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Critical Role of Zinc Ion on *E. coli* Glutamyl-Queuosine-tRNA^{Asp} Synthetase (Glu-Q-RS) Structure and Function

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Abstract Glutamyl-queuosine-tRNA^{Asp} synthetase (Glu-Q-RS) and glutamyl-tRNA synthetase (GluRS), differ widely by their function although they share close structural resemblance within their catalytic core of GluRS. In particular both *Escherichia coli* GluRS and Glu-Q-RS contain a single zinc-binding site in their putative tRNA acceptor stem-binding domain. It has been shown that the zinc is crucial for correct positioning of the tRNA^{Glu} acceptor-end in the active site of *E. coli* GluRS. To address the role of zinc ion in Glu-Q-RS, the C101S/C103S Glu-Q-RS variant is constructed. Energy dispersive X-ray fluorescence show that the zinc ion still remained coordinated but the variant became structurally labile and acquired aggregation capacity. The extent of aggregation of the protein is significantly decreased in presence of the small substrates and more particularly by adenosine triphosphate. Addition of zinc increased significantly the solubility of the variant. The aminoacylation assay reveals a decrease in activity of the variant even after addition of zinc as compared to the wild-type, although the secondary structure of the protein is not altered as shown by the Fourier transform infrared spectroscopy study.

Keywords Glu-Q-RS · Aggregation · Zinc binding site · FT-IR

Abbreviations

Glu-Q-RS	Glutamyl-queuosine-tRNA^{Asp} synthetase
GluRS	Glutamyl-tRNA synthetase
K_D	Dissociation constant
FTIR	Fourier transform infrared spectroscopy
ED-XRF	Energy dispersive X-ray fluorescence
TMAO	Trimethylamine *N*-oxide
IR	Infrared
E. coli	*Escherichia coli*
AlaRS	Alanyl-tRNA synthetase
ATP	Adenosine triphosphate
L-Glu	L-Glutamic acid
L-Arg	L-Arginine
D_2O	Deuterium oxide

1 Introduction

Aminoacyl-tRNA synthetases (aaRSs) are an ancient group of modular house-keeping enzymes which plays a crucial role during protein synthesis by catalyzing the aminoacylation of tRNAs [1]. Several aaRSs contain a zinc ion which is involved in diverse functions such as amino acid

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discrimination, aminoacylation or in reinforcing the structural stability of the protein [2–8].

The role of zinc ion in *Escherichia coli* glutamyl-tRNA synthetase (GluRS) has been extensively investigated. The metal ion was found to be a part of the SWIM domain, an integral part of the tRNA acceptor-stem-binding domain of the protein [9, 10]. In *E. coli* GluRS, the zinc ion is tetracoordinated to the Cys98, Cys100, Cys125 and His127 residues [9] and it is loosely attached to the C100Y GluRS variant, although the structure of the variant is very similar to that of the wild-type (WT) enzyme [10]. It was also reported that the zinc depleted *E. coli* GluRS exhibits a more open structure consisting in a higher proportion of β-sheets and random coils compared to the WT GluRS [11]. Apart from the important structural role, the zinc also exhibits a crucial role in the activity of GluRS [9–11].

Campanacci et al. [12] reported that glutamyl-Q-tRNA^{Asp} synthetase (Glu-Q-RS) from *E. coli*, a paralog of GluRS, contains, like GluRS one zinc ion. Glu-Q-RS presents significant structural similarities with the catalytic core of GluRS consisting in the N-terminal domain of the protein, however both enzymes differ widely by their functional properties [13, 14]. Glu-Q-RS glutamylates the Q34 residue of the anticodon of tRNA^{Asp} [15], hence it is a tRNA hypermodifying enzyme [16].

Escherichia coli GluRS and Glu-Q-RS also differ in their chemical environment of the zinc-binding domain. Although the zinc ion is tetracoordinated far from the enzyme active site by four amino acid residues, namely Cys101, Cys103, Cys119 and Tyr115 in Glu-Q-RS, and Cys98, Cys100, Cys125 and His127 in GluRS, however Glu-Q-RS lacks the SWIM domain [12, 16]. The significance of the zinc ion in Glu-Q-RS that is evolutionary closely related to GluRS is currently unknown. Moreover, there is no report of the structural and/or functional role of zinc ion in any tRNA-modifying enzyme.

To understand the role of the zinc ion in Glu-Q-RS, we constructed a Glu-Q-RS variant by site directed mutagenesis of Cys101 and Cys103, two residues of the zinc cluster and replaced both amino acids by Ser residues. Cys and Ser are isosteric amino acids, preventing an effect by bulkiness change on the stability of the protein [17, 18]. We report here that the Glu-Q-RS C101S/C103S variant acquires aggregation capacity. However the extent of aggregation is significantly reduced in presence of the small substrates [L-glutamic acid (L-Glu), adenosine triphosphate (ATP)] as well as by addition of zinc ions. The in vitro aminoacylation assay of the C101S/C103S Glu-Q-RS shows reduced activity as compared to that of the WT enzyme, but its activity is not altered by addition of free zinc. Fourier transformed infrared (FTIR) spectroscopic investigation of the WT and the mutated Glu-Q-RS does not show any significant difference in the β-sheet content, excluding the β-sheet mediated aggregation of the variant.

2 Materials

Restriction enzymes were purchased from Fermentas. The radioactive L-Glu was purchased from Perkin-Elmer. All other materials were of analytical grade.

3 Methods

3.1 Construction of *E. coli* C101S/C103S Glu-Q-RS Variant

For construction of C101S/C103S *E.coli* Glu-Q-RS variant, the following primer was used 5′-GGACTAAGTTATTAC AGCACCAGCACGCGTGCGCGTATTC-3′. The variant was constructed using in vitro site-directed mutagenesis according to the manufacturer's protocol (Stratagene). The mutation was confirmed by DNA sequencing. To isolate the C101S/C103S Glu-Q-RS variant, protein auto-induction media was used [19]. The mutated protein was purified like WT Glu-Q-RS as described by Campanacci et al. [12]. Glu-Q-RS was expressed in the BL21 (DE3) Rosetta II strain transformed by the recombinant pDest17 vector [14]. The tRNA^{Asp} was purified as described by Blaise et al. [18].

3.2 Determination of Zinc by ED-XRF Spectroscopy

The amount of zinc was analyzed by the energy dispersive X-ray fluorescence spectrometry technique using a Jordan Valley Ex-3600 Energy dispersive X-ray fluorescence (ED-XRF) spectrometer. The protein sample (20 µl) was spotted on a Whatman filter paper (Grade 1 circles, 90 mm). The concentrations of C101S/C103S Glu-Q-RS and WT GluRS were 0.42 and 1 mg/ml respectively. Before measurement, the solvent was evaporated under vacuum. Examination of the blank filter paper revealed absence of any zinc contamination. The measurement was carried out at a voltage of 15 kV and a current of 2,750 mA [20, 21].

3.3 Fluorescence Spectroscopy Method

The aggregation study was monitored by light scattering experiments in a HITACHI F7000 fluorescence spectrophotometer at 25 °C. The excitation and emission wavelength was 350 nm [22]. Both excitation and emission slits width were 5 nm.

In the presence of the substrates, 2 mM ATP, 5 mM L-Glu, 3 µM tRNA^{Asp} and 5 mM $MgCl_2$ and osmolytes [1 M

trimethylamine *N*-oxide (TMAO) and 500 mM L-arginine (L-Arg)], the concentration of the variant and WT proteins was kept at 5 μM. The aggregation study of the variant and WT Glu-Q-RS was conducted without the substrates, in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 10 % of glycerol in the presence of 0.5, 2 and 5 μM of protein. To study the effect of the added zinc on the WT and on the variant Glu-Q-RS, the concentration of zinc acetate was kept at 1 mM.

3.4 FTIR Spectroscopy

Soluble Glu-Q-RS or aggregated C100S/C103S Glu-Q-RS variant in the absence or in the presence of 1 mM zinc acetate, were taken in a microcon filter device fitted with a 10-kDa cut-off membrane and the deuterium exchange process was carried out as described earlier [23, 24]. The concentrated deuterium exchanged samples were then placed between two calcium fluoride windows separated by a 50 μm thick spacer. Infrared (IR) spectra were recorded at 25 °C on a Spectrun 100 Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer) with a resolution of 2 cm^{-1}. The spectra were collected in the region of 1,600–1,700 cm^{-1} with the accumulation of 16 scans. To resolve the overlapping infrared bands Fourier self-deconvolution was used. Curve fitting of the amide I region (raw spectra) was performed with the peak fitting function of mixed Lorentzian and Gaussian, using Thermo GRAMS AI software. The FWHM was initially set at 15 cm^{-1} and the iteration process was carried out till the Chi square value remained unchanged. Second derivative and Fourier self-deconvolved spectra were used as a peak position guide for the curve fitting procedure.

3.5 Aminoacylation Assay

The reaction mixture contained 50 mM Na-HEPES buffer pH 7.5, 16 mM $MgCl_2$, 2 mM ATP, 0.1 mM unlabeled L-Glu with trace amounts of [^{3}H]L-Glu (specific activity: 42.9 Ci/mmol), 0.8 mM β-mercaptoethanol and 5 μM purified $tRNA^{Asp}$. The concentration of Glu-Q-RS and C101S/C103S Glu-Q-RS was kept at 0.5 μM. When added, the concentrations of zinc acetate and Na_2 EDTA were 1 mM. The assay was carried out at 37 °C as described [25], 10 μl aliquots were withdrawn at each time point and precipitated with 10 % of trichloro acetic acid (TCA). The amount of glutamyl-tRNA formed was determined by counting the radioactivity in a scintillation counter.

4 Results

ED-XRF is a very well known analytical technique to detect Na to U elements in samples [20, 21]. The 3D

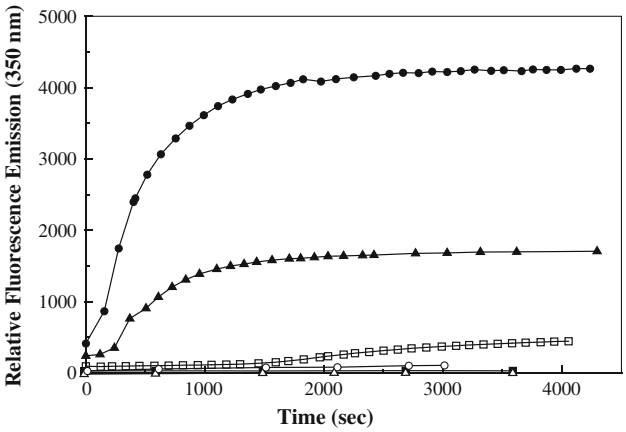

Fig. 1 Time-dependent aggregation study of the C101S/C103S Glu-Q-RS variant in the presence of increasing concentrations of protein. The concentrations of the WT and variant Glu-Q-RS were 0.5 μM (*open square*, *filled square*), 2 μM (*open triangle*, *filled triangle*) and 5 μM (*open circle*, *filled circle*) in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 10 % of glycerol. The excitation and emission wavelength was 350 nm. The light scattering study of WT Glu-Q-RS was done as a control experiment

structures of free Glu-Q-RS and Glu-bound Glu-Q-RS show presence in the protein of a zinc ion, tetracoordinated to residues Cys101, Cys103, Cys119 and Tyr115 (Fig. I, Supplementary information) [12, 26]. In both structures, solved at very high resolution, the occupancy of the zinc has been refined to 1 indicating that each protein molecule is bound to a zinc ion. Thus the native protein is saturated nearly to 100 % with the zinc. ED-XRF study (Fig. II, Supplementary information) shows that the C101S/C103S Glu-Q-RS variant still contains the zinc but in a strongly decreased stoichiometry (0.2 ion of zinc/mol of protein) compared to the WT Glu-Q-RS protein (Fig. III, Supplementary information). Therefore substitution of C101 and C103 by Ser residues decreases the affinity of the protein for zinc approximately by two to three orders of magnitude. Further, the absence of saturating zinc in solution displays dramatic change in the solubility of the protein. Unlike wild type Glu-Q-RS, the variant shows a strong tendency to aggregate in solution at room temperature. The extent of aggregation versus time was studied in the presence of 0.5, 2 and 5 μM of the enzyme variant. Figure 1 clearly shows that the extent of aggregation increases linearly with increasing protein concentrations of the variant. However, addition of a saturating concentration of the small substrate ATP and/or L-Glu decreases the rate of aggregation of C101S/C103S Glu-Q-RS. Among the ligands studied, ATP has the most efficient effect (Fig. 2a). In presence of $tRNA^{Asp}$ however, the extent of aggregation of Glu-Q-RS variant increases only marginally (Fig. 2a). Since both ATP and L-Glu decrease the rate of aggregation significantly, we examined the effect of osmolytes such as

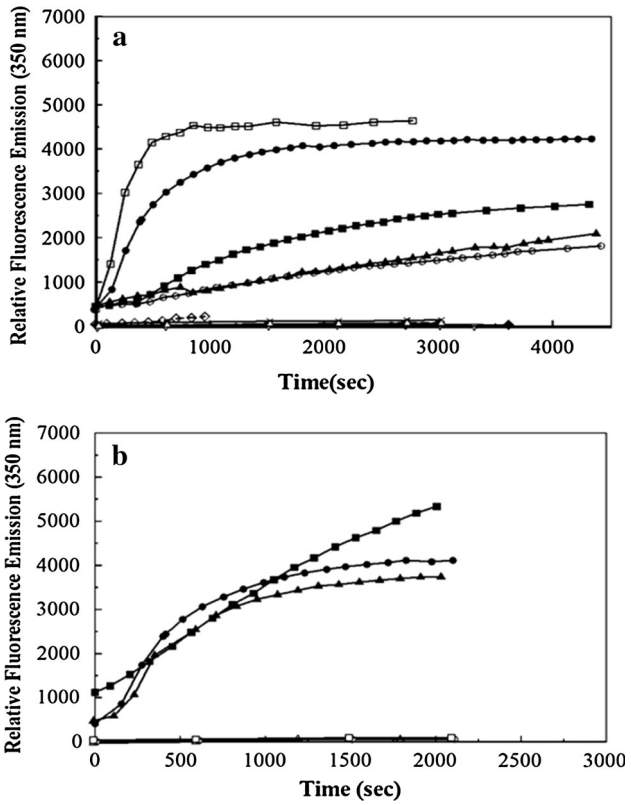

Fig. 2 Effects of substrates and osmolytes on the WT Glu-Q-RS and on the extent of aggregation of C101S/C103S Glu-Q-RS variant. The experiments were conducted in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 10 % of glycerol. **a** Time dependent aggregation of 5 μM of the C101S/C103S Glu-Q-RS variant in the presence of 5 mM L-Glu (*filled circle*), 2 mM ATP or 2 mM ATP + 5 mM L-Glu (*filled square*), 3 μM $tRNA^{Asp}$ (*open circle*). In the presence of 0.5 μM C101S/C103S Glu-Q-RS the time-dependent aggregation was studied with 2 mM ATP, 5 mM L-Glu, and 5 μM $tRNA^{Asp}$ (*open square*). The time dependent effect of the substrates on the WT Glu-Q-RS was studied with 5 μM of enzyme in the presence of 5 mM L-Glu (*star*), 2 mM ATP or 2 mM ATP + 5 mM L-Glu (*inverted triangle*), 3 μM $tRNA^{Asp}$ (*filled triangle*) or with 0.5 μM WT Glu-Q-RS and 2 mM ATP, 5 mM L-Glu, 5 μM $tRNA^{Asp}$ (*filled diamond*) or in the absence of substrates (*open circle*). **b** Time dependent aggregation of 5 μM C101S/C103S Glu-Q-RS variant in the presence of 1 M TMAO (*filled square*), or 500 mM L-Arg (*filled triangle*) and in absence of the osmolytes (*filled circle*). Time dependent effects of 1 M TMAO (*open square*), or 500 mM L-Arg (*open triangle*) on 5 μM WT Glu-Q-RS and in the absence of osmolytes (*open circle*). The excitation and emission wavelength was 350 nm

TMAO and L-Arg [27, 28]. Both are known osmolytes that stabilize the protein structure [27, 28]. It has been reported that TMAO acts as a "counteracting osmolyte" [29] whereas, L-Arg behaves as an additive to increase activation energy of protein–protein association [30]. However, no significant change is observed in the rate of aggregation of the C101S/C103S Glu-Q-RS variant in presence of either 1 M TMAO or 500 mM L-Arg (Fig. 2b). Under identical conditions no detectable aggregation was observed for WT Glu-Q-RS.

Fourier transform infrared spectroscopy is a well-established technique to determine the secondary structures of proteins [23, 24, 31]. Amide I band is highly sensitive to small variations of conformational changes of the protein structure [31, 32]. Figure 3a, b shows that the amide I region of the WT and the C101S/C103S variant Glu-Q-RS was centered at 1,653 cm^{-1} indicating that α helix is the predominant conformation in both proteins. Fourier-deconvolution of the amide I of wild-type Glu-Q-RS protein reveals seven bands corresponding to 1,625, 1,633, 1,642, 1,651, 1,659, 1,669 and 1681 cm^{-1}. After Fourier-deconvoluation, amide I region of C101S/C103S Glu-Q-RS protein shows seven bands corresponding to 1,618, 1,631, 1,639, 1,649, 1,668, 1,678 and 1,690 cm^{-1}. Analysis of the secondary structures from FTIR data shows that the wild type Glu-Q-RS consists of 38 % of α-helices, 33 % of β-sheets, 14 % of β-turns and 13 % of random coils; these values are nearly close to those in the crystal structure of the native Glu-Q-RS [12]. The presence of 1 mM zinc ion does not alter the secondary structure of this protein (Fig. 3c; Table 1). Indeed, the secondary structural percentages of the C101S/C103S Glu-Q-RS variant do not varied significantly compared to the WT protein, except the β-turn content, which shows a higher proportion (Table 1). However, in the presence of 1 mM zinc ion, the α-helix percentage of the variant increases whereas that of β-sheet decreases (41 % α-helix, 17 % β-sheet, 18 % β-turn, 24 % random coil) (Table 1; Fig. 3d).

Enzymatic activities of the WT and C101S/C103S variant of Glu-Q-RS were determined by in vitro aminoacylation assays in the presence of 0.5 μM of enzyme (Fig. 4); Since at this concentration the extent of aggregation decreased significantly in the presence of all substrates (Fig. 2a) the Glu-Q-RS variant remains essentially in soluble form. Figure 4 clearly indicates that the mutated Glu-Q-RS is significantly less active than the WT Glu-Q-RS. Addition of 1 mM of free zinc is unable to enhance the activity of the Glu-Q-RS variant, whereas it stimulates drastically its solubility (Fig. 5). The activity of the WT enzyme however decreased substantially in presence of 1 mM zinc and partial revival was observed when EDTA was added prior to the assay. This may be due to the displacement of Mg^{2+} from Mg-ATP complex by zinc. The activity of the variant remained unchanged under all these conditions.

5 Discussion

Zinc plays a crucial role in catalytic activity and substrate discrimination in various aaRSs [4, 5, 33]. Removal of zinc led to loss of activity from *E. coli* MetRS, ThrRS and GluRS [7, 11, 34]. Substitution of C145 involved in zinc

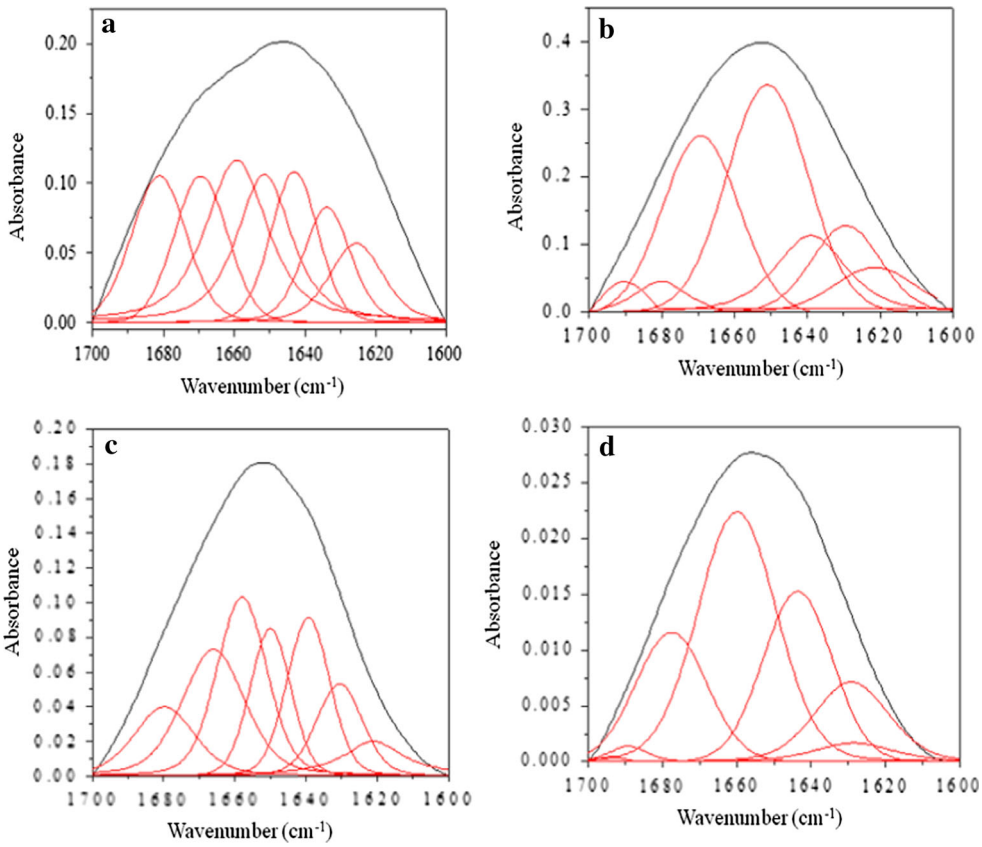

Fig. 3 Fourier self-deconvolved spectra of Glu-Q-RS and C101S/C103S Glu-Q-RS in the presence and in the absence of 1 mM zinc acetate. The spectra were recorded at 25 °C on a Spectrum 100 FT-IR spectrometer (Perkin Elmer) with a resolution of 2 cm^{-1}. The spectra were collected in the region 1,600–1,700 cm^{-1}. **a**, **c** Fourier self-deconvolved spectra of Glu-Q-RS in absence and in presence of 1 mM zinc acetate. The concentration of Glu-Q-RS was 7 mg/ml in 100 mM Tris–HCl buffer pH 8.0, containing 150 mM NaCl, and 10 % of glycerol. **b**, **d** Fourier self-deconvolved spectra of aggregated C101S/C103S Glu-Q-RS in the absence and in the presence of 1 mM zinc acetate

Table 1 Secondary structures of Glu-Q-RS and C101S/C103S Glu-Q-RS variant in the absence and in the presence of 1 mM zinc acetate, determined by Fourier transform infrared spectroscopy self-deconvolution

Secondary Structures	In absence of 1 mM zinc acetate		In presence of 1 mM zinc acetate	
	Glu-Q-RS	C101S/C103S Glu-Q-RS	Glu-Q-RS	C101S/C103S Glu-Q-RS
α-Helix (%)	38	35	37	41
β-Sheet (%)	33	28	27	17
β-Turn (%)	14	25	20	18
Random (%)	13	12	16	24

binding by Ser in *E. coli* MetRS led to loss of the aminoacylation capacity of the enzyme without significant alteration of its structure [35]. Banerjee et al. [10] reported that substitution in *E. coli* GluRS of C100 involved in zinc binding by Tyr decreases the affinity of the protein for L-Glu (K_m and K_D values increased 12- and 20-fold respectively) and decreases its k_{cat} of $tRNA^{Glu}$ charging. However, analysis of the secondary structures by far-UV CD spectra of the variant reveals the absence of major structural distortion of the protein [10]. Further removal of the zinc by the chelating agent 1, 10-phenanthroline led to a more opened conformation of the protein as revealed by a decreased electrophoretic mobility [11]. In contrast,

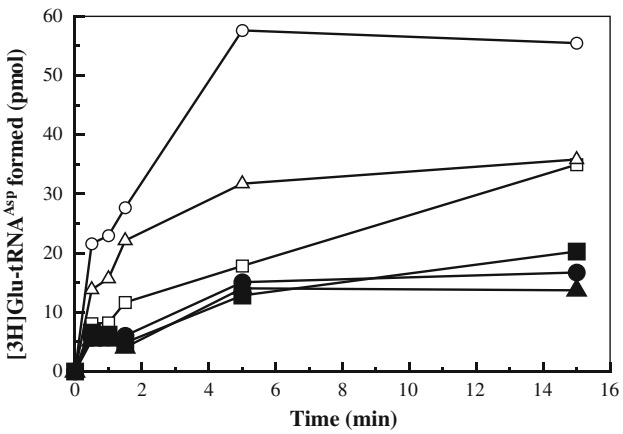

Fig. 4 Aminoacylation assays of Glu-Q-RS and C101S/C103S variant in the presence and in the absence of 1 mM zinc acetate. Aminoacylation was performed at 37 °C in the reaction mixture described in "Materials and Methods" containing 0.5 μM of Glu-Q-RS or C101S/C103S Glu-Q-RS. When added, the concentrations of zinc acetate and Na_2 EDTA were of 1 mM. 10 μl aliquots were withdrawn at each time point. The amount of radioactivity retained was determined by liquid scintillation counting. The curves of Glu-Q-RS WT and C101S/C103S Glu-Q-RS variant alone (*open circle*, *filled circle*) in presence of zinc acetate (*open square*, *filled square*) and EDTA and zinc acetate (*open triangle*, *filled triangle*) are shown

substitution in *E. coli* alanyl-tRNA synthetase (AlaRS) of the Cl78, Hl88 and H191 residues coordinated to the zinc ion by Ser, Gln and Gln residues respectively affects the

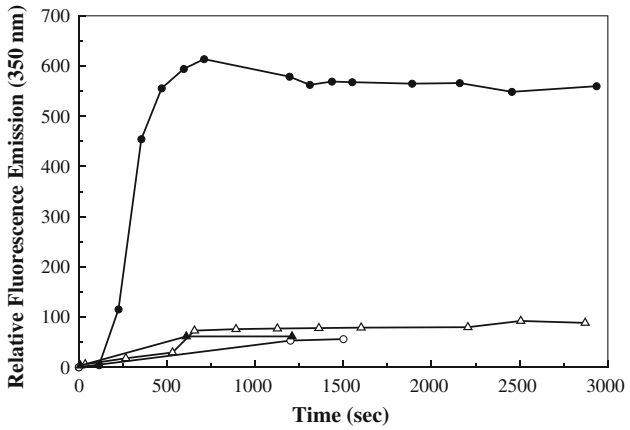

Fig. 5 Time-dependent aggregation of 0.5 μM of the C101S/C103S Glu-Q-RS variant in the absence (*filled circle*) and in the presence of zinc acetate (*open triangle*) and time-dependent light scattering of 0.5 μM WT Glu-Q-RS variant in the absence (*open circle*) and in the presence of zinc acetate (*filled triangle*). The experiments were conducted in 100 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and 10 % of glycerol. The excitation and emission wavelength was 350 nm

structural stability of the protein. Further the inability of the variant to complement the null strain, suggests a crucial role of the zinc in the catalytic activity of this enzyme [3].

Since *E. coli* Glu-Q-RS exhibits a remarkable structural similarity with *E. coli* GluRS indicating that the two proteins are evolutionary related, we compared the role of the zinc in the two proteins. In contrast to the GluRS mutated on the homologous residues, the C101S/C103S variant of Glu-Q-RS shows a concentration dependent aggregation of the protein. But the ED-XRF study reveals that the C101S/C103S Glu-Q-RS variant still contains zinc. Thus the mutation of the Cys residues involved in zinc binding does not abolish the zinc-binding capacity of the variant. However, the stoichiometry is significantly reduced as a consequence of the decreased affinity of the protein for the zinc because the oxygen has a much lower atomic radius for coordination than the sulphur. When free zinc was added, the extent of aggregation of the Glu-Q-RS variant decreased drastically (Fig. 5) and the secondary structures were significantly altered (Fig. 3d; Table 1), whereas its aminoacylation activity was not improved. This observation indicates that addition of free zinc might modulate the local conformation of the Glu-Q-RS variant, however it fails to promote the active-site reorientation necessary for $tRNA^{Asp}$ hypermodification.

The decrease of the extent of aggregation by ATP and L-Glu reveals substrate-induced conformation stability of the protein by these ligands. It is noteworthy to mention here that we observed previously that the K_D of L-Glu decreased drastically in the presence of a saturating ATP concentration, indicating a probable conformational change induced by this ligand [35]. Interestingly ATP is the most effective ligand for this effect among all of the ligands tested here. Finally, since the FTIR study indicates that the secondary structures of the C101S/C103S Glu-Q-RS variant remain roughly unchanged, a β-sheet mediated aggregation of the variant can be excluded.

Our structural and functional investigations indirectly underscore the fact that mutation of the residues involved in zinc binding either perturb the tertiary structure of Glu-Q-RS keeping the secondary structure intact or might modify the local conformation of the enzyme. In either instance, some of the hydrophobic groups might have been exposed to the solvent promoting the formation of aggregates. Our study clearly reveals that the zinc ion not only plays a functional role, but has also a strong influence on the structure of Glu-Q-RS.

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