BIOSYNTHESIS OF Δ -AMINOLEVULINATE IN GREENING BARLEY LEAVES. VIII: PURIFICATION AND CHARACTERIZATION OF THE GLUTAMATE-tRNA LIGASE

by

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Barley chloroplast glutamate-tRNA ligase was purified using salicylate-Sepharose and phosphocellulose chromatography. The purified enzyme amino-acylates δ -ALA-RNA, the other glutamate acceptor tRNAs of the chloroplast, as well as *E. coli* tRNA^{Glu}. An antibody was raised against the purified ligase and coupled to Sepharose. The enzyme purified by immunoaffinity chromatography has a subunit molecular weight of 54 kilodaltons. The dehydrogenase involved in δ -aminolevulinate synthesis was separated from the ligase by immunoaffinity chromatography. Reconstitution experiments confirmed that glutamate-tRNA ligase participates in δ -aminolevulinate synthesis and it is suggested that a single glutamate-tRNA ligase provides activated glutamate for both chlorophyll and protein biosynthesis. An antibody raised against *B. subtilis* glutamate-tRNA ligase cross reacted with the barley glutamate-tRNA ligase.

1. INTRODUCTION

The control of chlorophyll synthesis is achieved primarily by regulation of δ -aminolevulinate synthesis. In greening barley and other plants δ -aminolevulinate is synthesised from glutamate by an unusual pathway. The first step in the pathway activates glutamate by ligating it to δ -ALA-RNA. This step is catalysed by the enzyme glutamate-tRNA ligase in the presence of ATP and Mg^{2+} . Glutamyl- δ -ALA-RNA is then reduced to glutamate 1-semialdehyde by an NADPH-dependent dehydrogenase. Finally aminotransferase converts the

semialdehyde to δ -aminolevulinate. The enzyme glutamate 1-semialdehyde aminotransferase and the δ -ALA-RNA have been purified and characterised (5, 6, 15). In this paper we report the purification and characterisation of glutamate-tRNA ligase from the stroma of barley chloroplasts.

The dehydrogenase involved in δ -aminolevulinate synthesis co-purifies with the glutamate-tRNA ligase during affinity chromatography on Cibacron blue-Sepharose. Previous attempts to separate these two activities resulted in complete loss of the dehydrogenase activity. This paper

Abbreviations: δ -ALA-RNA = RNA involved in δ -aminolevulinate biosynthesis; HEPES = N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid; 1 M_c-RNA = total tRNA fraction of greening chloroplasts isolated as described in (6); SDS = Sodium dodecyl sulfate; Tris = trihydroxymethyl aminomethane; TCA = trichloroacetic acid.

also describes an immunoaffinity chromatography technique using an antibody raised against the purified ligase to separate it from the dehydrogenase without loss of activity of the latter.

2. MATERIALS AND METHODS

2.1. The starting material for purification

Intact chloroplasts were isolated from leaves of barley seedlings grown for six days in darkness and then illuminated for 12 hours. The plastids were disrupted by passing them through a French pressure cell and the soluble protein fraction was obtained by centrifugation. Thereafter the soluble proteins were subjected to Sephacryl S-300 gel filtration followed by affinity chromatography using Cibacron blue-Sepharose. The protein fraction that binds to the blue-Sepharose was eluted with salt and desalted using Sephadex G-50 gel filtration. The experimental details are as given in (6, 18). This protein fraction contained the glutamate-tRNA ligase and the dehydrogenase involved in δ -aminolevulinate synthesis (6) and was used as starting material for the purification.

2.2. Preparation of salicylate-Sepharose 4B

Salicylic acid was coupled via its carboxyl group to the primary amino group of AH-Sepharose 4B using N'-ethyl-N'-(3-dimethylaminopropyl) carbodiimide according to the instructions provided by Pharmacia Fine Chemicals in their hand book "Affinity Chromatography principles and methods". After coupling, excess salicylate was removed by three successive washes with 0.1 M-Tricine-NaOH pH 9.0 containing 0.3 M-glycerol, 25 mM-MgCl₂, 1 mM-dithiothreitol and with 0.1 M-sodium acetate pH 4.5 containing 0.5 M-NaCl.

2.3. Salicylate-Sepharose chromatography

A column (1.5×9 cm) was packed with salicylate-Sepharose 4B using 0.1 M-sodium acetate pH 4.5 containing 0.5 M-NaCl. The column was equilibrated with 0.1 M-Tricine-NaOH pH 9.0, 0.3 M-glycerol, 25 mM-MgCl₂ and 1 mM-dithiothreitol. The protein fraction purified by chro-

matography on blue-Sepharose was applied to this column and eluted with the above Tricine medium. The eluate was scanned for ultra-violet light absorption and collected in 2 ml fractions. After the unadsorbed ultra-violet light absorbing materials were eluted, a 150 ml linear gradient of 0 to 1 M-NaCl in the Tricine medium was applied to the column. Aliquots (25 μ l) from the fractions of the gradient were assayed in 1 ml reaction mixtures for glutamate-tRNA ligase activity. After chromatography the column was washed with two to three column volumes of 1 M-NaCl followed by 0.1 M-sodium acetate pH 4.5 containing 0.5 M-NaCl. The salicylate-Sepharose 4B column was stored in this acetate buffer. Column packing and chromatography were performed in a cold room at 4 °C.

2.4. Phosphocellulose P₁₁ chromatography

Phosphocellulose P₁₁ was equilibrated with 10 mM-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-NaOH, pH 7.2 containing 0.3 M-glycerol, 1 mM-MgCl₂ and 2 mM-dithiothreitol according to the instructions provided by the manufacturer. A column (1.5×12 cm) was packed. The glutamate-tRNA ligase containing fraction (or fractions) from the salicylate-Sepharose affinity chromatography was passed through a Sephadex G-50 column equilibrated with the above HEPES medium and applied to the phosphocellulose column. The phosphocellulose column was washed with the HEPES medium until all the unabsorbed, ultra-violet light absorbing materials were removed. The bound proteins were eluted using either a linear gradient of 0 to 0.3 M-KCl (150 ml) or a step gradient of 0.5 M and 1.0 M-KCl in the HEPES medium. With the linear gradient, the eluate was collected in 2 ml fractions, and with the step gradient the eluate showing ultra-violet light absorption was collected. Aliquots (25 μ l) from the fractions were assayed in 1 ml reaction mixtures for glutamate-tRNA ligase activity. The ligase containing fractions of the eluate from the linear gradient were pooled and dialysed against 10 mM-HEPES-NaOH, pH 7.2 containing 0.3 M-glycerol, 1 mM-MgCl₂ and 2 mM-dithiothreitol. In the step gradient, the relevant fractions were desalted using a Sephadex

G-50 column. The proteins were concentrated using an Amicon pressure dialysis cell and a PM30 filter. All operations, except for column packing and enzyme assays, were performed in a cold room at 4 °C.

2.5. Gel electrophoresis

Polyacrylamide gel electrophoresis was performed in slab gels 30 by 20 cm, 1 mm thick with 2.5 cm long stacking gels. Non-denaturing gel electrophoresis was carried out using 7.5% polyacrylamide and the Tris-glycine buffer system described in (4). Electrophoresis under denaturing conditions was performed in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate in the Tris-borate system as described in (2).

2.6. Immunological methods

2.6.1. Preparation of anti-glutamate-tRNA ligase IgG

An antibody was raised in rabbits against the glutamate-tRNA ligase purified up to the phosphocellulose cation exchange chromatography step. Immunization was performed by Dakopatts A/S, DK-2600 Glostrup, Denmark, in doses of 0.1 ml containing 100 μ g of antigen emulsified with Freund's incomplete adjuvant. IgG were purified from the immune serum according to (16).

2.6.2. Preparation of anti-glutamate-tRNA ligase-Sepharose

Anti-glutamate-tRNA ligase IgG was transferred into a medium containing 0.1 M-NaHCO₃ and 0.5 M-NaCl by passing through a Sephadex G-50 column equilibrated in this solution. CNBr activated Sepharose 4B (5 g) was swollen for 15 min in 1 mM-HCl and washed with 1 liter of 1 mM-HCl using a sintered glass funnel and a water aspirator. The gel was then washed with 50 ml of 0.1 M-NaHCO₃ containing 0.5 M-NaCl and immediately transferred into a 100 ml conical flask containing 80 mg of anti-ligase IgG in 30 ml of 0.1 M-NaHCO₃, 0.5 M-NaCl. The mixture was gently shaken for two hours at room temperature. Thereafter 1 ml of 1 M-glycine at

pH 8.0 was added and shaking continued for additional two hours. The gel was then washed four times successively with 0.1 M-NaHCO₃, 0.5 M-NaCl and 0.1 M-sodium acetate pH 4.5, 0.5 M-NaCl. The gel was stored in the acetate buffer at 4 °C.

2.6.3. Immunoaffinity chromatography

Anti-glutamate-tRNA ligase IgG-Sepharose 4B was packed into a column 1.5 by 10 cm using a 0.1 M-sodium acetate buffer pH 4.5 containing 0.5 M-NaCl. The column was washed with approximately 200 ml of 0.1 M-Tricine-NaOH pH 9.0, 0.3 M-glycerol, 1 mM-dithiothreitol and 25 mM-MgCl₂. The blue-Sepharose bound protein fraction (cf. 2.1) was applied to the antibody column. The column was washed with the Tricine-glycerol medium to remove all the unbound proteins and glutamate-tRNA ligase was eluted with 4 M-MgCl₂ dissolved in the same medium. The ligase containing fraction was transferred into 0.1 M-Tricine-NaOH pH 9.0, 0.3 M-glycerol, 1 mM-dithiothreitol and 25 mM-MgCl₂ by passing through a Sephadex G-50 column pre-equilibrated in this medium. The protein was then concentrated in an Amicon pressure dialysis cell using a PM30 filter. All these procedures were carried out in a cold room at 4 °C.

2.7. Glutamate-tRNA ligase assay and the assay for δ -aminolevulinate synthesis

The glutamate-tRNA ligase activity was measured by incubating either 0.2 or 1 ml reaction mixtures for 20 minutes at 28 °C. The incubation mixture contained 25 mM-HEPES-NaOH pH 6.8, 1 mM-dithiothreitol, 3 mM-MgCl₂, 1 mM-ATP, 5 μ Ci ³H(U)-L-glutamate, 0.05 to 2 OD₂₆₀ units of tRNA and 20 to 100 μ g of enzyme proteins. The reaction was stopped by placing the tubes on ice and adding 100% TCA to a final concentration of 10%. The precipitated material was transferred to glass fiber filter paper and washed thoroughly with 5% TCA followed by 96% ethanol. The filter was then dried under an infra-red lamp and radioactivity determined by scintillation counting.

Assay for δ -aminolevulinate synthesis was performed as described previously (6).

2.8. Other methods

Protein concentration was determined according to the method of LOWRY et al. (11). Radioactivity was measured by liquid scintillation counting.

2.9. Chemicals and Enzymes

L-glutamate- U - ^{14}C (275 mCi/mmol), L-(3,4- 3H)-glutamate (47.7 Ci/mmol), L-(2,3- 3H)-arginine (15 Ci/mmol) and (2- 3H)-glycine (41.2 Ci/mmol) were obtained from NEN, USA. Phosphocellulose P_{11} was obtained from Whatman, England. AH-Sepharose 4B and CNBr activated Sepharose 4B were obtained from Pharmacia, Sweden. N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride was obtained from Fluka AG, Switzerland. Barley tRNA used for this study was isolated as described previously (6). The total chloroplast tRNA fraction prepared by this procedure is referred to as 1 M_c -RNA. δ -ALA-RNA and the other two glutamate isoacceptor tRNAs were obtained from 1 M_c -RNA by HPLC fractionation as described previously (6). *E. coli* tRNA $_{2}^{Glu}$ was obtained from Boehringer Mannheim, West Germany. *E. coli* and *B. subtilis* glutamate-tRNA ligase were provided by Prof. JACQUES LAPOINTE, Laval University, Québec, Canada.

3. RESULTS

3.1. Purification of the glutamate-tRNA ligase

3.1.1. Salicylate-Sepharose chromatography

Approximately half of the proteins that bind to blue-Sepharose also bind to the salicylate-Sepharose column. When the bound proteins

Figure 1. Salicylate-Sepharose affinity chromatography.

The bound proteins were eluted with a linear gradient from 0 to 1 M-NaCl. 2 ml fractions were collected. Alternate fractions (25 μ l) were tested for glutamate-tRNA ligase activity in 1 ml assays as described in 2.7.

were eluted from the salicylate-Sepharose column using a gradient of NaCl, glutamate-tRNA ligase emerged as a single peak approximately at 0.25 M-NaCl (Figure 1). The peak fractions of activity gave a 4-5 fold purification of enzyme compared to the blue-Sepharose bound protein fraction. The active fractions were pooled and desalted. Several chromatographic runs were performed with the salicylate-Sepharose column by stepwise elution using 0.3 M-NaCl and 1 M-NaCl. All of the bound glutamate-tRNA ligase was eluted with 0.3 M-NaCl.

Table I. Reconstitution of δ -aminolevulinic acid synthesis using protein fractions separated by salicylate-Sepharose.

The 1 ml control reaction mixture contained 100 mM-Tricine NaOH, pH 7.9, 0.3 M-glycerol, 1 mM-dithiothreitol, 25 mM-MgCl $_2$, 1 mM-NADPH, 0.5 mM-ATP, 5 mM-levulinic acid, 2.5 μ Ci of L-(^{14}C -(U))glutamate (275 mCi/mmol), 0.5 mg of partially purified glutamate-1-semialdehyde aminotransferase and 2 OD $_{260}$ units of unfractionated RNA.

Assay	Conversion of L- ^{14}C -glutamate into δ -aminolevulinic acid (cpm)
1. Control	5,600
2. 1 + 0.6 mg blue-Sepharose bound proteins	2,647,000
3. 1 + 0.2 mg salicylate-Sepharose bound proteins	3,912,000
4. 1 + 0.5 mg salicylate-Sepharose bound and unbound proteins	4,290,000

Figure 2. Phosphocellulose P_{11} chromatography.

The ligase containing fractions from the salicylate-Sepharose chromatography were pooled, desalted and applied to the phosphocellulose column. After washing off the unbound proteins, the bound proteins were eluted with a linear gradient from 0 to 0.3 M-KCl. 2 ml fractions were collected. 25 μ l of alternate fractions were tested for glutamate-tRNA ligase activity in 1 ml assays as described in 2.7.

This fraction was desalted by passing through a Sephadex G-50 column and concentrated by pressure dialysis. Table I illustrates the ability of this fraction to reconstitute δ -aminolevulinate synthesis with δ -ALA-RNA and the amino-transferase containing fractions. The proteins that do not bind to the salicylate-Sepharose did not give a significant effect when added to the reconstituted assay mixture. It is concluded that the dehydrogenase involved in δ -aminolevulinate synthesis binds to salicylate-Sepharose and co-elutes with the ligase under the conditions of chromatography used.

3.1.2. Phospho-cellulose P_{11} chromatography

When the protein fraction obtained after salicylate affinity chromatography was chromatographed on the phosphocellulose column as described in section 2.4 more than 90% of the protein did not bind. However, the ligase bound to the phosphocellulose and was eluted as a single peak approximately at 0.25 M-KCl when a linear gradient of KCl was applied to the column (Figure 2). The phosphocellulose had a low capacity to adsorb ligase. It was necessary to apply the protein in a buffer of low ionic strength in order to have sufficient binding of the ligase. Increasing the concentration of HEPES from 10 mM to 25 mM prevented the binding of the ligase to phosphocellulose. Even in the medium chosen, (10 mM-HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid)-NaOH, pH 7.2 containing 0.3 M glycerol, 1 mM-MgCl₂ and 2 mM-dithiothreitol), the unbound fraction showed about 5% of the initial ligase activity. This was interpreted to be due to overloading of the column. When phosphocellulose chromatography was performed in 10 mM-KPO₄ buffer pH 7.4, 0.3 M-glycerol, 1 mM-MgCl₂ and 2 mM-dithiothreitol, ligase behaved exactly as in the HEPES system. However, the ligase lost its activity during pressure dialysis and it was concluded that ligase was not very stable in the phosphate buffer. Only a small fraction of the ligase bound to the phosphocellulose when chromatography was performed in 0.1 M-Tricine-NaOH pH 7.4, 0.3 M-glycerol, 1 mM-MgCl₂ and 2 mM-dithiothreitol.

Attempts to reconstitute δ -aminolevulinate formation with the bound and the unbound protein fractions from the phosphocellulose column were unsuccessful. It appears that phos-

Table II. Reconstitution of δ -aminolevulinate synthesis using protein fractions separated by anti-glutamate-tRNA ligase Sepharose 4B.

The assays were performed as described in Table I.

Assay	Conversion of ¹⁴ C-glutamate into δ -aminolevulinate (cpm)
1. Control	16,700
2. 1 + 0.6 mg blue-Sepharose bound proteins	1,920,000
3. 1 + 0.07 mg anti-ligase bound protein	20,300
4. 1 + 0.5 mg anti-ligase unbound proteins	49,100
5. 3 + 4	1,680,000

Table III. The dehydrogenase involved in δ -aminolevulinate synthesis binds to salicylate-Sepharose.

The blue-Sepharose bound proteins were chromatographed on anti-glutamate-tRNA ligase-Sepharose. The proteins that did not bind to this matrix were further chromatographed on the salicylate-Sepharose. The assays were performed as described in Table I.

Assay	Conversion of ^{14}C -glutamate into δ -aminolevulinate (cpm)
1. 0.07 mg anti-ligase bound protein	11,900
2. 1 + 0.3 mg salicylate-Sepharose unbound proteins	15,900
3. 1 + 0.2 mg salicylate-Sepharose bound proteins	1,715,000
4. 2 + 3	1,731,000

phocellulose chromatography inactivates the dehydrogenase involved in δ -aminolevulinate synthesis.

3.1.3. Separation of the glutamate-tRNA ligase from the dehydrogenase by immunoaffinity chromatography

The glutamate-tRNA ligase selectively bound to anti-ligase-Sepharose was eluted with 4 M- MgCl_2 . Table II illustrates the reconstitution of δ -aminolevulinate synthesis using the protein fractions separated by immunoaffinity chromatography. The addition of glutamate-tRNA ligase alone to a reaction mixture containing the δ -ALA-RNA and the aminotransferase containing fractions did not reconstitute δ -aminolevulinate synthesis. Both the protein fraction that does not bind to anti-ligase-Sepharose as well as the glutamate-tRNA ligase that binds to this column were required for enzymatic conversion of glutamate to δ -aminolevulinate. This observation confirms that glutamate-tRNA ligase participates in δ -aminolevulinate synthesis.

It is clear that the dehydrogenase involved in δ -aminolevulinate synthesis does not bind to the anti-ligase-Sepharose. The proteins that did not bind to the anti-ligase-Sepharose were loaded onto the salicylate-Sepharose column and separated into bound and unbound protein fractions. The bound proteins were eluted with 0.3 M- NaCl , desalted and concentrated. Under these conditions the dehydrogenase involved in δ -aminolevulinate synthesis bound to the salicylate-Sepharose column and remained fully active after elution (Table III).

Figure 4. Polyacrylamide gel electrophoretic analysis of the ligase purified by immunoaffinity-Sepharose chromatography.

SDS-polyacrylamide gel electrophoresis. Silver staining. Molecular weight markers (left lane) are indicated in kilodaltons.

3.1.4. Polyacrylamide gel electrophoresis

The glutamate-tRNA ligase purified by salicylate-Sepharose and phosphocellulose gave a single, diffuse band upon non-denaturing polyacrylamide gel electrophoresis (Figure 3A). Under denaturing conditions it gave three bands

Figure 3. Polyacrylamide gel electrophoretic analysis of the ligase purified by phosphocellulose chromatography.

A. Electrophoresis in a 7.5% non-denaturing polyacrylamide gel.

B. SDS-polyacrylamide gel electrophoresis. Coomassie blue staining. Molecular weight markers (right lane) are indicated in kilodaltons.

corresponding to apparent molecular weights of 53, 50 and 39 kilodaltons and many faint bands (Figure 3B). These faint bands are considered to be contaminants and breakdown products. The glutamate-tRNA ligase purified by immunoaffinity chromatography gave one major band (Figure 4) corresponding to molecular weight of 54 kilodaltons and several faint bands in the ranges of 90-95 and 30-40 kilodaltons. We think that 53 to 54 kilodaltons represent the subunit molecular weight of barley chloroplast glutamate tRNA ligase and that the 50 and 39 kilodalton polypeptides in the phosphocellulose purified ligase are either contaminants not recognized by the antiserum or breakdown products of the 53 kilodalton polypeptide.

3.2. Properties of the glutamate-tRNA ligase

3.2.1. Nucleotide requirement for the ligase activity

ATP was required for the aminoacylation reaction catalysed by the ligase. The other nucleotide triphosphates or ADP could not replace ATP when analysed using the ligase purified up to the phosphocellulose chromatography step (Table IV).

Table IV. The specificity of ATP in the aminoacylation with barley glutamate-tRNA ligase.

The reaction mixture contained in 1 ml the following: 25 mM-HEPES, pH 6.8, 3 mM-MgCl₂, 1 mM-dithiothreitol, 25 μ Ci of L-(3,4-³H)glutamate (47.7 Ci/mmole), 50 μ g of purified glutamate-tRNA ligase, 0.25 OD₂₆₀ unit of δ -ALA-RNA and 1 mM of nucleotides.

Nucleotide added	³ H-glutamyl-tRNA formation: TCA precipitable cpm
none	3,600
ATP	147,450
ADP	3,100
CTP	3,300
GTP	4,200

Table V. The purified glutamate-tRNA ligase loads all three glutamate acceptor tRNAs present in barley chloroplasts.

The ligase purified by phosphocellulose chromatography was used in enzyme assays with δ -ALA-RNA and the other two glutamate acceptor tRNAs purified by HPLC (6) as substrates.

substrate	^3H -glutamyl-tRNA formation: TCA precipitable cpm
1. δ -ALA-RNA	301,800
2. Glutamate acceptor tRNA 1	117,900
3. Glutamate acceptor tRNA 2	172,900

3.2.2. The ability of glutamate-tRNA ligases from barley chloroplast, *E. coli* and *B. subtilis* to aminoacylate different tRNA_S^{Glu}

The glutamate-tRNA ligase purified from

barley chloroplasts aminoacylated the δ -ALA-RNA and the two tRNA_S^{Glu} of the chloroplast (Table V) as well as tRNA_S^{Glu} from *E. coli* (Table VI). The *E. coli* glutamate-tRNA ligase did not efficiently aminoacylate the chloroplast tRNA_S^{Glu}. The *B. subtilis* glutamate-tRNA ligase was more efficient than *E. coli* glutamate-tRNA ligase in aminoacylating barley chloroplast tRNA_S^{Glu} (Table VI).

3.2.3. Inhibition of the glutamate-tRNA ligase by anti-glutamate-tRNA ligase

The polyclonal antibody obtained against the ligase inhibited the activity of this enzyme (Table VII). Ninety-two per cent inhibition was obtained when 114 μg of anti-ligase IgG was included in the assay mixture containing 0.1 mg of blue-Sepharose bound protein. The blue-

Table VI. tRNA substrate specificity of barley chloroplast, *E. coli* and *B. subtilis* glutamate-tRNA ligases.

The enzyme fractions were tested in 0.2 ml assays as described in Material and Methods, 2.7. δ -ALA-RNA refers to the RNA involved in δ -aminolevulinatase biosynthesis purified by HPLC from the total chloroplast tRNA fraction (1 M_c-RNA) isolated from greening barley chloroplasts as described in (6).

Enzyme	substrate	^3H -glutamyl-tRNA formation: TCA precipitable cpm
1. <i>E. coli</i> ligase	<i>E. coli</i> tRNA ₂ ^{Glu}	69,000
2. "	1 M _c -RNA	3,140
3. "	δ -ALA-RNA	1,070
4. Barley chloroplast ligase	<i>E. coli</i> tRNA ₂ ^{Glu}	435,600
5. "	1 M _c -RNA	635,900
6. "	δ -ALA-RNA	629,500
7. <i>B. subtilis</i> ligase	1 M _c -RNA	22,000

Table VII. Inhibition of glutamate-tRNA ligase by the antibody against the enzyme.

The blue-Sepharose bound proteins (0.1 mg) were pre-incubated at room temperature with different amounts of anti-ligase IgG and assayed for their ability to ligate glutamate, glycine and arginine to tRNA as described in section 2.7 using either L-(3,4- ^3H)-glutamate (47.7 mCi/mmol), L-(2,3- ^3H)-arginine (15 Ci/mmol) or L-(2- ^3H)-glycine (41.2 Ci/mmol).

Pre-incubated with:	Amino acid	^3H -glutamyl-tRNA formation TCA precipitable cpm	% Inhibition control
None (control)	^3H -glutamate	523,000	0
57 μg anti-ligase IgG	"	285,000	45
114 μg "	"	37,000	93
171 μg "	"	40,000	92
228 μg "	"	9,900	98
None (control)	^3H -arginine	228,000	0
114 μg anti-ligase IgG	"	232,000	0
None (control)	^3H -glycine	39,100	0
114 μg anti-ligase IgG	"	30,800	21

Table VIII. The antibody to barley chloroplast glutamate-tRNA ligase recognizes the *B. subtilis* glutamate-tRNA ligase.

The 0.2 ml reaction mixture contains 100 mM-HEPES pH 7.2, 10 mM-KCl, 10 mM-MgCl₂, 2 mM-ATP, 5 μ Ci of L-(3,4-³H) glutamate (47,7 Ci/mmol), 10 μ l of partially purified *B. subtilis* glutamate-tRNA ligase and 2 OD₂₆₀ of unfractionated barley chloroplast tRNAs. In assays 2 and 3, 60 μ g and 120 μ g, respectively, of IgG were added. Incubation was for 40 minutes at 30 °C.

Amount of anti-barley glutamate-tRNA ligase added	Amount of Glu-tRNA formed (TCA precipitable cpm)	% inhibition
1. 0 μ g IgG	89,800	0
2. 60 "	64,400	28
3. 120 "	29,600	67

Sephadex bound protein fraction contains other amino acid-tRNA ligases (6). The anti-ligase IgG (114 μ g) had no effect on the activity of the arginine-tRNA ligase and caused only 21% inhibition of glycine-tRNA ligase. The antibody against the barley glutamate-tRNA ligase also inhibited the *B. subtilis* ligase (Table VIII).

A polyclonal antibody to *B. subtilis* glutamate-tRNA ligase (provided by Prof. J. LA-POINTE) reacted with the barley chloroplast ligase and gave an immunoprecipitate. The enzyme, however, was not inactivated in the immunoprecipitate (Table IX).

4. DISCUSSION

The results presented in this paper confirm that glutamate-tRNA ligase catalyses the first step in the conversion of glutamate to δ -aminolevulinate. The purification procedure applicable to the ligase and its enzymatic and physical properties show a close similarity to the glutamate-tRNA ligases involved in protein synthesis.

Salicylate is an inhibitor of amino acid-tRNA ligases and the most sensitive among these is the glutamate-tRNA ligase (1). Therefore salicylate-Sepharose was used for affinity purification of the barley chloroplast glutamate-tRNA ligase. Phosphocellulose P₁₁ chromatography has been used in the purification of glutamate and glutamine-tRNA ligases (3, 8, 9, 17). This was also an effective step in the purification of barley chloroplast glutamate-tRNA ligase. The wheat chloroplast and *E. coli* glutamate-tRNA ligases have been purified using hydroxyapatite and phosphocellulose chromatography (8, 17). The

instability of the barley chloroplast enzyme in phosphate buffers prevented the use of hydroxyapatite columns which require phosphate anions for chromatographic separations.

The dehydrogenase involved in δ -aminolevulinate synthesis was partially purified in its active form using immunoaffinity chromatography followed by salicylate-Sepharose chromatography. This is an easy and quick method to obtain starting material for further purification of the dehydrogenase. Since dehydrogenase activity is completely lost during phosphocellulose chromatography we suggest that the dehydrogenase activity is due to an enzymatic complex which

Table IX. The *B. subtilis* glutamate-tRNA ligase antibody immunoprecipitates the barley glutamate-tRNA ligase without inhibiting its activity.

50 μ g of blue-Sepharose bound proteins were incubated with 0 (control), 2.5, 5 and 10 μ g of anti-*B. subtilis* glutamate-tRNA ligase IgG for 20 minutes at 28 °C. The immunoprecipitate was collected by centrifugation and washed once with 20 μ l of 25 mM-HEPES, pH 6.8 containing 1 mM-dithiothreitol and 3 mM-MgCl₂. The pellet was assayed for loading activity as described in material and methods.

Amount of anti- <i>B. subtilis</i> glutamate-tRNA ligase used for pre-incubation	Amount of barley glutamate-tRNA ligase precipitated (TCA precipitable cpm)
0 (control)	8,150
2.5 μ g IgG	9,185
5.0 "	11,980
10.0 "	62,000

irreversibly dissociates during phosphocellulose chromatography.

Wheat chloroplast glutamate-tRNA ligase is a dimer of 110 kilodaltons which dissociates on dilution to inactive 56 kilodalton monomers (14, 17). In *E. coli* and *B. subtilis* glutamate-tRNA ligases are monomers of 56 and 65 kilodaltons, respectively, (7, 8, 13) which associate with a 46 kilodalton protein. The 46 kilodalton protein is considered to be a regulatory factor because it increases the affinity of the ligase for glutamate and ATP (8, 13). The possibility of such a regulatory factor associated with the barley chloroplast glutamate-tRNA ligase cannot be excluded.

In the present study only one glutamate specific amino acid-tRNA ligase was identified in the stroma of greening barley chloroplasts. The purified enzyme aminoacylates δ -ALA-RNA and the other two glutamate acceptor tRNAs present in the chloroplast. Therefore it is likely that in greening barley chloroplasts a single glutamate-tRNA ligase provides activated glutamate for both chlorophyll and protein biosynthesis and plays a significant role in the coordination of the two biosynthetic pathways.

The glutamate-tRNA ligase from barley chloroplasts aminoacylates both the procaryotic tRNA^{Glu} from *E. coli* and the cytoplasmic eucaryotic tRNA^{Glu} from wheat germ. This indicates similarities between the cytoplasmic and chloroplast glutamate-tRNA ligases. These similarities may be the result of a single nuclear gene encoding both enzymes. In yeast a single nuclear gene encodes the histidine-tRNA synthetase destined for the cytoplasm as well as that imported into mitochondria, the latter being distinguished by a special transit peptide sequence (12).

Procaryotic glutamate-tRNA ligases were expected to aminoacylate chloroplast tRNA_S^{Glu} since the components of chloroplast protein biosynthesis resemble those of procaryotes in many ways. Two bacterial glutamate-tRNA ligases were tested for their ability to aminoacylate barley chloroplast tRNA_S^{Glu}. The enzyme from *B. subtilis* significantly aminoacylates the chloroplast tRNA_S^{Glu}, but the enzyme from *E. coli* shows little if any activity in this respect. This adds to the exceptional lack of specificity

which is displayed by the *B. subtilis* glutamate-tRNA ligase for tRNA^{Glu} and tRNA^{Gln} substrates (cf. 10).

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