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Purification and Characterization of Glutamyl-tRNA Synthetase[1]

An Enzyme Involved in Chlorophyll Biosynthesis

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ABSTRACT

Chlorophyll biosynthesis starts with the synthesis of glutamyl-tRNA (glu-tRNA) by a glutamyl-tRNA synthetase (Glu RS). The glu-tRNA is subsequently transformed to δ-aminolevulinic acid (ALA), which is a committed and regulated precursor in the chlorophyll biosynthetic pathway. The Glu RS from a green alga, *Chlamydomonas reinhardtii*, was purified and shown to be able to synthesize glu-tRNA and to participate in ALA synthesis in a coupled enzyme assay. Physical and chemical characterization of the purified Glu RS indicated that the enzyme had been purified to homogeneity. The purified enzyme has a native molecular weight of 60,000, an isoelectric point of 4.6, and it formed a single band of 32,500 daltons when analyzed by a silver stained denaturing gel. The N-terminal amino acid sequence of the 32,500 dalton protein was determined to be Asn-Lys-Val-Ala-Leu-Leu-Gly-Ala-Ala-Gly. The molecular weight analyses together with the unambiguous N-terminal amino acid sequence obtained from the purified enzyme suggested that the native enzyme was composed of two identical subunits. Polyclonal antibodies raised against the purified and denatured enzyme were able to inhibit the activity of the native enzyme and to interact specifically with the 32,500 dalton band on Western blots. Thus, the antibodies provided an additional linkage for the structural and functional identities of the enzyme. *In vitro* experiments showed that over 90% of the glu RS activity was inhibited by 5 micromolar heme, which suggested that Glu RS may be a regulated enzyme in the chlorophyll biosynthetic pathway.

The biosynthesis of ALA[2], the first committed intermediate in Chl synthesis, is highly regulated (6). There are three enzymatic steps converting glutamate to ALA; the enzymes have been partially purified from various plant sources. The current understanding of the ALA pathway is illustrated in Figure 1. In the first step, glutamate is charged onto a glutamate specific tRNA by a Glu RS (11). The activated glu-tRNA is transformed to GSA by a second enzyme, an NADPH-dependent dehydrogenase (24). Finally, an aminotransferase converts GSA to ALA by an intermolecular transamination (17).

To study the mechanism and regulation of the reaction steps leading from glutamate to ALA and to clone the genes coding for the three enzymes involved in ALA synthesis, we have embarked on the purification of these enzymes. In this paper, we report the purification and characterization of a Glu RS from *Chlamydomonas reinhardtii*. The purified enzyme can ligate glutamate to purified *Escherichia coli* $tRNA^{Glu}$ and to tRNA from *Chlamydomonas*. In addition, it is needed for ALA synthesis from glutamate. Part of the results has been reported in abstract form (7).

MATERIALS AND METHODS

Chemicals

Tricine was obtained from United States Biochemicals. Ammonium sulfate was obtained from Mallinckrodt. DEAE Trisacryl M was obtained from LKB, USA. Sephadex G-25 was obtained from Pharmacia. Matrex Gel B was obtained from Amicon, USA. Acrylamide, bis-acrylamide were obtained from Boehringer Mannheim Biochemicals. [^{3}H]Glutamate was obtained from Amersham. All the other chemicals were obtained from Sigma Chemical Co. unless otherwise indicated.

Enzyme Purification

Cell Culture and Ammonium Sulfate Fractionation

Wild-type *Chlamydomonas reinhardtii* (strain 137C) cells were grown in liquid HSA media as previously described (23). Cells were grown up to 2×10^6 cell/mL and harvested by continuous centrifugation. The following procedure was performed at 0 to 4°C. The concentrated cells were washed with Tricine buffer (50 mM Tricine [pH 8.3], 1 mM DTT, and 1 mM $MgCl_2$) and centrifuged at 9,800*g* for 5 min to pellet the cells. The cells were resuspended in 55% saturated $(NH_4)_2SO_4$ (prepared in Tricine buffer) at a 1:4 (w/v) ratio, then ruptured by passing through a French pressure cell at 8,000 psi. The resulting dark green slurry was stirred for 15 min and centrifuged at 27,000*g* for 15 min. The clear supernatant was collected and $(NH_4)_2SO_4$ adjusted to 85% saturation (55/85% cut). The stirring and centrifugation steps were repeated to

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[2] Abbreviations: ALA, δ-aminolevulinic acid; Glu-tRNA, glutamyl-tRNA; Glu RS, glutamyl-tRNA synthetase; GSA, glutamate-1-semialdehyde; DI, DI*, DIII, and DR and fractions eluted from the DEAE column as labeled in Figure 2; Bis, *N,N'*-methylenebisacrylamide; TEMED,*N,N,N',N'*-tetramethylethylenediamine; IEF, isoelectric focusing; IgG, immunoglobulin G.

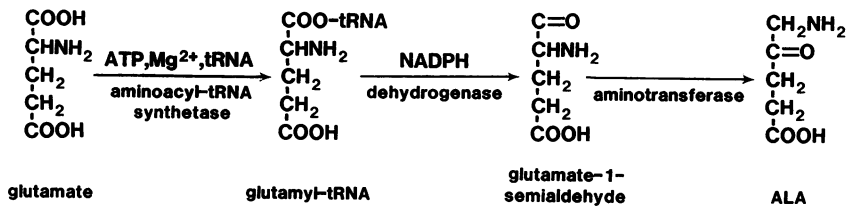

Figure 1. Current version of ALA biosynthetic pathway in plants (modified from Huang and Wang [12]).

collect the protein pellet which was kept in a −70°C freezer if not used immediately.

DEAE Column Chromatography

The DEAE anion exchanger (Trisacryl M, LKB) was packed in a 4.8 × 12 cm column and equilibrated with 500 mL of Tricine buffer before use. Protein samples in Tricine buffer were loaded onto the DEAE column at a flow rate of 2 mL/min. Elution of proteins was monitored by UV absorbance at 280 nm. After all the unbound proteins had passed through the column, the bound proteins were eluted by a step gradient of $(NH_4)_2SO_4$ or NaCl prepared in Tricine buffer. The bound protein fractions were then desalted by passing through a G-25 column and their enzyme activities determined. The column was regenerated by washing successively with 1.5% Triton X-114 and 1 M NaCl and then equilibrating with Tricine buffer.

Blue B Affinity Gel Column Chromatography

Protein sample in Tricine buffer was loaded onto a 2.5 × 10 cm column packed with blue B affinity gel. After unbound proteins were eluted, the column was washed with 5 bed volumes of Tricine buffer. Bound proteins were eluted by 1 M NaCl in Tricine buffer, desalted by passing through a G-25 column, and assayed for enzyme activity.

Native-Protein PAGE

A nondenaturing polyacrylamide gel was prepared by omitting SDS, 2-mercaptoethanol and the stacking gel from the procedures of (26). A slab gel (0.3 × 12 × 16 cm) of 5% acrylamide and 2.7% Bis, 0.1% (v/v) TEMED, and 1 mg/mL ammonium persulfate was cast 24 h before use. The electrode buffer used was 50 mM Tris, 380 mM glycine (pH 8.3) and the resolving gel buffer was 375 mM Tris-HCl (pH 8.8). The gel was run with a Hoefer model SE600 apparatus maintained at 4°C. Protein samples were prepared in the loading buffer of 10 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue. A constant current of 5 mA was applied until the dye had entered 0.5 cm into the gel, then 20 mA was applied until the dye front was 2 cm from the bottom of the gel. Protein bands were located by staining a longitudinally cut strip while the rest of the gel was kept at 4°C. Based on the R_f values of protein bands the slab gel was cut into horizontal strips. The enzyme activities were determined after electrophoretic elution of the proteins from the gel strips.

Electrophoretic Elution of Proteins from Nondenaturing Gels

The proteins in the nondenaturing gel strips were electrophoretically eluted based on the method of (1). A sample concentrator (ISCO model 1750) was used with sample buffer of 10 mM Tris/acetate (pH 8.6) and an electrode buffer of 40 mM Tris/acetate (pH 8.6). The polyacrylamide gel was cut into pieces and submerged in the sample buffer. Electrophoretic elution was carried out at 3 W for 3 h at 4°C. The eluted proteins were collected and kept in aliquots at −20°C.

Protein Assays

The protein assays used were based on the method of Bradford (3). The Bio-Rad protein assay kit (Bio-Rad Laboratories) was used with BSA as a standard.

Aminoacylation Assays

Aminoacylation assays were performed in a 15 μL reaction mixture containing 0.05 (A_{260}) unit $tRNA^{Glu}$(II) (*Escherichia coli*), 1 mM ATP, 10 mM $MgCl_2$, 2 mM DTT, 2.5 μCi [^{3}H] glutamate (35 Ci/mmol), and enzyme preparation in Tricine buffer. After incubation at 29°C for 30 min, the reaction mixture was collected on a fiberglass filter (Whatman, GF/C). Unincorporated radioactive glutamate was removed by washing the filter for 15 min, two times each in 5% trichloroacetic acid and in 95% ethanol, at 4°C. The radioactivity on the dried filter was measured in liquid scintillation counting cocktail with a Beckman LS-5801 counter.

ALA Synthesizing Activity Assays

ALA synthesizing activity was assayed in 1.5 mL microcentrifuge tubes. The 100 μL reaction mixture contained 1 mM ATP, 1 mM $MgCl_2$, 1 mM NADPH, 2 mM 4,6-dioxoheptanoic acid, 5 μCi of [^{3}H]glutamic acid, and enzyme preparation. After a 30-min incubation at 29°C, the reaction was stopped by adding 10 μL of 300 mM unlabeled (carrier) ALA and 10 μL of 70% perchloric acid, and was then kept on ice for 15 min. Precipitated proteins were removed by centrifugation. The supernatant was recovered and partially neutralized by adding 10 μL of 7 N KOH. The sample was cooled and centrifuged to remove precipitates. The supernatant was collected and 20 μL was injected onto a C18 reverse phase HPLC column (4.6 × 150 mm, Phenomenex Ultremex 5C18). The C18 column was run at room temperature with a buffer of 50 mM NaH_2PO_4 (pH 3.0), 5 mM SDS, and 10 mM *t*-amylalcohol at a flow rate of 2.5 mL/min. The elution of carrier ALA was monitored by a refractive index detector (Differential-Refractometer, Knauer). Labeled ALA was collected together with the carrier ALA and radioactivity was measured with a liquid scintillation counter.

Heme Inhibition Analysis

Hemin was dissolved in a solvent of ethanol and 0.01 N KOH (1:1 v/v), and was added to the aminoacylation assays in different concentrations to test its effects on the Glu RS activities. The same volume of solvent without heme was added to a reaction as control.

Native Protein Mol Wt Determination

Native protein mol wt determination was performed according to the procedure of Sigma Technical Bulletin No. MKR-137 which was based on the method of Ferguson (9). In addition, the mol wt of the active enzyme was determined by a calibrated HPLC gel permeation column (Bio-Sil TSK-250, 0.5 × 30 cm, Bio-Rad).

SDS-PAGE Mol Wt Determination

The mol wt of the denatured enzyme was determined by SDS-PAGE based on the procedure of Laemmli (15). A 12.5% gel was electrophoresed at 10 V/cm until the bromophenol blue dye reached the bottom of the gel and was stained with silver stain (Bio-Rad). The R_f values of the mol wt markers (SDS-7, Sigma) were plotted against their log(M_r) to construct the standard curve.

Isoelectric Point Determination

For analytical isoelectric focusing, precast slab gels of pH 3.5 to 9.5 (Selectragel, Integrated Separation Systems) were used. The gels were run at 4°C and at 3 W constant power for 3 h and then stained with Coomassie blue. Protein markers of different isoelectric points (from Sigma and Bio-Rad) were used to construct the pH gradient curve.

Amino Acid Sequence Analysis

To ensure purity, proteins isolated from nondenaturing gels were purified further by a preparative SDS polyacrylamide gel (3 × 120 × 160 mm). The protein band of interest was cut out and electroeluted in a buffer of 10 mM *n*-ethylmorpholine/acetate (pH 8.6) according to the method of Bhown *et al.* (1). The eluted proteins were lyophilized and subjected to N-terminal amino acid sequence analysis based on Edman degradation (8) using an Applied BioSystems model 470A amino acid sequenator.

Table II. *Separation of ALA Synthesizing Activities by DEAE Column Chromatography.*

Protein fractions from the DEAE column were combined to reconstitute ALA synthesizing activity. The assays were performed in 100 μL reaction mixtures containing 1 mM ATP, 1 mM $MgCl_2$, 1 mM NADPH, 2 mM 4,6-dioxoheptanoic acid, and 5 μCi of [^{3}H]glutamic acid. After a 30 min incubation at 29°C, ALA synthesized from radioactive glutamate was purified and the radioactivity determined by a liquid scintillation counter (details in "Materials and Methods"). All three fractions are needed for maximum activity in converting glutamate to ALA.

Fractions	Activity
	dpm
DR + DI + DIII	6,593
DI + DIII	2,569
DR + DIII	1,861
DR + DI	1,061

Antibody Preparation

Protein sample in phosphate buffer (20 mM $NaHPO_4$, 15 mM NaCl [pH 7.0]) was homogenized with an equal volume of complete adjuvant. One mL of the protein-adjuvant mixture (100 μg antigen) was injected subcutaneously into New Zealand White rabbits to induce antibodies. Two booster injections were administered at 2-week intervals, and blood was collected in the 5th week. Crude serum was obtained after blood cells were removed from the blood sample by centrif-

Table I. *Summary of Purification*

Steps[a]	Total Protein	Specific Activity[b]		Total Activity		Yield		Purification Glu RS
		Glu RS	ALA	Glu RS	ALA	Glu RS	ALA	
	mg	*×10⁻⁶*	*×10⁻⁴*	*×10⁻⁶*	*×10⁻⁴*	%		*-fold*
Crude	960	3	0.18	2,880	170	100	100	1
$(NH_4)_2SO_4$	647	27	0.4	17,470	260	600	150	9
DI	65	380	8.3	24,700	540	860	310	127
Bb	4	1,590	33.1	6,360	130	220	77	530
GE	0.1	16,000	66.2	1,600	6.6	56	4	5,330

[a] Proteins extracted from *C. reinhardtii* cells were used to purify Glu RS involved in ALA synthesis. Total soluble proteins were used as crude. Other separation steps and corresponding active fractions were: 55–85% ammonium sulfate precipitated proteins ($[NH_4]_2SO_4$), DEAE column bound fraction I (DI), blue B column bound (Bb), and protein eluted from nondenaturing polyacrylamide gel (GE). [b] The specific activities of the active fractions are expressed in dpm mg^{-1} min^{-1} for glutamyl-tRNA synthetase activity (Glu RS) and ALA synthesizing activity (ALA), both at 29°C. ALA synthesizing activity of DI, Bb, and GE were measured by a reconstituted assay supplemented with constant amounts of the DR and DIII fractions. Specific activities calculated for the ALA synthesizing activity of DI, Bb, and GE fractions were thus based on their respective protein concentrations only. The yield calculation is based on the total activity of each step compared to crude as 100%. Fold purification for ALA was not calculated.

Table III. *Cofactor Requirements of Glutamyl-tRNA Synthetase*

Assay[a]	Activity
	cpm
All cofactors	159,000
−ATP	2,130
$-Mg^{2+}$	2,206
−tRNA	1,393
Boiled enzyme	546

[a] Aminoacylation assays were performed using tRNA from *Chlamydomonas* as described in the text. Cofactors omitted are indicated by a (−). Boiled enzyme (0.5 mg/mL) plus all cofactors was assayed as a negative control.

ugation (5000*g*, 15 min at 4°C). IgG was purified from the crude serum by $(NH_4)_2SO_4$ precipitation and DE52 anion exchange chromatography according to the method of (10). Purified antibodies were screened for their ability to recognize Glu RS as a specific antigen by Western blot analysis and their ability to inhibit Glu RS activity.

Western Blot Analysis

Protein samples were separated by SDS-PAGE and electrophoretically blotted onto a nitrocellulose (NC) filter according to the method of (22). The NC filter was developed colorimetrically based on the method of Towbin *et al.* (2). First, the filter was incubated overnight with TBS-BSA (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween-20, 0.02% NaN_3, and 0.3% BSA) to block remaining protein binding sites on the NC filter. The blocked filter was rinsed briefly and incubated for 2 h with 20 μg/mL rabbit antibodies prepared in TBS-BSA. The filter was then washed three times in TBS-BSA for 20 min each and incubated with 1:1,000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase. After a 1-h incubation, the filter was washed three times in TBS-BSA for 15 min each and two times with substrate buffer (10 mM K_2CO_3, 2 mM $MgCl_2$ adjusted to pH 10.0 with HCl) for 10 min each. Alkaline phosphatase activity was localized on the NC filter by the presence of a dark purple color when incubated with nitro blue tetrazolium (2 mg/mL), and 5-bromo-4-chloroindoxyl phosphate (5 mg/mL).

RESULTS

Enzyme Purification

Enzyme Assays and Purification Strategy

Both $tRNA^{Glu}$ aminoacylation and ALA synthesis were assayed at each step of the purification procedure to ensure that we were purifying the Glu RS that was involved in ALA synthesis. Aminoacylation assays were carried out by using *Chlamydomonas* tRNA or *Escherichia coli* tRNA. Our results indicated that *E. coli* tRNA was a valid substitute for *Chlamydomonas* tRNA in these assays (Tables I, II, and III). Based on this finding, *E. coli* $tRNA^{Glu}$(II) was used for routine aminoacylation assays during purification. After total cell extracts active in ALA synthesis were fractionated, the fraction containing Glu RS was identified through an aminoacylation assay. All other fractions were tested for their ability to participate in ALA synthesis by trials, with the fraciton containing Glu RS as a constant. All the other essential fractions thus identified were used together in a reconstituted ALA synthesizing assay to monitor Glu RS during purification.

Differential Ammonium Sulfate Precipitation of ALA Synthesizing Enzymes

Ammonium sulfate was directly incorporated into the cell resuspension buffer because examination by microscope revealed that the cells were broken by French Pressure Cell more efficiently in the presence of $(NH_4)_2SO_4$. The presence of $(NH_4)_2SO_4$ also made it possible to use a high speed centrifuge (27,000*g*, 15 min) instead of an ultracentrifuge (220,000*g*, 30 min) to remove insoluble materials. After the initial (55%) $(NH_4)_2SO_4$ cut to remove cell debris and one-third of the total proteins (Table I), most proteins remaining in the supernatant were precipitated by a second (85%) $(NH_4)_2SO_4$ cut. The protein precipitated by the 55 to 85% $(NH_4)_2SO_4$ cut included all the components necessary for ALA synthesis from glutamate. Higher specific activity and enzyme yield were obtained by using $(NH_4)_2SO_4$, compared to the crude extracts prepared in the absence of $(NH_4)_2SO_4$ (Table I). Over 100% yield was obtained, probably because some unknown interfering factors of the enzyme assay were removed.

DEAE Column Chromatography of ALA Synthesizing Activities

The $(NH_4)_2SO_4$ (55/85%) precipitated proteins were redissolved in Tricine buffer and passed through a G-25 column

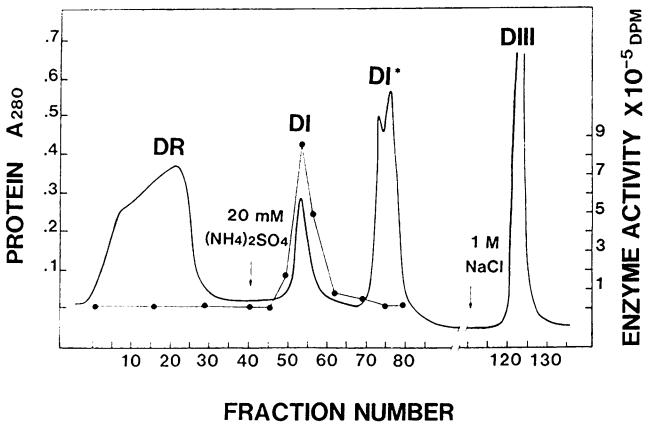

Figure 2. DEAE column chromatography fractionation of ALA synthesizing enzymes. Proteins extracted from *C. reinhardtii* cells were first fractionated by $(NH_4)_2SO_4$ and desalted, then loaded onto a DEAE ion exchange column in Tricine buffer. Proteins were monitored at A_{280} (solid line). The unbound proteins were collected as DR, and bound proteins were first eluted by 20 mM $(NH_4)_2SO_4$ which resulted in two peaks, DI and DI*. The rest of the proteins were eluted by 1 M NaCl as DIII. Glu RS activity (line with black dots) was analyzed and found to coincide with DI. Fractions after number 80 had only a baseline level of glutamyl-tRNA synthetase activities. DR and DIII together with DI were necessary to make ALA from glutamate in a reconstituted assay (see Table II).

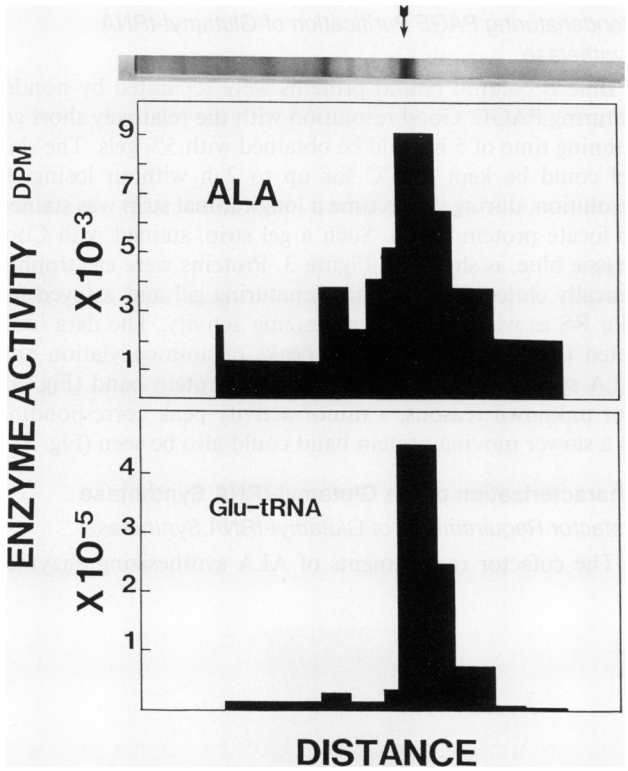

Figure 3. Purification of glutamyl-tRNA synthetase by nondenaturing PAGE. Partially purified glutamyl-tRNA synthetase (Bb fraction) was further purified by preparative nondenaturing PAGE. A center longitudinal strip was cut off from the slab gel and stained with Coomassie blue to visualize protein bands. A picture of a gel strip is shown at the top of the figure. The arrow indicates the protein band containing glutamyl-tRNA synthetase activity. The remaining gel was cut into horizontal strips according to the R_f values of stained bands. Proteins were eluted from the gel strips and were analyzed for glutamyl-tRNA synthetase (lower panel) and ALA synthesizing activity in a reconstituted assay by adding DR and DIII (upper panel). The figures show that the major activity peaks of glutamyl-tRNA synthetase and ALA synthesis coincide in the same protein band.

to remove the salt before being loaded onto the DEAE column. The run off proteins from the DEAE column were pooled as DR. Bound proteins were eluted by continuous salt gradients and over 90% of the enzyme activity was eluted before 20 mM $(NH_4)_2SO_4$ (data not shown). In preparative scale enzyme isolations with step salt gradient, two A_{280} absorbance peaks (DI and DI*) were eluted with 20 mM $(NH_4)_2SO_4$ (Fig. 2). The fractions corresponding to the first peak (DI) contained Glu RS while the DI* fractions did not. After the 20 mM $(NH_4)_2SO_4$ wash, the rest of the DEAE bound proteins were eluted by 1 M NaCl as one peak (DIII). It was found that ALA synthesizing activity could be reconstituted if DI fraction was supplemented with DR and DIII (Table II). Therefore, DR and DIII were collected to supplement active fractions obtained from further purification of DI in reconstituted ALA synthesis assays. Glu RS was the only active component in the DI fraction which was necessary for ALA synthesis because after being purified to homogeneity, it could replace the DI fraction in reconstituted ALA synthesis assays.

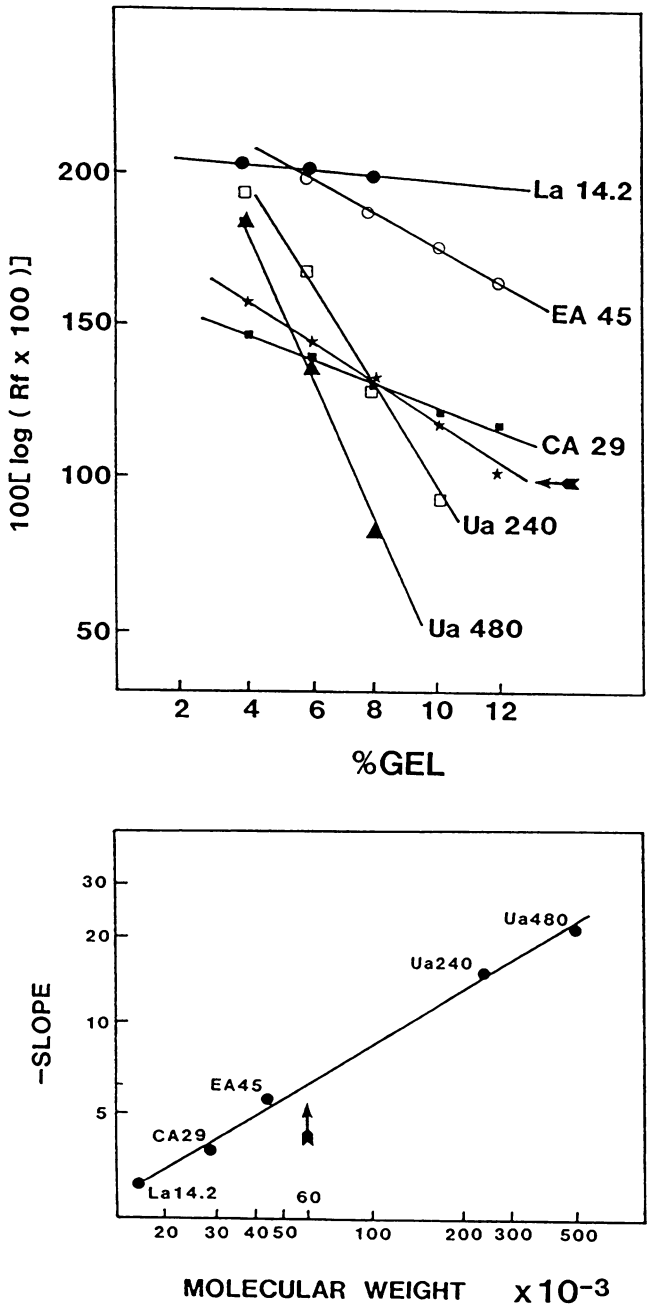

Figure 4. Native protein mol wt determination of glutamyl-tRNA synthetase by Ferguson's plot. A Ferguson's plot was constructed based on the methods of Ferguson (9). Glutamyl-tRNA synthetase purified by non-denaturing gel as well as marker proteins were run on non-denaturing gels of 2, 4, 6, 10, and 12% acrylamide, all with 2.7% Bis. The R_f values of each protein at different gel concentrations were calculated and 100(log[R_f × 100]) *versus* percent gel were plotted (upper panel). The log(-slope) of each line in the upper panel was determined and plotted against the logarithm of the mol wt of its corresponding marker protein (lower panel). The mol wt of glutamyl-tRNA synthetase was determined in the lower panel to be 60,000 (arrow). The native protein markers (Sigma) used and their mol wt were α-lactalbumin (La 14,200), carbonic anhydrase (CA 29,000), chicken egg albumin (EA 45,000), and urease monomer (Ua 240,000) and urease dimer (Ua 480,000).

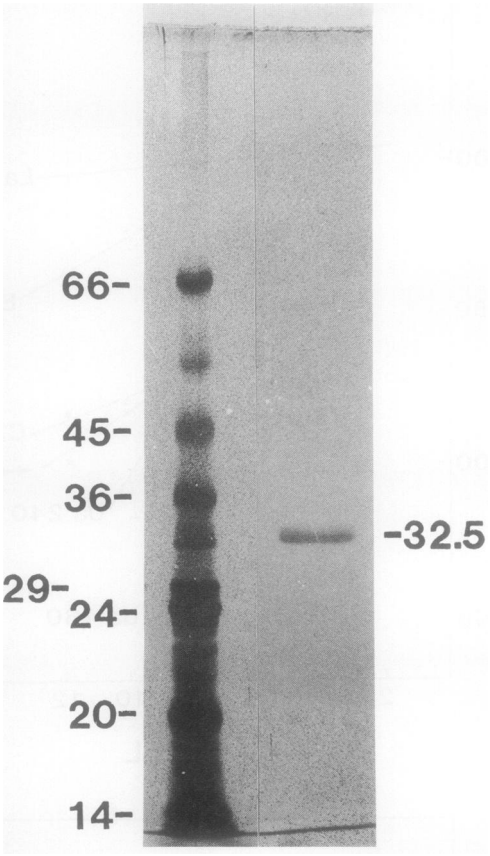

Figure 5. SDS-PAGE mol wt determination of glutamyl-tRNA synthetase. Glutamyl-tRNA synthetase purified by a nondenaturing gel was analyzed by SDS-PAGE to determine the mol wt of the subunits. The 12.5% gel was stained with silver stain. Lanes from a slab gel loaded with SDS-7 (Sigma) mol wt marker and purified glutamyl-tRNA synthetase are shown. The lane loaded with purified enzyme shows only one band, which is at 32,500 D. SDS-7: Albumin (bovine, 66 kD), albumin (egg, 45 kD), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 36 kD), carbonic anhydrase (bovine erythrocyte, 29 kD), trypsinogen (bovine pancreas, PMSF treated, 24 kD), trypsin inhibitor (soybean, 20 kD), α-lactalbumin (bovine milk, 14 kD).

The DEAE column was thus able to separate Glu RS from other enzymes involved in ALA synthesis. DI fractions were pooled for further purification. DI* fractions were not needed for ALA synthesis and were discarded.

Blue Column Chromatography

Active fractions from the DEAE column (DI) were loaded onto a blue B (Matrex Gel B) affinity column and the unbound proteins were washed off with Tricine buffer. The bound proteins (Bb) were eluted by 1 M NaCl. After removal of NaCl by a G-25 column, Bb was found to be active in glu-tRNA synthesis (Table I). In addition, the Bb fraction was active in ALA synthesis when supplemented with DR and DIII (Table I). The unbound proteins from the blue B column were discarded because they were not active in either aminoacylation or ALA synthesis.

Nondenaturing PAGE Purification of Glutamyl-tRNA Synthetase

Blue B column bound proteins were separated by nondenaturing PAGE. Good resolution with the relatively short gel running time of 5 h could be obtained with 5% gels. The slab gel could be kept at 4°C for up to 2 h without losing its resolution, during which time a longitudinal strip was stained to locate protein bands. Such a gel strip, stained with Coomassie blue, is shown in Figure 3. Proteins were electrophoretically eluted from the nondenaturing gel and assayed for Glu RS as well as ALA synthesizing activity. The data indicated that the major activity peaks of aminoacylation and ALA synthesis coincided in the same protein band (Fig. 3). For unknown reasons, a minor activity peak corresponding to a slower moving protein band could also be seen (Fig. 3).

Characterization of the Glutamyl-tRNA Synthetase

Cofactor Requirements of Glutamyl-tRNA Synthetase

The cofactor requirements of ALA synthesizing enzymes

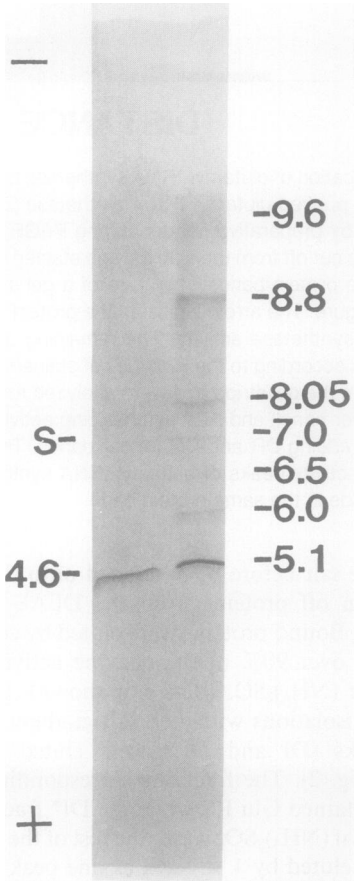

Figure 6. Isoelectric point determination of glutamyl-tRNA synthetase. Glutamyl-tRNA synthetase purified by a nondenaturing gel was loaded onto a 3.5–9.5 IEF gel, focused and stained with Coomassie blue to locate protein bands. Glu RS was focused as a single band at pH 4.6. The loading slot position is marked (S-). The pI markers (Bio-Rad) used and their (pI) values are: β-lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), equine myoglobin (7.0), whale myoglobin (8.05), α-chymotrypsin (8.8), and Cyt *C* (9.6).

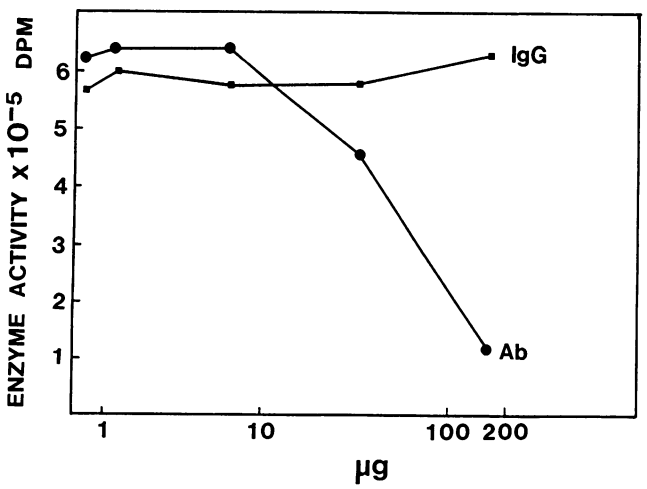

Figure 7. Enzyme inhibition by anti-glutamyl-tRNA synthetase antibodies. Glutamyl-tRNA synthetase activity was assayed in the presence of different amounts of purified anti-glutamyl-tRNA synthetase antibodies (Ab) or control antibodies (IgG). The results show that induced antibodies inhibit enzyme activity while the same amount of control IgG has no effect.

have been determined to be ATP, NADPH and Mg^{2+} (24). It was established in this work that ATP and Mg^{2+} are essential for the Glu RS activity (Table III). A partially purified enzyme from *Chlamydomonas* was reported to be able to charge $tRNA^{Glu}$ from *E. coli* and this charged $tRNA^{Glu}$ could be used for ALA synthesis (12). Glu RS purified from *Chlamydomonas* was active in charging glutamate to $tRNA^{Glu}$(II) from *E. coli* as well as tRNA extracted from *Chlamydomonas* (Table I and III).

Apparent Mol Wt of the Native Enzyme is 60,000

The apparent mol wt of the native Glu RS determined by the Ferguson's plot is 60,000 (Fig. 4). In addition, partially purified fraction (DI) was also separated by an HPLC gel permeation column, and Glu RS activity was eluted as a peak corresponding to a mol wt of 60,000 (data not shown).

Dimeric Structure of the Glu RS

When the purified enzyme eluted from nondenaturing gels was analyzed by SDS-PAGE under reduced conditions and the gel silver stained, only one band was observed (Fig. 5). This band had a mol wt of 32,500 and its presence always correlated to the activities during purification. Based on the mol wt of the native enzyme (60,000) and the single protein band on the SDS gel (32,500), we concluded that the active enzyme is composed of two subunits, each with a mol wt of 32,500.

Isoelectric Point of the Glu RS is 4.6

Analytical isoelectric focusing of the purified Glu RS was run on a pH gradient gel of 3.5 to 9.5. Only one band was observed on the sample lane, corresponding to an isoelectric point of 4.6 (Fig. 6). Preparative flat bed IEF separation of partially purified enzyme also resulted in the elution of the enzyme activity in the pH zone of 4.6 (data not shown).

Partial N-Terminal Amino Acid Sequence of Glutamyl-tRNA Synthetase

The 32.5 kD protein eluted from the SDS gel was analyzed to determine its partial N-terminal amino acid sequence. The first 10 amino acids from the N-terminus were analyzed twice and gave the same sequence: Asn-Lys-Val-Ala-Leu-Leu-Gly-Ala-Ala-Gly. The N-terminal amino acid sequence data from the 32.5 kD protein suggests that the two subunits of Glu RS are identical.

Production of Specific Antibodies against Glutamyl-tRNA Synthetase

Antibodies induced by SDS gel isolated 32.5 kD protein were able to inhibit native Glu RS activity while control

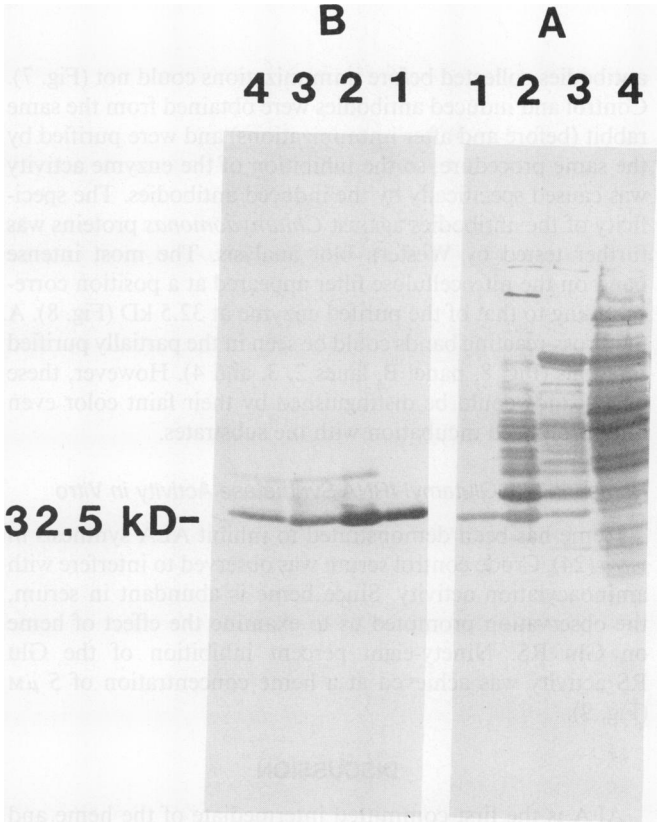

Figure 8. Western blot analysis of anti-glutamyl-tRNA synthetase antibodies. Glutamyl-tRNA synthetase at different purification stages was separated by SDS-PAGE and electrophoretically blotted onto a nitrocellulose filter. Half of the nitrocellulose filter was stained for total proteins with amido black (panel A). The other half of the filter was immunostained with antibodies against glutamyl-tRNA synthetase (panel B). The antibodies interacted primarily with a band at 32.5 kD which corresponds to that of the purified glutamyl-tRNA synthetase (lane 1). Lane 1, Nondenaturing PAGE purified glutamyl-tRNA synthetase; lane 2, blue B column bound fraction; lane 3, DEAE column bound fraction; lane 4, 55 to 85% ammonium sulfate precipitated proteins.

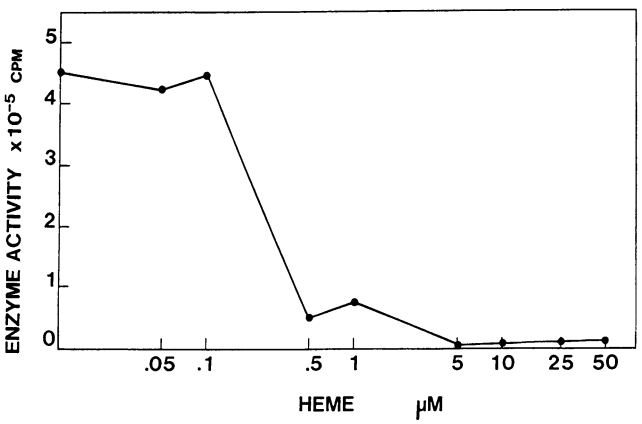

Figure 9. Heme inhibition of glutamyl-tRNA synthetase activity. Heme was dissolved in a mixture of ethanol:0.01 N KOH (1:1 v/v) and added to aminoacylation assays in different concentrations to examine the inhibition of enzyme activity. Solvent alone was used as control. Inhibition of 98% was reached at a heme concentration of 5 μM.

antibodies collected before immunizations could not (Fig. 7). Control and induced antibodies were obtained from the same rabbit (before and after immunizations) and were purified by the same procedure, so the inhibition of the enzyme activity was caused specifically by the induced antibodies. The specificity of the antibodies against *Chlamydomonas* proteins was further tested by Western blot analysis. The most intense band on the nitrocellulose filter appeared at a position corresponding to that of the purifed enzyme at 32.5 kD (Fig. 8). A few cross-reacting bands could be seen in the partially purified fractions (Fig. 8, panel B, lanes 2, 3, and 4). However, these extra bands could be distinguished by their faint color even after prolonged incubation with the substrates.

Heme Inhibits Glutamyl-tRNA Synthetase Activity in Vitro

Heme has been demonstrated to inhibit ALA synthesis *in vitro* (24). Crude control serum was observed to interfere with aminoacylation activity. Since heme is abundant in serum, the observation prompted us to examine the effect of heme on Glu RS. Ninety-eight percent inhibition of the Glu RS activity was achieved at a heme concentration of 5 μM (Fig. 9).

DISCUSSION

ALA is the first committed intermediate of the heme and Chl biosynthetic pathway. Increasing number of organisms have been shown to use the 5-carbon pathway to synthesize ALA. In the 5-carbon pathway of ALA formation, the first reaction is the ligation of glutamate onto its cognate tRNA catalyzed by a Glu RS. Glu RS activity involved in ALA synthesis has been reported in barley (13), *Chlamydomonas* (24), and Chlorella (25). The enzyme has been partially purified from chloroplasts of wheat (18) and barley (5). We now report the purification of a Glu RS from *Chlamydomonas*. This Glu RS is needed for the synthesis of ALA from glutamate. It can aminoacylate $tRNA^{Glu}$ from *Escherichia coli* as well as tRNA from *Chlamydomonas* and it requires Mg^{2+} and ATP as cofactors.

The Glu RS from *Chlamydomonas* has a native mol wt of about 60,000. This value was obtain by analyzing the purified enzyme with native PAGE followed by Ferguson plots and with a HPLC gel filtration column. The isoelectric point of the purified enzyme is 4.6. When analyzed with SDS-PAGE, the purified enzyme showed a single protein band with a mol wt of about 32,500. It was concluded that this Glu RS consists of two subunits of the same mol wt. The unambiguous N-terminal amino acid sequence obtained from the 32.5 kD protein band suggested that the two subunits may be identical. The enzyme from *Chlamydomonas* has smaller mol wt than any other reported aminoacyl-tRNA synthetases (19).

Polyclonal antibodies were raised with the purified Glu RS from *Chlamydomonas*. The antibodies reacted with the enzyme protein band in Western blot analysis. In addition, the antibodies were able to inhibit Glu RS activities. Therefore, we have purified and identified a protein band as that of a Glu RS. While we have shown that this Glu RS is the first enzyme of ALA synthesis, we do not know whether the same enzyme is also involved in protein synthesis. However, it is known that there is only one aminoacyl-tRNA synthetase for each amino acid in *E. coli* (19); and there seems to be one Glu RS in chloroplasts of barley (5) and wheat (18). Using *E. coli* $tRNA^{Glu}$ as substrate, we found one major Glu RS activity peak during each step of the enzyme purification until the enzyme was purified. This observation suggests that there is only one Glu RS in the chloroplast of *Chlamydomonas*. In barley the single $tRNA^{Glu}$ from chloroplast has been identified (21) and it has been suggested that a single $tRNA^{Glu}$ is used for protein synthesis and ALA synthesis in the chloroplast (14). In *Synechocystis* it also has been shown that the same glu-$tRNA^{Glu}$ can be used as substrates for both ALA synthesis and protein synthesis (20). Therefore, it is not unreasonable to assume that one Glu RS is making a single pool of glu-$tRNA^{Glu}$ which is used for both ALA and protein synthesis in the chloroplast.

Recently, it has been shown that *E. coli* also used the 5-carbon pathway for ALA synthesis (16). The complete amino acid sequence of the *E. coli* Glu RS, a 56 kD protein, has been reported (4). It is interesting that, despite their identical functions and the fact that they can replace each other in aminoacylation assays, Glu RS from *Chlamydomonas* and *E. coli* shared no homology in their N-terminal amino acid sequence. In addition, antibodies raised against the *Chlamydomonas* enzyme did not recognize the *E. coli* enzyme (our unpublished observation).

Heme has been reported to inhibit ALA synthesis in many organisms. Using partially purified enzyme fractions and assays which monitor specific steps of glutamate to ALA conversion, Huang and Wang (12) were able to identify dehydrogenase as the enzyme sensitive to heme inhibition. In the present study, using the purified enzyme and its specific assay, we found that Glu RS is also inhibited by heme. If, as discussed above, the same Glu RS is serving both ALA synthesis and protein synthesis in the chloroplast, the observed heme inhibition of Glu RS activity would mean that those two functions in the chloroplast are regulated coordinately by

heme. However, lower heme concentration has been observed to inhibit Glu to ALA synthesis (12). Therefore, the significance of heme inhibition on Glu RS requires further study.

Note Added in Proof

In a recent report, M. Chen, D. Jahn, A. Schon, G. P. O'Neill, and D. Söll ([1990] J Biol Chem 265: 4054–4057) purified Glu RS from *Chlamydomonas* and found it to be a monomeric enzyme of 62 kD. The discrepancy will have to be resolved with further work.

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