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Purification, Characterization, and Fractionation of the δ-Aminolevulinic Acid Synthesizing Enzymes from Light-Grown *Chlamydomonas reinhardtii* Cells[1]

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ABSTRACT

The synthesis of δ-aminolevulinate from glutamate by *Chlamydomonas reinhardtii* membrane-free cell homogenates requires Mg^{2+}, ATP, and NADPH as cofactors. The pH optimum is about 8.3. When analyzed by a Fractogel TSK gel filtration column the δ-aminolevulinate synthesizing enzymes, including glutamate-1-semialdehyde aminotransferase, elute with an apparent molecular weight of about 45,000. The enzymes obtained from the gel filtration column were separated into three fractions by affinity column chromatography. One fraction binds to heme-Sepharose, one to Blue Sepharose, while the enzyme converting the putative glutamate-1-semialdehyde to δ-aminolevulinic acid is retained by neither column. All three fractions are necessary for the conversion of glutamate to δ-aminolevulinate. The δ-aminolevulinate synthesizing enzymes from *Chlamydomonas* are sensitive to inhibition by heme but not sensitive to inhibition by protoporphyrin.

The synthesis of ALA[2] is a rate-limiting step for Chl biosynthesis (14) and is believed to be regulated by heme and perhaps other intermediates of the Chl pathway (5, 7, 10, 11, 22, 30). In greening higher plants, radioactive tracer experiments indicated that the five-carbon skeleton of ALA was derived from the intact carbon skeleton of the glutamate or α-ketoglutarate molecule (2, 24). This is called the C_5 pathway of ALA biosynthesis. Recently, ^{13}C nuclear magnetic resonance spectroscopy of Chl formed from [1-^{13}C]glutamate and [2-^{13}C]glycine in greening maize has shown that ^{13}C incorporation is consistent with the formation of ALA by the C_5 pathway but not from glycine and succinate (27). In plastids isolated from greening spinach (12), barley (19), cucumber (31), and maize (24) ALA is synthesized by the C_5 pathway.

In one proposed C_5 pathway, glutamate is converted via glutamate-1-semialdehyde to ALA (2) and requires the enzyme glutamate-1-semialdehyde aminotransferase (20). Another C_5 pathway proposes that α-ketoglutarate is metabolized to γ,δ-dioxovalerate and then converted by a transaminase to ALA. Glyoxylate transaminase appears to catalyze this process in *Euglena* (8). The glutamate-1-semialdehyde aminotransferase was detected in barley (20) and maize (26) plastids. The aminotransferase from barley has been purified and characterized (21). The formation of ALA from glutamate by soluble enzymes from barley plastids (13) and from wheat leaves (9) required Mg^{2+}, ATP, and NADPH. The conversion of α-ketoglutarate to ALA by a soluble preparation from maize required Mg^{2+}, NADH, pyridoxal phosphate, and an amino donor (16). With the more active barley preparations it has been possible to analyze in detail the steps in the conversion of glutamate to ALA. The presence of soluble enzymes converting glutamate into glutamate-1-semialdehyde has been demonstrated in greening barley plastids (28). At least two proteins are required for the conversion of glutamate to glutamate-1-semialdehyde (28).

A C_5 pathway for ALA synthesis has also been demonstrated in greening single celled organisms, *e.g., Cyanidium caldarium* (18), *Chlorella vulgaris*, and *Fremyella diplosiphon* (25), and in *Scenedesmus obliquus* (23). Greening *Chlamydomonas* cells contain enzymes able to synthesize ALA from glutamate and from glutamate-1-semialdehyde (29). In this report we present evidence that, in *Chlamydomonas*, ALA is made by a set of enzymes similar to those in barley plastids. Properties of these enzymes in *Chlamydomonas reinhardtii* are described.

MATERIALS AND METHODS

Culture Conditions. Wild type stocks of *Chlamydomonas reinhardtii* used in this study were derived from strain 137C. Cells were pregrown on agar plates with HSA (30) under the light (about 5000 lux, provided by cool-white fluorescent lamps, as measured with an LI-185 photometer, Lambda Instrument Corp.). Cells were inoculated into 500-ml flasks containing 300 ml of liquid HSA and grown for 2 to 3 d on a shaker under the light. When cell concentration reached 2 to 3 × 10^6 cells/ml (determined by hemacytometer counts), the culture was diluted to a cell concentration of about 0.5 × 10^6 cells/ml in a 2800-ml Fernbach flask with 1400 ml liquid HSA. This culture was grown on a shaker under the light, until cell concentration reached about 2 × 10^6 cells/ml.

Enzyme Preparation. All steps of enzyme isolation were carried out at 0 to 4°C. *Chlamydomonas* cells were harvested by centrifugation in a continuous flow rotor, (J-CF-Z Beckman) at 10,000*g* and washed once with elution buffer (0.3 M glycerol, 0.1 M Tricine, adjusted to pH 8.3 with fresh NaOH solution unless stated otherwise). The washed cells were pelleted by centrifugation at 10,000*g* for 5 min then resuspended to a concentration of 5 × 10^8 cells/ml in the same buffer which was also 1 mM in DTT and $MgCl_2$. The cells were disrupted by passing through an

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[2] Abbreviations: ALA, δ-aminolevulinic acid; HSA, high salt acetate medium.

Aminco French pressure cell at 8,000 p.s.i. or a Yeda press at 2,000 p.s.i. twice. The cell homogenates were centrifuged at 220,000*g* for 20 min. The supernatant containing soluble proteins was collected and used as crude enzymes.

Protein Determination. The protein concentration of enzyme preparations was determined according to Bradford (3) using Bio-Rad dye reagent concentrate.

Gel Filtration of the Crude Enzyme Preparation. A Fractogel (MCB) TSK HW-55 (F) column 5 × 49 cm was equilibrated with elution buffer adjusted to 1 mM DTT and 1 mM $MgCl_2$. Crude enzyme solution (between 250–400 mg of protein) was loaded onto the column and the eluate monitored for light absorption at 280 nm. The flow rate was about 9 $ml \cdot cm^{-2} \cdot h^{-1}$, controlled by a peristaltic pump; 6-ml fractions were collected, portions of which were used for different enzyme assays.

The void volume of the column was determined by elution of Blue Dextran. The column was calibrated with mol wt standards from Pharmacia Chemicals. The elution constant (K_{av}) for each protein standard was calculated according to the manufacturer's suggestions.

Fractionation of the ALA Synthesizing Enzymes by Affinity Chromatography with a Heme-Sepharose Column or a Blue Sepharose Column (28). The fractions of the gel filtration column known to contain ALA synthesizing activity were pooled and loaded onto either a Blue Sepharose CL-6B (Pharmacia) column (2.5 × 10 cm) or a heme-Sepharose column (2.5 × 10 cm). The columns were washed with elution buffer until all unbound proteins had been removed and collected as the runoff fractions. Proteins bound to either column were then eluted with elution buffer which was made 1 M in NaCl. The proteins eluted by the salt solution were desalted with a Sephadex G-50 column (2.5 × 24 cm). Alternatively, the runoff fraction of the Blue-Sepharose column was loaded subsequently onto the heme-Sepharose column. The proteins bound were eluted and desalted as described above.

Assay for δ-Aminolevulinate Synthesizing Activity with Radioactive Glutamate. For the standard assay of ALA synthesizing activity, a 1-ml enzyme preparation in elution buffer containing 10 μmol levulinic acid, 25 μmol $MgCl_2$, 1 μmol NADPH, 5 μmol ATP, and 2.5 μCi L-[U-^{14}C]glutamate (10 nmol) was incubated in a shaking water bath for 20 min at 29°C.

After incubation, 100 μg of ALA was added to the enzyme assay mixture as a carrier, and the reaction stopped with 40 μl of 70% $HClO_4$. The protein precipitate was removed by centrifugation and the supernatant loaded onto a Dowex 50W-X8, 200 to 400 mesh (Bio-Rad) cation exchange column (8 × 14 mm) which had been prewashed twice with 2 ml 1 N NaOH, then 3 ml Na-citrate buffer (pH 3.08, 0.2 M Na^+). After loading, the column was washed again with 5 ml of the pH 3.08 Na-citrate buffer which eluted the unreacted glutamate. The adsorbed ALA was eluted with 5 ml of Na-citrate buffer (pH 5.10, 0.2 M Na^+).

The pH of the final eluate was adjusted to 6.9 to 7.0 with 0.5 M Na_3PO_4 and 1 N NaOH. After the addition of 100 μl of ethyl acetoacetate, ALA pyrrole was formed by heating at 100°C for 20 min. The pH of the cooled sample was adjusted to 7.4 and excess ethyl acetoacetate removed by 3 × 1 ml extractions with chloroform. The chloroform layer was discarded. After a final pH adjustment to 4.0, the sample was extracted with 3 × 3 ml ether and the ether extracts pooled. After centrifuging the ether extract at low speed, excess water was removed, one drop of concentrated NH_4OH was added to the ether, and the ether extract evaporated to dryness under N_2 at 55°C.

The ALA-pyrrole was further purified by TLC. The dried ether extract was eluted with a few drops of alkaline acetone (NH_4OH:acetone, 1:100, v/v) and chromatographed on a 5 × 20 cm microcrystalline cellulose plate with 0.5% (w/w) fluorescent indicator. After developing to about 10 cm with 1-propanol:1-butanol:5% NH_4OH (1:2:1, v/v/v), the chromatogram was allowed to air-dry. The purified ALA-pyrrole was located under a UV lamp (254 nm) as a dark band, scraped off and eluted with 1.0 ml of H_2O:methanol:NH_4 (5:4:1, v/v/v). By this stage the ALA-pyrrole was already quite pure, as indicated by the lack of radioactivity on the TLC plate other than in the ALA-pyrrole band. Radioactivity of 0.5 ml of the eluate was measured in a liquid scintillation counter. Twenty-five μl of the purified ALA-pyrrole was used to estimate the recovery of ALA with modified Ehrlich reagent using a millimolar extinction coefficient (553 nm) of 58 (15). The enzyme activity, corrected for the recovery of carrier in the purifed pyrrole, is given as cpm in purified ALA-pyrrole/mg protein·20 min. The purification procedure consistently recovered 30% to 40% of the added carrier, so differences in recovery did not significantly affect the calculated enzyme activities.

For studies of pH optimum, the crude enzyme preparation was prepared in pH 9 elution buffer and adjusted to the desired pH with 1.0 M Tricine, 1.0 N HCl, 1.0 N acetic acid, or 1.0 N NaOH (for pH 10.0) before adding the cofactors. To determine the effect of pH on the stability of the enzyme, the pH of the enzyme solution was adjusted and the solution kept on ice (0°C) up to 4 h before the assay.

Formation of ALA from Nonradioactive Glutamate. The assay mixture for ALA synthesizing activity with nonradioactive glutamate was the same as that for the radioactive assay except 200 nmol of glutamic acid was used in place of radioactive glutamate. After the reaction was stopped, ALA was purified by cation exchange column chromatography then converted into ALA-pyrrole which was extracted into ether. The ether containing the ALA-pyrrole was dried with N_2 and the amount of ALA determined (15).

Preparation of Heme-Sepharose Column. Heme was joined via a six-carbon linker to Sepharose (AH-Sepharose, Pharmacia) as given in (28). After washing alternately with acidic and alkaline solutions of 50% (v/v) methanol containing 1 M NaCl, the gel was further washed with 96% (w/v) acetic acid followed by elution buffer until the eluate was clear. The heme-Sepharose column (2.5 × 10 cm) was regenerated by washing either with 96% acetic acid or 4 M urea followed by at least 150 ml of elution buffer.

Assays for Glutamate-1-Semialdehyde Aminotransferase, ALA Dehydratase, and Porphobilinogen Deaminase. All enzyme preparations were in elution buffer (0.3 M glycerol, 0.1 M Tricine, 1 mM DTT, 1 mM $MgCl_2$). Glutamate-1-semialdehyde aminotransferase activities were assayed (pH 8.3) by adjusting 1 ml of enzyme preparation to 75 μM in glutamate-1-semialdehyde (prepared according to [20]) and incubating the mixture for 20 min at 29°C. A reaction mixture without enzyme was used as the control. The reaction was stopped by adding 40 μl of 70% $HClO_4$. After removing the precipitated proteins by centrifugation, the reaction mixture was adjusted to pH 6.9 and incubated with 100 μl of ethyl acetoacetate for 20 min at 100°C to make ALA-pyrrole. The pyrrole was reacted with equal volumes of modified Ehrlich reagent (15) for 20 min and the absorption spectrum recorded using the control as the reference. The ALA formed was identified by the absorption spectrum and its level determined using ϵ_{mM} 553 nm of 58 (15).

ALA dehydratase was assayed by adding 2 μmol of ALA to 1 ml of enzyme preparation and incubating the mixture for 20 min at 29°C. The reaction was stopped by 40 μl of $HClO_4$ and the protein removed by centrifugation. The porphobilinogen formed was measured with a spectrophotometer after reacting with equal volumes of modified Ehrlich reagent for 20 min. Porphobilinogen deaminase was assayed by incubating 1 ml of enzyme preparation with 0.4 μmol of porphobilinogen for 20 min at 29°C. After the reaction was stopped and the protein

precipitate removed, the uroporphyrin formed was scanned with a spectrophotometer. Absorption at 400 nm (Soret peak of the absorption spectrum) was used to determine the relative activity of different enzyme fractions.

RESULTS

Cofactor Requirements for the ALA Synthesizing Enzymes from *Chlamydomonas*. Crude enzymes, prepared as described in "Materials and Methods," were used in experiments determining optimal conditions for the enzyme assay. An absolute requirement for Mg^{2+}, ATP, and NADPH was observed (Table I). The inhibitory effect of pyridoxyl phosphate is ascribed to its reaction with ALA. The optimal pH for the enzyme activity is 8.3 (Fig. 1).

The activities of the crude enzymes declined at similar rates over a period of 4 h when tested at different pH values between 8.3 and 9.0 (Fig. 2). Storage for 4 h at 0°C resulted in a 50% loss of activity, irrespective of the pH used. However, crude enzyme preparations could be kept at −90°C for weeks with almost fully retained activities. After partial purification by gel filtration, the enzymes became more stable.

The optimal concentration of ATP for the crude enzyme was determined to be 2 to 5 mM (Fig. 3A). When the ATP level was maintained at 5 mM, the optimal concentration for NADPH was

Table I. *Cofactor Requirements for the Synthesis of δ-Aminolevulinate from [^{14}C]Glutamate by the Crude Enzyme Preparations from Light-Grown, Wild Type Cells of C. reinhardtii*

Cofactor concentrations are 5 mM for ATP, 1 mM for NADPH, 25 mM for Mg^{2+}.

	ALA Synthesizing Activity
	cpm in purified ALA-pyrrole/mg protein·20 min
1. 1 ml enzyme + all cofactors	20667
2. 1 ml enzyme—ATP	106
3. 1 ml enzyme—NADPH	1078
4. 1 ml enzyme—Mg^{2+}	101
5. 1 ml enzyme—all cofactors	54
6. 1 ml boiled enzyme + all cofactors	245
7. 1 ml enzyme—NADPH + 1 mM NADH	10105
8. 1 ml enzyme + all cofactors + 1 mM pyridoxyl-P	2150

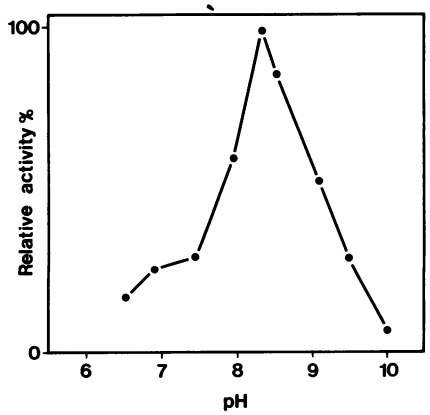

FIG. 1. The pH optimum of crude ALA synthesizing enzymes prepared from light-grown, wild type cells of *C. reinhardtii*. The pH of the reaction mixtures was adjusted with 1 M Tricine and 1 M NaOH. Activities are given as percentages of the activity at pH 8.3.

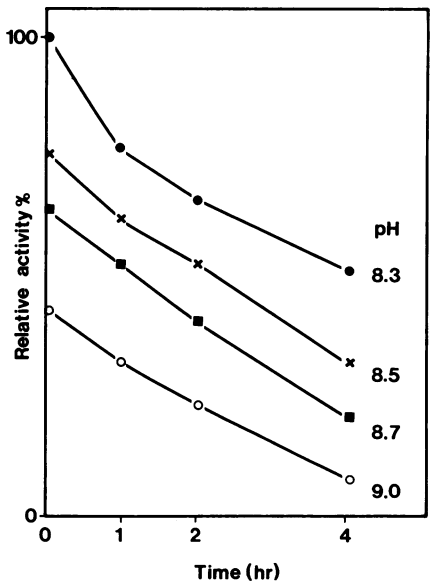

FIG. 2. Stability of crude ALA synthesizing enzymes in different pH. The enzymes were prepared from light-grown, wild type cells of *C. reinhardtii*. Relative activities were plotted against time. The maximum activity was 50,000 cpm in purified ALA-pyrrole/mg protein·20 min.

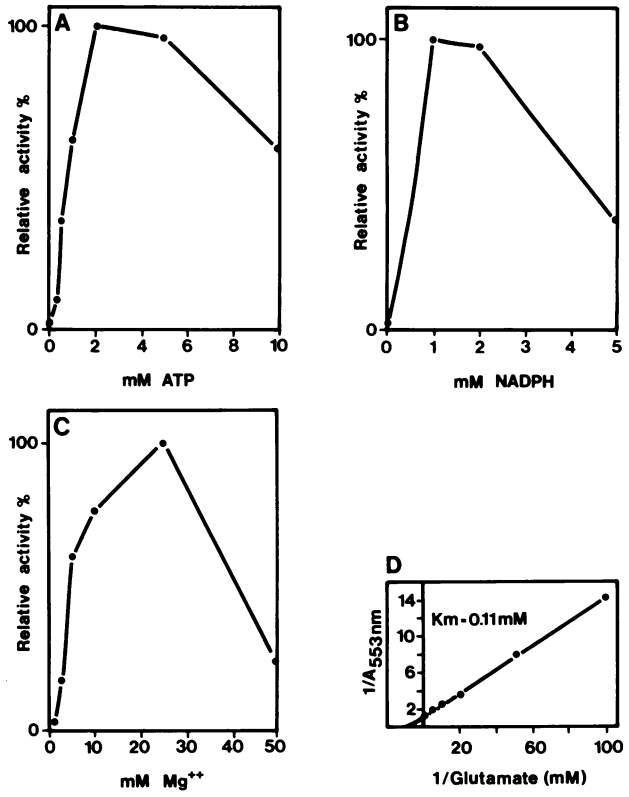

FIG. 3. Optimum concentrations of cofactors for the crude ALA synthesizing enzymes. A, ATP; B, NADPH; C, Mg^{2+}; and D, glutamate, whose K_m value was calculated to be 0.11 mM. The maximum activity for A, B, and C is about 60,000 cpm in purified ALA pyrrole/mg protein·20 min.

1 to 2 mM (Fig. 3B). At 5 mM ATP and 1 mM NADPH, the optimal Mg^{2+} concentration for ALA synthesis was 25 mM (Fig. 3C). Higher concentrations of ATP, NADPH, or Mg^{2+} all caused some inhibition of activity.

To determine the K_m value of glutamate for the ALA synthe-

sizing enzymes, the crude enzymes were passed through a Sephadex G-50 column (2.5 × 24 cm) to remove small molecules and the eluted enzymes assayed with different concentrations of cold glutamate. As shown by the double reciprocal plot of the results, a K_m value of 0.11 mM for glutamate was obtained by extrapolation (Fig. 3D). The crude enzymes convert glutamate to ALA linearly for up to 90 min of incubation. Thereafter, no additional increase in ALA synthesis occurred (Fig. 4).

Partial Purification of the ALA Synthesizing Enzymes and Some Other Enzymes of the Chl Pathway with a Gel Filtration Column. The elution profile of crude enzymes through a TSK column monitored at 280 nm showed three distinct peaks (Fig. 5). All of the chloroplast pigments were eluted off the column in the first peak. The ALA synthesizing enzyme activity was eluted at the left shoulder of the third peak. The mol wt of the protein fraction having the highest ALA synthesizing activity was estimated to be around 45,000 D. The glutamate-1-semialdehyde aminotransferase eluted immediately after the peak of ALA synthesizing enzyme activity and was estimated to have a mol wt of about 30,000 D. ALA dehydratase, which converts ALA to porphobilinogen, eluted off the column at the second peak and has an estimated mol wt of about 150,000 D. Porphobilinogen deaminase, which synthesizes uroporphyrinogen from 4 molecules of porphobilinogen, was eluted at the latter half of the third peak and has an estimated mol wt of about 15,000 D.

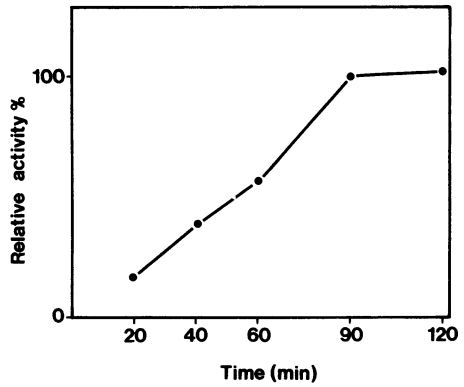

FIG. 4. The time course of ALA formation by the crude ALA synthesizing enzymes incubating at 29°C. The maximum activity was 80,000 cpm in purified ALA-pyrrole/mg protein·20 min.

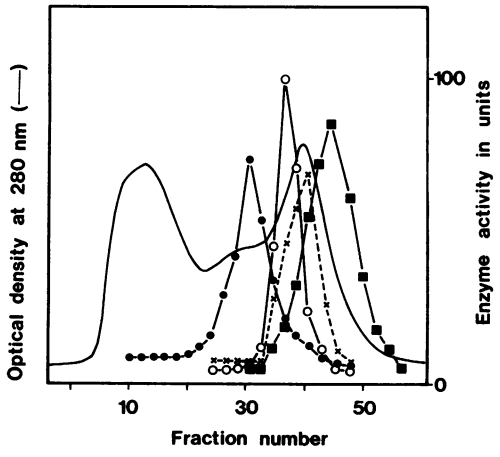

FIG. 5. Gel filtration chromatography of crude enzyme preparation from light-grown, wild type *C. reinhardtii* cells showing elution profiles of ALA synthesizing enzyme activity (O——O), glutamate-1-semialdehyde aminotransferase (x---x), ALA dehydratase (●——●), and porphobilinogen deaminase (■——■). A column of Fractogel TSK 55F (5 × 48 cm) was used. Fraction volumes were 6 ml. Enzyme activities were measured as described in "Materials and Methods."

Fractionation of the ALA Synthesizing Enzymes with a Blue Sepharose Column or a Heme-Sepharose Column. Gel filtration column fractions active in ALA synthesis were pooled, concentrated, and passed through a heme-Sepharose column. Some of the proteins bound to the column and some did not. The bound and the unbound protein fractions tested individually showed only weak ability to convert glutamate to ALA (Table II). When the fractions were combined, the ALA synthesizing activity was more than 6-fold higher than the sum of the individual activities. In the same way, fractions from the gel filtration active in ALA synthesis were also passed through Blue-Sepharose and results similar to those obtained with heme-Sepharose were seen (Table II). The bound and the unbound fraction could be reconstituted into a 17-fold more active preparation. Most of the glutamate-1-semialdehyde aminotransferase activity was not bound to either of the two affinity columns. These results indicated that both the heme-Sepharose column and the Blue Sepharose column could separate the ALA synthesizing enzymes into two fractions.

When fractions from the two columns were recombined, the results were less clear (Table II). However, it was obvious that the two columns were not retaining the same components of the ALA synthesizing enzymes, since the different fractions could not replace one another. It was concluded that, similar to what was observed in barley (28), the ALA synthesizing enzymes have three components. Glutamate-1-semialdehyde aminotransferase was not bound to either column and the Blue Sepharose column retained one of the other two components. The heme-Sepharose column retained both of the other two components of the ALA synthesizing enzyme which explained the high activity obtained from combining the fraction bound to the heme-Sepharose column with the runoff fraction of the Blue Sepharose column (assay 9). Based on this deduction, it was decided that the partially purified ALA synthesizing enzymes should be passed through the Blue Sepharose column first, then the heme-Sepharose column. In all the assays where runoff protein fractions from the heme-Sepharose column were used, the ALA synthesis was lower than expected. This could be due to a slow leakage of inhibitory heme from the column.

Synthesis of ALA from Glutamate by Three Separable Protein Fractions of the ALA Synthesizing Enzymes. Because the Blue Sepharose bound fewer components of the ALA synthesizing enzymes, the active fractions from the gel filtration were first

Table II. *δ-Aminolevulinate Synthesizing Activity and Glutamate-1-semialdehyde Aminotransferase Activity of Enzyme Fractions Separated by a Heme-Sepharose Column or a Blue Sepharose Column*

	ALA Synthesizing Activity	Glutamate-1-semialdehyde Aminotransferase Activity
	cpm in purified ALA-pyrrole/mg protein·20 min	*nmol ALA formed/mg protein·hr*
Heme-Sepharose column		
1. Bound enzymes	6776	1.7
2. Runoff enzymes	1980	16.2
3. 1 + 2	52166	—[a]
Blue Sepharose column		
4. Bound enzymes	6353	2.0
5. Runoff enzymes	3191	16.5
6. 4 + 5	160763	—
7. 1 + 4	16185	—
8. 2 + 4	3355	—
9. 1 + 5	65326	—
10. 2 + 5	2801	—

[a] —, Not done.

passed through the Blue Sepharose column. The proteins not bound were then passed through the heme-Sepharose column. This yielded three fractions containing different components of the ALA synthesizing enzymes. These were: heme-Sepharose bound proteins (H), the Blue Sepharose bound proteins (B), and the runoff proteins (R). As indicated by the data in Table III, the three fractions were incapable of converting glutamate to ALA either alone or in combinations of two. We do not know why H + R was less active than H or why B + R was less active then B. All three fractions were needed for the active synthesis of ALA from glutamate. The obvious exception was the combination of H and B fractions. By analogy with the results from barley (28) (also see "Discussion"), it was assumed that the two fractions (H + B) could convert glutamate to glutamate-1-semialdehyde which would co-purify with ALA in our purification procedure and would have lower extinction than ALA when determined with Ehrlich reagent. Therefore, even though the radioactivity present in the H + B combination was 60% of that in the H + B + R combination, the measurable "ALA-pyrrole derivative" (presumably formed by glutamate-1-semialdehyde) in the cold assay for the H + B combination was only 24% of that in the H + B + R combination (Table III). To verify this notion, nonradioactive glutamate was incubated with H + B with all the cofactors for 20 min. At the end of incubation, the proteins were precipitated by acid, the pH of the supernatant adjusted to 8.3, and incubated again after adding R which contains the glutamate-1-semialdehyde aminotransferase (Table III). Addition of elution buffer instead of R was used as control. The results (Fig. 6) showed that H + B made a compound (assumed to be glutamate-1-semialdehyde) which formed an Ehrlich-positive pyrrole with a low extinction (Fig. 6, spectrum 1). The same compound in protein-free supernatant, after incubation with R was converted to ALA which formed pyrrole with much higher extinction (Fig. 6, spectrum 2) and co-purified with authentic ALA-pyrrole on a thin-layer chromatogram (data not shown).

Sensitivity of ALA Synthesis to Protoporphyrin and Heme. Protoporphyrin and heme, two tetrapyrroles proposed to have regulatory roles in the synthesis of ALA, were tested as inhibitors of the crude ALA synthesizing enzymes. Solutions of protoporphyrin (0.5 mM) and heme (0.5 mM) were made by dissolving them in a 1:1 (v/v) mixture of ethanol and 0.01 M KOH. The solutions were diluted so that different amounts of pigment in 100 μl of ethanolic KOH could be added to each 1-ml reaction mixture. Solvent alone was added to the control. Heme was highly inhibitory even at low concentrations while protoporphyrin was without effect on the reaction (Fig. 7).

Table III. *Formation of δ-Aminolevulinate from Labeled or Unlabeled Glutamate and from Glutamate-1-semialdehyde Catalyzed by the Three Enzyme Fractions Separated by Serial Affinity Column Chromatography*

	ALA Synthesizing Activity		Glutamate-1-semialdehyde Aminotransferase Activity
	cpm in purified ALA-pyrrole/mg protein/20 min	*nmol of ALA formed/mg protein/20 min*	*nmol of ALA formed/mg protein/hr*
H[a]	5537	Trace	Trace
B[a]	1667	Trace	Trace
R[a]	401	Trace	4.5
H + R	1969	Trace	—[b]
B + R	413	Trace	—
H + B	65520	1.05[c]	—
H + B + R	85743	4.3	—

[a] H, enzyme fraction which binds to the heme-Sepharose column; B, enzyme fraction which binds to the Blue Sepharose column; R, enzyme fraction which does not bind to either the heme-Sepharose column or the Blue Sepharose column.

[b] —, Not done.

[c] Considered to be the pyrrole formed by glutamate-1-semialdehyde which co-purifies with the ALA-pyrrole. See text and Figure 6.

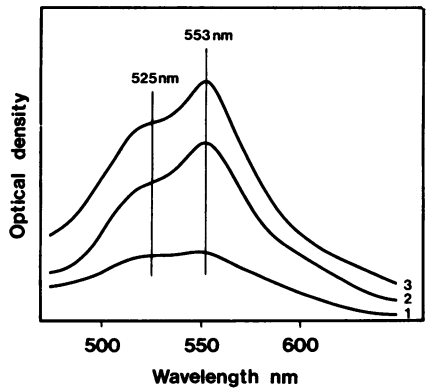

FIG. 6. Absorption spectra of ALA-pyrrole derivatives formed by incubating cold glutamate with the following enzyme combinations. 1, H + B for 20 min, precipitate the proteins, adjust the pH to 8.3, add elution buffer, incubate for another 20 min; 2, same as (1) except elution buffer was replaced with R proteins; 3, H + B + R, incubate for 20 min. See text for more detail.

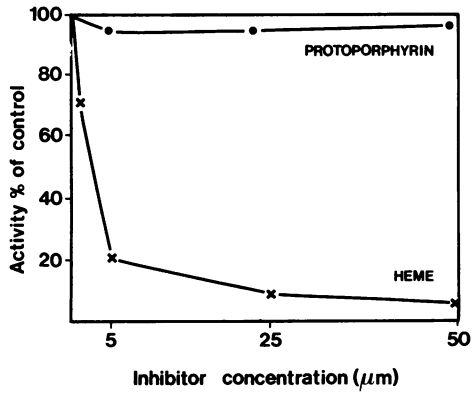

FIG. 7. Sensitivity of crude ALA synthesizing enzymes of *C. reinhardtii* to heme and protoporphyrin. Percentages of the control activity were plotted against various concentrations of heme and protoporphyrin.

DISCUSSION

The soluble protein fractions obtained by ultracentrifugation of disrupted *C. reinhardtii* cells actively convert glutamate to ALA. This conversion requires ATP, Mg^{2+}, and NADPH. The requirement for NADPH is less stringent and this could be caused by the presence of DTT in the enzyme preparation. At the pH used (8.3), DTT has a much higher reduction potential than NADPH and could probably regenerate NADPH from NADP (6). Replacing NADPH with 1 mM NADH reduced the enzyme activity by half. A boiled enzyme preparation has no activity. The optimal pH for the enzyme activity is 8.3 and, at 29°C, the enzyme activity is linear with time for up to 90 min. It was also found that the cells need to adapt to growth in liquid medium before active ALA synthesizing enzymes are found. Cells which have been transferred from solid medium to liquid medium for 2 d have only about 10% of the ALA synthesizing enzyme activity compared to the well adapted cells (more than 3 d in liquid medium) (data not shown). We do not know the reason for this change.

Light-grown, wild type cells were used as the source for ALA synthesizing enzymes because these cells were actively dividing (8 h/cell division) and synthesizing Chl (91 μmol/10^{10} cells). This means that each cell on an average would have to synthesize 5.5 $\times$ 10^9 molecules of Chl every 8 h. Since each molecule of Chl is made of 8 ALA molecules, this could be translated into 5.5 $\times$ 10^9 molecules of ALA synthesized/cell·h.

As shown in Figure 5, the elution profiles of ALA dehydratase and porphobilinogen deaminase overlap with that of the ALA synthesizing enzyme. As a result, even after partial purification with gel filtration, ALA dehydratase and porphobilinogen deaminase were still present in ALA synthesizing enzyme fractions. Therefore, levulinic acid was added to all assay mixtures to inhibit further metabolism of ALA. Without levulinic acid, the yield of ALA was reduced and uroporphyrin could be detected in the reaction mixture (data not shown).

The apparent mol wt of the ALA synthesizing enzymes based on gel filtration with a TSK Fractogel column was 45,000 D. Our preliminary results indicated that the smallest of the three enzymes has an apparent mol wt of 35,000 D. Therefore, the three enzymes could not have been in a complex during gel filtration. The 45,000-D value of the peak of activity could just happen to be the overlapping point for the three enzymes with different mol wt. We do not know whether the ALA synthesizing enzymes exist as a complex *in vivo*.

The mol wt of the *Chlamydomonas* aminotransferase, ALA-dehydratase, and porphobilinogen deaminase analyzed by the Fractogel were smaller compared to the corresponding barley enzymes analyzed by gel filtration on Sephacryl S-300. This may be due to a higher degree of subunit association in the barley enzymes.

The crude ALA synthesizing enzymes are very sensitive to inhibition by heme but not by protoporphyrin. This result agrees with the observation by others (4, 11). Heme is the end product of the iron branch of the porphyrin pathway and could act as a feedback inhibitor of ALA synthesizing enzyme(s) (5, 11). It is not clear whether heme also serves as a repressor for ALA synthesizing enzymes.

In an earlier paper, we have shown that the ALA synthesizing enzymes from greening barley plastids could be separated into 3 different enzymes by affinity column chromatography: one bound to heme-Sepharose (H), one bound to Blue Sepharose (B), and one bound to neither (R) (28). All three fractions together were needed for the conversion of glutamate to ALA. One of the three, glutamate-1-semialdehyde aminotransferase present in R, was positively identified (28). The other two combined (H + B) were shown to convert glutamate to a compound which co-purified with the organically synthesized, putative glutamate-1-semialdehyde on HPLC. The compound synthesized by H + B as well as the putative glutamate-1-semialdehyde could be converted by R to ALA which was identified by HPLC and TLC against authentic samples of ALA (28).

We have now separated the ALA synthesizing enzymes from light-grown *Chlamydomonas* cells into three enzyme fractions. This fractionation was accomplished in the same way as with the barley by affinity column chromatography with Blue Sepharose and heme-Sepharose. Like the glutamate-1-semialdehyde aminotransferase from barley, the enzyme from *Chlamydomonas* capable of converting the putative glutamate-1-semialdehyde to ALA was not bound to either column (R) (Table III). Furthermore, the two bound *Chlamydomonas* enzyme fractions combined (H + B) were able to convert glutamate to glutamate-1-semialdehyde. Even though we have not compared the enzymically formed 'glutamate-1-semialdehyde' with the organically synthesized, putative glutamate-1-semialdehyde as we have done in the barley experiments (28), we feel that by analogy with the result obtained in barley and by the data presented in Figure 6, the enzymes and the reaction sequence are quite similar in the two organisms. We conclude that in *C. reinhardtii*, ALA is synthesized from glutamate via glutamate-1-semialdehyde by a set of enzymes similar to those found in greening barley plastids. A recent paper (17) reported that a solubilized ALA synthesizing system from maize plastids resembled that from barley. Final elucidation of the reaction sequence from glutamate to ALA awaits the identification of the chemical structure of glutamate-1-semialdehyde.

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LITERATURE CITED

1. BEALE SI 1971 Studies on the biosynthesis and metabolism of δ-aminolevulinic acid in *Chlorella*. Plant Physiol 48: 316–319
2. BEALE SI, SP GOUGH, S GRANICK 1975 Biosynthesis of δ-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. Proc Natl Acad Sci USA 72: 2719–2723
3. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248–254
4. CASTELFRANCO PA, BM CHERESKIN 1982 Biosynthesis of Chlorophyll *a*. *In* JA Schiff, ed, On the Origins of Chloroplasts, Elsevier/North-Holland, New York, pp 199–218
5. CASTELFRANCO PA, OTG JONES 1975 Protoheme turnover and chlorophyll synthesis in greening barley tissue. Plant Physiol 55: 485–490
6. CLELAND WW 1964 Dithiothreitol, a new protective reagent for SH groups. Biochemistry 3: 480–482
7. DUGGAN J, M GASSMAN 1974 Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. Plant Physiol 53: 206–215
8. FOLEY T, SI BEALE 1982 δ-Aminolevulinic acid formation from γ,δ-dioxovaleric acid in extracts of *Euglena gracilis*. Plant Physiol 70: 1495–1502
9. FORD SH, HC FRIEDMANN 1979 Formaton of δ-aminolevulinic acid from glutamic acid by a partially purified enzyme system from wheat leaves. Biochim Biophys Acta 569: 153–158
10. GOUGH S 1972 Defective synthesis of porphyrins in barley plastids caused by mutation in nuclear genes. Biochim Biophys Acta 286: 36–54
11. GOUGH SP, CR GIRNTH, CG KANNANGARA 1981 Δ-Aminolevulinate synthesis in greening barley. 1. Regulation. *In* G Akoyunoglou, ed, Proc. Fifth International Congress in Photosynthesis, Vol 5. Balaban Int Sci Ser, Philadelphia, pp 107–116
12. GOUGH SP, CG KANNANGARA 1976 Synthesis of δ-aminolevulinic acid by isolated plastids. Carlsberg Res Commun 41: 183–190
13. GOUGH SP, CG KANNANGARA 1977 Synthesis of δ-aminolevulinate by a chloroplast stroma preparation from greening barley leaves. Carlsberg Res Commun 42: 459–464
14. GRANICK S 1959 Magnesium porphyrins formed by barley seedlings treated with δ-aminolevulinic acid. Plant Physiol 34: xviii
15. GRANICK S, S SASSA, JL GRANICK, RD LEVENE, A KAPPAS 1972 Assays for porphyrins, δ-aminolevulinic acid dehydratase and porphobilinogen synthetase in microliter samples of whole blood: Application to metabolic defects involving the heme pathway. Proc Natl Acad Sci USA 69: 2381–2385
16. HAREL E, E MELLER, M ROSENBERG 1978 Synthesis of δ-aminolevulinic acid [^{14}C] by cell-free preparations from greening maize leaves. Phytochemistry 17: 1277–1280
17. HAREL E, E NÉEMAN 1983 Alternative routes for the synthesis of δ-aminolevulinic acid in maize leaves. II. Formation from glutamate. Plant Physiol. 72: 1062–1067
18. JURGENSEN JE, SI BEALE, RF TROXLER 1976 Biosynthesis of δ-aminolevulinic acid in the unicellular rhodophyte Cyanidium Caldarium. Biochem Biophys Res Commun 69: 149–157
19. KANNANGARA CG, SP GOUGH 1977 Synthesis of Δ-aminolevulinic acid and chlorophyll by isolated chloroplast. Carlsberg Res Commun 42: 441–457
20. KANNANGARA CG, SP GOUGH 1978 Biosynthesis of Δ-aminolevulinate in greening barley leaves: glutamate-1-semialdehyde aminotransferase. Carlsberg Res Commun 43: 185–194
21. KANNANGARA CG, SP GOUGH, CR GIRNTH 1981 Δ-aminolevulinate synthesis in greening barley. 2. Purification of enzymes. *In* G Akoyunoglou, ed, Proc. Fifth International Congress in Photosynthesis, Vol 5. Balaban Int Sci Ser, Philadelphia, pp 117–127
22. KANNANGARA CG, SP GOUGH, D VON WETTSTEIN 1978 Biosynthesis of δ-aminolevulinate and chlorophyll and its genetic regulation. *In* G Akoyunoglou, JH Argyroundi-Akoyunoglou, eds, Chloroplast Development. Elsevier/North Holland Biomedical Press, New York, pp 147–160
23. KLEIN O, H SENGER 1978 Two biosynthetic pathways to δ-aminolevulinic acid in a pigment mutant of the green alga, *Scenedemus obliquus*. Plant Physiol 62: 10–13
24. MELLER E, S BELKIN, E HAREL 1975 The biosynthesis of δ-aminolevulinic acid in greening maize leaves. Phytochemistry 14: 2399–2402

25. MELLER E, E HAREL 1978 The pathway of δ-aminolevulinic acid synthesis in *Chlorella vulgaris* and in *Fremyella diplosiphon*. *In* G Akoyunoglou, JH Argyroundi-Akoyunoglou, eds, Chloroplast Development, Elsevier/North Holland Biomedical Press, New York, pp 51–57
26. MELLER E, E HAREL, CG KANNANGARA 1979 Conversion of glutamate-1-semialdehyde and 4,5-dioxovaleric acid to 5-aminolevulinic acid by cell-free preparations from greening maize leaves. Plant Physiol 63: S-548
27. PORRA J, O KLEIN, PE WRIGHT 1982 The proof by ^{13}C-NMR spectroscopy of the predominance of the C_5 pathway over the Shemin pathway in chlorophyll biosynthesis in higher plants and the formation of the methyl ester group of chlorophyll from glycine. Eur J Biochem 130: 509–516
28. WANG WY, SP GOUGH, CG KANNANGARA 1981 Biosynthesis of Δ-aminolevulinate in greening barley leaves. IV. Isolation of three soluble-enzymes required for the conversion of glutamate to Δ-aminolevulinate. Carlsberg Res Commun 46: 243–257
29. WANG WY, SP GOUGH, CG KANNANGARA 1982 Δ-Aminolevulinate synthesizing enzymes of *Chlamydomonas reinhardtii*. Plant Physiol 69: S-133
30. WANG WY, WL WANG, JE BOYNTON, NW GILLIAM 1974 Genetic control of chlorophyll biosynthesis in *Chlamydomonas*: Analysis of mutants at two loci mediating the conversion of protoporphyrin IX to magnesium protoporphyrin. J Cell Biol 63: 806–823
31. WEINSTEIN JD, PA CASTELFRANCO 1978 Mg-protoporphyrin IX and δ-aminolevulinic acid synthesis from glutamate in isolated greening chloroplasts. δ-Aminolevulinic acid synthesis. Arch Biochem Biophys 186: 376–382