Purification of the Glutamyl-tRNA Reductase from \textit{Chlamydomonas reinhardtii} Involved in \(\delta\)-Aminolevulinic Acid Formation during Chlorophyll Biosynthesis\(^*\)

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The formation of \(\delta\)-aminolevulinic acid, the first committed precursor in porphin biosynthesis, occurs in certain bacteria and in the chloroplasts of plants and algae in a three-step, tRNA-dependent transformation of glutamate. Glutamyl-tRNA reductase, the second enzyme of this pathway, reduces the activated carboxyl group of glutamyl-tRNA (Glu-tRNA) in the presence of NADPH and releases glutamate-1-semialdehyde (GSA). We have purified Glu-tRNA reductase from \textit{Chlamydomonas reinhardtii} by employing six different chromatographic separations. The apparent molecular mass of the protein when analyzed under both denaturing (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and non-denaturing conditions (rate zonal sedimentation on glycerol gradients) was 130,000 Da; this indicates that the active enzyme is a monomer.

In the presence of NADPH Glu-tRNA reductase catalyzed the reduction to GSA of glutamate acylated to the homologous tRNA. Thus, the reductase alone is sufficient for conversion of Glu-tRNA to GSA. In the absence of NADPH, a stable complex of Glu-tRNA reductase with Glu-tRNA can be isolated.

\(\delta\)-Aminolevulinic acid (ALA),\(^\dagger\) the first common precursor in the biosynthesis of the porphin nucleus of chlorophyll, hemes, and bilins is formed by at least two biosynthetic pathways (Castelfranco and Beale, 1983; Kannangara et al., 1988). As first demonstrated in purple bacteria (Shemin and Russell, 1953) ALA is synthesized by a condensation of glycine and succinyl-CoA catalyzed by ALA synthase (EC 2.3.1.37). A second pathway, the C\(\text{\&}\) pathway, detected in the chloroplasts of plants and algae (Beale et al., 1975; Kannangara et al., 1988) begins with GSA, and the first committed precursor in porphin biosynthesis, occurs in porphyric biosynthesis (Wang et al., 1981, 1988). Their enzymatic activities can be separated into enriched fractions by conventional protein purification techniques (Wang et al., 1981; Bruyant and Kannangara, 1987; Wang et al., 1984; Weinstein et al., 1987). Glu-tRNA synthetase (Bruyant and Kannangara, 1987) and GSA aminotransferase (Wang et al., 1981) have been purified from barley chloroplasts and partially characterized. However, the Glu-tRNA reductase has proven difficult to purify due to its reported instability (Kannangara et al., 1988) and the difficulty in separating Glu-tRNA reductase from the Glu-tRNA synthetase. Because of these observations and because of the finding that GTP enhances the overall yield of \textit{in vitro} conversion of glutamate to ALA it was suggested that the Glu-tRNA reductase and Glu-tRNA synthetase are complexed with Glu-tRNA and that this complex is the “substrate” for the enzyme which initiates the reduction (Kannangara et al., 1988). Glu-tRNA reductase is a very interesting enzyme. It has the unusual property of requiring tRNA as “cofactor” and is able to discriminate between different glutamate tRNA species (Peterson et al., 1988) apparently recognizing nucleotides that are not important for recognition by the Glu-tRNA synthetase. In addition, the enzyme is subject to feedback regulation by heme and appears to be a major control point in porphin biosynthesis (Wang et al., 1987).

In order to investigate the mechanism of Glu-tRNA reductase and the regulation of its expression we embarked on the purification of this enzyme. Here we report the partial purification and an initial characterization of the Glu-tRNA reductase from the chlorophycean alga \textit{Chlamydomonas reinhardtii}.

\section*{EXPERIMENTAL PROCEDURES}\(^2\)

\section*{RESULTS}

\textbf{Assay for Glu-tRNA Reductase Activity—}The direct assay for Glu-tRNA reductase activity is the measurement of the activity...
Fig. 1. Scheme of ALA formation via the C5 pathway. The first steps of chlorophyll biosynthesis.

**Chloroplast Glutamyl-tRNA Reductase**

**FIG. 2. Scheme for the purification of Glu-tRNA reductase from C. reinhardtii.** Whole cell extracts (S-100) from approximately 300 liters of C. reinhardtii grown photosynthetically were used as starting material. Column sizes, elution volumes, and protein concentrations are described in the "Miniprint Section" and in Table I. The molar concentrations of the KC1 step gradient elutions are indicated for each column.

conversion of Glu-tRNA into GSA. While the preparation of the tRNA substrate (even in radioactive form) is easily accomplished, the high concentrations needed to get an observable reaction may lead to enzyme inhibition by the tRNA. This situation is obviated in vivo where Glu-tRNA synthetase constantly renews Glu-tRNA from discharged tRNA. Furthermore, the reaction product GSA is difficult to assay in vitro, since the reconstitution assay could not be performed due to the presence of all three enzymes and tRNA in the S-100.

Because of the high concentrations of tRNA, Glu-tRNA synthetase and GSA-aminotransferase in the S-100 preparation, the reconstitution assay for Glu-tRNA reductase activity could not be used to determine the specific activity of the reductase in the S-100. Given the overall yields of enzyme recovery we assumed a 50% recovery of enzyme activity and a 1-fold purification after the DEAE-cellulose column.

The next step was a phosphocellulose column which excluded 90% of the applied protein and yielded a 3-fold increase in the specific activity (Fig. 1B, Miniprint Section, and Table I). The reductase was eluted with a high ionic strength buffer (1 m KC1) from the P-11 column. The Phenyl-Sepharose matrix gave a very good purification of the enzyme. Glu-tRNA reductase was eluted at 0.2 m KC1 (Fig. 1C, Miniprint Section) with a 0-fold increase in specific activity (Table I). The next purification step took advantage of the observation that Glu-tRNA reductase binds to the NADPH analogue dye Cibacron Blue (Wang et al., 1981; Kannangara et al., 1984).

As expected the NADPH-dependent reductase was adsorbed to the matrix. The enzyme activity was eluted with 2 m KC1 yielding a 3-fold increase in specific activity (Fig. 1D, Miniprint Section, and Table I).

The anion exchange resin Mono S resin afforded a 5-fold purification of the enzyme. It may be pertinent to mention that this step completely separated the Glu-tRNA reductase (eluting with 250 mM KC1) from Glu-tRNA synthetase (eluting 100 mM KC1), a separation which was very difficult to achieve in the past (Wang et al., 1987). As the Mono S fraction was still composed of several proteins, we applied gel filtration on the FPLC resin Superose 12 as a final purification step. In order to obtain reproducible and optimal resolution on the Superose 12 column, we found it necessary to exceed a flow rate of 0.25 ml/min and to use freshly regenerated resin. The reductase activity was eluted in a single, sharp peak (Fig. 3A). Subsequent analysis by SDS-gel electrophoresis showed that fraction 24 contained highly purified enzyme (Fig. 4).

**Physical Properties of Glu-tRNA Reductase**—The molecular weight of Glu-tRNA reductase was determined for the native and denatured protein. Two independent methods, rate zonal sedimentation in a glycerol gradient and gel filtration on the FPLC molecular sieving matrix Superose 12, were used to determine the molecular weight of the native enzyme. For the rate zonal sedimentation analysis 20 μg of protein (Mono S fraction) was centrifuged at 45,000 rpm in a Beckman SW 50.1 rotor at 0 °C for 21 h through a 10–35% glycerol gradient

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<th>Total specific activity</th>
<th>Yield</th>
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<td>13.4</td>
<td>880</td>
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* One unit is defined as 1 nmol of ALA formed under the described assay conditions.

† Not determined. The enzyme activity was not determined in the S-100, since the reconstitution assay could not be performed due to the presence of all three enzymes and tRNA in the S-100.

‡ 90% recovery of enzyme activity was assumed for this step.

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containing 1 M KCl to prevent nonspecific aggregation. The peak of reductase activity was obtained in fraction 7, which corresponded to the position of phosphorylase b (Mr = 97,000) in the marker gradient run (Fig. 3C). A sedimentation coefficient of $s_{20,w}$ of 6.5 ± 0.2 for the reductase was deduced from a standard plot of the known $s_{20,w}$ values of different marker proteins (Fig. 3D). A native molecular mass of 130,000 ± 5,000 was calculated for the reductase using the relationship between the sedimentation coefficient of a protein and its molecular mass (Martin and Ames, 1961). Molecular sieve chromatography on Superose 12 (Fig. 3A) gave a similar molecular weight for the native enzyme (see Fig. 3B). The determination of the molecular weight of the protein by SDS-polyacrylamide gel electrophoresis gave a value of 130,000 ± 5,000 (Fig. 4). Based on these results we conclude that *Chlamydomonas reinhardtii* Glu-tRNA reductase is a 130-kDa protein of monomeric quaternary structure.

From the results of the gel filtration experiment we calculated an apparent Stokes radius (Siegel and Monty, 1965) of 47.5 ± 1.0 for the protein. This high value suggests a slightly asymmetric shape for the native Glu-tRNA reductase.

Given the enzyme's cofactor requirement of a tRNA we wondered if the purified enzyme was associated with nucleic acids. Absorbance measurements at 260 and 280 nm indicate that the purified reductase is not associated with RNA or DNA nor with NADPH. This conclusion was further supported by experiments in which pretreatment of the reductase with micrococcal nuclease had no effect on enzyme activity (data not shown).

**Purified Glu-tRNA Reductase Reduces Glu-tRNA to GSA**

As there is uncertainty about the exact mechanism of the reduction of glutamate and about the number of proteins involved in this process (Kannangara et al., 1988), we wanted to know whether our purified Glu-tRNA reductase could reduce *E. coli* Glu-tRNA to GSA with the aid of NADPH as cofactor. This required that a reliable method be found which...
separates glutamate from GSA, ALA, and Glu-tRNA. This is complicated by the fact that GSA is very labile (Hoober et al., 1988). Following earlier work (Mau et al., 1987) we chose HPLC analysis on a reversed-phase octadecyl silane column, which was demonstrated to separate Glu, GSA, and ALA. The elution position of the markers are shown in Fig. 5A. Glutamate elutes in fraction 14, Glu-tRNA in fraction 8, GSA, and ALA in fractions 45 and 70, respectively. When the products of the reaction of purified Glu-tRNA reductase (Superose 12 fraction) with [14C]Glu-tRNA were analyzed the major radioactive product was GSA in fraction 45 (Fig. 5B). This shows that Glu-tRNA reductase can convert Glu-tRNA to GSA in the absence of any other protein. The appearance of small amounts of side products may be due to spontaneous chemical conversion of GSA into other compounds or may suggest the existence of other intermediates in the reduction. GSA is highly unstable at physiological pH values, with a half-life of 3–4 min at pH 8.0 (Hoober et al., 1988). While our reductase assays were performed at neutral pH values, the assays were terminated by acidification and then analyzed at an acidic pH value at which GSA is stable.

*Glu-tRNA Reductase Forms a Stable Complex with Glu-tRNA.—*In light of the postulated complex of proteins with the reductase and Glu-tRNA (Kannangara et al., 1988) we wanted to investigate the existence of possible complexes of Glu-tRNA reductase by density gradient centrifugation in a 10–35% glycerol gradient. We reasoned that stable complexes may be obtained in the absence of NADPH as the enzymatic reduction by Glu-tRNA reductase could not proceed. As seen in Fig. 6C Glu-tRNA reductase does form a complex with Glu-tRNA which is not present when the enzyme or the tRNA are sedimented alone (Fig. 6, A and B). The complex is formed by the 130-kDa protein as confirmed by SDS gel analysis of the peak fraction (data not shown). These data show that complex formation between Glu-tRNA reductase and Glu-tRNA does not require GTP or other proteins.

**DISCUSSION**

The experiments presented above show that purified native *Chlorella reinhardtii* Glu-tRNA reductase is a single polypeptide chain (apparent *M*<sub>r</sub> = 130,000). This contrasts with the earlier suggestion that this enzyme has a molecular mass of 51 kDa (Wang et al., 1987). In the presence of NADPH the purified enzyme catalyzes the reduction to GSA of glutamate acylated to the homologous tRNA. The purified enzyme can form a stable complex with its substrate, Glu-tRNA. While a larger complex composed of the reductase, Glu-tRNA synthetase and Glu-tRNA can also be detected (data not shown) it is clear that the proposed large complex (Kannangara et al., 1988) containing additional proteins and GTP is not a requirement for GSA formation.

It is interesting to consider the mechanism of this reduction. The reaction is formally analogous to the back reaction of glyceraldehydephosphate dehydrogenase where the activated (by phosphorylation) carboxyl group of 3-phosphoglycerate is reduced to 3-phosphoglycerate (see, e.g. Stryer, 1988). This reaction proceeds through an acylenzyme intermediate. The similarity with Glu-tRNA reductase is obvious; the enzyme reduces a carboxyl group of glutamate (activated by aminoacyl-tRNA formation) to glutamate 1-semialdehyde in the presence of NADPH. Activation
Fig. 6. Glycerol gradient sedimentation analysis of complex formation of the Glu-tRNA reductase and Glu-tRNA. Aliquots of Glu-tRNA reductase (2 pmol) of the Superose 12 fraction were mixed with precharged Escherichia coli [14C]Glu-tRNA (60 pmol) and then sedimented through a 10-35% glycerol gradient by centrifuging the gradients in a Beckman SW 50.1 rotor at 45,000 rpm for 21 h at 4 °C. After centrifugation, 0.15-ml fractions were collected and assayed for trichloroacetic acid-precipitable radioactivity and/or reductase activity. The great excess of Glu-tRNA was used in order to maximize complex formation with the enzyme. A, Glu-tRNA reductase. B, E. coli [14C]Glu-tRNA. C, Glu-tRNA reductase preincubated with E. coli [14C]Glu-tRNA in the absence of NADPH.

Acknowledgment—We thank Astrid Schön for her help and continuing interest in the project.

REFERENCES


of amino acids by esterification with tRNA is, of course, well known and provides the basis for protein biosynthesis. Further studies with Glu-tRNA reductase should provide evidence whether this mechanism is correct and what significance the unusual carboxyl activation by tRNA may have.
EXPERIMENTAL PROCEDURES

All operations were performed at 0–4°C except noted otherwise.

Materials

Bovine thymocyte nucleoprotein was purchased from Sigma Chemical Co. (St. Louis, MO), fast green from E. Merck (Darmstadt, Germany), ethidium bromide from Miles Laboratories (Kankakee, IL), and [3H]thymidine from Amersham (Oakville, On.). All other reagents were from commercial sources.

Preparation of thymocytes

Thymocytes were isolated from fetal calf thymus glands using a modification of the method of DeKruyff et al. (1977). The thymus was minced and digested for 30 min at 37°C in a medium containing 0.2% trypsin, 0.02% DNase, and 1% bovine serum albumin. The digested thymus was filtered through 100 μm and 40 μm nylon mesh to remove un-digested tissue. The cell suspension was then layered onto a Percoll gradient and centrifuged for 40 min at 4°C at 200 × g. The thymocytes were obtained from the interface of the Percoll and pooled, washed twice, and resuspended in a 1:1 mixture of 33% RPMI and 67% 10% FCS. The cell concentration was determined using a hemocytometer. The viability of the cells was determined by trypan-blue exclusion and was > 90% for all experiments. The thymocytes were cultured in T150 flasks in RPMI 1640 and incubated at 37°C with 5% CO2.

Preparation of DNA

DNA was isolated from cultured thymocytes using the method of Birnboim and Doly (1979). The DNA concentration was determined spectrophotometrically at 260 nm. Distilled water was used for all dilutions. When necessary, DNA was dialyzed against physiological saline (0.9% NaCl) for 2 days to remove ethidium bromide.

Preparation of RNA

RNA was isolated from cultured thymocytes using the guanidinium thiocyanate/acid phenol method of Chirgwin et al. (1979). The RNA concentration was determined spectrophotometrically at 260 nm. Distilled water was used for all dilutions. When necessary, RNA was dialyzed against physiological saline (0.9% NaCl) for 2 days to remove chloroform.

Preparation of protein

Protein was isolated from cultured thymocytes using the method of Lowry et al. (1951). The protein concentration was determined spectrophotometrically at 280 nm. Distilled water was used for all dilutions. When necessary, protein was dialyzed against physiological saline (0.9% NaCl) for 2 days to remove trichloroacetic acid.

Protein conjugation

When necessary, proteins were conjugated with the trinitrophenyl (TNP) group using the method of Kung and Chen (1977). The TNP concentration was determined spectrophotometrically at 400 nm. Distilled water was used for all dilutions. When necessary, TNP was dialyzed against physiological saline (0.9% NaCl) for 2 days to remove trinitrobenzene.

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