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Complex formation between glutamyl-tRNA synthetase and glutamyl- tRNA reductase during the tRNA-dependent synthesis of 5-aminolevulinic acid in *Chlamydomonas reinhardtii*

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The formation of a stable complex between glutamyl-tRNA synthetase and the first enzyme of chlorophyll biosynthesis glutamyl-tRNA reductase was investigated in the green alga *Chlamydomonas reinhardtii*. Apparently homogenous enzymes, purified after previously established purification protocols were incubated in various combinations with ATP, glutamate, $tRNA^{Glu}$ and NADPH and formed complexes were isolated via glycerol gradient centrifugation. Stable complexes were detected only after the preincubation of glutamyl-tRNA synthetase, glutamyl-tRNA reductase with either glutamyl-tRNA or free $tRNA^{Glu}$, ATP and glutamate, indicating the obligatory requirement of aminoacylated $tRNA^{Glu}$ for complex formation. The further addition of NADPH resulting in the reduction of the tRNA-bound glutamate to glutamate 1-semialdehyde led to the dissociation of the complex. Once complexed to the two enzymes $tRNA^{Glu}$ was found to be partially protected from ribonuclease digestion. *Escherichia coli*, *Bacillus subtilis* and *Synechocystis 6803* $tRNA^{Glu}$ were efficiently incorporated into the protein–RNA complex. The detected complexes provide the chloroplast with a potential channeling mechanism for Glu-$tRNA^{Glu}$ into chlorophyll synthesis in order to compete with the chloroplastic protein synthesis machinery.

Chlorophyll biosynthesis; Glutamyl-tRNA synthetase; Glutamyl-tRNA reductase; Complex formation; tRNA channeling

1. INTRODUCTION

In the green alga *Chlamydomonas reinhardtii* 5-aminolevulinic acid (ALA), the general precursor for chlorophyll biosynthesis is synthesized from aminoacylated glutamyl-tRNA (Glu-$tRNA^{Glu}$) [1–3]. Glutamyl-tRNA synthetase (GluRS) initially forms Glu-$tRNA^{Glu}$ for chlorophyll- and protein biosynthesis [4–6] before glutamyl-tRNA reductase (GluTR) reduces the activated glutamate to glutamate 1-semialdehyde (GSA) [7,8]. Glutamate 1-semialdehyde aminotransferase (GSA-AT) finally transfers in a pyridoxal 5-phosphate-dependent reaction the amino group located at the C2 atom of GSA to the C1 position releasing 5-aminolevulinic acid (ALA) [9,10]. All three enzymes from *C. reinhardtii* have been purified and characterized previously [4,7,9,11]. Partial amino acid sequences of the GluRS demonstrated its structural relationship to known prokaryotic GluRS enzymes [6]. The $tRNA^{Glu}$ cofactor has been isolated and sequenced and two duplicated genes were cloned from the chloroplast genome of *C. reinhardtii* [12,13]. In vitro transcription of one of the genes was independent of its 5'-region and did not seem to be regulated by light [14]. All isolated *C. reinhardtii* chloroplastic $tRNA^{Glu}$ species equally sustained chlorophyll and protein biosynthesis [6]. Purification of GluTR was difficult due to its low cellular abundance [7,8]. These findings raise the question of how chlorophyll synthesis can compete with plastitic protein biosynthesis for aminoacylated $tRNA^{Glu}$. The availability of purified enzymes and tRNA led to the investigation of a potential channeling mechanism via complex formation between the different enzymes and the tRNA. This communication describes the formation, isolation and initial characterization of complexes between GluRS and GluTR in the presence of aminoacylated $tRNA^{Glu}$, which may provide the basis for a tRNA channeling mechanism into chlorophyll biosynthesis.

2. MATERIALS AND METHODS

Biochemicals were from Sigma Chemical Co. [^{14}C]Glu (285 mCi/mmol) and [^{32}P]ATP (400 Ci/mmol) were obtained from Amersham. Purified *E. coli* $tRNA^{Glu}$ was purchased from Boehringer Mannheim. *Synechocystis* tRNA was a gift from Dr. G.P. O'Neill. Purified *B. subtilis* $tRNA^{Glu}$ was a gift from Dr. Y. Ishino. For the tests of the various tRNAs in the *C. reinhardtii* system 15 pmol [^{14}C]Glu-$tRNA^{Glu}$, previously aminoacylated with homologous GluRS, were employed [8]. The *C. reinhardtii* cell wall reduced strain CC-400 cw-15mt+ was a kind gift from Dr. Elizabeth H. Harris, Duke University, North Carolina, USA. *C. reinhardtii* cells were grown in the light and a cell-free extract was prepared as described in detail before [4,14]. GluRS, GluTR and GSA-AT were purified from a cell free extract as

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described [4,7,9]. All employed enzymes were concentrated by additional chromatography on Mono-Q to a final concentration of 5–10 mg protein/ml [4]. Isolation of *C. reinhardtii* $tRNA^{Glu}$ on Mono-Q was performed as described recently [15–17]. Endlabelled [^{32}P]tRNA was obtained by standard methods [15]. GluRS and GluTR activities were assayed as described [4,8]. Complexes composed of purified components were formed in GluTR assay buffer (20 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 3 mM DTT, 8% (v/v) glycerol) at 30°C for 15 min. Preincubation mixes (0.1 ml) contained 5 pmol GluTR, 5 pmol GluRS, 7–14 pmol [^{14}C]Glu-$tRNA^{Glu}$ or [^{32}P]$tRNA^{Glu}$ or 2 nmol [^{14}C]Glu, 0.5 A_{260} *C. reinhardtii* $tRNA^{Glu}$ and 2 mM ATP. In some experiments, 2 mM NADPH or 20 pmol GSA-AT and 0.1 mM pyridoxal 5′-phosphate were added. Purified enzymes, tRNAs and protein–RNA complexes were separated by centrifugation through 10–35% glycerol gradients formed in GluTR assay buffer in a Beckman SW 50.1 rotor at 45,000 rpm and 4°C for 13.5 h as detailed before [5,7,8,15]. The positions of the enzymes were determined by activity tests. Aminoacylated [^{14}C]Glu-$tRNA^{Glu}$ and endlabelled [^{32}P]$tRNA^{Glu}$ were identified and quantified by acid precipitation and scintillation counting [4]. For the RNase A protection assays samples containing 10 pmol GluRS, 10 pmol GluTR with either 25 pmol [^{14}C]Glu-$tRNA^{Glu}$ or with 0.2 A_{260} $tRNA^{Glu}$, 500 pmol [^{14}C]Glu and 2 mM ATP were incubated for the time points indicated before 5 μg/ml RNase A were added. RNase A treatment was performed for 5 min on ice before the formed complexes were sedimented through a glycerol gradient and the amount of aminoacylated tRNA in the complex containing fraction 13 of the gradient was determined as described above.

3. RESULTS AND DISCUSSION

3.1. *GluRS and GluTR form a stable complex in the presence of aminoacylated* $tRNA^{Glu}$

During the initial characterization of purified GluTR from *C. reinhardtii* stable complexes between the enzyme and Glu-$tRNA^{Glu}$ were isolated by glycerol gradient centrifugation [7]. In the course of the experiments higher molecular complexes were observed while incubating crude enzyme fractions containing both GluRS and GluTR activities in combination with free $tRNA^{Glu}$, ATP and glutamate (discussed in [7]). In order to gain further insights into the nature of those complexes, experiments with purified GluRS and GluTR were performed. The same analytical system of glycerol gradient centrifugation was employed. Single marker runs with purified components revealed the sedimentation position for free endlabelled tRNA, GluRS and GluTR in fractions 3, 6 and 9, respectively (Fig. 1A). Parallel centrifuged marker proteins of M_r = 66,000 (bovine serum albumin) and 116,000 (β-galactosidase) were detected in fraction 6 and 9, confirming the previously observed M_r-values for GluRS (62,000) and GluTR (130,000) from *C. reinhardtii* (data not shown). Preincubation of GluRS, GluTR and endlabelled $tRNA^{Glu}$ in the presence of ATP shifted part of the employed tRNA into the position of the synthetase, indicating its complexation with the enzyme (Fig. 1B). Similar results were observed earlier with GluRS from *E. coli* [18]. In good agreement with earlier experiments, no binding of the free tRNA by GluTR was observed [7]. Moreover, we failed to detect the formation of a stable complex between the two enzymes in the presence (Fig. 1B) or absence (data not shown) of free $tRNA^{Glu}$. Addition of glutamate to the preincubation, allowing the aminoacylation reaction to proceed, drastically changed the sedimentation position of the employed components. A new high molecular weight complex of approximately M_r = 200,000 was detected in fraction 13 consisting of GluRS, GluTR and aminoacylated $tRNA^{Glu}$ (Fig. 1C). Almost all aminoacylated tRNA and most of the employed enzyme activities were part of the complex. Similar results were obtained with the preincubation of GluRS, GluTR and [^{14}C]Glu-$tRNA^{Glu}$ (data not shown). Incubation of partially purified *C. reinhardtii* chloroplastic methionyl-tRNA synthetase with GluTR in the presence of $tRNA^{Met}$, methionine and ATP failed to produce detectable complexes and served as control for the specificity of the complex formation (data not shown).

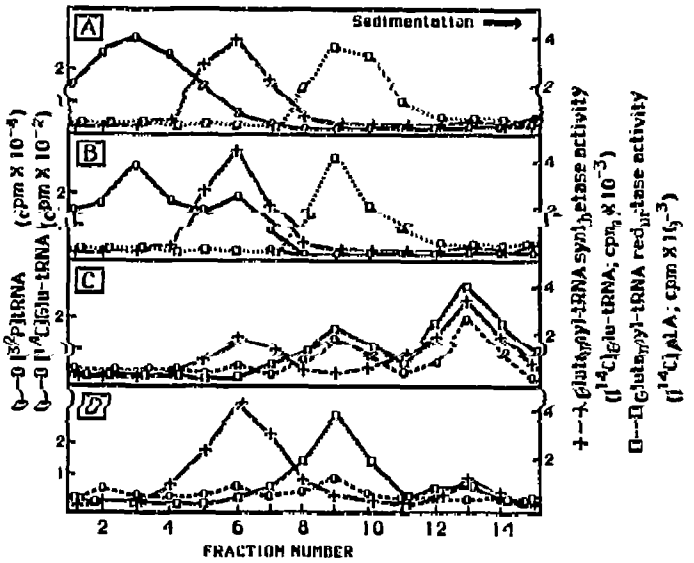

Fig. 1. Isolation of complexes between *C. reinhardtii* GluRS and GluTR in the presence of aminoacylated $tRNA^{Glu}$ by glycerol gradient sedimentation. Amounts of employed enzymes, cofactors and tRNAs, conditions of preincubation, separation via glycerol gradient centrifugation and the determination of the position of the enzymes and tRNAs are given in detail in section 2. (A) Separate marker centrifugations with purified GluRS, GluTR and [^{32}P]$tRNA^{Glu}$. (B) Separation of preincubated GluRS, GluTR and [^{32}P]$tRNA^{Glu}$. (C) Separation of preincubated GluRS, GluTR and $tRNA^{Glu}$, [^{14}C]Glu and ATP. (D) Same as (C) with the addition of NADPH.

3.2. *Dissociation of the GluRS–GluTR–RNA complex upon addition of NADPH*

Almost no GluRS–GluTR–Glu-$tRNA^{Glu}$ complexes (fraction 13) were detected when NADPH was present during the preincubation. The enzymes sedimented as single proteins into fraction 6 (GluRS) and 9 (GluTR) as shown in Fig. 1D. Addition of NADPH allowed the reductase reaction to proceed and lead to the deacylation of $tRNA^{Glu}$ with the subsequent release of GSA as

described earlier [7,8]. This result suggests that the previously discovered necessary integrity of the aminoacyl bond between $tRNA^{Glu}$ and glutamate for the efficient recognition and binding of $tRNA^{Glu}$ by GluTR alone [7] is also a basic requirement for its higher molecular complex with GluRS and GluTR.

Complex formation of purified *C. reinhardtii* GSA-AT either with the high molecular complex described above or at least with GluTR alone might prevent the release of GSA, which is unstable under physiological conditions. However, attempts to integrate GSA-AT activity into any kind of stable complex failed.

3.3. *Complex formation protects $tRNA^{Glu}$ from RNase digestion*

For an initial characterization of the process of complex formation the sensitivity of complexed Glu-$tRNA^{Glu}$ to digestion by RNase A was analyzed at various time points during complex formation as outlined in section 2. The detected complexed $tRNA^{Glu}$ in fraction 13 of the gradient after 15 min preincubation time without the addition of RNase served as control and was defined as 100%. All other obtained values for complexed tRNA in fraction 13 of the gradient were related to that. Complex formation for 15 min protected up to 35% of $tRNA^{Glu}$ from RNase A (data not shown). This result was used in the time course of Fig. 2 to measure complex formation by protection of the complexed tRNA from RNase digestion. Moreover, the experiment compared complex formation of previously aminoacylated $tRNA^{Glu}$ with the reaction of free tRNA and glutamate in the presence of ATP. Fig. 2 shows that

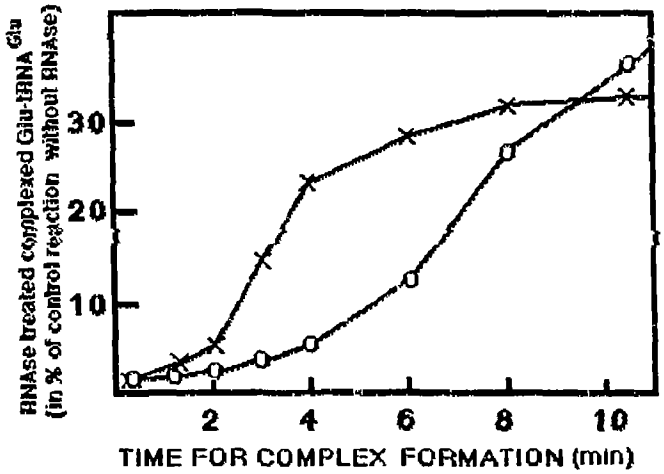

Fig. 2. Time course of complex formation analyzed by the protection of tRNA from RNase A digestion. Samples (0.1 ml) containing GluTR, GluRS and either [^{14}C]Glu-$tRNA^{Glu}$ (x—x) or [^{14}C]Glu, $tRNA^{Glu}$ and ATP (o—o) were incubated under standard conditions outlined in section 2. RNase A was added at the indicated times after the preincubation start and complexes were isolated by glycerol gradient centrifugation. The amount of radioactive aminoacylated tRNA in fraction 13 of the gradient was determined and related to samples without RNase treatment (=100%) as described in section 3.

Table I

Transfer RNA specificity of complex formation

Source of [^{14}C]Glu-$tRNA^{Glu}$	Complexed [^{14}C]Glu-$tRNA^{Glu}$ (pmol)
C. reinhardtii	12.3
E. coli	13.1
Synechocystis 6803	10.2
B. subtilis	8.4

Assays (0.1 ml) containing 15 pmol [^{14}C]Glu-$tRNA^{Glu}$, 20 pmol *C. reinhardtii* GluRS and GluTR were carried out with additions and under standard conditions as described in section 2. Complexes were isolated and quantitated as described in detail in section 2.

complex formation with previously aminoacylated tRNA occurred after 3–4 min while complex formation from free tRNA and glutamate takes approximately 5 min longer due to the initial aminoacylation reaction required for the formation of Glu-$tRNA^{Glu}$. These results demonstrate the tight nature of the complex and emphasize again the importance of aminoacylated tRNA for its formation.

3.4. *Transfer RNA specificity of the complex formation*

Complex formation with aminoacylated $tRNA^{Glu}$ from various species was tested in order to gain insights to the tRNA specificity of the reaction. The amount of complexed aminoacylated $tRNA^{Glu}$ served as an indication of its acceptance by the enzymes. The *C. reinhardtii* enzymes formed a complex with all of the charged tRNAs tested, although the degree of complex formation varied slightly (Table I). These findings are in good agreement with the broad tRNA-specificity of the *C. reinhardtii* enzymes [1,4,19].

3.5. *Complex formation for tRNA channeling into chlorophyll synthesis*

C. reinhardtii GluTR seemed to be of low cellular abundance [7]. Both chloroplastic $tRNA^{Glu}$ species sustain protein and chlorophyll synthesis equally well [6]. If aminoacylated $tRNA^{Glu}$ was released from GluRS, then GluTR would have to compete with the abundant protein synthesis machinery for the Glu-$tRNA^{Glu}$. The highly specific complex between GluTR and GluRS that was detected in the presence of aminoacylated $tRNA^{Glu}$ could be the basis for a tRNA channeling mechanism into chlorophyll synthesis in order to satisfy the tRNA requirements for this biosynthetic route. This is similar to the proposed channeling of arginyl-tRNA into the ubiquitin-dependent protein degradation system in mammalian cells [20].

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