

Charging of Both, Plastidal tRNA^{gln} and tRNA^{glu} with Glutamate and Subsequent Amidation of the Misacylated tRNA^{gln} by a Glutamyl-tRNA Amidotransferase in the Unicellular Green Alga *Scenedesmus obliquus*, Mutant C-2A'

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C₅-Pathway, Glutamyl-tRNA^{glu}-Synthetase (E.C.6.1.1.17), Misacylation of tRNA^{glu}, Amidotransferase, *Scenedesmus obliquus*

In a previous paper we described the purification of a glutamyl-tRNA synthetase from the unicellular green alga *Scenedesmus obliquus*, mutant C-2A'. We now demonstrate that, firstly, this enzyme is capable of mischarging plastidal tRNA^{gln} from barley with glutamate, as well as it regularly charges the plastidal tRNA^{glu} from *Scenedesmus*. Secondly, we show that the mischarged glutamyl-tRNA^{gln} is subsequently amidated by a glutamyl-tRNA amidotransferase to form the glutaminyl-tRNA^{gln} required for plastidal protein biosynthesis. This phenomenon could already be demonstrated for higher plant chloroplasts, mitochondria, cyanobacteria and gram-positive bacteria, as far as investigated. As recently shown the applied glutamyl-tRNA synthetase from *Scenedesmus* is a plastidal enzyme. In this paper we prove by treatment with monobromobimane and cyanogen bromide that the „regular“ substrate of the enzyme, tRNA^{glu} from *Scenedesmus*, is a plastidal tRNA with the plastid-specific sulfur modification in the anticodon. In the case of cyanogen bromide treatment, a total inactivation of the tRNA was achieved, revealing the presence of a sulfur modification in the plastid-tRNA^{glu} anticodon.

Introduction

Glutamyl-tRNA synthetase catalyses the first step of the C₅-pathway to 5-aminolevulinate (ALA) by charging the corresponding tRNA^{glu} with glutamate. So far, only a few synthetases could be purified and characterized (wheat, Rati-*naud et al.*, 1983; barley, Bruyant and Kannangara, 1987; *Chlamydomonas reinhardtii*, Chen *et al.*, 1990; *Bacillus subtilis*, Proulx *et al.*, 1983; *E.coli*, Lapointe and Söll, 1972). Recently, we characterized and purified this enzyme from the green alga *Scenedesmus obliquus*, mutant C-2A', to apparent homogeneity (Vothknecht *et al.*, 1994). Like from chloroplasts of higher plants and from those

bacteria which synthesize ALA via the C₅-pathway, also from the green alga *Scenedesmus* only one glutamyl-tRNA synthetase could be isolated. It has thus to be concluded that like in the above mentioned cases also in *Scenedesmus* a single enzyme provides both, the glutamyl-tRNA^{glu} for ALA-formation and for protein biosynthesis. For the glutamyl-tRNA synthetases from chloroplasts (Schön *et al.*, 1988) and gram-positive bacteria (Wilcox and Nirenberg, 1968) it was furthermore shown that they are capable of mischarging tRNA^{gln} with glutamate. By this reaction and the subsequent amidation of the bound glutamate to glutamine the absence of a glutaminyl-tRNA synthetase is compensated and the glutaminyl-tRNA^{gln}, required for protein biosynthesis, provided. In the present paper we will demonstrate the presence of a corresponding glutamyl-tRNA amidotransferase in *Scenedesmus*, too, and thus that glutaminyl-tRNA^{gln} is synthesized in this organism in the same manner. It has already been shown in our recent contribution on glutamyl-tRNA synthetase (Vothknecht *et al.*, 1994) that the isolated *Scenedesmus* enzyme is a plastidal protein. The proof for the plastidal origin of the ap-

Abbreviations: ALA, 5-aminolevulinate; gln, glutamine; glu, glutamate; gly, glycine; leu, leucine; mam⁵s² U, 5-[(methyl-amino)methyl]-2-thiouridine; TCA, trichloroacetic acid; TLC, thin layer chromatography.

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plied catalytic components is of great importance, as it is impossible to isolate intact chloroplasts from *Scenedesmus* (like it is from many other green algae). This implies the risk of cytoplasmic or mitochondrial contaminations in the assays, possibly leading to wrong conclusions. Therefore we here will furthermore demonstrate that also the applied tRNA^{glu} from *Scenedesmus*, the „regular“ substrate of the enzyme, is of plastidal origin.

Materials and Methods

Chemicals

Monobromobimane and methionine sulfoximine were obtained from Sigma (St. Louis, U.S.A.), cyanogen bromide from Aldrich (Deisenhofen, F.R.G.). All other applied chemicals were p.a. grade or of the highest available purity. Protein determinations were performed with Biorad-reagent (Biorad, Bad Soden, FRG.) following the method of Bradford (1979). Yeast- and *E.coli* total tRNA, as well as *E.coli* tRNA^{glu}, were supplied by Sigma (Deisenhofen, F.R.G.) and *E.coli*-tRNA^{gln} by Subriden RNA (Rollingbay, U.S.A.). The plastidal tRNA-preparation and the purified plastidal tRNA^{gln} from barley were a kind gift from Dr. C.G. Kannangara (Carlsberg Laboratory, Copenhagen, Denmark).

Plant material and cell growth conditions

For all experiments cells of the X-ray induced mutant C-2A' of *Scenedesmus* (Bishop, 1971) were employed. As greening of the mutant is light-dependent (Senger and Bishop, 1972), after 72–96 h of heterotrophic growth the cells were exposed to white fluorescent light of an intensity of 20 W m⁻² for 6 h. Further culturing conditions, determination of the cell density and harvesting were described earlier (Vothknecht *et al.*, 1994).

Origin of *Scenedesmus* enzyme- and tRNA preparations

After harvesting cells were broken and a 300 000×g supernatant prepared (Vothknecht *et al.*, 1994). For loading of the different tRNAs the eluate of a Blue Sepharose affinity column or, alternatively, purified glutamyl-tRNA synthetase was employed. Both preparations were obtained as recently described (Vothknecht *et al.*, 1994). The

required glutamyl-tRNA amidotransferase activity is present in the Blue Sepharose eluate which in turn was used as the enzyme source. The total tRNA-preparation from *Scenedesmus* was obtained from the eluate of a chlorophyllin Sepharose affinity column as described by Breu and Dörnemann (1988).

Enzyme assays

Charging of the different tRNAs with labelled glu and gln

Loading of the different total tRNA preparations (100 µg per assay) and specific tRNAs (5 µg per assay) with labelled substrates as well as the determination of the bound radioactivity was performed as described earlier (Vothknecht *et al.*, 1994).

Determination of glutamyl-tRNA amidotransferase activity

Measurement of the enzyme activity of glutamyl-tRNA-amidotransferase was performed following the modified method of Wilcox and Nirenberg (1968) in 100 mM Tricine-buffer, pH 7.9. The assay mixture contained total tRNA from barley (100 µg), 1 mM ATP, 1 mM unlabelled gln, 5 mM methionine sulfoximine, 100 µl of Blue Sepharose preparation from *Scenedesmus* and 10 µCi of L-[2,3-³H]-glu in a total volume of 500 µl. After incubation for 30 min at 28 °C the reaction was stopped by the addition of 50 µl of icecold 100% TCA. The precipitate was collected by centrifugation for 5 min in an Eppendorf-centrifuge (Eppendorf, Hamburg, F.R.G.) and the supernatant discarded. After hydrolysis of the bound amino acids from the tRNA by adding 1 ml of 20 mM KOH, samples were adjusted to pH 6–6.5 with 1 M HCl, administered to Dowex 1x8 columns (3 ml bedvolume) and the columns washed with 2 ml of bidistilled water. The collected eluates were lyophilized over night and then dissolved in 40 µl of bidistilled water. 10 µl of the concentrate were then applied as a spot to a TLC-plate coated with cellulose (20 x 20 cm, coating 0.25 mm, Merck, Darmstadt, F.R.G.) together with reference spots of unlabelled plus labelled glu and gln. The chromatogram was developed with 2-propanol/formic acid/H₂O (80/20/4; v/v/v). The references were visu-

alized by spraying with ninhydrin and additionally by radio-TLC-scanning with a TLC-scanner (Model Rita, Raytest, Straubenhardt, F.R.G.). The tracks with the labelled compounds were also analyzed by TLC-scanning. For quantitative analysis the track was scraped off in patches of 0.5 cm high, transferred to scintillation vials with Ready Safe (Beckmann, München, F.R.G.) as scintillation cocktail and the radioactivity determined in a liquid scintillation counter (Betamatic II, Kontron, Neufahrn, F.R.G.).

Treatment of total tRNA with monobromobimane

Following the method of Kosower (1978) 1 mg of a total tRNA-sample from *Scenedesmus* were dissolved in 350 µl of 1 M Tris-buffer, pH 8.8, and 2 µl of a monobromobimane solution (200 mM, in methylcyanide) added. After an incubation time of 15 min at room temperature the excess of the reagent was removed by extracting the tRNA with phenol/chloroform/isoamyl alcohol (25/25/1 v/v/v). Subsequently, the tRNA was precipitated with ethanol, collected by centrifugation (Eppendorf-Centrifuge) and redissolved in 50 µl of bidistilled water. Fluorescence was detected with a Shimadzu RF 540 fluorometer (Shimadzu-Europa, Duisburg, F.R.G.). An untreated sample was used as reference. Excitation wavelength was 220 nm (5nm bandwidth), emission was determined between 290 and 400 nm (10 nm bandwidth). Finally, loading experiments with labelled glutamate were performed as described above.

Treatment of total tRNA with cyanogen bromide

Following the method of Saneyoshi and Nishimura (1970) 50 µl of 1 M sodium carbonate buffer, pH 8.9, were mixed with 5 mg of a total tRNA preparation from *Scenedesmus*. 2.5 mg of cyanogen bromide, dissolved in 100 µl of 10% (v/v) aqueous ethanol were added and the reaction mixture incubated for 10 min under vigorous stirring at room temperature. To remove excess cyanogen bromide the tRNA was twice precipitated with ethanol, redissolved in bidistilled water, and finally taken up again in 500 µl of the same solvent. Subsequently, loading experiments with labelled glu, gly and leu were performed as described above.

Results and discussion

In this paper more evidence for the plastidal origin of the glutamyl-tRNA synthetase from *Scenedesmus*, which was purified to apparent homogeneity (Vothknecht *et al.*, 1994) will be given by the fact that it is highly specific for tRNA^{glu} from plastids and bacteria. Furthermore, it will be demonstrated that the enzyme is capable of mischarging plastidal tRNA^{gln} with glu which is again characteristic for the plastidal glutamyl-tRNA synthetase. Finally, it will be shown that the regular substrate of glutamyl-tRNA synthetase, tRNA^{glu}, is also of plastidal origin.

Charging of different tRNA-preparations with glu by Scenedesmus glutamyl-tRNA-synthetase

Highly purified glutamyl-tRNA synthetase (Vothknecht *et al.*, 1994) was used for the charging experiments. The charging of total tRNA from *Scenedesmus*, barley and yeast, purified tRNA^{glu} from *E.coli* and tRNA^{gln} from barley and *E.coli* in the presence of labelled glutamate is shown in Table Ia. Insignificant or no charging could be observed in the case of the total tRNA from yeast and with tRNA^{gln} from *E.coli*, while all other tRNAs could be loaded.

From the fact that both, tRNA^{glu} and tRNA^{gln} from barley plastids, and also tRNA^{glu} from *E.coli* were charged (Table Ib), it can be concluded that the *Scenedesmus* enzyme is a plastidal one since, so far, no cytoplasmic glutamyl-tRNA synthetase is described that misacylates tRNA^{gln}. That the cytoplasmic tRNA from yeast is loaded much less efficiently by the purified *Scenedesmus* glutamyl-tRNA synthetase is in agreement with results from *Chlamydomonas* where the purified plastidal glutamyl-tRNA synthetase (Chen *et al.*, 1990) was not able to charge tRNA from yeast and further confirms the plastidal origin of the purified *Scenedesmus* enzyme.

Mischarging of tRNA^{gln} by the Scenedesmus glutamyl-tRNA synthetase

Furthermore, also tRNA^{gln} from barley is charged with glu by the *Scenedesmus* glutamyl-tRNA-synthetase (Table Ia). This 'mischarging' of gln-specific tRNA with glu was already reported for the glutamyl-tRNA synthetases from gram-

Table I. Charging of tRNA-preparations of different origin with glu and gln.

tRNA-sample	labelled substrate	tRNA-bound substrate [$\mu\text{moles}\cdot\text{min}^{-1}$]
a) charging with glu by highly purified glutamyl-tRNA synthetase		
total tRNA, <i>Scenedesmus</i>	glutamate	1.6×10^{-6}
total tRNA, barley plastids	glutamate	1.4×10^{-6}
total tRNA, yeast	glutamate	0.09×10^{-6}
tRNA ^{glu} , <i>E. coli</i>	glutamate	2.5×10^{-6}
tRNA ^{gln} , barley plastids	glutamate	3.6×10^{-6}
tRNA ^{gln} , <i>E. coli</i>	glutamate	–
b) charging with gln by a crude Blue Sepharose protein preparation		
total tRNA, <i>Scenedesmus</i>	glutamine	0.03×10^{-6}
total tRNA, yeast	glutamine	–
tRNA ^{gln} , barley plastids	glutamine	–
tRNA ^{gln} , <i>E. coli</i>	glutamine	–

positive bacteria (Wilcox and Nirenberg, 1968), *Halobacterium volcanii* (Gupta, 1984) and higher plant organelles (Schön *et al.*, 1988; Schön and Söll, 1988b). However, the mischarging does not take place in gram-negative bacteria like *E. coli*.

In parallel experiments the loading of the different tRNA-preparations with gln was tested with crude *Scenedesmus* enzyme extracts. Only traces of glutaminyl-tRNA synthetase activity could be found when *Scenedesmus* total tRNA was used. The same result was obtained by using a Blue Sepharose enzyme preparations (Table Ib) which contain all aminoacyl-tRNA synthetases, as partially shown in Fig. 2 by the controls. This result indicates the absence of a plastidal glutaminyl-tRNA synthetase in *Scenedesmus obliquus* and is in agreement with data from chloroplasts, mitochondria, cyanobacteria, archaebacteria and gram-positive bacteria, as already stated above (Wilcox and Nirenberg, 1968; Gupta, 1984; Schön *et al.*, 1988b). The traces of glutaminyl-tRNA activity are probably due to the presence of cytoplasmic glutaminyl-tRNA synthetase in these preparations.

In all the above documented cases the absence of a glutaminyl-tRNA synthetase and the resulting mischarging of tRNA^{gln} with glu by a glutamyl-tRNA synthetase is compensated by the presence of a glutamyl-tRNA amidotransferase which converts the glutamyl-tRNA^{gln} into glutaminyl-tRNA^{gln}. In *in vitro* experiments gln is the amino-donor for this reaction (Schön *et al.*, 1988; Strauch *et al.*, 1988, Jahn *et al.*, 1990). The involved en-

zyme, glutamyl-tRNA amidotransferase, has only been purified from *Chlamydomonas* (Jahn *et al.*, 1990).

Amidotransferase activity in *Scenedesmus*

Thus we tried to prove the existence of this particular glutamyl-tRNA amidotransferase also in *Scenedesmus*. Following the modified method of Wilcox and Nirenberg (1968) total tRNA-preparations from barley plastids were incubated with labelled glu, a Blue Sepharose enzyme preparation and unlabelled gln as aminodonor. As a control, unlabelled gln as the amino donor of the transamidation reaction was omitted. The bound amino acids were subsequently split off the tRNAs by hydrolysis and glu and gln separated by ion exchange chromatography. The gln containing fraction was analyzed by TLC with labelled and unlabelled glu and gln as references, additionally visualizing the amino acids by spraying with ninhydrin. As shown in Fig. 1, only in the case of added, unlabelled gln as aminodonor labelled gln could be detected. As glutamine synthetase was inhibited by methionine sulfoximine this clearly demonstrates the presence of a glutamyl-tRNA amidotransferase in the Blue Sepharose enzyme preparation from *Scenedesmus*. From these results it has to be concluded that also in *Scenedesmus*-chloroplasts, like in other so far examined plastids, a glutaminyl-tRNA synthetase is missing and that this deficit is compensated by the mischarging of tRNA^{gln} with glu by glutamyl-

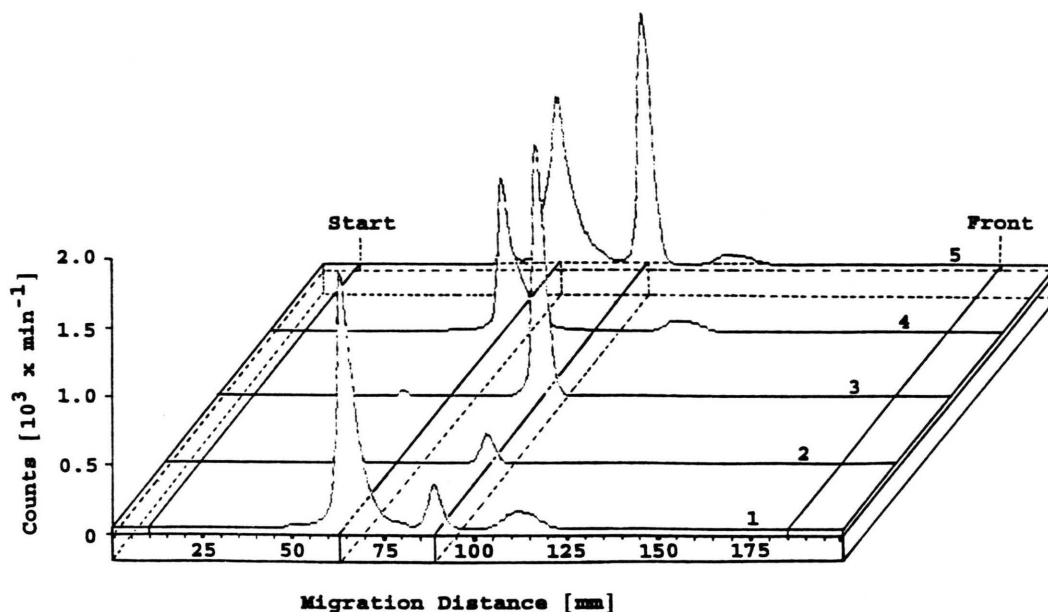


Fig. 1. Identification of gln by TCL. Gln was formed by transamidation of glutamyl-tRNA^{gln} to glutamyl-tRNA^{glu} by a *Scenedesmus* glutamyl-tRNA amidotransferase. Track 1: labelled gln formed by the amidotransferase reaction in the presence of unlabelled gln as amino donor; Track 2: Control without amino donor; Tracks 3, 4, and 5 are labelled references: glu, gln, and glu + gln.

tRNA synthetase and subsequent transamidation of the bound glu to gln by a glutamyl-tRNA amidotransferase. Further attempts will be made to isolate, purify and characterize this enzyme from *Scenedesmus*.

Sulfur modification in the the *Scenedesmus*-tRNA^{glu}-anticodon

To prove that the main part of the tRNA^{glu} in our experiments is of plastidal origin, attempts were made to show that like in the barley plastid tRNA^{glu} (Schön *et al.*, 1986) a sulfur modification is present in the *Scenedesmus* tRNA^{glu}-anticodon, too.

Common to all tRNAs are posttranslational modifications of nucleotides. From experiments with purified tRNA^{glu} from barley chloroplasts it is known that ten modified nucleotides are present (Schön *et al.*, 1986). Most striking was the existence of a 5-[methylamino)-methyl]-2-thioridine (mam⁵s²U) in the first position of the UUC-anticodon, a modification which is known from tRNAs from eubacteria (Sprinzl *et al.*, 1989). When these at the anticodon sulfur-modified tRNAs are

treated with cyanogen bromide and by this converted into their thiocyanate derivatives their charging with glu is nearly completely inhibited (Saneyoshi and Nihimura, 1971; Agris *et al.*, 1973). Hybrid-tRNAs missing this modification also showed only reduced loading with glu (Sylvers *et al.*, 1993)

Taking into account these data we tested the total tRNA preparation from *Scenedesmus* for the presence of a sulfur modification and thus the inhibition of its charging with glu by treatment with monobromobimane and cyanogen bromide. Following the method of Kosower (1978) total tRNA-preparations were treated with monobromobimane. Afterwards sample and untreated control were examined for their fluorescence (for details see Methods). Compared to the control the treated sample showed an approx. 6-fold increase in fluorescence at 307 nm, clearly indicating the presence of a sulfur modification in the *Scenedesmus*-tRNA. Charging experiments of treated and untreated probes with labelled glu, however, only revealed a 10% inhibition in the treated sample. From this quite poor inhibitory effect it cannot be

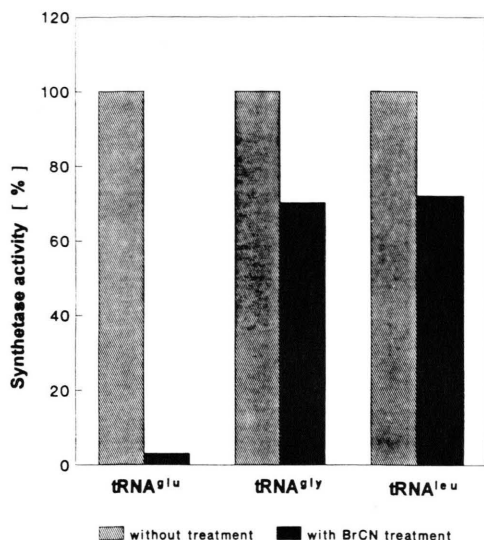


Fig. 2. Charging capacities of the different tRNAs after treatment with cyanogen bromide. Controls without treatment were set as 100% in each case.

concluded that the specific sulfur modified anticodon is present also in the *Scenedesmus* tRNA^{glu}. The effect might rather be due to some chemical destruction during the purification procedure after the reaction or to other sulfur modifications in the molecule, not related to the anticodon. From other eucaryotic tRNAs such modifications with sulfur are known (Björk *et al.*, 1987). From this experiment it can thus only be concluded that also in *Scenedesmus*-tRNA^{glu} sulfur modifications might be present, however, their location in the anticodon cannot be derived from these data.

As the above method did not yield unambiguous results, treatment of the tRNA with cyanogen bromide was employed. The reagent reacts quantitatively with thiouridine to form the corresponding thiocyanate (Saneyoshi and Nishimura, 1970). After treatment with BrCN and purification of the sample, only 3% of the original charging capacity of tRNA^{glu} with glu as substrate could be reco-

vered (Fig. 2). This is in accordance with results from *E. coli* tRNA (Saneyoshi and Nihimura, 1971), where an inhibition of 6% of the original charging capacity for tRNA^{glu}, tRNA^{gln} and tRNA^{lys} was found. All these tRNAs contain this specific thiouridine modification in the anticodon. Thus the dramatic inhibitory effect of the BrCN-treatment on *Scenedesmus*-tRNA^{glu} can only be attributed to the presence of a sulfur modification in the anticodon. To exclude that the inhibition is solely due to chemical destruction of the tRNA-preparation, as a control charging experiments with glycine and leucine were performed. In these studies the original charging capacity was retained by about 70% (Fig. 2), indicating that the inhibitory effect on tRNA^{glu} is highly specific and due to the presence of the anticodon-sulfur-modification. The presented data thus make the presence of the mam⁵s²U-anticodon in *Scenedesmus* most likely. Final evidence, however, can only be given by sequencing of the tRNA which is the aim of our present work on the compound.

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