/ml)				
250	238.9	0.31	1.31	1.62
750	222.2	0.35	1.39	1.74

Etiolated radish seedlings grown in the dark for 4 days were used. One hundred cotyledons with hypocotyls (excised 2 cm from the top) were incubated in a vessel containing 20 ml of antibiotic solution, after which they were illuminated at 18°C for 20 hr. The cotyledons were then harvested and homogenized as described in the text. The homogenate was centrifuged at 24,000×*g* for 20 min. Its pellet and supernatant were designated the 24,000×*g* fraction and the "Supernatant", respectively.

Effect of antibiotics on light induced changes in ALAD activity

Hypocotyls, from radish seedlings grown in the dark for 4 days, were cut 2 cm below the cotyledons. The excised shoots were incubated in petri dishes with various antibiotics in the light for 20 hr (Table 5). The accumulation of chlorophyll was inhibited by cycloheximide, a specific inhibitor of protein synthesis on the 80S ribosomes, as its concentration increased. Cycloheximide also inhibited the light induced increase in ALAD activities of the "Supernatant" fraction and the 24,000×*g* fraction. D-*threo* Chloramphenicol or kanamycin, inhibitors of 70S ribosome directed protein synthesis, caused a decrease in chlorophyll accumulation. However, these inhibitors did not affect the normal increase in ALAD activities in the 24,000×*g* fraction. It must be noted that there was a slight decrease in the ALAD activities of the "Supernatant" fraction on the addition of these antibiotics to the incubation medium. In the present experiments, L-*threo* chloramphenicol, which is known to have no effect on protein synthesis on the 70S ribosomes (*4*), had no effect on ALAD activiteis, but it did cause a decrease in chlorophyll accumulation to some extent. From the results shown in Table 5, only cycloheximide, of the antibiotics used in this experiment, appears to inhibit the light induced increase of

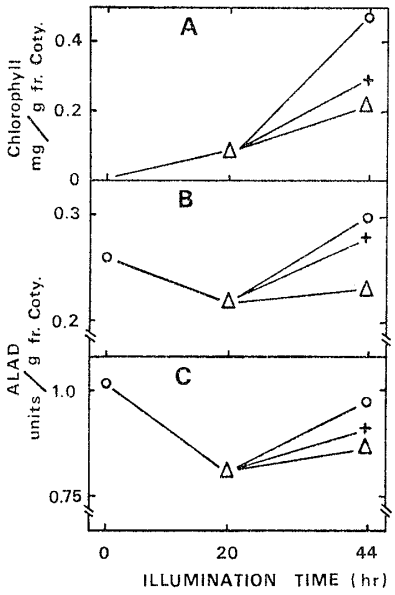

Fig. 1.

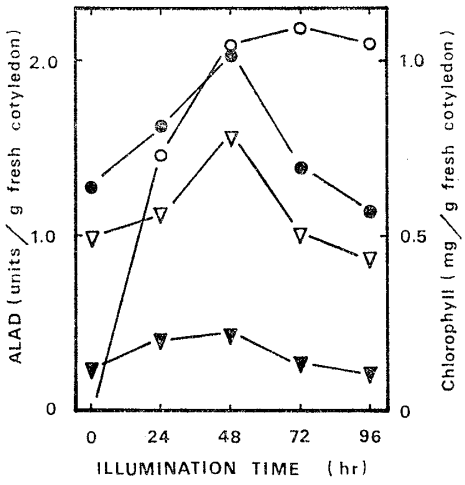

Fig. 2.

Fig. 1. *Effect of antibiotics on the formations of chlorophyll and ALAD.* One hundred etiolated cotyledons with hypocotyls were incubated with cycloheximide (0.5 μg/ml) in the light for 20 hr, then washed once with water and incubated with 2 mM K-phosphate buffer (pH 7.0), D-*threo* chloramphenicol (250 μg/ml) or cycloheximide, respectively, for another 24 hr in the light. The chlorophyll (inset A) and ALAD (inset B for the 24,000×*g* fraction; C for the "Supernatant" fraction) in the cotyledons were then determined. ○—△: treated with cycloheximide △—○: treated with K-phosphate buffer △—+: treated with D-chloramphenicol △—△: treated with cycloheximide

Fig. 2. *Time courses of chlorophyll accumulation and ALAD activity.* Etiolated radish seedlings grown in the dark for 4 days were illuminated continuously for an additional 4 day period. Changes in the chlorophyll content (○), ALAD activities of the "24,000×*g*" (▼), the "Supernatant" (▽) and the total (●).

ALAD activities in the chloroplasts and cytoplasm. These observations were further confirmed with the following experiment.

The cotyledons with hypocotyl hooks described above were first incubated with cycloheximide for 20 hr in the light, then incubated with cycloheximide, D-*threo* chloramphenicol or phosphate buffer as the control, for another 24 hr in the light (Fig. 1). ALAD activities in both the 24,000×*g* and "Supernatant" fractions, which were depressed by the treatment with cycloheximide, increased after replacing the cycloheximide with phosphate buffer or D-*threo* chloramphenicol, as compared with activities in the cotyledons treated with cycloheximide. This suggests that the ALAD located in the cytoplasm and that in the chloroplasts are synthesized on the cytoplasmic 80S ribosomes.

ALAD and chloroplast development

Fig. 2 shows the time courses of chlorophyll accumulation and ALAD activities in greening radish cotyledons. Continuous illumination of the etiolated seedlings caused a rapid accumulation of chlorophyll up to 2 days. From 2 days after illumination, the chlorophyll content remained constant, indicating that the chloroplasts became almost fully developed on the third day under the conditions employed. ALAD activities in both the 24,000×*g* fraction and "Supernatant" fraction as well as the total activity increased for 2 days, as did the chlorophyll accumulation. During this period, the activity of ALAD in the 24,000×*g* fraction appeared to increase at a faster rate than that of the "Supernatant". Interestingly, after the chlorophyll content reached a constant level, the ALAD activities tended to decrease.

Discussion

The experiments described in this paper were used to study the subcellular localization and biosynthetic site of ALAD in relation to chloroplast development in greening radish cotyledons. Data obtained using fractionation techniques show that ALAD is located in the chloroplasts of greening cotyledons. ALAD activity was also found in the proplastid fraction prepared from etiolated radish cotyledons. Granick (*3*) reported that the transformation of exogenous ALA to some porphyrin compounds occurred in proplastids isolated from tobacco, bean and barley leaves. Rebeiz and Castelfranco (*15*) have also shown that etioplasts are capable of synthesizing labelled protochlorophyll from ^{14}C-ALA. All this indicates that the ALAD situated in the pathway for chlorophyll synthesis is located in proplastids or chloroplasts. As compared with the distribution and specific activity of the chloroplast marker enzyme, NADPH-diaphorase, it was concluded that ALAD is also present in the cytoplasm of radish cotyledon cells.

As shown in Table 3, most of the ALAD in the chloroplasts was readily released by osmotic shock, which suggests that the enzyme might be in a soluble form in the stroma or be loosely bound to the lamellae.

Results in Table 5 and Fig. 1, show that both the chloroplastic and cytoplasmic ALAD are presumably synthesized on the cytoplasmic 80S ribosomes. Chloroplastic 70S ribosomes appear to be unable to synthesize ALAD. This suggests that cytoplasmic ALAD might be transferred into plastids during chloroplast

development. It has been observed that a number of chloroplast proteins are apparently synthesized outside the chloroplast (*1*). Ellis and Hartley (*2*) inferred that a specific mechanism must exist for transporting the chloroplast proteins produced on cytoplasmic ribosomes across the outer membranes of the plastids.

Under the experimental conditions employed, greening of the cotyledons was almost completed by continuous illumination for 2 days, as shown in Fig. 2. After that time, the chlorophyll content on a fresh cotyledon basis remains constant but, interestingly, the total ALAD activity of the green cotyledons begins to decrease. This indicates that there is a regulatory system for ALAD activity which may participate in regulating the synthesis of chlorophyll.

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