Invariant amino acids essential for decoding function of polypeptide release factor eRF1

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ABSTRACT

In eukaryotic ribosome, the N domain of polypeptide release factor eRF1 is involved in decoding stop signals in mRNAs. However, structure of the decoding site remains obscure. Here, we specifically altered the stop codon recognition pattern of human eRF1 by point mutagenesis of the invariant Glu55 and Tyr125 residues in the N domain. The 3D structure of generated eRF1 mutants was not destabilized as demonstrated by calorimetric measurements and calculated free energy perturbations. In mutants, the UAG response was most profoundly and selectively affected. Surprisingly, Glu55Arg mutant completely retained its release activity. Substitution of the aromatic ring in position 125 reduced response toward all stop codons. This result demonstrates the critical importance of Tyr125 for maintenance of the intact structure of the eRF1 decoding site. The results also suggest that Tyr125 is implicated in recognition of the 3d stop codon position and probably forms an H-bond with Glu55. The data point to a pivotal role played by the YxCxxxF motif (positions 125–131) in purine discrimination of the stop codons. We speculate that eRF1 decoding site is formed by a 3D network of amino acids side chains.

INTRODUCTION

High fidelity of termination of protein synthesis ensures the formation of normal-sized polypeptide chains as encoded in the genome. Translational termination is known to be mediated by class-1 polypeptide release factors (RF1 and RF2 in prokaryotes, eRF1 and aRF1 in eukaryotes and Archaea, respectively) [reviewed in (1–5)]. When a nucleotide triplet (referred to as termination, or stop, or nonsense codon) in mRNA occupies the ribosomal A site, it is decoded by RFs at the small ribosomal subunit.

For prokaryotes, it has been suggested that the second and third nucleotides of the stop codons are decoded directly by a linear ‘protein anticodons’ (PAT in RF1 and SPF in RF2) (6–8).

For eukaryotes, it is now established that the specificity of stop codon recognition is associated with the eRF1 rather than the ribosome (9) and the recognition site is located in the N domain of eRF1 as is evident from genetic in vivo data (10), biochemical studies in vitro (11,12) and ‘molecular chimera’ approach (13). Data on multiple alignments of eRF1 amino acid sequences with different stop codon specificities are also consistent with this conclusion (14). The computer-assisted hypothesis suggesting the existence of so-called ‘terminator’ tRNAs inside the large ribosomal RNA (15) is inconsistent with the experimental data (9,13,16). Cross-linking experiments showing the close proximity of the A-site-located stop codon and eRF1 (17,18) and in particular cross-linking with a NIKS tetrapeptide of the N domain (19) are also consistent with the above mentioned conclusion.

There are significant differences between the data obtained by various approaches regarding the nature and position of amino acid residues of eRF1 implicated in decoding of the stop codons within the ribosome (10–14). This diversity of opinions is probably not associated with the origin of eRF1 since the eRF1 protein family is known to be highly conserved (20–22) and, for instance, the data obtained with yeast and...
human eRF1 should be fully compatible. Rather, the apparent controversies may be derived by a methodological distinction between in vivo and in vitro strategies or between genetic versus biochemical approaches.

Here, we introduced various amino acid substitutions into four positions in the N domain of human eRF1. Two of them, 55 and 125, are invariant in all class 1 eRF1s and aRFs and occupied by Glu and Tyr, respectively. Two other positions, 51 and 126, are variable in eRF1s from different species. All these positions were mentioned earlier as potentially essential for stop codon recognition based mostly on indirect evidence (10,14,22,23). The generated mutants were examined in vitro with respect to alterations of their functional and physical properties. Ability of mutated forms of eRF1 to trigger hydrolysis of fMet–tRNA (mimicking peptidyl–tRNA) in the ribosome was tested. Mutants were also studied by differential scanning calorimetry to evaluate stability of the protein globule after mutagenesis.

MATERIALS AND METHODS

Cloning and mutagenesis of human eRF1

The full-length cDNA encoding eRF1 with C-terminal His6-tag fusion was cloned into pET23b(+) vector (Novagen) under the control of phage T7 RNA polymerase promoter as described previously (11,24).

The mutagenesis procedure was simplified by introducing into human eRF1 cDNA a unique Bst98I site affecting neither amino acid sequence nor the reading frame of human eRF1. GeneEditor in vitro site-directed mutagenesis kit (Promega) was used with the RFBst primer 5′-CCATTCTTAAGGGGCAAAACGCAAGG-3′ (the Bst98I site underlined). The resulting construct pERF4B containing the unique Bst98I site within the gene encoding human eRF1 at positions 576–581 (T576C substitution) from the start ATG codon was used for mutagenesis of human eRF1.

The mutagenesis procedure was performed according to the PCR-based ‘megaprimer’ method (25). Two rounds of PCR were performed with the same PCR mixture. The PCR primers used for the generation of eRF1 mutants are available on request (kissel@eimb.relarn.ru). For the M51 and E55 mutants the direct primer (RFNde), 5′-GAGATATACA-TATGGCGGACGACC-3′ (the NdeI site underlined) together with one of the reverse primers for these mutants were used in the first round of PCR. In the second round the ‘megaprimer’ synthesized in the first round served as the direct primer together with the reverse primer (RFBst) 5′-CC-ATTCTTAAGGGGCAAAACGCAAGG-3′ (the Bst98I site underlined). For the Y125 and L126 mutants the direct RFBst primer together with one of the reverse primers for these mutants was used in the first round. In the second round the ‘megaprimer’ synthesized in the first round served as the direct primer together with the reverse RFNde primer. The resulting 590 bp PCR product was purified in low-melting NuSieve GTG agarose (FMC Bioproducts) using DNA extraction kit (Bio-Rad), hydrolyzed with NdeI and Bst98I, and ligated with pERF4B plasmid, treated with the same endonucleases. The ligated mixture was used for the transformation of Escherichia coli, strain Z85. The cloned DNAs were sequenced and appropriate clones were used for the expression of the mutant eRF1s. The first rounds of DNA amplifications were carried out in 25 μl reaction mixtures containing 20 ng of pERF4B DNA, 0.48 μM direct primer and 0.6 μM reverse primer, 0.2 mM each of four deoxyribonucleoside triphosphates, 1× of Pfu DNA polymerase reaction buffer and 0.75 U of PfuTurbo DNA polymerase (Stratagene). The denaturation was carried out at 95°C (3 min) for the first cycle, and at 94°C (30 s) for the next 20–25 cycles. The amplification included 30 s of the primer annealing (60°C) and 40 s for elongation (72°C). The second round of PCR was performed after addition to the PCR mixture of 12 pmol of the reverse primer (RFBst/RFNde) and 1.25 U of PfuTurbo DNA polymerase. Amplification with ‘megaprimer’ was performed for 28 cycles at 94°C for 30 s, the appropriate annealing temperature Tm for the reverse primer for 40 s and at 72°C for 140 s for elongation.

Expression and purification of human eRF1 and eRF3Cp

The wild-type human eRF1 and its mutants and the human eRF3Cp containing His6-tags at the C-termini were produced in E.coli, strain BL21(DE3), and purified using Ni-NTA Superflow (Qiagen), as described previously (26,27). The N-terminal amino acids (1–138) were absent in eRF3Cp without any influence on its GTPase activity.

Ribosomes

Rabbit reticulocyte 80S ribosomes washed with 0.5 M KCl were treated with puromycin and GTP for dissociation into subunits which were subsequently resolved by centrifugation in a 10–25% (w/v) sucrose gradient containing 0.3 M KCl, 3 mM MgCl2, 1 mM DTT and 20 mM Tris–HCl, pH 7.6. Before addition to the incubation mixtures, the subunits were combined in an equimolar ratio.

In vitro RF activity assay

The eRF1 activity was measured as described previously (20,28) at saturating levels (50 μM) of one out of the three stop-codon-containing tetraplets. The incubation mixture (25 μl) contained 20 mM Tris–HCl, pH 7.5, 15 mM MgCl2, 8 mM NH4Cl, 1.5 pmol [35S]Met–tRNA2Met–AUG–ribosome complex and 4 pmol of eRF1. The background was measured without tetraplet and subtracted from all values. AUG and ribotetraplets were synthesized by A. Veniaminova and M. Ryabkova (Institute of Chemical Biology and Fundamental Medicine, Novosibirsk).

GTPase activity assay

GTPase activity was followed by accumulation of [32P]P using a modified charcoal precipitation assay as described previously (29). Incubation mixture (50 μl) contained 20 mM Tris–HCl, pH 7.5, 30 mM NH4Cl, 15 mM MgCl2, 0.16 μM ribosomes, 0.16 μM human Cp and 10 μM [γ-32P]GTP (800–2000 c.p.m./pmol), human eRF1 was added in 0.04, 0.08 and 0.16 μM (final concentrations). Reaction was run at 30°C. 12.5 μl aliquots were withdrawn every 4 min and mixed with 0.5 ml of 5% activated charcoal suspension in NaH2PO4, cooled on ice. The mixture was vortexed and centrifuged at 9 300 g for 15 min at 4°C. Aliquot of supernatant (375 μl) was counted on scintillation counter.
Differential scanning calorimetry

Measurements were performed using differential adiabatic scanning microcalorimeter SCAL-1 (Scal Company, Pustchino, Russia) in 0.32 ml glass cells. The rate of heating was 1 K/min, in some cases 0.125, 0.5 and 2.0 K/min. Protein concentration ranged from 0.3 to 2.0 mg/ml. Partial molar heat capacity, \( C_p \) of proteins was determined as described previously (30). Partial molar protein volume was calculated from its amino acid composition as recommended (31). The precision of calorimetric enthalpy determination was 7 ± 1%; experimental error of \( T_d \) value did not exceed ±0.2°C. For analysis of melting curves the MinScal programme (SCAL Company) was used. Other details could be found elsewhere (32).

Calculation of free energy changes in human eRF1 mutants

We performed the analysis of free energy perturbation (FEP) caused by point mutations in human eRF1 as described previously (33,34). Briefly, we implemented our analysis basing on NAMD (http://www.ks.uiuc.edu/Research/namd/) FEP module and self-designed script for ICM (www.molsoft.com). We selected the N domain of wt-eRF1 (positions 5–146) from the eRF1 crystal structure (1d9) for introducing point mutations. Free energy calculations were performed as described previously (35,36). The N129A mutant was modeled by 3D-JIGSAW programme (www.bmm.icnet.uk/servers/3djigsaw/) and used as the model for introduction of double mutation N129A+F131A.

RESULTS

Selection of amino acids positions for mutagenesis

Multiple alignments of amino acid sequences of eRF1 and aRF1 are shown in Figure 1. The reasons to combine RFs from different kingdoms of living matter relay on the close structural homology between eRF1 and aRF1 (21). Evolutionarily distant eRF1s are selected including organisms with variant genetic codes, e.g. Euplotes, Tetrahymena, etc. (22).

In recently suggested models for decoding site of eRF1 several residues are mentioned (10,14,22,23), including positions 51, 55, 125 and 126 (Figure 1). Some of these positions are located on the surface of the eRF1 molecule as is evident from crystal structure (37) and for these reasons amino acid side chains in these positions are exposed to interaction with ligands, e.g. mRNA. Moreover, positions 125 and 126 are proximal to Cys127 which is known to be essential for decoding function of eRF1 (12). Positions 55 and 126 are proximal in space in eRF1 crystals (37). Collectively, these data point to the necessity to analyze possible involvement of positions mentioned above in decoding function of eRF1.

Positions 51 and 55

Position 51 is occupied in human eRF1 by Met residue which is not conserved and varies considerably in both eRF1 and aRF1 families (Figure 1). The RF activities of the M51L, M51Y, M51D, M51R and M51E mutants have been tested in an in vitro assay. All these eRF1 mutants retain full activity.

![Figure 1. Alignment of the N domain fragments from eRF1 and aRF1 primary structures. Residue numbering is based on human wild-type eRF1. The shortened Latin names are given. Accession numbers are in brackets. Hundred percent identical positions are black-shadowed; 80% identical positions are gray-shadowed. Similarity scoring is disabled.](image-url)
with any of the three stop codons indistinguishable from that of the wild-type eRF1 (data not shown). Since the substituting amino acids are very different from the initial Met residue and at the same time distinct among themselves, it implies that the eRF1 function is tolerant to the nature of amino acid in position 51.

In a sharp contrast with Met51, position 55 is occupied exclusively by Glu (Figure 1). This may point to an essential structural and/or functional role of this residue in eRF1/aRF1 protein superfamily. In fact, in E55A, E55S and E55Y mutants the UAG response was entirely abolished, whereas only about 2- to 3-fold reduction of the RF activity took place with UGA (Table 1). The UAG-selective inactivation was not due to inability of the mutant proteins to bind to the ribosome as the GTPase activity of eRF3 which was a measure of eRF1 function toward UAG. Clearly, this selective inactivation is not due to damage of binding to the ribosome: two other activities are not affected at all and the GTPase activity of eRF3 which is entirely dependent on the binding of eRF1 and eRF3 to the ribosome as mentioned above remains as in the presence of the wild-type eRF1 (Figure 2). Most likely, invariant Tyr125 is essential for the UAG stop codon recognition.

The neighboring variable L126 has been found to be non-essential for function since L126E, L126F and L126I mutants possess the same activity toward all three stop codons as the wild-type eRF1 (data not shown).

### Hydrogen bonding between Glu55 and Tyr125

As follows from the structural analysis (Figure 3A) positions 55 and 125 are close in space and can form a hydrogen bond between the hydrogen atom of OH group of Tyr125 and the oxygen atom of the COO group of Glu55. The length of the bond is equal to 1.73 Å (Figure 3B). This suggestion is consistent with the functional alterations observed with mutant eRF1. When in the E55A mutant the acceptor group disappears, the RF activity is completely lost toward the UAG stop codon (Table 1). It is also five times diminished if Glu is substituted with a shorter amino acid residue (Asp) leading to increase in the distance between the acceptor and donor groups forming the H-bond. The E55Q mutant has also a 3-fold reduced RF activity, possibly because the acceptor COO group is converted into a potential donor CONH$_2$ group. This group theoretically is able to form an H-bond with OH group of Tyr125 causing an increase of the distance between the interacting atoms (not < 2 Å). Moreover, due to the neighborhood of the aromatic ring the donor property of the Tyr125 OH group is significantly weakened making an H-bond between the Tyr125 and Gln55 unstable.

Our suggestion that Glu55 and Tyr125 form an H-bond is consistent also with other observations. All Tyr125 mutants tested so far (Table 1) reduce their RF activity if the donor group of the amino acid side chain is lost. To explain a rather surprising observation that the E55R mutant is completely active (Table 1), we have inserted Arg instead of Glu in the 3D structure of eRF1 (Figure 3C). It turned out from the model that an ‘inversion’ of the H-bond formation takes place. Instead of H-bond acceptor in position 55, Arg becomes a donor of H while oxygen of the Tyr OH group becomes an acceptor. The distance between H of the NH group (Arg55) and O of Tyr125 is equal to 1.82 Å (Figure 3C) which permits H-bond formation. This is possible because the strong NH group of Arg55 is able to bind even to weak OH acceptor group of Tyr125.

### Thermal denaturation of wild-type eRF1 and its mutants

In principle, point mutations of invariant amino acids if they play an essential role in maintenance of 3D structure of the protein may cause changes in protein stability. The destabilization

| Mutant eRF1 | Release activity (%) | UAAA | UAGA | UGAA |
|-------------|----------------------|------|------|------|
| E55A        | 59                   | 0    | 48   |      |
| E55D        | 72                   | 21   | 78   |      |
| E55Q        | 75                   | 35   | 80   |      |
| E55R        | 98                   | 97   | 104  |      |
| E55Y        | 11                   | 2    | 56   |      |
| E55S        | 27                   | 1    | 36   |      |
| Y125A       | 5                    | 6    | 8    |      |
| Y125E       | 12                   | 14   | 19   |      |
| Y125F       | 100                  | 34   | 100  |      |
| Y125S       | 17                   | 2    | 54   |      |

The table shows the release activity of human Glu55 and Tyr125 eRF1 mutants. The release activity of mutant eRF1s was measured according to the in vitro Caskey’s assay as described previously (20,28). Data are given in % versus the wild-type eRF1. Average values from three independent experiments run in duplicates are presented. One-letter amino acid code is used. An error in RF activity measurements varied from 8.5 to 10% for different mutants. Background values (without any stop codon) were subtracted everywhere.
of protein conformation can, in turn, causes a decrease or even abolishment of RF activity.

To examine this possibility, we have measured a thermal stability of eRF1 mutants in comparison with wild-type protein. As shown in Table 2, melting temperatures of the mutants differ insignificantly from the $T_d$ of wt-eRF1. The results mean that protein globule after point mutagenesis remains stable enough and the functional alterations (Table 1) are not associated with a destabilization of protein 3D structure. This conclusion is supported by analogous analysis performed with other eRF1 mutants (32).

The only apparent exception is Y125A mutant with $T_d = 2.8^\circ C$ (Table 2). However, most likely, it is not the cause of functional damage because, for example, a double mutant N129P + K130Q exhibits strong destabilization ($T_d = 7.9^\circ C$) (32), whereas its RF activity was only partially diminished toward UAG and was fully preserved versus two other stop codons (12).

Changes in the calculated free energy of eRF1 mutants

We have calculated the changes in free energy induced by point mutations in eRF1 as described in Materials and Methods. In addition to E55 and Y125 mutants, we have performed similar analysis for some previously described (12) mutants (positions 129 and 131). As seen from Figure 4, changes in free energy of mutants in positions 55 and 125 are very small or negligible and do not exceed 20 kcal/mol. This result means that mutations in these positions do not destabilize the 3D structure of eRF1 in full agreement with calorimetric measurements (Table 2). At the same time the strong alteration of free energy in double N129AF131A mutant (Figure 4) is consistent with known involvement of these residues in intramolecular interactions (37) and with known profound changes of functional activity of this mutant (12). The case of this double mutant demonstrates the sensitivity of the used methods: free energy change calculated for the N129AF131A mutant is 90 kcal/mol (Figure 2), which correlates with $\Delta T_d = -6^\circ C$ (Table 2, N 10). In contrast, for E55 and Y125 mutants this free energy difference is $\sim 20$ kcal/mol and consistent with very small $\Delta T_d$ values (Table 2). The Y125F is the only mutation tested so far that slightly stabilizes eRF1 molecule (Figure 4).

DISCUSSION

Invariant amino acids, such as Glu55 and Tyr125, may be essential for maintenance of spatial protein structure and/or for decoding function of eRF1. We attempted to follow the consequences of point mutagenesis of Glu55 and Tyr125 using three strategies. First, we measured the thermal stability of eRF1 mutants by differential scanning microcalorimetry and found that the destabilization of protein conformation is in most cases negligible with one exception for Y125A mutant where decrease in melting temperature is meaningful (Table 2). This observation implies that these positions in protein structure are not critical for protein stability as anticipated from surface location of both residues in protein globule (37). Second, we calculated possible changes of free energy of eRF1 mutants in comparison with wild-type protein and found very small alterations (Figure 4). This indicates that the introduced substitutions do not disturb the protein conformation. These data are fully consistent with the calorimetric

![Figure 2](image-url)
Third, we measured the ability of eRF1 mutants to induce GTPase activity of eRF3 within the ribosome (Figure 2). We have shown earlier that this assay reflects the ability of mutated eRF1 to bind to the ribosome (29) and is sensitive to some mutations in the N domain of eRF1 (12, 24). For all Glu55 and Tyr125 mutants tested so far, their ability to activate eRF3 GTPase within the ribosome was the same as for wt-eRF1. It means that codon-independent binding ability of mutated eRF1 forms toward the ribosome remains undamaged.

Collectively, all three approaches speak against the possibility that functional consequences of eRF1 mutagenesis are associated with protein destabilization or distortion of the 3D structure.

Substitution of the aromatic ring in position 125 makes the eRF1 almost silent toward two or three stop codons (Table 1).
This result shows the initial importance of Tyr125 for maintenance of the intact structure of the decoding site.

The UAG response is affected most strongly in the Glu55 and Tyr125 mutants (Table 1). This is the only stop codon which has G in the third position while it has common U with two other stop codons and shares A with UAA. Consequently, Glu55 and Tyr125 are implicated in the recognition of the G base in the UAG stop codon.

We assume that between Glu55 and Tyr125 an H-bond is formed. This interaction may help to preserve close proximity of the two remote regions of the N domain. Parallel UAG-selective loss of RF activity in E55A and Y125F mutants suggests that preservation of the H-bond between these residues is a prerequisite for UAG-dependent RF activity. The role of Glu55 is less essential than Tyr125 because other amino acids in position 55 are able to partially substitute Glu and, most surprisingly, Arg55 is as active as Glu55 (Table 1). Therefore, we suggest that in the Glu-Tyr pair the major role is played by Tyr, whereas Glu is most likely required for H-bond formation with Tyr125 to fix it in a proper orientation.

Since the functioning eRF1 is located within the ribosome the Glu55-Tyr125 pair is probably surrounded by hydrophobic environment and therefore water molecules will probably not interfere with H-bond formation. Similar behavior of intramolecular H-bonds in proteins has recently been discussed (38,39).

Although we consider E55 as invariant amino acid (Figure 1) it appeared very recently that in ciliate Loxodes striatus eRF1 (Swiss-Prot accession no. Q5CD84) this residue is substituted by Ala (40). L.striatus eRF1 corresponds in this position to one of our mutated forms of human eRF1 which has no RF activity toward UAG (Table 1). Based on our results we infer that this ciliate eRF1 possesses an altered stop codon recognition pattern lacking UAG response. This prediction is fully consistent with the fact that in this organisms only UGA servers as a single stop codon while two other stop codons are reassigned from sense codons (40).

The sets of amino acids recognizing A and G are different, for example, Ser and Thr form H-bonds with A, while G prefers Asn, Glu and Gly (41). We suggest that A and G in the same position are recognized by non-identical sets of amino acids.

In the previous work (12), an omnipotent human eRF1 has been converted into unipotent factor recognizing only UGA stop codon. The major role in this conversion is played by Cys127 and/or Phe131. From these data, it is evident that these amino acid residues can be implicated in recognition of A in the second stop-codon position. The Cys residue can form an H-bond with A while the aromatic ring of Phe can be involved in van der Waals interactions with a purine heterocycle (41).

Thus, two purines in the UAG stop codon require for specific discrimination at least four amino acids probably with a dominant role of Cys127 and Tyr125. The U base should be discriminated by no less than two amino acids as predicted by structural analysis (42). Therefore, we assume that stop codon triplet requires for its specific recognition more than three amino acid residues.

In previously published recognition models (14,23) Glu55 has been assumed to be involved in recognition of U, the first stop codon base. Our data clearly show that this amino acid residue is not implicated in discrimination of U. The most striking example is the E55R mutant, which is as active as the wt-eRF1 toward all three stop codons (Table 1). Tyr125 and Cys127 have been also assumed to recognize the first U (14). This hypothesis contradicts our previous data (12) showing the role of Cys127 in 2nd base discrimination but not the first. As shown here, mutations at Tyr125 do not profoundly impair UGA response (Table 1) and therefore, most likely, Tyr125 is not involved in U discrimination.

Leu126 has been proposed as an amino acid residue which recognizes the second stop-codon base (10,14). However, substitutions in this position as shown here exhibit no effect on RF activity toward all three stop codons.

In summary, the suggested models (10,14,23) are not supported by previous (12) and new (this work) data obtained by directed point mutagenesis followed by in vitro determination of RF activity versus all three stop codons. Previous models were based on indirect genetic data (10), bioinformatics approach (14,22) or molecular modeling (10,14,23).

We suggest that stop codon recognition is controlled by many amino acid residues (at present only part of them are identified) via a 3D network rather then by one amino acid–one nucleotide interaction as proposed in the prokaryotic recognition model (6). It is noteworthy that amino acid residues identified as being implicated in stop codon recognition by eRF1s (Glu, Tyr, Cys and Phe) are distinct from those involved in stop codon recognition in bacteria (Pro, Ala, Thr, Ser and Phe) with one exception of Phe. This remarkable difference most likely reflects profound dissimilarities between eRF1/aRF1 and RF1/RF2 protein families in their amino acid sequences (20,21,37) and 3D structures (37,43).

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