Mutational analysis of Glu^{272} in elongation factor 1A of *E. coli*

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Received 1 May 1998

Abstract In our previous work (Mansilla et al. (1997) Protein Eng. 10, 927–934) we showed that Arg^7 of *Escherichia coli* elongation factor Tu (EF1A) plays an essential role in aminoacyl-tRNA (aa-tRNA) binding. Substitution of Arg^7 by Ala or Glu lost this activity. We proposed that Arg^7 forms a salt bridge with the charged conserved amino acid Glu^{272} (Asp^{284} in *Thermus aquaticus*) thereby binding the N-terminal region of the protein to domain 2 and thus completing the conformational rearrangement needed for binding aa-tRNA. In this work we have mutated Glu^{272} to arginine, either alone ($Glu^{272}Arg$), or in combination with one of the above mentioned mutations ($Arg^7Glu/Glu^{272}Arg$) in order to test this hypothesis. Our results show that, in confirmation of our thesis based on structural knowledge, the substitution of Glu^{272} (Asp^{284}) decreases the ability of EF1A:GTP to bind aa-tRNA.

Key words: Elongation factor; Aminoacyl-tRNA binding; Site directed mutagenesis; Salt bridge formation

1. Introduction

Elongation factor 1A (EF1A, formerly EF-Tu), a representative example of a GDP/GTP binding protein, plays an important role in protein biosynthesis carrying aminoacyl-tRNA (aa-tRNA) to the A site of the mRNA-programmed ribosomes. After correct codon-anticodon interaction guanosine triphosphate (GTP), bound as a cofactor, is hydrolysed to guanosine diphosphate (GDP). The elongation factor thus changes its conformation and thereby loses its affinity for the ribosome and dissociates in complex with GDP. The amino acid of the newly delivered aa-tRNA is incorporated into the growing polypeptide chain. EF1A is reactivated by another elongation factor, EF1B (formerly EF-Ts), which catalyses the exchange of GTP for GDP.

The structures of EF1A in complex with either GDP, GTP, EF1B or GTP:aa-tRNA are known [2–7], but the role for the N-terminal region remained uncertain until recently when we started to analyze the role of several conserved basic residues, Lys^2, Lys^4 and Lys^9 and Arg^7, in this region in EF1A from *E. coli*. These four residues are all located at the random coil N-terminal region of EF1A. We first determined that Arg^7 and Lys^9 have a role in maintaining the very compact three-dimensional structure of the GTP form by keeping domains 1 and 2 together and constraining domain 1, respectively, in order to bind aa-tRNA [1,8].

Previously we specifically mutated Arg^7 to alanine or glutamate. From the results we concluded that Arg^7 is involved in the binding of aa-tRNA. Based on the three-dimensional structure of the ternary complex EF1A:GTP:$PhetRNA^{Phe}$ from *Thermus aquaticus* [6] we proposed the formation of a salt bridge between Arg^7 and the very well conserved Asp^{284} (Glu^{272} in *E. coli*). Arg^7 is located in a random coil part of the EF1A structure, while Glu^{272} is positioned at the beginning of a beta strand in domain 2. The residues are more than 10 Å apart when EF1A is in the inactive GDP form, but when the structural rearrangement of the molecule takes place upon GTP binding [2], the nitrogens of the guanidine group of Arg^7 and the oxygen of the carboxyl group of Glu^{272} are brought closer together and they are in a position very suitable to maintain a salt bridge which will stabilise the interactions between domains 1 and 2.

In the present work, we have mutated Glu^{272} from EF1A of *E. coli* to arginine and then have combined this mutation with the previously introduced mutation Arg^7Glu thus creating the double mutant $Arg^7Glu/Glu^{272}Arg$. The first recombinant protein was designed to give information about the direct functional and structural contribution from the acidic residue. The second was made in order to test whether aa-tRNA binding could be recovered by exchanging the amino acids responsible for the proposed salt bridge.

2. Materials and methods

2.1. Construction of mutants

Site-directed mutagenesis was performed both on the pGEX vector containing the wild type *tufA* gene encoding EF1A [10] and in the previous construction harbouring the Arg^7Glu mutation using the USE mutagenesis kit (Pharmacia Biotech) following the method of Unique site elimination [9]. The mutagenesis primer had the sequence 5′-GCCGTGCTGGTAGGAACGTAGGTG-3′ and the *Pst*I/*Sac*II selection primer had the sequence 5′-GCGTGACACCACGATGCCCGCGGCAATGGCAACAACG-3′. The mutations were verified by sequencing the resulting DNA with the dye terminator cycle sequencing ready reaction kit (Perkin Elmer).

2.2. Expression and purification

Basically we followed the procedure described by Knudsen et al. [10] but the cells were opened by lysozyme treatment as follows: 2.5 ml ice cold buffer S (50 mM Tris-HCl, pH 7.6, at 4°C, 10 mM $MgCl_2$, 15 μM GDP, 1% Triton X-100) per gram of cells was used to resuspend the cell pellet. Then 1.2 mg lysozyme/g of cell pellet was added and the slurry stirred very slowly for 10 min at room temperature. Finally 0.1 ml of 2% sodium deoxycholate and 2.5 μg DNAse I were added per gram of cell paste. After purification of the proteins, the total protein concentration was measured using the Bio-Rad protein assay method.

2.3. Protein activity

The concentration of active protein was determined by measuring the maximum GDP exchange following Miller and Weissbach [11]. 5 μM [^{3}H]-GDP (1000 dpm/pmol) was incubated with 0.5 μM EF1A for 20 min at 30°C in binding buffer (50 mM Tris-HCl, pH 7.6, at 4°C, 100 mM NH_4Cl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT). The samples were placed on ice for 10 min and 100-μl portions spotted onto cellulose acetate filters (Gelman Science) previously soaked in washing buffer (10 mM Tris-HCl, pH 7.6, at 4°C, 10 mM $MgCl_2$, 10 mM NH_4Cl). The filters were washed with 3 ml of washing buffer. The EF1A complex bound to the filters was counted in a scintillation

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PII S0014-5793(98)00646-2

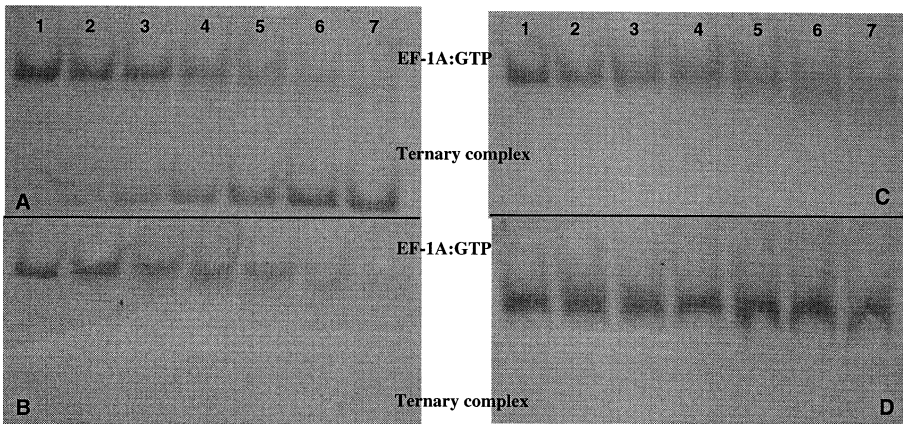

Fig. 1. Native PAGE: A: wild-type EF1A; B: $Arg^{7}Glu/Glu^{272}Arg$; C: $Arg^{7}Glu$; D: $Glu^{272}Arg$. Lanes 1–7: 0, 25, 50, 75, 100, 200 and 400 pmol Phe-$tRNA^{Phe}$ added to 100 pmol EF1A:GTP.

counter. The resulting concentrations expressing the active concentration of the protein were used from this point on. This experiment was also repeated varying the incubation time from 0 to 90 min to determine an equilibrium time for the proteins.

2.4. Temperature stability of the GDP form

EF1A:GDP stability was measured by incubating 5 μM [^{3}H]-GDP (1000 dpm/pmol) (Amersham/USB) with 0.1 μM EF1A:GDP in binding buffer. The solution was incubated for 20 min at different temperatures from 0 to 60°C and then put on ice. 85-μl samples were then filtered, washed with 4 ml washing buffer and counted as described above.

2.5. Conversion of EF1A:GDP into EF1A:GTP

To measure EF1A:GTP activity, EF1A:GDP had to be converted into EF1A:GTP, its active form. In order to do this, we incubated 0.5 μM EF1A:GDP with 5 μM GTP (3000 dpm/pmol), 0.5 mM phosphoenolpyruvate (PEP), and 0.066 μg/μl pyruvate kinase at 30°C. 85-μl portions of the samples were withdrawn at different time points from 0 to 120 min, spotted onto nitrocellulose filters (Schleier and Schuell), washed with washing buffer and counted as before.

2.6. Temperature stability of the GTP form

The experiment is performed as described above but the time of preincubation for the conversion of EF1A:GDP to EF1A:GTP is fixed at 60 min and the samples are then incubated for 15 min at different temperatures from 0 to 50°C. After this last incubation is finished, the samples from the different temperatures are placed on ice and then filtered and counted as before.

2.7. Determination of the ternary complex stability

As we were basically interested in the mutants' aa-tRNA binding behavior, we performed three different assays to help us evaluate any possible ternary complex formation: native PAGE [12], the ribonuclease digestion rate assay [13], and the non-enzymatic hydrolysis of the aminoacyl bond assay [14,15]. Native PAGE was performed by complexing 100 pmol EF1A:GTP with increasing amounts of Phe-$tRNA^{Phe}$ (0–400 pmol) by incubation at 37°C for 10 min in a total volume of 30 μl, the samples were then cooled on ice and 15 μl loaded. The gels were 5% polyacrylamide (19:1), pH 6.8 (50 mM Tris-HCl, pH 6.8, 10 mM Mg-acetate, 65 mM NH_4-acetate, 5 mM EDTA, 10 μM GTP and 1 mM DTT) and were run at 4°C. The gels were stained with Coomassie blue.

The ribonuclease digestion assay was performed as described [13] with the following final concentrations of the various components: 2.0 μM EF1A, 400 μM GTP, 8 mM PEP, 0.05 μg/μl pyruvate kinase and 0.70 μM [^{14}C]Phe-$tRNA^{Phe}$ (550 dpm/pmol). The final concentration of RNase A was 10 μg/ml in buffer PP6 (60 mM Tris-HCl, pH 7.6, at 4°C, 6 mM $MgCl_2$, 30 mM NH_4Cl, 30 mM KCl).

In the non-enzymatic assay we used 1 μM EF1A, 1 mM GTP, 2 mM PEP, 0.11 μg/μl PK and 0.3 μM [^{14}C]Phe-$tRNA^{Phe}$ (285 dpm/pmol) in buffer PP6. In the two last mentioned assays we carried out a control containing only aa-tRNA to test the behavior of free aa-tRNA in solution and compare it with the protection derived from aa-tRNA:EF1A:GTP complex formation.

3. Results

The yield of protein for $Glu^{272}Arg$ and $Arg/Glu/Glu^{272}Arg$ was 1.9 and 2.4 mg protein/g of cell pellet, respectively, and corresponding GDP binding activities were 50% and 40% of the total protein. The purities were 95% for both as determined by SDS-PAGE.

All of the assays conducted to test the GDP and GTP binding capacity of the mutants showed that the proteins were perfectly capable of binding the nucleotides in a fashion similar to that of the wild type. The temperature denaturation assay showed no significant differences from the wild type. The $\theta_{1/2}$ (temperature at which the activity of the protein is 50% of the maximum under the given conditions) was calculated. Wild-type EF1A values were 46.5 and 40.2°C for the GDP and the GTP forms, respectively. A decrease of only 3–4°C and 2°C, respectively, was observed for the mutants. The assays related to aa-tRNA binding clearly showed that $Glu^{272}Arg$ was unable to bind aa-tRNA. In the native PAGE, two bands can be seen for the wild type (Fig. 1, panel A). The upper band corresponds to the EF1A:GTP complex, and the

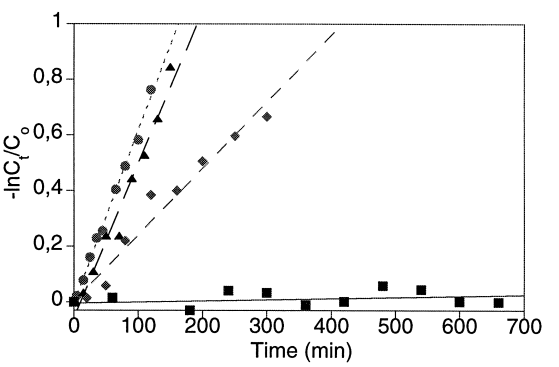

Fig. 2. Protection of the Phe-$tRNA^{Phe}$ against non-enzymatic hydrolysis at 20°C. Symbols: ■, wild-type EF1A; ▲, $Glu^{272}Arg$; ◆, $Arg^{7}Glu/Glu^{272}Arg$; ●, control (free Phe-$tRNA^{Phe}$).

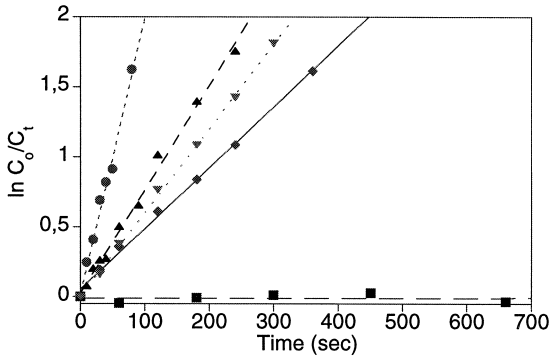

Fig. 3. Apparent dissociation rate constants for the ternary complexes at 0°C determined using the RNase-protection assay. Symbols: ■, wild-type EF1A; ▲, Glu^{272}Arg; ▼, Arg^{7}Glu; ♦, Arg^{7}Glu/Glu^{272}Arg; ●, control (free Phe-tRNA^{Phe}).

lower band to the ternary complex EF1A:GTP:aa-tRNA, that migrates faster due to the negative charges of the phosphate backbone of tRNA. For Glu^{272}Arg only the upper bands corresponding to EF1A:GTP were present (Fig. 1, panel D).

The double mutant Arg^{7}Glu/Glu^{272}Arg showed a slight recovery of activity (Fig. 1, panel B). In the native PAGE assay, which is very sensitive to even weak interactions, it can be seen that for this mutant a weak interaction between EF1A:GTP and Phe-tRNA^{Phe} is taking place. The bands in the last lanes (5, 6 and 7), corresponding to the highest concentrations of aa-tRNA, are more diffuse, which means that an unstable interaction is present though not strong enough to sustain a stable ternary complex, i.e. the binary complex is released gradually during electrophoresis. Our previous mutant protein Arg^{7}Glu was also tested and shows a very slight temporary interaction only visible with 200 and 400 pmol Phe-tRNA^{Phe} (Fig. 1, panel C) whereas Arg^{7}Glu/Glu^{272}Arg reflects this slight interaction already at 100 pmol aa-tRNA. When the four native gels (Fig. 1) are compared, there is a gradual loss of interaction from the fully stable wild type (panel A) to the non-interacting Glu^{272}Arg (panel D). The results of the two other assays (non-enzymatic hydrolysis of the aminoacyl bond and ribonuclease digestion rate assay) also show this tendency. Fig. 2, presenting the results of the non-enzymatic assay, shows the plot of ln C_o/C_t vs. time, where C_t and C_o represent the concentration of ternary complex at time t and the initial concentration of the complex, respectively. From this graph the time at which Phe-tRNA^{Phe} presents 50% spontaneous deacylation, $T_{1/2}$, can be obtained. The values for the experiment are presented in Table 1. Correlation coefficients for the mutants are all above 0.98. Wild-type aa-tRNA:EF1A:GTP remains unhydrolysed for at least 24 h.

Fig. 3, displaying the results of the RNase assay, presents the plot of ln C_o/C_t vs. time with C_t and C_o being the concentration of ternary complex at time t and the initial concentration of the complex, respectively. The apparent dissociation rate constant k_{-1} for aminoacyl-tRNA can be obtained as the slope of the plotted line. The results of the determination of k_{-1} are presented in Table 1.

When the double mutant is compared with Glu^{272}Arg or Arg^{7}Glu it can be seen that some aa-tRNA-binding capacity has been reestablished. The differences in the k_{-1} and $T_{1/2}$ values (Table 1) are reproducible and show that some significant interaction takes place. However, the resulting protection is still weak and not sufficient to stabilize a ternary complex as was already seen in the native PAGE (Fig. 1).

4. Discussion

This study shows that Glu^{272} (Asp^{284} *T.aq.* EF1A) is needed, indirectly, for aa-tRNA binding and enforces our hypothesis that both Arg^{7} and Glu^{272} (Asp^{284}) take part in a tight non-covalent binding between domains 1 and 2 of EF1A:GTP. The lack of this contribution to the stability of the overall structural rearrangement impairs aa-tRNA binding, even though both Arg^{7} and Glu^{272} are situated at the opposite face of the aa-tRNA site on EF1A [1,6].

The mutations introduced in the protein did not abolish the main properties of the protein with respect to binding of GDP or GTP, since all of the assays conducted to test this function showed no significant differences when compared to wild-type EF1A. Thus the overall spatial structure of the protein is maintained. This is of particular interest when we consider the drastic effect produced on binding of aa-tRNA, the major function of the protein, especially for the Glu^{272}Arg mutant.

The results of the native gels shown in Fig. 1 reflect clearly how Glu^{272}Arg cannot sustain ternary complex formation and suggest this need for a perfect overall structure to accomplish aa-tRNA binding. This idea is confirmed by the non-enzymatic hydrolysis of the aminoacyl bond assay and the ribonuclease digestion rate assays, shown in Figs. 2 and 3, where Glu^{272}Arg behaves as the control (no EF1A added) clearly suggesting that there is no direct interaction between aa-tRNA and the mutant. Native PAGE can reflect very subtle interactions ranging from a complete absence of binding to strong formation of the ternary complex, so it is very suitable for revealing the extent of the interactions between EF1A and aa-tRNA. These different degrees of interaction can be seen for example in our previous reports [16,17].

The results obtained by characterization of the double mutant Arg^{7}Glu/Glu^{272}Arg suggest that a weak interaction is

Table 1
Apparent dissociation rate constant k_{-1} for aa-tRNA and $T_{1/2}$, the time at which Phe-tRNA^{Phe} presents 50% spontaneous deacylation

	$k_{-1}\times10^4$ (s^{-1})	$T_{1/2}$ (min)
Control (without EF1A)	196 ± 9.00	113 ± 10
Wild-type EF1A	0.08 ± 0.50	> 1400
Glu^{272}Arg	74.40 ± 2.00	135 ± 4
Arg^{7}Glu	60.08 ± 0.80	n.d.
Arg^{7}Glu/Glu^{272}Arg	43.40 ± 1.00	289 ± 6

The values of the mean ± S.D. were obtained from several independent assays.
n.d., not determined.

possible when arginine and glutamate have their positions exchanged. We would have expected that, if there were no relationships between Arg^{7} and Glu^{272}, two mutations that separately give no interaction, as we have shown for $Glu^{272}Arg$ and $Arg^{7}Glu$, when combined, should be at least as deleterious. Instead, we observed an improvement in aa-tRNA binding though not as strong as the affinity observed for wild-type EF1A. The lack of full re-establishment of the salt bridge broken by the introduction of single-point mutations is not surprising, since salt bridge formation requires a precise geometry of the side chains involved, as shown by others [18]. The swapping of mutations probably leads to slight distortions of the backbone conformation thus disturbing the precise re-formation of the disrupted bond.

The experiments carried out in both this and previous work indicate that amino acids placed in loops or turns can have an important role in the function of the protein, at least in its active form. They are not only concerned in folding and stability [19].

The study of these amino acids also shows how very minor disruptions of the many different interactions that prepare the protein for its final function, binding and transport of aa-tRNA, prevent the attainment of the full function of the protein, even though they are situated in distant parts of the molecule, in flexible environments and at the opposite face of EF1A from the aa-tRNA contact surface.

Acknowledgements: We thank Associate Professor Jens Nyborg and Post-doctoral Fellow Asgeir Björnson for their valuable discussions and comments on the manuscript and Karen Margrethe Nielsen, Gitte Hartvigsen and Ph.D. student Gitte Pedersen for their technical assistance and patience. This work was funded by the Danish Biotechnology Programme, Centre for Interaction, Structure, Function and Engineering of Macromolecules (CISFEM). F.M.C. was funded by a FEBS short term fellowship and C.R.K. was funded by the Carlsberg Foundation.

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