BIOSYNTHESIS OF Δ -AMINOLEVULINATE IN GREENING BARLEY LEAVES IV. ISOLATION OF THREE SOLUBLE ENZYMES REQUIRED FOR THE CONVERSION OF GLUTAMATE TO Δ -AMINOLEVULINATE

by

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The soluble enzymes converting glutamate into δ -aminolevulinate and subsequently into uroporphyrinogen were partially purified from the stroma of greening barley plastids using Sephacryl S-300 gel filtration. By affinity chromatography employing sequentially Blue Sepharose, Matrex Gel Red A and heme-Sepharose the partially purified enzymes were separated into three fractions which together are required to catalyze the synthesis of δ -aminolevulinate from glutamate: proteins binding to Blue Sepharose, proteins binding to heme-Sepharose and run-off proteins not retained by the three columns. By analysing the characteristics of these fractions the following conclusions are reached:

1. Conversion of glutamate into glutamate-l-semialdehyde in the presence of ATP, Mg^{2+} and NADPH requires at least two proteins, one binding to heme-Sepharose and one binding to Blue Sepharose.
2. Glutamate-l-semialdehyde is converted into δ -aminolevulinate by glutamate-l-semialdehyde aminotransferase, which is not retained on the affinity columns.
3. The run-off protein fraction also contains δ -aminolevulinate dehydratase and porphobilinogen deaminase.
4. The heme-Sepharose bound protein(s) probably converts glutamate to glutamate-l-phosphate in the presence of ATP and Mg^{2+} and the Blue-Sepharose bound protein(s), glutamate-l-phosphate to glutamate-l-semialdehyde, in the presence of NADPH.

1. INTRODUCTION

In the developing chloroplast δ -aminolevulinic acid is synthesised from glutamate by soluble enzymes (4, 7, 11, 20). The rate limiting step in the chlorophyll pathway is the synthesis of δ -aminolevulinic acid (3, 5, 11, 15). Its formation in vitro is strongly inhibited by heme (8) and has been proposed to proceed in the following way: Glutamate is converted into glutamate-1-phosphate, which is reduced to glutamate-1-semialdehyde and the aldehyde transaminated to δ -aminolevulinic acid (14). The enzyme catalysing the last step, glutamate-1-semialdehyde aminotransferase has been isolated and purified (16).

This paper describes how we separated the δ -aminolevulinic acid synthesising plastid stroma preparation into three components and studied the characteristics of these components.

2. MATERIALS AND METHODS

2.1. Chemicals

N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride was obtained from Fluka AG, Switzerland. Na-lauryl sulphonate (specialty pure for biochemical studies) and t-amyl alcohol from British Drug Houses, UK. Hemin, NADPH, L-methionine DL-sulphoximine and ATP were obtained from Sigma, USA. L-glutamate- U - ^{14}C (275 mCi·mmole $^{-1}$) was obtained from Amersham, UK. Sephacryl S-300, Blue Sepharose CL-6B and AH Sepharose-4B were obtained from Pharmacia, Sweden. Matrex Gel Red A was from Amicon, USA. Glutamate-1-semialdehyde was synthesised from N-carbo-benzoxy-L-glutamic acid γ -benzyl ester (Bachem, Switzerland) as described previously (14). Porphobilinogen was obtained from Porphyrin Products, USA.

2.2. Preparation of heme-Sepharose

Heme was linked to the amino group of the six-carbon spacer of AH-Sepharose-4B in the following manner. Hemin (134 mg, 200 μ mole) was mixed with 0.5 ml of 1 M-NaOH and dissolved by the addition of 100 ml methanol. Forty ml of 0.01 M-HCl were added to the heme solution while stirring, thus changing the reading of a pH meter to pH 7.2. Water (60 ml) was then added. It was found essential to

maintain the pH reading above 7.0 to prevent precipitation of heme. AH-Sepharose-4B (5 g) was swollen in 200 ml of 0.5 M-NaCl and washed with 1000 ml of 0.5 M-NaCl followed by 1000 ml of distilled water. The heme solution was added to the gel suspension. N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (38 mg, 250 μ moles, in 1 ml water) was added dropwise to the heme gel mixture. The suspension was shaken at room temperature for at least 24 hours in darkness. In the first hour of incubation the pH of the solution was maintained above 7.0 using 0.1 M-NaOH. At the end of the incubation the gel beads appeared dark brown. The heme-Sepharose gel was removed from the solution by centrifugation at 600 g for 5 min (representing standard procedure unless otherwise mentioned) and washed alternately with acidic (10 % acetic acid) and alkaline (1 % NaOH) solutions of 50 % methanol containing 1 M-NaCl. The washing was continued until no more heme leached out from the gel beads.

2.3. Plant material

Three kg of barley seeds (*Hordeum vulgare* L.cv. Svalöfs Bonus) were germinated and grown on plastic trays containing moist vermiculite in a dark room kept at 23 °C and 70 to 80 % relative humidity. After six days of growth the seedlings were greened under »Osram Fluora« fluorescent light for six hours. Leaves were harvested, wrapped in aluminium foil and cooled in ice.

2.4. Isolation of plastids and stroma proteins

All steps of enzyme isolation were carried out at 0 to 4 °C. The leaves were homogenised in 0.6 M-glycerol, 0.1 M-Tricine-NaOH (pH 9.0) using a blender fitted with replaceable razor blades (17). The homogenate was squeezed through two layers of nylon mesh (31 μ) and centrifuged at 1,400 g for five minutes. The plastid pellet was suspended in a solution of 0.3 M-glycerol, 0.1 M-Tricine-NaOH (pH 9.0), 1 mM-dithiothreitol and 1 mM-MgCl $_2$ then disrupted by passing through an Amicon French pressure cell at 8000 psi. The disrupted plastids were centrifuged at 226,000 g for 20 minutes and the supernatant containing the stroma proteins collected.

2.5. Purification of glutamate-l-semialdehyde aminotransferase

The glutamate-l-semialdehyde aminotransferase was purified (16) by sequential chromatography on DEAE-Sephadex A25, Sepharose 6B, Biogel A 0.5 and Sephacryl S-200. The equilibration and basic elution medium used was 0.3 M-glycerol, 0.1 M-Tricine-NaOH (pH 7.9), 1 mM-dithiothreitol and 1 mM-MgCl₂. Stroma proteins (3.84 g protein) were applied batchwise to the DEAE-Sephadex A 25 column (38 × 2.5 cm) and washed with 320 ml of elution medium and eluted then with a linear gradient of NaCl (0 to 0.5 M) in the same medium. Glutamate-l-semialdehyde aminotransferase eluted at about 0.23 M-NaCl. The active fractions were pooled and concentrated by pressure dialysis to about 10 ml using an Amicon PM30 filter. The concentrate was applied to a Sepharose 6B column (5 × 48 cm). Eight ml fractions were collected. The active fractions eluted around fraction 90 and were pooled and concentrated to 10 ml. The proteins were then applied to a Biogel A 0.5 column (5 × 50 cm) and eluted as a broad peak around fraction 60 (8 ml fractions). After the fractions containing aminotransferase were pooled and concentrated to 10 ml they were fractionated on a Sephacryl S-200 column (80 × 2.5 cm). The fraction size were eight ml. The activity eluted around fraction 33 and was concentrated to give purified aminotransferase (specific activity, 70 nmole δ -aminolevulinate · mg protein⁻¹ · minute⁻¹). Gel electrophoresis on a 7.0% polyacrylamide column (5 × 110 mm) of the purified aminotransferase revealed five bands staining with Comassie blue. Enzyme assays with unstained gels showed that one of the bands was aminotransferase.

2.6. Column elution medium and the desalting and concentration of proteins

The elution medium consisted of 0.3 M-glycerol, 0.1 M-Tricine-NaOH (pH 9), 1 mM-dithiothreitol and 25 mM-MgCl₂ and was used as the standard medium unless otherwise mentioned. The proteins were desalted by gel filtration through a Sephadex G-50 column (25 × 30 cm). The proteins were concentrated by pressure dialysis using an Amicon Pressure Dialyser

fitted with a PM30 filter. This filter retains molecules larger than 30,000 dalton.

2.7. Partial purification of the δ -aminolevulinate synthesising enzymes by gel filtration on Sephacryl S-300

A Sephacryl S-300 column (5 × 49 cm) was equilibrated with elution medium. The stroma protein solution was adjusted to 25 mM-MgCl₂ and then loaded onto the column. The eluate was monitored for absorption of light at 280 nm and collected in 8 ml fractions. Portions from the fractions were assayed with glutamate-U-¹⁴C for δ -aminolevulinate synthesising activity. The active fractions were pooled and concentrated.

2.8. Fractionation of the δ -aminolevulinate synthesising enzymes by affinity chromatography on heme-Sepharose, Blue Sepharose and Matrex Gel Red A

The heme-Sepharose column (1 × 4 cm), the Blue Sepharose column (1 × 6 cm) and the Matrex Gel Red A column (1 × 6 cm) were pre-equilibrated with the elution medium used for gel-filtration. Ten ml (30 mg protein) of partially purified δ -aminolevulinate synthesising enzymes were passed through each column. The columns were washed with the elution medium until no further proteins eluted. The run-off fraction (proteins that do not bind) was concentrated. Bound proteins were eluted from the heme-Sepharose column with 0.3 M-NaCl in the elution medium and from the other two columns with 1 M-NaCl in the elution medium. Each of these fractions was desalted and concentrated to 10 ml. The affinity columns were regenerated by the following procedures. The heme-Sepharose column was washed three times, alternately with 96% acetic acid and elution medium. The Blue Sepharose column was washed with 0.5 M-NaCl in 0.1 M-Na acetate pH 4.5 and thereafter with elution medium. Matrex Gel Red A was washed with 7 M-urea in 0.5 M-NaOH and with elution medium.

Figure 1. Elution pattern of δ -aminolevulinic acid synthesising activity from a Sephacryl S-300 column.

2.9. Serial affinity chromatography of the partially purified δ -aminolevulinic acid synthesising proteins

Stroma proteins obtained from isolated plastids were fractionated on a Sephacryl S-300 column as described in section 2.7. When the fractions that were known to contain the δ -aminolevulinic acid synthesising proteins began to appear (cf. Figure 1), the eluate of the S-300

column was passed through the following columns in series: Blue Sepharose (2.5×8.5 cm), Matrex Gel Red A (1×6 cm) and heme-Sepharose (2×10 cm). The final eluate from the column series was scanned for absorption of light at 280 nm and the proteins (run-off proteins) were collected and concentrated to 10 ml. After the δ -aminolevulinic acid synthesising proteins were completely eluted from the S-300 column it was disconnected from the series of subsequent columns. The elution medium was then pumped directly through the Blue Sepharose column until no further run-off proteins eluted from the heme-Sepharose column. The run-off protein fraction was concentrated to 10 ml. Proteins bound to the Blue Sepharose column and the heme-Sepharose column were eluted with 1 M-NaCl in the elution medium. These fractions were then desalted. After desalting, the protein fraction binding to heme-Sepharose was concentrated to 10 ml. The desalted proteins binding to Blue Sepharose were passed through a small heme-Sepharose column (1×4 cm) and the fraction not binding to the heme column was collected and concentrated to 10 ml. The procedure is illustrated in Figure 2.

Figure 2. Diagram illustrating partial purification and serial affinity chromatography of the δ -aminolevulinic acid synthesising enzymes.

2.10. Assay for δ -aminolevulinate synthesising activity with radioactive or unlabelled glutamate

The following standard assay was used unless otherwise stated: To the enzyme in 2 ml of elution medium were added 2 μ mole of NADPH, 1 μ mole of ATP and 200 nmoles of L-glutamate or 2.5 μ Ci L-glutamate U- 14 C. The incubation was for 20 min at 27 °C.

2.11. Glutamate-l-semialdehyde aminotransferase assay

The assay was carried out in 1 ml of elution medium containing approximately 2 mM-glutamate-l-semialdehyde and 100 μ g of protein. The reaction proceeded for 20 minutes at 27 °C and was stopped by adding 20 μ l of 70 % perchloric acid. After removal of the precipitated protein by centrifugation, the supernatant was adjusted to pH 6.9 and incubated with 100 μ l of ethyl acetoacetate, at 100 °C for 20 minutes. The pyrrole formed was treated with modified Ehrlich's reagent (9) and the absorption at 525 and 553 nm was measured after 20 minutes. The amount of δ -aminolevulinate was estimated using $\epsilon_{\text{mM } 553 \text{ nm}}$ of 58 l $\cdot$ mmole $^{-1}$ (9).

2.12. Determination of radioactive δ -aminolevulinate

The assay mixture was cooled on ice, 100 μ g of δ -aminolevulinate hydrochloride added as carrier and proteins precipitated by the addition of 40 μ l of 70 % perchloric acid. After removal of the precipitated protein by centrifugation, the sample was loaded onto a Dowex 50W X 8 column (0.6 $\times$ 2 cm) which was prewashed with 2 ml of Na-citrate buffer (pH 3.1, 0.2 M-Na $^{+}$). After loading, the column was washed again with 2 ml of the pH 3.1 Na-citrate buffer. The δ -aminolevulinate which had adsorbed to the column was eluted with 5 ml of Na-citrate buffer (pH 5.1, 0.2 M-Na $^{+}$). The δ -aminolevulinate was converted to a pyrrole with ethyl acetoacetate and the pyrrole purified by thin layer chromatography as described previously (6, 13). The purified pyrrole was eluted from the chromatogram and analysed for the recovery of carrier. The radioactivity was measured and corrected for the recovery. This procedure is modified

from the one described previously (13). It will not discriminate between 14 C-glutamate-l-semialdehyde and 14 C- δ -aminolevulinate although recoveries of the aldehyde may be low due to the absence of added cold carrier.

2.13. Assay for δ -aminolevulinate formed from unlabelled glutamate

Samples were prepared and loaded onto Dowex columns as described in 2.12. The fraction eluted at pH 5.1, which contained δ -aminolevulinate (and also glutamate-l-semialdehyde if present), was adjusted to pH 6.9. After the addition of 100 μ l of ethyl acetoacetate, pyrrole was formed by heating at 100 °C for 20 minutes. The samples were cooled and adjusted to pH 7.5 and the ethyl acetoacetate removed by three extractions with chloroform. The samples were adjusted to pH 3.8 and pyrrole obtained by three extractions with ether. After addition to the ether of one drop of 25 % ammonia it was evaporated to dryness. Equal volumes of distilled water and modified Ehrlich's reagent (9) were added. After 20 minutes, the absorption at 525 and 553 nm was determined with a Zeiss PMQ spectrophotometer or the absorption spectra scanned using an Aminco DW2A spectrophotometer. The amount of δ -aminolevulinate was estimated using a $\epsilon_{\text{mM } 553 \text{ nm}}$ of 58 (9). Under these conditions, glutamate-l-semialdehyde also forms a pyrrole which gives a pink colour with Ehrlich's reagent. But this pyrrole has a $\epsilon_{\text{mM } 553 \text{ nm}}$ at least one order of magnitude smaller and will not interfere with δ -aminolevulinate estimations.

2.14. Separation of glutamate, glutamate-l-semialdehyde and δ -aminolevulinate by cation exchange chromatography on Dowex 50W X 8

In order to determine the presence of glutamate-l-semialdehyde in incubated samples it was necessary to separate the aldehyde from δ -aminolevulinate and NADPH. This was done by cation exchange chromatography on Dowex 50W X 8 (200–400 mesh) (14). A Dowex column (0.6 $\times$ 2 cm) was washed with 2 ml of 1 M-NaOH and equilibrated with 2 ml Na-citrate buffer (0.2 M-Na $^{+}$) pH 3.1. After deproteinising with perchlo-

ric acid the sample (2 ml) was adjusted to pH 1.9 with 5 M-KOH and the precipitated KClO_4 removed by centrifugation. The supernatant was loaded onto the cation exchange column. The column was then washed sequentially with sodium citrate buffers (0.2 M- Na^+) at pH 3.1 (3 ml), 3.6 (3 ml), 4.1 (1 ml), 4.6 (2 ml + 1 ml) and 5.1 (3 ml). In this procedure NADPH does not bind to Dowex and glutamate elutes in the fractions at pH 3.1 and 3.6. Most of the glutamate-l-semialdehyde elutes in the fractions at pH 4.1 and 4.6 and most of the δ -aminolevulinate in the pH 5.1 fraction.

The relative concentrations of glutamate-l-semialdehyde present in fractions were estimated by incubating the fractions with glutamate-l-semialdehyde aminotransferase for 20 minutes at 27 °C, after adjusting the pH to 8.0 with phosphate buffer. The δ -aminolevulinate from the pH 5.1 fraction and that formed from glutamate-l-semialdehyde were then estimated as described in 2.11.

2.15. High pressure liquid chromatographic separation of glutamate, glutamate-l-semialdehyde and δ -aminolevulinate

These compounds were separated on a reverse phase octadecyl silane column (Waters μ Bondapak C_{18}) using a Waters 6000A solvent delivery system. The mobile phase used was as follows: 50mM- NaH_2PO_4 , adjusted to pH 3.0 with phosphoric acid and made up to 5mM-sodium lauryl sulphonate and 1% t-amyl alcohol. This solvent system and column is a modified version of one reported by HANCOCK et al. (10) for the separation of amino acids. Before injection of samples the column was washed with mobile phase for at least 30 minutes. It was important to equilibrate and operate at 20 °C.

2.16. Preparation of samples and high pressure liquid chromatography

After incubation the samples were cooled on ice and carrier glutamate-l-semialdehyde and δ -aminolevulinate added to give a concentration of 3mM of each. Proteins were precipitated by the addition of 20 μ l of 70% perchloric acid per ml of sample. After removal of proteins, the pH of the supernatant was adjusted to 3.0 with 5 M-

KOH and the precipitated KClO_4 removed by centrifugation. A portion of the supernatant (20 μ l) was injected into the high pressure liquid chromatograph and collection of 2 ml fractions started immediately. From each fraction one ml was used to measure radioactivity, and from appropriate fractions, 0.5 ml was assayed for glutamate-l-semialdehyde, using glutamate-l-semialdehyde aminotransferase as in 2.11 except that the pH was 8.0, and 0.5 ml was used for δ -aminolevulinate determination.

2.17. Glutamine synthetase (5-glutamyl kinase) and 1-glutamyl kinase assays

These activities were assayed in elution medium (pH 7.9) containing 25mM L-glutamate, 5mM ATP, 12.5 mM- MgCl_2 and 50mM NH_2OH . Incubation was for 20 minutes at 27 °C. After incubation glutamate hydroxamates formed were determined according to HESTRIN (12). The volume was adjusted to 2.0 ml with distilled water and 0.5 ml of 10% FeCl_3 , in 0.1 M-HCl and 3.3% trichloroacetic acid, was added. The pH was then adjusted to 1.2 ± 0.2 with 12% HCl, the solution centrifuged and the absorption at 540 nm measured against a control which had been incubated in the absence of enzyme.

1-Glutamyl kinase activity was measured in the presence and absence of 10 mM L-methionine-DL-sulphoximine, a strong inhibitor of pea leaf glutamine synthetase (19) and other 5-glutamyl kinase activities (1, 18).

2.18. Assay for δ -aminolevulinate dehydratase and porphobilinogen deaminase

These activities were assayed in 1 ml of 0.3 M-glycerol, 0.1 M-Tricine- NaOH (pH 8) with 10mM- δ -aminolevulinate for the dehydratase and 100 μ g of porphobilinogen for the deaminase. After incubation for 20 min at 27 °C the samples were deproteinised by adding 20 μ l of 70% perchloric acid and centrifugation. Porphobilinogen formed in the dehydratase assay was determined with modified Ehrlich's reagent (9). Uroporphyrinogen formed in the deaminase assay was converted to uroporphyrin when the acidified samples were left for 15 min at room temperature. Uroporphyrin was determined by spectrophotometry using $\epsilon_{\text{mM } 406} = 541$ a value determined in 0.5 N HCl (2).

3. RESULTS AND DISCUSSION

3.1. Partial purification of the δ -aminolevulinate synthesising enzymes from the plastid stroma by gel-filtration on Sephacryl S-300

The δ -aminolevulinate synthesising proteins present in the plastid stroma preparation were partially purified by gel-filtration. Three main ultraviolet light absorbing peaks were eluted from the Sephacryl S-300 column (Figure 1). The first peak contained membrane proteins and was green. The later half of the second band and the third band were able to convert glutamate and glutamate-l-semialdehyde to δ -aminolevulinate.

3.2. Affinity chromatography of the partially purified δ -aminolevulinate synthesising enzymes on heme-Sepharose and separation into two fractions

Heme strongly inhibits δ -aminolevulinate synthesis from glutamate (8).

Advantage was taken of this fact and a heme-Sepharose column prepared to which the δ -aminolevulinate synthesising enzymes or a component thereof could be bound. As shown in Table I, the heme-Sepharose column binds a small amount of protein when the partially

purified δ -aminolevulinate synthesising enzymes were passed through. Neither the bound protein fraction nor the proteins which passed through the column and contained the glutamate-l-semialdehyde aminotransferase activity were able to convert large amounts of glutamate to δ -aminolevulinate. However, a 15 fold stimulation of δ -aminolevulinate synthesis from glutamate was seen when these two fractions were combined. We conclude that a protein binding to the heme-Sepharose column is required for δ -aminolevulinate synthesis. The retained protein is most likely the component of the δ -aminolevulinate synthesising system which is regulated by heme.

3.3 Affinity chromatography of the partially purified δ -aminolevulinate synthesising enzymes on Blue Sepharose CL 6B and Matrex Gel Red A

Blue Sepharose and Matrex Gel were used as affinity material to separate the partially purified δ -aminolevulinate synthesising enzymes. As with the heme-Sepharose chromatography, glutamate-l-semialdehyde aminotransferase activity was not retained by Blue Sepharose (Table I). The protein fractions separated by either the Blue Sepharose or the Matrex Gel synthesised little δ -aminolevulinate from glutamate (Table I). When

Table I

Reconstitution of the δ -aminolevulinate synthesising activity by combining the two fractions from the partially purified δ -aminolevulinate synthesising enzymes separated on three different affinity columns.

ALA = δ -aminolevulinate. — = not done.

Affinity column	Protein fraction (mg)	Aminotransferase activity (nmole ALA formed)	cpm incorporated into ALA
Heme-Sepharose	run-off (1.380)	34	6,900
	bound (0.044)	1.6	2,200
	run-off + bound	—	141,000
Blue Sepharose	run-off (1.050)	21	1,200
	bound (0.295)	trace	2,200
	run-off + bound	—	401,000
Matrex Gel Red A	run-off (0.250)	—	1,100
	bound (0.780)	—	1,700
	run-off + bound	—	29,100

Table II

Synthesis of δ -aminolevulinate by protein fractions obtained from heme-Sepharose and Blue Sepharose affinity chromatography of partially purified δ -aminolevulinate synthesising enzymes.

Protein concentrations used in the assays are as follows: Heme-Sepharose bound fraction (0.044 mg), heme-Sepharose run-off fraction (1.38 mg), Blue Sepharose bound fraction (0.295 mg), Blue Sepharose run-off fraction (1.05 mg) and purified glutamate-l-semialdehyde aminotransferase (1.2 mg).

Assay	cpm incorporated into δ -aminolevulinate
Heme-Sepharose run-off + Heme-Sepharose bound	141,000
Blue Sepharose run-off + Blue Sepharose bound	401,000
Heme-Sepharose run off + Blue Sepharose bound	22,000
Heme-Sepharose bound + Blue Sepharose run-off	3,300
Heme-Sepharose bound + Aminotransferase	2,100
Blue Sepharose bound + Aminotransferase	3,500
Blue Sepharose bound + Heme-Sepharose bound + Aminotransferase	72,100

the two protein fractions separated on the Blue Sepharose column were combined, a 113 fold stimulation of δ -aminolevulinate synthesis from glutamate was observed. Reconstituting the two protein fractions separable on the Matrex Gel column yielded a 10 fold stimulation. Affinity

chromatography with Blue Sepharose and Matrex Gel, like that with heme-Sepharose, can separate the δ -aminolevulinate synthesising enzymes into two fractions which upon reconstitution actively convert glutamate into δ -aminolevulinate.

Table III

Synthesis of δ -aminolevulinate from glutamate by protein fractions obtained from serial affinity chromatography of partially purified δ -aminolevulinate synthesising enzymes.

Assay with	nmoles of δ -aminolevulinate formed	
	- Levulinate	+ 5mM-Levulinate
1. Run-off proteins (1.7 mg)	0	0
2. Heme-Sepharose bound proteins (0.36 mg)	0	0
3. Blue Sepharose bound proteins (1.1 mg)	0	0
4. 1. + 2.	0	0
5. 1. + 3.	0	0
6. 2. + 3.	trace	trace
7. 1. + 2. + 3.	7.4	17

Figure 3. Formation of δ -aminolevulinate from cold glutamate. Absorption spectra of Ehrlich's reagent treated pyrroles derived from the products formed in the assays of Table III. δ -Aminolevulinate is formed in assay 7 in larger amounts in the presence of levulinate than its absence.

3.4. Experiments combining fractions from the Blue Sepharose column with fractions from the heme-Sepharose column

Assays for δ -aminolevulinate synthesis were performed after combining fractions from the Blue Sepharose column and the heme-Sepharose

column. Table II shows that in no case could a fraction from Blue Sepharose be combined with a fraction from heme-Sepharose to give activities comparable with those obtained by combining fractions from the same column. Even though aminotransferase activity was present in the fractions not bound to the columns, purified aminotransferase could not replace the run-off fractions in reconstitution experiments with a bound fraction. However, aminotransferase was able to substitute for the run-off fractions when combined with both of the bound fractions.

It is concluded that the two columns segregate the components of the δ -aminolevulinate synthesising enzymes differently and that there are more than two components required for the conversion of glutamate into δ -aminolevulinate. In addition the two columns retain different proteins which together with purified glutamate-semialdehyde aminotransferase can synthesize δ -aminolevulinate from glutamate.

3.5. Requirement of three different proteins for the synthesis of δ -aminolevulinate from glutamate

Separation of the δ -aminolevulinate synthesising enzymes into three separate protein fractions was achieved by serial affinity chromatography using Blue Sepharose CL 6B, Matrex Gel Red A and heme-Sepharose 4B as described in

Figure 4. Formation of uroporphyrin from glutamate by combining the three protein fractions isolated by serial affinity chromatography.

The spectra were recorded from the assays given in Table III. Assay No. 7 without levulinate gives a spectrum characteristic for uroporphyrin which is largely absent when levulinate is present.

Method section 2.9. (cf. Figure 2). These three protein fractions, one bound to Blue Sepharose, one bound to heme-Sepharose and one which did not bind to the three columns were tested individually and in combination for δ -aminolevulinate synthesis from glutamate.

In the experiments using unlabelled glutamate (Table III, Figure 3), all three protein fractions were needed for δ -aminolevulinate synthesis from glutamate. When the three fractions were incubated together, twice as much δ -aminolevulinate was formed in the presence of levulinate than in its absence. Furthermore, in the absence of levulinate an additional product was formed which after perchloric acid treatment fluoresced red under ultraviolet light and had an absorption spectrum characteristic of uroporphyrin (Figure 4). It appears that glutamate was converted into uroporphyrinogen in addition to δ -aminolevulinate in the absence of levulinate. An increased δ -aminolevulinate accumulation in the presence of levulinate is due to the inhibition of the δ -aminolevulinate dehydratase. When the Blue Sepharose bound protein fraction and the heme-Sepharose bound protein fraction were combined

(Assay 6 in Table III), glutamate was converted into a product which after pyrrole formation, gave a weak Ehrlich reaction (Figure 3).

In similar experiments using radioactive glutamate, the requirement of all three protein fractions for δ -aminolevulinate synthesis from glutamate was confirmed (Table IV). Radioactive δ -aminolevulinate formation was again increased in the presence of levulinate. The run-off protein fraction, which has all the aminotransferase activity (Table V), could be replaced by purified aminotransferase. Therefore, the product formed by combining the protein fractions bound to Blue Sepharose and heme-Sepharose is expected to be glutamate-l-semialdehyde (Assay 6, Table IV). This is confirmed by experiments described in the following sections.

Significant δ -aminolevulinate dehydratase and porphobilinogen deaminase activities were found in the run-off protein fraction (Table V). These results explain the increased δ -aminolevulinate formation in the presence of levulinate (Assay 7, Tables III and IV) and the accumulation of uroporphyrinogen in the absence of levulinate (Figure 4).

Table IV

Synthesis of δ -aminolevulinate from L-glutamate-U- ^{14}C by protein fractions obtained from serial affinity chromatography of partially purified δ -aminolevulinate synthesising enzymes.

Assay	cpm incorporated into δ -aminolevulinate (in sample 6, glutamate-l-semialdehyde)			
	I		II	III
	- Lev	+ Lev	+ Lev	+ Lev
1. Run-off proteins	700	900	3,100	2,300
2. Heme-Sepharose bound proteins	1,100	1,300	1,400	1,800
3. Blue Sepharose bound proteins	1,800	2,100	3,500	4,900
4. 1 + 2	1,300	800	1,800	1,500
5. 1 + 3	4,000	5,300	6,400	16,800
6. 2 + 3	505,000	740,000	640,000	570,000
7. 1 + 2 + 3	1,120,000	1,980,000	2,620,000	2,210,000
8. 2 + 3 + aminotransferase	—	—	2,920,000	1,520,000

In experiment I, proteins used for samples 1, 2 and 3 were 1.7mg, 0.36mg and 1.1mg.

In experiment II, proteins used for samples 1, 2 and 3 were 1.86mg, 0.24mg and 1.05mg.

In experiment III, proteins used for samples 1, 2 and 3 were 1.26 mg, 0.26mg and 0.88mg.

Aminotransferase was purified as described in section 2.5, 0.62mg was used. Lev = 5mM-levulinate. — = not done.

Table V

Glutamate-l-semialdehyde aminotransferase, δ -aminolevulinate dehydratase and porphobilinogen deaminase activities associated with the three protein fractions obtained by serial affinity chromatography.

Protein fraction	Glutamate-l-semialdehyde aminotransferase	ALA-dehydratase	PBG-deaminase
Run-off proteins	160	100	4.7
Heme-Sepharose bound proteins	7.2	13	0.5
Blue Sepharose bound proteins	0	0.6	0.6

Glutamate-l-semialdehyde aminotransferase activity is given in nmoles ALA $\cdot$ mg protein⁻¹ $\cdot$ 20 min⁻¹. ALA-dehydratase activity is given in nmoles PBG $\cdot$ mg protein⁻¹ $\cdot$ 20 min⁻¹. PBG-deaminase activity is given in nmoles of uroporphyrin $\cdot$ mg protein⁻¹ $\cdot$ 20 min⁻¹. ALA = δ -aminolevulinate. PBG = porphobilinogen.

3.6. Glutamate-l-semialdehyde as an intermediate in δ -aminolevulinate synthesis from glutamate

In order to demonstrate that glutamate-l-semialdehyde is an intermediate in δ -aminolevu-

Figure 5. Enzymic synthesis of glutamate-l-semialdehyde from glutamate.

Absorption spectra are given for the pyrrole derivatives of the assay products after treatment with Ehrlich's reagent. Glutamate was incubated with the heme-Sepharose bound protein fraction and Blue Sepharose bound protein fraction. The incubation mixture was deproteinised and incubated with elution medium as control (1), with purified aminotransferase (2) and with the run-off fraction (3).

linate synthesis, the following experiment was carried out. Blue Sepharose bound fraction (1.1 mg protein) and heme-Sepharose bound fraction (0.36 mg protein) were incubated with 0.1mM-glutamate, 1mM-NADPH, 0.5mM-ATP 25mM-MgCl₂ and 5mM-Na-levulinate in 2ml of elution medium for 20 minutes at 27 °C. The reaction was stopped by adding 40 μ l of cold perchloric acid (70%). The precipitated proteins were removed by centrifugation and the supernatant was carefully adjusted to pH 8.0 using 1M-NaOH and 0.5M-Na₃PO₄. The supernatant was then incubated for another 20 minutes at 27 °C with either purified glutamate-l-semialdehyde aminotransferase (0.6 mg) or the run-off fraction (1.7 mg protein). The control was performed by adding elution medium prior to the second incubation. Figure 5 illustrates that the Blue Sepharose bound fraction and the heme-Sepharose bound fraction together make a product which can be converted into δ -aminolevulinate either by glutamate l-semialdehyde aminotransferase or the run-off protein fraction.

3.7. Identification of the intermediate as glutamate-l-semialdehyde by cation exchange chromatography on Dowex 50W X 8

As has been previously shown, glutamate-l-semialdehyde, glutamate and δ -aminolevulinate bind to the cation exchanger Dowex 50W X 8 equilibrated to pH 3.1 (14). These amino-

Table VI**Identification of enzymically synthesised glutamate-l-semialdehyde by purification on Dowex 50W X 8.**

The protein fractions bound to heme-Sepharose (0.52 mg) and to Blue Sepharose (1.75 mg) were incubated with unlabelled glutamate as given in section 2.9 except that the assay was carried out at pH 7.9 in 4 ml. The sample was then divided in two and fractionated on two cation exchange columns and pairs of homologous samples were collected and combined. These samples were used for δ -aminolevulinate determination before and after incubation with purified glutamate-l-semialdehyde aminotransferase.

pH of eluting buffer	ml of fraction	nmoles of δ -aminolevulinate in each fraction	
		before incubation with aminotransferase	after incubation with aminotransferase
3.1	3	—	—
3.6	3	—	—
4.1	1	0.8	4.3
4.6	2	1.9	7.4
4.6	1	0.7	1.5
5.1	3	1.5	—

compounds can be separated in this way from carboxylic acids such as α -ketoglutarate and 4,5-dioxovalerate which do not bind. From the column, glutamate can be eluted at pH 3.6,

glutamate-l-semialdehyde at pH 4.1 to 4.6 and δ -aminolevulinate at pH 5.1 (14). The intermediate formed from glutamate by incubation with a mixture of the protein fractions bound to Blue Sepharose and heme-Sepharose behaved like glutamate-l-semialdehyde on the Dowex column. The presence of glutamate-l-semialdehyde in the fractions eluted at pH 4.1 and 4.6 where it should elute, was demonstrated by assaying for δ -aminolevulinate directly and after incubation with purified glutamate-l-semialdehyde aminotransferase (Table VI). The traces of Ehrlich positive compounds present in the pH 4.1 and 4.6 fractions before incubation with glutamate-l-semialdehyde aminotransferase are probably due to pyrrole formed from glutamate-l-semialdehyde with ethyl acetoacetate. The trace of Ehrlich positive compound present in the pH 5.1 fraction may be δ -aminolevulinate formed by residual aminotransferase present in bound, protein fractions (Table I).

3.8. The formation of ^{14}C -glutamate-l-semialdehyde by the protein fractions bound to Blue Sepharose and heme-Sepharose and its subsequent conversion to δ -aminolevulinate by glutamate-l-semialdehyde aminotransferase

In the high pressure liquid chromatographic system described in 2.15 and 2.16, glutamate, glutamate-l-semialdehyde, and δ -aminolevulinate could be completely separated from each other (Figure 6A). In this system, α -ketoglutarate elutes before glutamate. The radioactive products formed by the protein fractions bound

Figure 6. High pressure liquid chromatography and the identification of radioactive glutamate-l-semialdehyde formed from L-glutamate- ^{14}C by protein fractions bound to heme-Sepharose and Blue Sepharose.

6A presents the elution pattern of authentic glutamate-l-semialdehyde, δ -aminolevulinate and L-glutamate- ^{14}C . 6B shows the formation of radioactive glutamate-l-semialdehyde by incubating heme-Sepharose bound and Blue Sepharose bound proteins (—). When the run-off protein fraction was incubated with the other two fractions radioactive δ -aminolevulinate is formed (---).

to Blue Sepharose and heme-Sepharose (Table IV, experiment II, assay 6) were separated into two main peaks (Figure 6B). The first peak (4–8 ml) had the retention time of glutamate as determined by injection of a sample of authentic radioactive glutamate. This peak had 40 % of the total radioactivity. The second radioactive peak (14–18 ml) co-chromatographed with carrier glutamate-l-semialdehyde. This peak contained 56 % of the total radioactivity. Four per cent of the total radioactivity co-chromatographed with authentic δ -aminolevulinate (in fraction 24–26 ml). Comparing the above results with that of Table IV, experiment II, assay 6, it is seen that only 12 % of the incubated radioactive glutamate is recovered as glutamate-l-semialdehyde. This discrepancy may be explained by losses of radioactive semialdehyde during purification due to absence of cold carrier glutamate-l-semialdehyde.

It is concluded that the protein fractions bound to Blue Sepharose and heme-Sepharose convert 56 % of the glutamate to glutamate l-semialdehyde and only 4 % to δ -aminolevulinate. When the run-off protein fraction, which contains glutamate-l-semialdehyde aminotransferase, was added and incubated with the other two protein fractions (Table IV, experiment II, assay 7) 52 % of the total radioactivity was present in δ -aminolevulinate whereas only 1 % appeared in glutamate-l-semialdehyde (Figure 6B) (compare Table IV, where 53 % of the incubated radioactivity is found in ^{14}C - δ -aminolevulinate). In these analyses all of the injected radioactivity was recovered in the three peaks.

The experiments described in sections 3.6, 3.7 and 3.8 reveal that glutamate-l-semialdehyde is an intermediate in the synthesis of δ -aminolevulinate from glutamate and that glutamate-l-semialdehyde aminotransferase is one of the three soluble proteins of the δ -aminolevulinate synthesising enzyme system. Furthermore, aminotransferase is not necessary for the function of the other two enzymes.

3.9. Evidence suggesting that the protein fraction bound to the heme-Sepharose column contains glutamate-l-phosphate kinase activity

A direct assay of glutamate-l-phosphate kinase activity by trapping the phosphate with hydroxylamine and measuring the red colour formed on addition of FeCl_3 is complicated by the presence of glutamine synthetase and possibly other glutamyl kinases. Methionine sulfoximine (10mM) strongly inhibits glutamine synthetase (19) and 5-glutamyl kinases (1, 18). The synthesis of δ -aminolevulinate from glutamate by partially purified enzyme was not inhibited by this inhibitor. When the three protein fractions separated by serial affinity chromatography were assayed for their glutamine synthetase activity, most of the activity was found in the run-off protein fraction and the heme-Sepharose bound protein fraction (Table VII). In the presence of methionine sulfoximine, the heme-Sepharose bound fraction had five times higher activity per mg protein than that of the Blue Sepharose bound fraction. The

Table VII

Glutamate kinase activities associated with the three protein fractions obtained by serial affinity chromatography.

Assay with	Protein fraction	Hydroxamate formed	
		OD 540nm	OD 540nm · mg protein ⁻¹
	Heme-Sepharose bound	0.25	4.8
	Blue Sepharose bound	0.13	0.70
	Run-off protein	0.52	2.0
10mM-methionine sulfoximine	Heme-Sepharose bound	0.21	0.80
	Blue Sepharose bound	0.14	0.15

kinase activity found in the heme-Sepharose bound fraction is conceivably responsible for the synthesis of glutamate-1-phosphate and that associated with the run-off fraction is conceivably due to glutamine synthetase.

In another experiment, we attempted to use either the Blue Sepharose bound fraction or the heme-Sepharose bound fraction to convert glutamate in the presence of ATP and Mg^{2+} into a product which was then incubated in the presence of NADPH with appropriate fractions for enzymic formation of δ -aminolevulinate. The heme-Sepharose bound fraction (3ml, 1.56mg protein) and the Blue Sepharose bound fraction (3ml, 5.28mg protein) were separately incubated with 0.5mM-ATP, 25mM- $MgCl_2$ and 10 μ Ci of L-glutamate- U - ^{14}C for 20 minutes at 27 °C. The incubation mixtures were cooled on ice and filtered by pressure dialysis using a PM10 filter which retains molecules larger than 10,000 dalton. The filtrate from the assay with the heme-Sepharose bound fraction was then incubated with 3ml of the Blue Sepharose bound fraction, 3 ml of the run-off fraction (7.56 mg protein) and 1mM-NADPH for 20 minutes at 27 °C and analysed for ^{14}C - δ -aminolevulinate. After purification 6800 cpm were recovered in δ -aminolevulinate. When the filtrate from the assay with the Blue Sepharose bound fraction was incubated with 3ml of heme-Sepharose bound fraction, 3ml of the run-off fraction and 1mM-NADPH, only 1900 cpm were recovered in δ -aminolevulinate. These experiments indicate that the heme-Sepharose column binds an ATP utilising component of the δ -aminolevulinate synthesising enzyme system which could be glutamate-1-phosphate kinase. The Blue Sepharose bound protein fraction then is likely to contain the glutamate-1-phosphate dehydrogenase. These indications will be followed up in further experiments.

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