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BIOSYNTHESIS OF △-AMINOLEVULINATE IN GREENING BARLEY LEAVES: GLUTAMATE 1-SEMIALDEHYDE AMINOTRANSFERASE

by

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L-Glutamate-1-semialdehyde was synthesized by catalytic hydrogenation of N-carbobenzoxy-L-glutamyl-1-chloride-5-benzyl ester. Soluble protein extracts of chloroplasts isolated from greening barley leaves enzymically converted L-glutamate-1-semialdehyde to δ-aminolevulinate. The enzyme was partially purified by gel filtration on a Biogel column excluding proteins larger than 500,000 daltons. The enzyme had a broad pH optimum around 8.0 and required no specific cofactors for activity. Aminooxyacetate (20 mM), cycloserine (20 mM), p-chloromercuribenzoate (0.1 mM), glyoxylate (20 mM) and pyridoxal phosphate (5 mM) inhibited δ-aminolevulinate formation from L-glutamate-1-semialdehyde. However, β- hydroxyglutamate (1 mM) a potent inhibitor of L-glutamate-U-^{14}C conversion to δ-aminolevulinate, had no effect on L-glutamate-1-semialdehyde aminotransferase. The aminotransferase activity was eluted from the Biogel column together with the enzyme activity that converted L-glutamate-U-^{14}C into δ-aminolevulinate. Soluble proteins prepared from etiolated plastids and mature chloroplasts of barley had a low specific activity of L-glutamate-1-semialdehyde aminotransferase compared to soluble proteins from greening plastids. It is proposed that L-glutamate-1-semialdehyde aminotransferase catalyses a part reaction in the conversion of L-glutamate to δ-aminolevulinate in greening barley plastids.

1. INTRODUCTION

Previous studies have demonstrated that chloroplasts and cell free systems from greening leaves convert ^{14}C labelled L-glutamate and/or α-ketoglutarate into δ-aminolevulinate (4, 5, 6, 8). △-aminolevulinate formation from glutamate in stroma preparations of greening barley plastids has been shown to require ATP, NADPH and Mg^{2+}. The biosynthesis of δ-aminolevulinate from glutamate is therefore considered to proceed according to Figure 1 involving the following three steps: (I) glutamate is phosphorylated with ATP in the presence of Mg^{2+}, (II) the glutamate-1-phosphate is reduced by NADPH to glutamate-1-semialdehyde and (III) the aldehyde is transaminated to δ-aminolevulinate. In this paper we report a synthesis of glutamate-1-semialdehyde, the postulated precursor of δ-aminolevulinate. Greening barley is shown to have an enzyme which catalyses the conversion of glutamate-1-semialdehyde to δ-aminolevulinate.

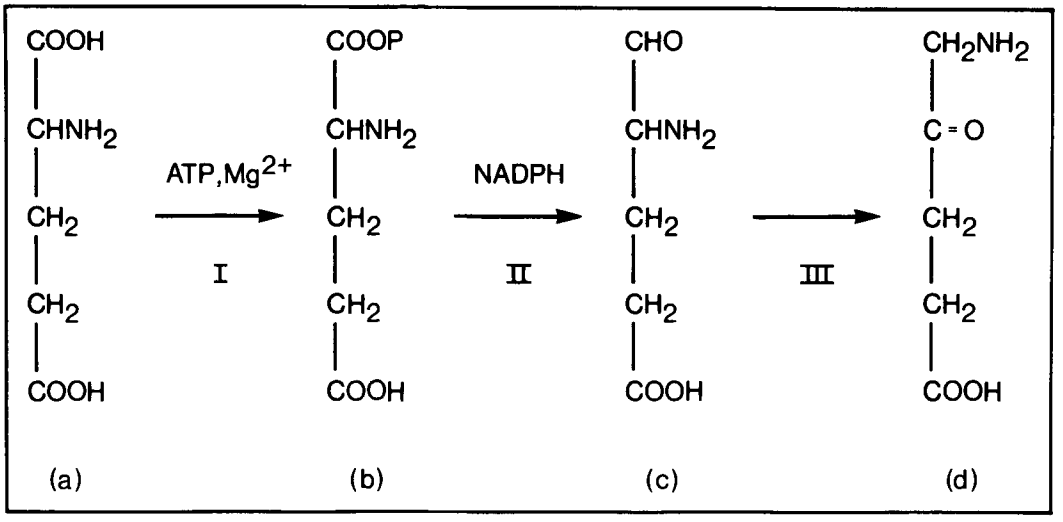

Figure 1. Proposed scheme for δ-aminolevulinate biosynthesis from L-glutamate in greening barley plastids. a) glutamate, b) glutamate-1-phosphate, c) glutamate 1-semialdehyde, d) δ-aminolevulinate. The reaction I is catalysed by a kinase and the reaction II by a dehydrogenase. Glutamate-1-semialdehyde aminotransferase catalyses the reaction III.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of barley (Hordeum vulgare L. cv. Svalöf's Bonus) were germinated and grown on moist vermiculite at 21°C for 6 days either in complete darkness or in light. Greening seedlings were obtained by illuminating the dark grown seedlings for 6 hours with »Osram Fluora« fluorescent light.

2.2. Chemicals

N-Carbobenzoxy-L-glutamate-γ-benzyl ester was obtained from Bachem Inc., Marina del Rey, California, U.S.A. L-Glutamate-U-^{14}C of specific activity 290 mCi/mmole was obtained from Radiochemical Centre, Amersham. DL-β-hydroxyglutamate was supplied by ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.

2.3. Plastid isolation

Leaves were harvested, cooled on ice, and homogenized in a precooled homogenizer fitted with razor blades (9). Approximately 160 g fresh weight of pre-cooled leaves were homogenized with 400 ml of ice cold medium containing 0.6 M-glycerol, 0.1 M-Tricine, 3 mM-$Ca(NO_3)_2$ and the pH adjusted to 7.9 with NaOH. The homogenate was squeezed through a single layer of nylon gauze (31μ mesh) and filtered through another layer of similar nylon gauze. The filtrate was centrifuged for 5 minutes at 0-4°C in a Sorval RC 2B centrifuge at 3000 rpm using a GSA rotor (1500 x g). The plastid pellet was gently suspended in approximately 40 ml of ice cold suspension medium containing 0.3 M-glycerol, 0.1 M-tricine, and 1 mM-dithiothreitol. The pH of the suspension medium was adjusted with NaOH to 7.9. Plastids were again pelleted by centrifugation for 5 minutes at 3500 rpm in the Sorval centrifuge using a SS34 rotor (1400 x g). The final plastid pellet was resuspended in a small volume (ca. 10 to 15 ml) of medium containing 1 mM-$MgCl_2$ and the other components of the suspension medium. Uniform suspension of plastids was achieved employing a Potter Elvehjem homogenizer.

2.4. Extraction of soluble protein from isolated plastids and purification of enzyme activity

Plastid suspensions were passed through an ice cold French pressure cell at 16000 p.s.i. and centrifuged for 1 hour at 50000 rpm in a Beckman L5/75 ultracentrifuge at 0-4°C using a

Ti 50 rotor. The clear supernatant contained the soluble proteins of the plastids and was used either directly in enzyme assays or after partial purification using a Biogel A 0.5 column. A column (2.5 times 85 cm) was loaded with 10 to 15 ml of protein solution containing 3 to 6 mg protein per ml and eluted with a medium containing 0.3 M-glycerol, 0.1 M-Tricine, 1 mM-dithiothreitol, 1 mM-$MgCl_2$ and adjusted to pH 7.9 with NaOH. The eluate was scanned for ultraviolet light absorption using a LKB uvicord II and 8 ml fractions were collected.

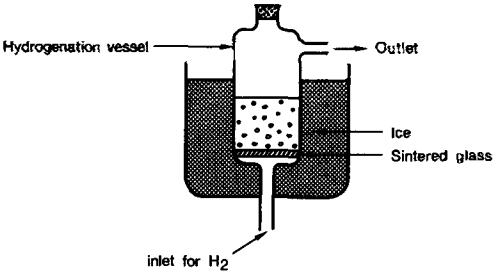

Figure 2. Hydrogenation set-up.

2.5. Synthesis of L-glutamate 1-semialdehyde

N-Carbobenzoxy L-glutamate γ-benzyl ester (500 mg) was dissolved in a minimal volume (4 ml) of dry ether in a 50 ml centrifuge tube and cooled on ice. Phosphorus pentachloride (500 mg) was added to this solution and gently shaken. Within 5 to 10 minutes the formation of a white crystalline mass of the acid chloride takes place and the reaction is allowed to run to completion by keeping the tubes for an additional 30 minutes on ice. The crystals were washed three times with 25 ml aliquots of hex-

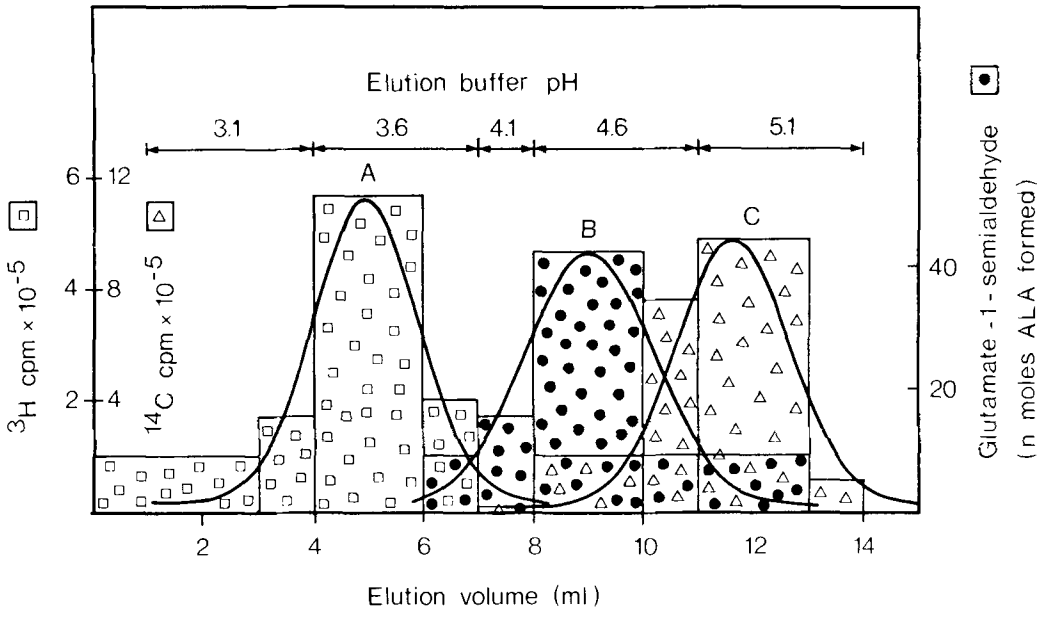

Figure 3. Purification of glutamate-1-semialdehyde from glutamate and δ-aminolevulinic acid. Elution profiles are A. glutamate, B. glutamate-1-semialdehyde, C. δ-aminolevulinate. The normal distribution curves were matched by eye to the histograms.

A pre-equilibrated Dowex 50X8 column (2 x 0.6 cm) was loaded with 1.2 ml of a mixture containing L-glutamate-4-^3H, δ-aminolevulinate-5-^{14}C and glutamate-1-semialdehyde preparation. The pH of the mixture was 1.6. The column was washed stepwise with sodium citrate buffers in the following order: 3 ml of buffer at pH 3.1, 2 plus 1 ml buffer at pH 3.6, 1 ml of buffer at pH 4.1, 2 plus 1 ml of buffer at pH 4.6 and 3 ml of buffer at pH 5.1. All citrate buffers were 0.2 N for Na^+. The washings were collected separately and the ^3H and ^{14}C counts determined. Glutamate-1-semialdehyde was assayed enzymically as given in 2.6. ALA = δ-aminolevulinate.

ane and finally dissolved in 40 ml of dry ether. The ether solution was placed in the hydrogenation vessel (Figure 2) with 500 mg of freshly prepared dry palladium black and 10 µl of quinoline poison (cf. 2.7.). The mixture was cooled on ice. The ice cold mixture was bubbled with N_2 for 1 to 2 minutes and then vigorously with H_2. Liberation of HCl during hydrogenation was monitored by holding a filter paper moistened with ammonia to the outlet of the hydrogenation vessel. The formation of aldehyde was also followed by spot tests on filter paper using Schiff's reagent. Hydrogenation was allowed to proceed at 0°C until no further HCl was liberated. Ether was then separated from the palladium black and the latter was extracted three times with 5 ml aliquots of water. The ether fraction was extracted twice with 3 ml aliquots of water. The aqueous extracts contained the glutamate-1-semialdehyde and were pooled and purified by ion exchange chromatography. The aqueous extract was acidic (pH between 1 and 2) and was found to contain two major ninhydrin positive substances when analysed on an amino acid analyser. One of the peaks corresponded to glutamate and the other was eluted close to the δ-aminolevulinate standard. The yield of semialdehyde was 11%, determined as given in *2.5.2.*

2.5.1. Purification of glutamate-1-semialdehyde

Preparations were absorbed on a Dowex 50X8 (200-400 mesh) ion exchange column (2×0.6 cm) pre-equilibrated with sodium citrate buffer having a pH of 3.1. The column was then washed stepwise with different quantities of sodium citrate buffers of increasing pH as follows: 3 ml at pH 3.1, 2 plus 1 ml at pH 3.6, 1 ml at pH 4.1, 2 plus 1 ml at pH 4.6 and 3 ml at pH 5.1. All sodium citrate buffers were prepared by adjusting 0.2 M-NaOH with solid citric acid. As evident from the results in Figure 3 this procedure separates glutamate-1-semialdehyde from glutamate and δ-aminolevulinate. Glutamate-1-semialdehyde migrated with an R_f of 0.27 in descending chromatograms on Whatmann 3 MM paper developed with *N*-butanol:acetic acid:water (25:4:10 v/v/v) and was well separated from glutamate (R_f 0.12) and δ-aminolevulinate (R_f 0.35). Glutamate-1-semialdehyde on chromatograms was identified by spraying with ninhydrin (yellow spot), spraying with Schiff's reagent (characteristic aldehyde colour), and spraying with dinitrophenylhydrazine reagent (yellow spot), as well as its ability to be converted into δ-aminolevulinate on incubation with enzyme.

2.5.2. Estimation of glutamate-1- semialdehyde concentration

The amount of glutamate-1-semialdehyde in purified preparations was determined by titration for carbonyl groups (14).

2.6. Glutamate-1-semialdehyde aminotransferase assay

The enzyme assay was performed in 15 ml tubes. The incubation mixture contained in a total volume of 1 ml the following: 0.3 M-glycerol, 0.1 M-Tricine-NaOH, 0.25 mM-dithiothreitol, 1 mM-glutamate-1-semialdehyde and 50 to 100 µg enzyme protein. The pH of the incubation mixture was 7.9. The mixtures were incubated for 15 minutes at 22°C. Reactions were stopped by placing the tubes in ice and addition of 50 µl of 70% perchloric acid. Precipitated protein was centrifuged off and 50 µl of 5 N-KOH added. The precipitate of $KClO_4$ was removed by centrifugation. Δ-aminolevulinate present in the supernatant was condensed with ethyl acetoacetate and the pyrrole formed was estimated. These procedures have been described before (11).

2.7. Other methods

Protein was measured according to LOWRY et al. (10). Optical density was measured using a Zeiss PMQ 3 spectrophotometer. Assays for L-glutamate-U-^{14}C incorporation into δ-aminolevulinate and determinations of radioactivity were performed as described previously (4). Palladium black was prepared according to the method described in (12) and dried by washing with acetone and dry ether. Quinoline poison was prepared according to the method described in (7).

Table I

Cofactor requirement for glutamate l-semialdehyde aminotransferase.

Experiment	δ-Aminolevulinate formed n moles
1. Control	7.53
2. + 0.5 mM-ATP	6.50
3. + 0.5 mM-ATP + 0.25 mM-NADPH	6.74
4. + 0.5 mM-ATP + 0.25 mM-NADPH + 25 mM-$MgCl_2$	6.00
5. + 5 mM-L-glutamate	7.24
6. + 5 mM-L-phenylalanine	7.57
7. + 5 mM-L-alanine	7.20
8. + 5 mM-aspartate	7.32
9. - Enzyme	0
10. Boiled enzyme	0

Incubations were carried out in a total volume of 1 ml in 15 ml tubes for 15 minutes at 22°C. The control incubation mixture in experiment 1 contained 89 μg partially purified enzyme, 1 mM-glutamate-1-semialdehyde, 0.25 mM-dithiothreitol, 0.3 M-glycerol and 0.1 M-Tricine-NaOH at pH 7.9. In experiment 10 the enzyme was placed in a boiling water bath for 5 minutes and cooled on ice before adding the incubation mixture.

3. RESULTS

3.1. Properties of glutamate-1-semialdehyde aminotransferase

Table I illustrates the cofactor requirement for δ-aminolevulinate synthesis from glutamate-1-semialdehyde. None of the cofactors which stimulate the conversion of L-glutamate-U-^{14}C to δ-aminolevulinate (5) are required for glutamate-1-semialdehyde aminotransferase activity. Glutamate and aspartate function as amino donors of several plant transamination reactions. Phenylalanine and alanine were reported as amino group donors in a dioxovalerate transaminase reaction (3). All four amino acids were without effect on glutamate-1-semialdehyde aminotransferase activity. This makes it likely that the enzyme catalyses a transfer of the amino group at the C-2 position of the semialdehyde molecule to the C-1 position. The enzyme has a broad pH optimum around 8.0 (Figure 4) and within the range of the three temperatures tested δ-aminolevulinate formation was highest at 30°C (Table II). Figure 5 illustrates the effect of increasing amounts of substrate in the incubation mixture. Glutamate-1-semialdehyde concen-

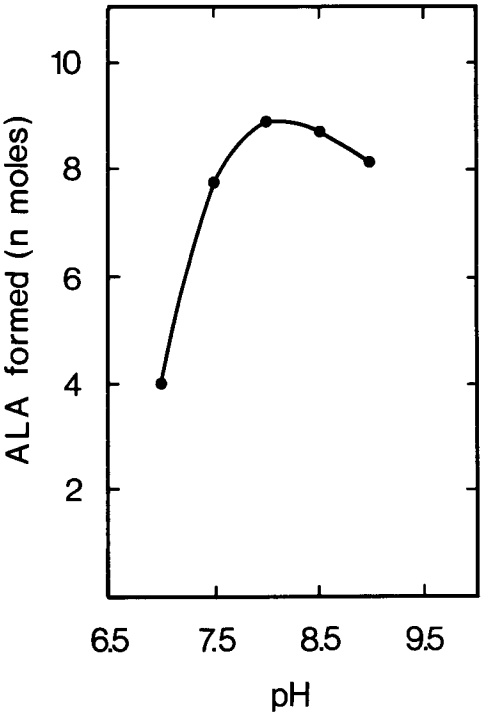

Figure 4. The effect of pH on glutamate-1-semialdehyde aminotransferase. 0.1 M-tricine-NaOH buffers were used and the assays were performed as given under Table I. ALA = δ-aminolevulinate.

Table II

The effect of temperature on glutamate 1-semialdehyde aminotransferase.

Incubation temperature °C	δ-Aminolevulinate formed n moles
14	4.06
22	7.53
30	9.85

Assays were performed under the conditions given for experiment 1 in Table I except for variations in temperature.

trations below 2 mM stimulated δ-aminolevulinate formation, whereas higher concentrations inhibited the glutamate-1-semialdehyde aminotransferase activity. These data result in a K_m value for the substrate of about 0.4 mM. Under the assay conditions described in section 2.6 δ-aminolevulinate formation from glutamate-1-semialdehyde is approximately linear up to 30 minutes (Figure 6).

3.2. Inhibitors of glutamate-1-semialdehyde aminotransferase

Inhibitor studies with plastids isolated from greening barley leaves have shown that biosynthesis of δ-aminolevulinate from L-glutamate-U-^{14}C involves a transamination step (8). The effect of several known inhibitors of transamination on the aminotransferase activity is given in Table III. The transaminase inhibitors aminooxyacetate (20 mM), cycloserine (20 mM) and D-penicillamine (20 mM) inhibited glutamate-1-semialdehyde aminotransferase by 57, 43 and 9% respectively. Glyoxylate inhibited the enzyme by 27%. The δ-aminolevulinate synthesis from L-glutamate-U-^{14}C in isolated plastids is inhibited by very low concentrations of β-hydroxyglutamate reaching 96% inhibition at 50 μM (8). This compound at 1 mM concentration had, however, no effect on δ-aminolevulinate synthesis from glutamate-1-semialdehyde.

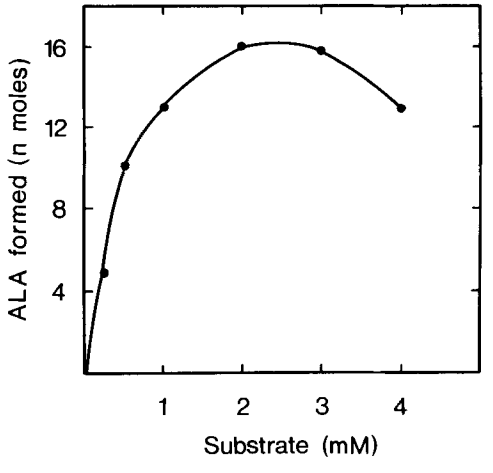

Figure 5. The effect of substrate concentration on glutamate-1-semialdehyde aminotransferase. Assay conditions are as given under Table I. ALA = δ-aminolevulinate.

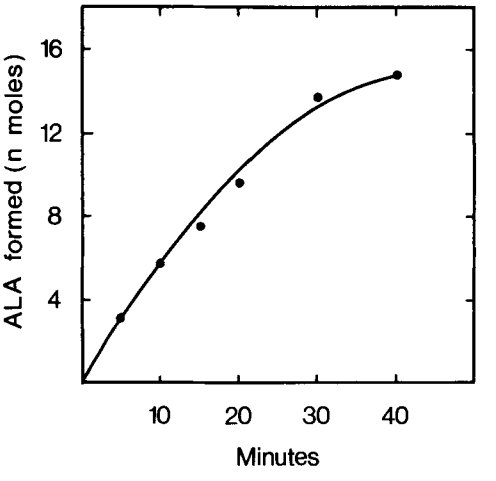

Figure 6. Time course of δ-aminolevulinate formation by glutamate-1-semialdehyde aminotransferase. Assay conditions are given under Table I. ALA = δ-aminolevulinate.

Pyridoxal phosphate at a concentration of 5 mM gave a 47% inhibition while pyridoxamine phosphate at the same concentration is stimulatory (Table III). The inhibitor of sulfhydryl containing enzymes, p-chloromercuribenzoate (0.1 mM), inhibited the aminotransferase by 45% when added to the incubation mixture (Table III). In another experiment the enzyme was preincubated with 0.1 mM-p-chloromercuribenzoate for 30 minutes at 22°C and 100% inhibition of activity occurred.

Table III

Effects of inhibitors on δ-aminolevulinate synthesis from glutamate 1-semialdehyde.

Inhibitor	Concentration mM	δ-aminolevulinate formation % of control
Aminooxyacetate	1.0	89
	20.0	43
D-Cycloserine	1.0	109
	20.0	57
D-Penicillamine	1.0	82
	20.0	91
Glyoxylate	1.0	100
	20.0	73
β-Hydroxyglutamate	0.1	104
	1.0	100
p-Chloromercuribenzoate	0.1	55
	1.0	0
Pyridoxal phosphate	0.025	95
	5.0	53
Pyridoxamine phosphate	5.0	111

Assay conditions are given in section 2.6.

3.3. △-aminolevulinate synthesis from L-glutamate-U-^{14}C and glutamate-1-semialdehyde

Table IV compares glutamate-1-semialdehyde aminotransferase activity with δ-aminolevulinate synthesizing activity from L-glutamate-U-^{14}C in different plastid types. Both activities were detected in soluble protein preparations from etioplasts, greening plastids and mature chloroplasts of barley. In greening plastids the two activities were respectively 5 to 20 fold higher than in etioplasts and mature chloroplasts when compared on a per mg protein basis.

3.4. Fractionation of enzyme activites

Fractionation of soluble proteins of greening barley plastids on a Biogel A 0.5 column gave a partial purification of the two enzymatic activities that synthesize δ-aminolevulinate

Table IV

Comparision of δ-aminolevulinate synthesizing activities in etioplasts, greening chloroplasts and mature chloroplasts of barley.

Soluble protein extract from	δ-aminolevulinate synthesis from	
	glutamate-1-semialdehyde	L-glutamate-U-^{14}C
	n moles/mg protein	cpm fixed/mg protein
1. Etioplasts	5.38	266
2. Greening chloroplasts	28.6	11387
3. Mature chloroplasts	6.26	459

Plastids were isolated from 6 day old seedlings grown in darkness (for 1), 6 day old dark grown seedlings illuminated for 6 hours (for 2) and 6 day old seedlings grown under continuous illumination (for 3). Isolated plastids were disrupted by passing through a French pressure cell and soluble proteins were separated from membranes by ultracentrifugation as given under 2.4. These soluble protein preparations were assayed directly for δ-aminolevulinate formation. The assay conditions for the two activities are given under Table I and Figure 7.

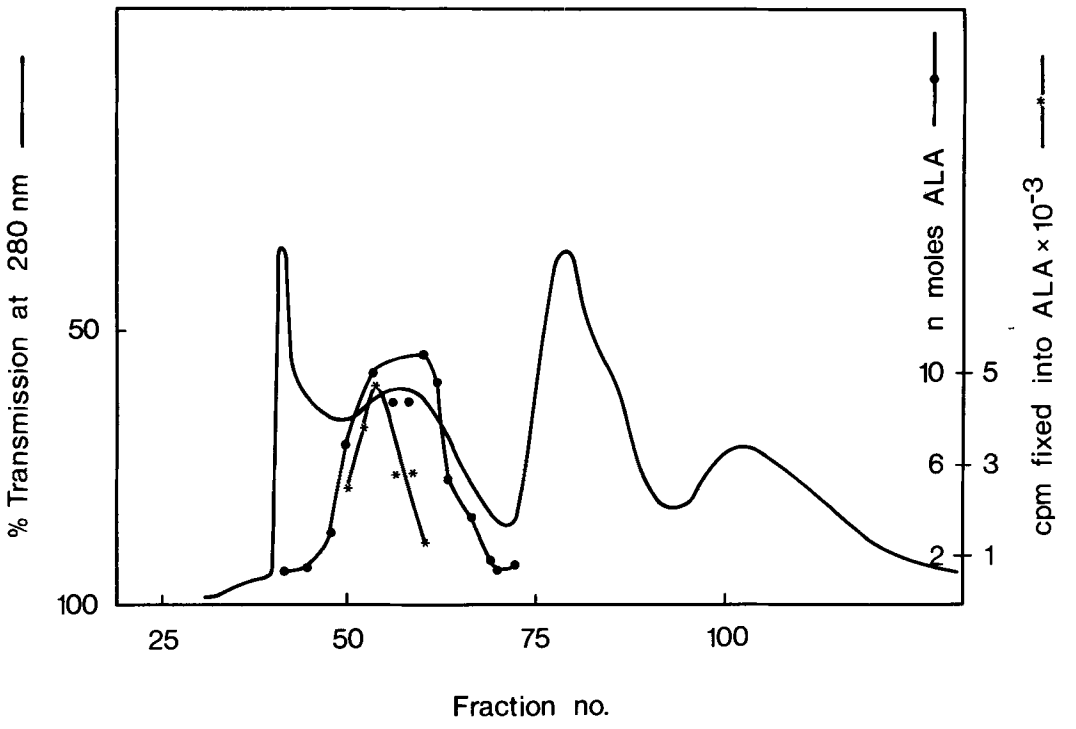

Figure 7. Partial purification of δ-aminolevulinate synthesizing activities of greening barley chloroplasts.

Soluble proteins of greening barley chloroplasts were fractionated on a Biogel A 0.5 column (2.5 x 85 cm) as given in 2.4. Glutamate-1-semialdehyde amniotransferase was assayed as in 2.6. The incubation mixture for L-glutamate-U-^{14}C incorporation into δ-aminolevulinate contained in 1.2 ml the following: 0.3 M-glycerol, 0.1 M-Tricine-NaOH, 1 mM-dithiothreitol, 20 mM-$MgCl_2$, 0.5 mM-ATP, 0.25 mM-NADPH, 1.5 μCi L-glutamate-U-^{14}C and 1 ml of column fraction. The pH of the incubation mixture was 7.9. The mixture was incubated for 15 minutes at 22°C and assayed for incorporation of radioactivity into δ-aminolevulinate (4). ALA = δ-aminolevulinate.

(Figure 7). In the column eluate all the fractions which contained the ability to synthesize δ-aminolevulinate from L-glutamate-U-^{14}C also contained glutamate-1-semialdehyde aminotransferase activity. However the tail end fractions of the aminotransferase peak had no activity for the synthesis of δ-aminolevulinate from L-glutamate-U-^{14}C. The results given in Figure 7 indicate that the two enzyme activities can be separated with glutamate-1-semialdehyde aminotransferase having a lower molecular weight than the enzyme that converts glutamate to δ-aminolevulinate. Column fractions containing glutamate-1-semialdehyde aminotransferase could be stored frozen for several weeks without a noticeable loss of activity. In sharp contrast, the ability to convert L-glutamate-U-^{14}C into δ-aminolevulinate was completely lost when the active fractions from the column were stored frozen for two days.

4. DISCUSSION

Soluble protein extracts of plastids isolated from greening barley leaves convert glutamate-1-semialdehyde to δ-aminolevulinate. The results presented in this paper show that this conversion is due to the presence in developing chloroplasts of an enzyme catalysing the transfer of the amino group from the C-2 position of glutamate-1-semialdehyde substrate to the C-1 position. There are no previous reports on intramolecular transamination reactions in higher plants. It will be of interest to determine if the new enzyme is related to aminomutases (cf. 13) which function with a

vitamin B_{12} catalysed intramolecular transfer of an amino group. High concentrations of pyridoxal phosphate inhibit δ-aminolevulinate synthesis from glutamate-1-semialdehyde by the partially purified enzyme. This could be due to the formation of a Schiff base between pyridoxal phosphate and a free lysine residue necessary for enzyme activity (2). Further experiments will have to be carried out in order to decide whether or not pyridoxal phosphate participates in the glutamate-1-semialdehyde aminotransferase reaction.

It is of interest to compare glutamate-1-semialdehyde aminotransferase with the previously recognized activity synthesizing δ-aminolevulinate from L-glutamate (5). The soluble protein preparations previously shown to be capable of synthesizing δ-aminolevulinate from L-glutamate-U-^{14}C in the presence of ATP, NADPH and Mg^{2+} ions were also able to synthesize it from glutamate-1-semialdehyde in the absence of added cofactors. Both enzymes are found with their highest specific activities in developing chloroplasts where active δ-aminolevulinate synthesis occurs. Glutamate-1-semialdehyde has been considered as a possible intermediate in δ-aminolevulinate synthesis (1). The present studies lend support to this route with glutamate-1-semialdehyde aminotransferase activity being a part reaction in the conversion of L-glutamate into δ-aminolevulinate. We propose that the conversion of glutamate to δ-aminolevulinate involves at least three enzymatic activities as illustrated in Figure 1. Glutamate is first phosphorylated by a kinase in the presence of ATP and Mg^{2+} to give glutamate-1-phosphate which is then reduced to glutamate-1-semialdehyde in the presence of NADPH by a dehydrogenase. The last step of δ-aminolevulinate synthesis is catalysed by glutamate-1-semialdehyde aminotransferase. The three enzymes may exist as a complex or separate proteins in the stroma of developing chloroplasts. The ability to carry out the overall reaction, the conversion of L-glutamate-U-^{14}C to δ-aminolevulinate, is very unstable in partially purified preparations. This has hindered our attempts to purify this activity to homogeneity. In future attempts, purification of partial activities and reconstitution of the overall reaction should therefore be considered. This will help to determine which step is regulatory and also the mode of regulation of δ-aminolevulinate synthesis in greening barley.

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