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Role of the individual domains of translation termination factor eRF1 in GTP binding to eRF3

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ABSTRACT

Eukaryotic translational termination is triggered by polypeptide release factors eRF1, eRF3, and one of the three stop codons at the ribosomal A-site. Isothermal titration calorimetry shows that (i) the separated MC, M, and C domains of human eRF1 bind to eRF3; (ii) GTP binding to eRF3 requires complex formation with either the MC or M + C domains; (iii) the M domain interacts with the N and C domains; (iv) the MC domain and Mg^{2+} induce GTPase activity of eRF3 in the ribosome. We suggest that GDP binding site of eRF3 acquires an ability to bind γ-phosphate of GTP if altered by cooperative action of the M and C domains of eRF1. Thus, the stop-codon decoding is associated with the N domain of eRF1 while the GTPase activity of eRF3 is controlled by the MC domain of eRF1 demonstrating a substantial structural uncoupling of these two activities though functionally they are interrelated.

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Key words: *termination of translation; GTPase; ribosome; guanine nucleotide binding; isothermal titration calorimetry; protein–protein interactions.*

INTRODUCTION

At termination of protein biosynthesis in eukaryotes one of the three stop codons, UAA, UAG, or UGA, located at the ribosomal A-site is decoded by class-1 polypeptide release factor eRF1 which triggers ester bond hydrolysis in peptidyl-tRNA at the ribosomal peptidyl-transferase centre (PTC) (for review see Refs. 1 and 2). eRF1 acts cooperatively with class-2 eRF3[3] which is a ribosome- and eRF1-dependent GTPase.[4] Human eRF1 consists of three domains—N, M, and C[5] (Fig. 1), which correlate with its three different functions. Highly conserved YxCxxxF and NIKS motifs of the N domain of eRF1 (Fig. 1) are implicated in stop codon decoding at the small ribosomal subunit.[1,6] The M domain of eRF1 is implicated in triggering the ester bond hydrolysis in peptidyl-tRNA at the large ribosomal subunit mediated by the invariant GGQ motif (Fig. 1).[5,7–13] The C domain of eRF1 binds to the C domain of eRF3[14,15]; this interaction is critically essential for GTPase activity of termination complex and fast termination reaction.[3] eRF3 in solution binds GDP while in the presence of eRF1 and Mg^{2+} it binds both GTP and GDP.[16,17] Presumably, a quaternary eRF1·eRF3·GTP·Mg^{2+} complex formed in cytoplasm enters the ribosomal A-site carrying a stop codon while the P-site is occupied by peptidyl-tRNA.[17]

The objective of this work is to clarify the following yet unanswered questions: (i) are the separated domains of eRF1 able to bind eRF3 in solution? (ii) which eRF1 domain(s) is (are) sufficient to induce GTP binding to eRF3? (iii) are the individual domains of eRF1 able to interact with each other in solution? (iv) which domain(s) of eRF1 is (are) able to induce GTPase activity of the eRF3·ribosome complex?

For quantitative thermodynamic characterization of interactions between the separated domains of eRF1 and between eRF1 domains and eRF3, isothermal titration calorymetry (ITC) has been applied, which is a sensitive and reliable technique for evaluation of protein–protein interactions.[18] Earlier, the ITC has been successfully applied to quantify binding of guanine nucleotides to eRF3.[17]

We have found that the N domain of human eRF1 is not involved in interaction with eRF3 whereas the MC domain binds as efficiently as a whole eRF1 molecule and induces GTP binding to eRF3 and GTPase activity of the eRF3·ribosome complex. The M plus C domains bind eRF3 and this ternary complex also acquires the ability to bind GTP.

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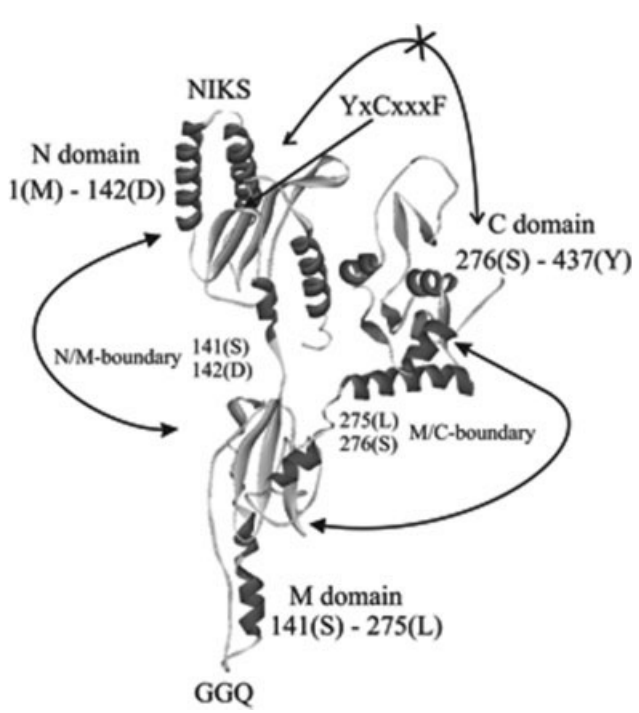

Figure 1

Domain organization of human eRF1 (PDB entry 1DT9). The boundaries between domains are shown. The functionally essential YxCxxxF, GGQ and NIKS motifs are indicated. Arrows show interactions between the domains or their absence (cross) as revealed by ITC in this paper.

METHODS

Cloning of the eRF1 domains

To obtain the separated eRF1 domains, fragments of the human *erf1* gene were PCR-amplified and cloned to pET23b(+) vector.[19] The resulting plasmids were used to express the gene fragments coding for the N, M, C, NM, and MC domains of eRF1 (Fig. 1), residues 1–142, 140–275, 275–437, 1–275, and 275–437, respectively.

Preparation of human eRF3, eRF1, and eRF1 domains

Human truncated eRF3 (amino acids 139–637), encoded by an essential gene GSPT1 (eRF3a), eRF1, and eRF1 MC, NM, N, M, and C domains, all of them tagged with a C terminal hexahistidine (for details see Ref. 19), were overproduced in *E. coli* C41(DE3) and purified using affinity chromatography on Ni-NTA agarose (Qiagen), HiTrap Q HP 16/25 5-mL anion-exchange, and HiTrap S HP 16/25 5-mL cation-exchange columns (Amersham Pharmacia Biotech). Peak fractions were collected and dialyzed against 0.1*M* KCl, 25 m*M* potassium phosphate, pH 7.5, 10% glycerol, 1 m*M* DTT, and 2 m*M* $MgCl_2$ (phosphate buffer) or against 0.15*M* KCl, 50 m*M* Tris-HCl, pH 7.5, 10% glycerol, 5 m*M* β-mercaptoethanol, and 2 m*M* $MgCl_2$ (Tris buffer). After dialysis the samples were used in ITC experiments. If necessary, the samples were concentrated by Centricon UFV4BGC25 tubes (Millipore). All protein preparations produced single bands after denaturing gel electrophoresis and corresponded to the molecular weight values deduced from the amino acid sequences.

GTPase activity of eRF3 in the ribosomes was inferred from the accumulation of $[^{32}P]P_i$, using adsorption of nucleotides on activated charcoal.[4] The reaction was run at 30°C. The amount of released $[^{32}P]P_i$ was estimated by scintillation counting. The $[^{32}P]P_i$ release resulted from spontaneous decomposition of $[\gamma\text{-}^{32}P]GTP$ was subtracted from all values. Free $[^{32}P]P_i$ accounted for less than 1% of the total radioactivity at time 0. In controls $[^{32}P]P_i$ was assayed in mixtures containing various combinations of two out of three essential components: eRF3 plus ribosomes, or eRF1 plus ribosomes, or eRF1 plus eRF3.

Isothermal titration calorimetry

The thermodynamic parameters of eRF1 binding to eRF3 and its domains, GDP and GTP were measured using a MicroCal VP-ITC instrument (MicroCal, Northampton, MA) as described elsewhere.[17] Experiments were carried out at 25°C in phosphate or Tris buffers. About 10-μL aliquots of ligands were injected into the 1.42-mL cell containing protein solution to achieve a complete binding isotherm. Protein concentration in the cell ranged from 6 to 30 μ*M* and ligand concentration in the syringe ranged from 50 to 300 μ*M*. The heat of dilution was measured by injecting the ligand into the buffer solution or by additional injections of ligand after saturation; the values obtained were subtracted from the heat of reaction to obtain the effective heat of binding. The resulting titration curves were fitted using MicroCal Origin software. Affinity constants (K_a) and enthalpy variations (ΔH) were determined.

RESULTS

Binding of eRF3 to individual domains of eRF1

Figure 1 shows the three-domain organization of human eRF1[5] and the sites where the polypeptide chain was cleaved to obtain individual domains.

On Figure 2 the upper panel presents the raw calorimetric data for the titration of eRF3 by ligands and the lower panel presents the binding isotherms. Complex formation between the MC domain and eRF3 is an enthalpy-favorable process and thermodynamic parameters of this binding are virtually the same as for the eRF1 binding to eRF3 (Table I). The dissociation constant (K_d) for the eRF1·eRF3 complex is 0.7 μ*M* and decreases 2.5-fold when eRF1 lacks its N domain. The C and M domains bind to

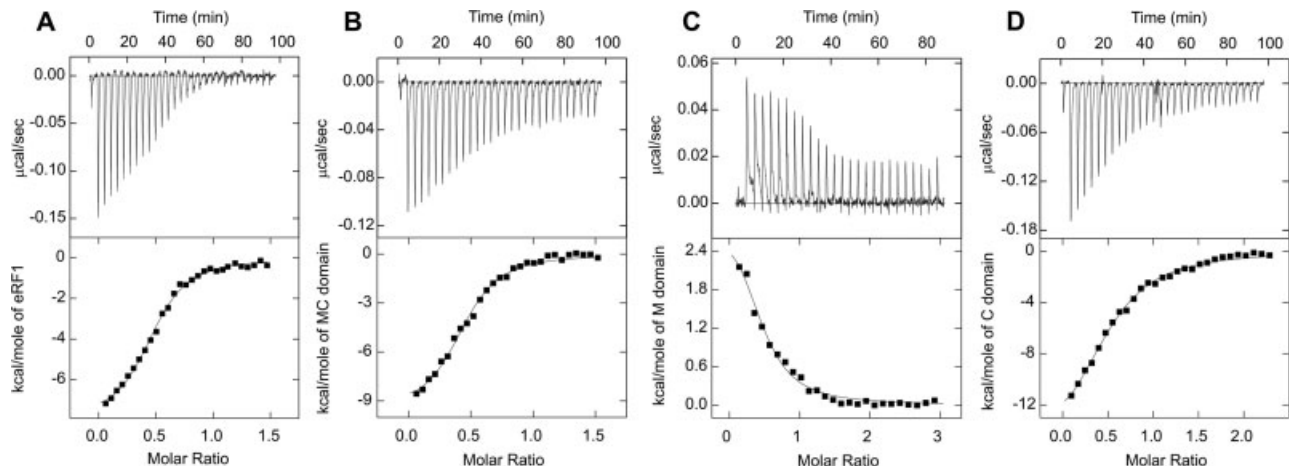

Figure 2

ITC curves (upper panel) and binding isotherms (lower panel) of eRF3 interactions with eRF1 (A), MC (B), M (C) and C domains (D) at 25°C in phosphate buffer, pH 7.5.

eRF3 with $\sim 1 \times 10^6\ M^{-1}$ association constants (K_a). However, complex formation between C domain and eRF3 is an enthalpy-favorable process, while binding of the M domain to eRF3 is entropy-favorable (Table I). The sum of enthalpy and entropy of M and C domains binding to eRF3 is roughly equal to the enthalpy and entropy of the MC domain binding. When the equimolar mixture of the M and C domains is titrated by eRF3, the binding parameters are very similar to those for eRF3 binding to the MC domain (Table I). Remarkably, the enthalpy of bind-

Table I

Thermodynamic Parameters of Human eRF3 Binding to GDP, GTP, eRF1, and its Separated Domains Determined by Isothermal Titration Calorymetry[a]

Sample[b]	Ligand	K_a[b] (M^{-1})	K_d[c] (µM)	ΔH[d] (kcal/mol)	$T\Delta S$[e] (kcal/mol)	ΔG[f] (kcal/mol)
eRF3	eRF1	1.4×10^6	0.7	−7.2[g]	1.2	−8.4
eRF3	N domain			ND		
eRF3	M domain	6.7×10^5	1.5	3.7[g]	11.6	−7.9
eRF3	C domain	1.0×10^6	1	−16.2	−8.0	−8.2
eRF3	MC domain	3.5×10^6	0.3	−8.8[g]	−0.1	−8.9
eRF3·M	C domain	1.3×10^6	0.8	−13.5	−5.2	−8.3
M + C	eRF3	2.7×10^6	0.4	−7.7	1.1	−8.8
eRF3	NM domain			ND		
eRF3	GDP	5.6×10^5	1.9	−9.2	−1.4	−7.8
eRF1·eRF3	GDP	5.1×10^5	2.0	−11.8	−4.0	−7.8
eRF3·C	GDP	4.8×10^5	2.1	−12.3	−4.5	−7.8
eRF3·M	GDP	7.7×10^5	1.3	−12.8	−4.8	−8.0
eRF3·MC	GDP	6.4×10^5	1.6	−11.2	−3.3	7.9
eRF1·eRF3	GTP	2.0×10^6	0.5	−2.2	6.4	−8.6
eRF3·C	GTP			ND		
eRF3·M	GTP			ND		
eRF3·MC	GTP	2.6×10^6	0.4	−2.8	5.9	−8.7
eRF3·(M + C)	GTP	7.5×10^5	1.3	−0.5	7.5	−8.0

ND, not detected; N, M, C, individual domains of eRF1; NM and MC, two domain eRF1 ones; M + C, equimolar mixture of two individual domains.

[a]All measurements were performed three or four times in 25 m*M* K_2HPO_4, 10% glycerol, 1 m*M* DTT, 0.1*M* KCl, and 2 m*M* $MgCl_2$. The stoichiometry of eRF3 binding to eRF1, its M and C domains and guanine nucleotides, calculated using ITC data, was below one and equal to 0.52 ± 0.15. This may be caused by partial inactivation of eRF3 during synthesis in *E. coli* or purification.[17,18]

[b]K_a, affinity constant; standard deviation did not exceed ±20%.

[c]K_d, dissociation constant; calculated as $1/K_a$.

[d]ΔH, enthalpy variation; standard deviation did not exceed ±8%.

[e]ΔS, entropy variation; calculated from the equation $\Delta G = \Delta H - T\Delta S$.

[f]ΔG, Gibbs energy; calculated from the equation $\Delta G = -RT\ln K_a$.

[g]Calculated taking into account the effect of protonation as described.[17]

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Table II
Thermodynamic Parameters of Interactions Between Individual Domains of Human eRF1 Determined by Isothermal Titration Calorymetry

Domains of eRF1						
Sample	Ligand	K_a (M^{-1})	K_d (μM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
C	M	4.8×10^5	2.1	1.5	9.2	−7.7
N	M	4.2×10^5	2.4	1.0	8.7	−7.7
N	MC	3.6×10^5	2.8	1.0	8.6	−7.6
C	N			ND		
NM	C	3.5×10^5	2.9	1.9	9.5	−7.6

Symbols, abbreviations and all other details are given in footnote to Table I. Stoichiometry of binding between domains of eRF1 was equal to 1.0 ± 0.1.

ing of the C domain to the eRF3·M domain complex is higher than the enthalpy of C domain binding to eRF3 and is roughly equal to the sum of enthalpies of the C domain interactions with M domain and eRF3. The NM and the N domains do not bind to eRF3 (Table I).

The alteration in the enthalpy of eRF1 binding to eRF3 ($\Delta H_{obs}(P_i) = -8.2$ kcal/mol) in phosphate buffer is significantly lower than in Tris buffer (ΔH_{obs}(Tris) = −19.2 kcal/mol).[17] This difference is caused by the changes in the protonation state of protein amino acid residues upon binding, because the enthalpy of ionization of Tris buffer is significantly higher than that of phosphate buffer. The observed binding enthalpy comprises association enthalpy (ΔH_b) and ionization enthalpy (ΔH_i) of the buffer according to equation $\Delta H_{obs} = \Delta H_b + (\Delta n)\Delta H_i$, where Δn is the number of protons released or taken by the protein on binding. For eRF1·eRF3 complex $\Delta n = -1.1$.[17] Hence, association of the factors is accompanied by proton release from the complex into solution caused by the changes of pK values of amino acid residue(s) upon complex formation. Similarly, ΔH_{obs} for the MC domain and eRF3 binding in phosphate, is much lower (−9.6 kcal/mol) than in Tris (−18.1 kcal/mol). Here $\Delta n = -0.85$, that is one proton is released into solution. When eRF3 binds the M domain, $\Delta n = -0.5$ [($\Delta H_{obs}(P_i) = 3.2$ kcal/mol, ΔH_{obs}(Tris) = −1.8 kcal/mol]. The binding enthalpies in phosphate and Tris buffers are similar for the complex formation between eRF3 and the C domain.

Interactions between separated domains of eRF1

The N domain binds to the M and MC domains but not to the C domain; the M domain binds to the N and C domains; the C domain binds to the M and NM domains (Table II). The binding constants for pairs of separated domains are similar and equal to ~4×10^5 M^{-1}. Interactions between domains are entropy-driven and thermodynamic profiles of binding are nearly the same (Table II). An increase of the entropic component during complex formation is frequently associated with structural effects based on the burial of hydrophobic surface area.[18] The large positive entropy of binding between separated domains of eRF1 indicates that these reactions are dominated by solvent rearrangement and hydrophobic forces.

Role of the M and C domains of eRF1 in the GTP binding to eRF3

The M, C, or MC domains of eRF1 exhibit no effect on GDP binding to eRF3 (Table I). GTP binds to eRF3 only if the MC·eRF3 complex is formed. Moreover, the constant of GTP binding to the MC·eRF3 complex is virtually the same as for the eRF1·eRF3 complex and is equal to 2.6×10^6 M^{-1}. The ternary complex composed of eRF3, M, and C domains binds GTP by 3.5 times weaker ($K_a = 7.5 \times 10^5$ M^{-1}).

GTPase activity of eRF3 measured in the presence of the ribosomes, eRF1, or its MC domain increases with increasing eRF1 or MC domain concentration (Fig. 3) and is nondetectable in the absence of either eRF1 or the MC

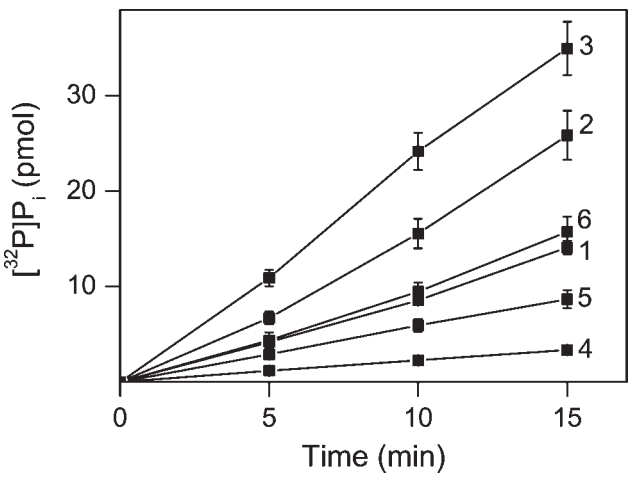

Figure 3
GTPase activity of eRF3 in the presence of ribosomes and eRF1, or its MC domain as inferred from [^{32}P]P_i accumulation. Curves 1-3 – correspond to 0.5, 1 and 2 pmol of eRF1; curves 4-6 – correspond to 0.5, 1 and 2 pmol of MC domain. The results were averaged from three independent experiments.

domain, eRF3, or the ribosomes. The lack of GTPase activity in the absence of ribosomes (data not shown) shows that the protein preparations were free from admixtures of exogenous GTPases, for example, *E. coli* GTPases. In the presence of the MC domain, the GTPase activity of eRF3 is about four times lower than with full-length eRF1 (Fig. 3). The N, M, C, and NM domains did not induce GTPase activity under the same conditions (data not shown).

DISCUSSION

We uncovered for the first time that the M domain of eRF1 binds to eRF3 (Fig. 2 and Table I). A common view based on *in vivo* double-hybrid and *in vitro* truncation experiments was that only the C domain of eRF1 is able to interact with eRF3.[14,15] Interaction of the C domain of eRF1 indeed considerably contributes to eRF1 binding to eRF3 but the M domain is clearly involved (Table I). The role of the M domain was not recognized earlier mostly because this interaction was masked by the major contribution of the C domain.

Since binding of the separated M and C domains to eRF3 is independent and additive (Table I), it means that the M domain binds directly to eRF3 protein, not via interaction with the C domain which takes place in the absence of eRF3 (Table II). Involvement of two out of three eRF1 domains shows that the interface of the eRF1·eRF3 complex are larger than it was assumed before.

In the M domain of yeast eRF1, the Gln residue in the GGQ motif can be methylated *in vitro* only in the presence of eRF3 and GTP.[20] It may indicate that eRF3 affects the conformation of the GGQ motif making it accessible to catalytic action of N^5-glutamine methylase. Interestingly, the NM domain is unable to bind eRF3 in contrast to the isolated M domain (Table I). Most likely, the N and M interaction (Table II) blocks or interferes with the eRF3 and M domain binding, for example, if the same molecular surface of the M domain is involved in both the N domain and eRF3 binding. The MC and N domains binding revealed by ITC (Table II) is undoubtedly due to the M and N domains interaction since the N and C domains do not interact (Table II). The NM domain interaction with the C domain (Table II) is due to the M and C binding because the N and C domains do not interact (Table II).

Discussing the data summarized in Table II, one has to keep in mind two essential aspects: all above mentioned interactions are revealed in solution, not inside the ribosome where the conformation could be altered significantly at least for eRF1.[21] Apart of that the mobility of isolated domains not bound covalently as in the whole molecule of eRF1 should be higher. Hence, some interactions can appear unfavorable in the whole eRF1 due to restricted flexibility of the domains. On the other hand, melting of the eRF1 molecule revealed by differential scanning calorimetry[22] argues in favor of interdomain interactions.

A common feature of class-2 termination factors RF3 and eRF3 is their ability to bind GDP.[16,17,23] GTP does not bind to eRF3 unless eRF1 is added to form a quaternary complex in the presence of Mg^{2+} which stimulates GTP binding to the eRF1·eRF3 complex.[17] What is the role of eRF1 in GTP binding to eRF3? The inability of the N domain of eRF1 to interact with eRF3 is consistent with the observation that the MC domain not only effectively binds to eRF3 but is also able to induce GTP binding to eRF3 (Table I). Apart of that, the MC domain efficiently stimulates GTPase activity of eRF3 within the ribosome (Fig. 3, curves 4–6). Furthermore, the eRF3, M, and C domains of eRF1 form a ternary complex which is also capable to bind GTP (Table I). Moreover, parameters of eRF3 binding to the M and C domains are roughly equal to the MC interaction with eRF3. Binding enthalpy of the C domain with the eRF3·M complex is approximately equal to the sum of enthalpies of interactions of the C and M domains and the C domain with eRF3. It implies that the C domain is potentially able to bind at once with the M domain to eRF3. Thus, ITC data argue in favor of specific complex formation between the M and C domains of eRF1, which can bind eRF3 (Table I). This ternary complex acquire the ability to bind GTP in the presence of Mg^{2+} (Table I).

Formation of the GTP binding site is not dependent on the decoding properties of eRF1 mediated by its N domain in accord with early observation[4] that GTPase activity of the ribosome bearing eRF1 and eRF3 is entirely independent of the presence of stop codons in the ribosome. The uncoupling between stop codon decoding and ribosomal GTPase activity means that the energy of GTP hydrolysis is not required for stop codon decoding but probably ensures fast termination reaction where eRF1 and eRF3 act cooperatively.[3] How to reconcile this apparent contradiction? The N domain of eRF1 is tightly fixed at the small ribosomal subunit via binding to the stop codon.[24] However, eRF1 molecule is flexible enough and its shape is Y-like, (not L-like as tRNA) both in crystal and in solution. Therefore, to place the GGQ motif properly at the PTC, one has to induce a significant conformational change of eRF1 to adopt it to the cavity of the A site. In fact, this large conformational change was observed experimentally in a reconstituted *in vitro* system of translation termination[3] and modeled by molecular dynamics.[21] We suggest that the GTP hydrolysis is necessary to induce conformational changes of eRF1 for proper positioning of the GGQ loop by changing the angle between the N and M domains of eRF1 at the PTC. This could be achieved indirectly by changing eRF3 conformation from GTP- to GDP-bound form with subsequent change of interaction with eRF1, or by using free energy of GTP hydrolysis to change the eRF1 conformation.

How the MC domain or the M plus C domains affect the eRF3 conformation making it capable of GTP binding? The GDP binding site is preformed in eRF3[25] because it

 DOI 10.1002/prot

binds GDP. Hence, the problem is to place the γ-phosphate of GTP in the enzyme active site. Two possible mechanisms could be considered. Either the MC domain of eRF1 changes the GDP-binding site of eRF3 in such a way that it acquires a property to bind GTP (for example, one of the positively charged side groups of eRF3 near the active site becomes exposed to γ-phosphate of GTP) or the MC domain is a donor of some chemical group(s) that via interaction with the GDP-binding site of eRF3 converts it into GTP/GDP-binding site. This latter suggestion considers eRF1 as a GAP (GTPase activating protein). Further studies will have to shed light on those mechanisms.

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