The life and death of translation elongation factor 2

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Abstract
eEF2 (eukaryotic elongation factor 2) occupies an essential role in protein synthesis where it catalyses the translocation of the two tRNAs and the mRNA after peptidyl transfer on the 80 S ribosome. Recent crystal structures of eEF2 and the cryo-electron microscopy reconstruction of its 80 S complex now provide a substantial structural framework for dissecting the functional properties of this factor. The factor can be modified by either phosphorylation or ADP-ribosylation, which results in cessation of translation. We review the structural and functional properties of eEF2 with particular emphasis on the unique diphthamide residue, which is ADP-ribosylated by diphtheria toxin from Corynebacterium diphtheriae and exotoxin A from Pseudomonas aeruginosa.

Introduction
In the elongation cycle of protein synthesis, elongation factor 1A delivers aminoacylated tRNA to the ribosomal A-site, whereafter the peptidyl transferase centre catalyses formation of the peptide bond leaving a deacylated tRNA in the ribosomal P-site and the newly formed peptidyl-tRNA in the A-site. This pre-translocational state of the ribosome is the substrate of the GTPase eEF2 (eukaryotic elongation factor 2) (reviewed in [1]), which catalyses the co-ordinated movement of the two tRNA molecules, the mRNA and conformational changes in the ribosome.

The bacterial factor EF2 (formerly EF-G) and the archaeal aEF2 are structurally and functionally homologous with eEF2. Both eEF2 and EF2 contain six structural domains (Figures 1 and 2). Domains I–V have folds as first described for the bacterial EF2, although insertions are found in domains I, II and IV of eEF2 [2,3]. Like other GTP-binding proteins, domain I contains five conserved sequence elements (G1–G5), forming the binding pocket for GTP/GDP. The G′ domain in eEF2 is somewhat bigger and shares no structural similarity with the corresponding domain in EF2. eEF2 is a highly elongated molecule with interesting dynamic properties [2]. Crystal structures have shown two conformations related by a quite large conformational change involving domain rotations of up to 75° (Figure 2). The apo-conformation of eEF2 is rather similar to the conformation of bacterial EF2 in complex with GDP, while binding of the fungicide sordarin to eEF2 induces a conformation so far not observed in EF2 [2]. The molecule contains three structural blocks (domains I–II, domain III and domain IV–V), which can move relative to each other. No structure is yet known of either eEF2 or EF2 in complex with GTP, but, since the G3 region (also called switch II) is expected to change significantly in response to GTP hydrolysis and is located at the interface between the three structural blocks, it appears likely that GTP hydrolysis will be correlated with conformational changes in the factor. This is in agreement with elegant kinetic experiments on EF2 showing that GTP hydrolysis precedes and accelerates translocation [4], and that an EF2 molecule cross-linked across the domain I–V interface can still hydrolyse GTP, but is inactive in both translocation and turnover [5]. Therefore the energy derived from GTP hydrolysis is used for the promotion of conformational changes in the ribosome preceding translocation. A rather complete kinetic scheme for bacterial translocation has been deduced [6]. Unfortunately, similar kinetic studies of eukaryotic translocation is currently lacking, but the very high conservation of both ribosomes, tRNA and EF2 makes it likely that the mechanism of translocation is quite similar in all kingdoms.
Figure 1 | Structural alignment of eEF2 from Saccharomyces cerevisiae (EF2_YEAST) and EF2 from Thermus thermophilus (EFG_THETH)
The α-helices (circles) and β-strands (arrows) are shaded according to the domain structure. Secondary-structure elements from domains I, III and V are shaded light grey, while those of domains G′, II, and IV are dark grey. The G1-G5 motifs are indicated by black boxes, the two threonine residues phosphorylated by the eEF2 kinase are marked with 'P', and the diphthamide (encoded as histidine) is labelled with an asterisk (*).

Cryo-EM (electron microscopy) reconstructions of both sordarin-stabilized eEF2–80 S [7] and antibiotic/GDPNP-stabilized EF2–70 S complexes [8–10] have been conducted, and support a similar mechanism of translocation in bacteria and eukaryotes. The docking of the sordarin-induced conformation into the 12 Å (1 Å = 0.1 nm) density of eEF2 required only rigid body rotation of two large structural blocks, whereas docking of EF2 into cryo-EM reconstruction maps of EF2–70 S complexes at 11–18 Å resolution required docking of individual small domains, which may be less reliable at low resolution. In the 80 S complex, eEF2 domains I and V interact with the 60 S subunit, and, in particular, the rRNA sarcin–ricin loop contacts the nucleotide binding pocket in domain I. Domain II interacts with the 40 S subunit, while domains III and IV are in contact with both ribosomal subunits. An important functional role of eEF2/EF2 domain IV is also evident from kinetic experiments with a domain-IV-deletion mutant of EF2, which still has ribosome-stimulated GTPase activity, but only catalyses translocation slowly and fails to be released from the ribosome after GTP hydrolysis [4].

The diphthamide
In contrast with bacterial EF2, eEF2 and eEF2 contain a unique and strictly conserved post-translationally modified histidine residue located at the tip of domain IV (Figure 2). Previously, NMR analysis suggested the residue to be a 2-[3-carboxamido-3-(trimethylammonio)-propyl] histidine, also referred to as diphthamide [11,12]. The biosynthesis of diphthamide is achieved by a complex stepwise addition to the
Figure 2 | Conformations of eEF2 from Saccharomyces cerevisiae

(A) eEF2-apo. (B) ADP-ribosylated eEF2 having both the antifungal inhibitor, sordarin (black), GDP (yellow) and Mg$^{2+}$ (cyan) bound to the factor. The three N-terminal domains of the two structures are superimposed and then translated horizontally. GDP and Mg$^{2+}$ shown with the eEF2-apo structure are modelled from the ADP-ribosylated eEF2 by superposition. The structural domains are coloured in different blue shades and indicated by numerals I-V, except for G’. ADPR-DIPH, ADP-ribosylated diphthamide.

Figure 3 | ADP-ribosylated diphthamide of eEF2 with interactions between the ADP-ribose (ADPR) and eEF2

Hydrogen bonds are shown in yellow, while potential interactions are shown in light blue.

histidine residue [13–19]. The first step is the transfer of the 3-amido-3-carboxypropyl group of AdoMet (S-adenosylmethionine) to the imidazole CE1 of the histidine residue and requires the action of the four proteins, referred to in yeast as Dph1–Dph4, of which Dph1–3 seem to associate to form a multimeric complex. In the next step, Dph5 is responsible for the trimethylation of the resulting amino group, again using AdoMet as the methyl donor. Finally, the carboxyl group of the diphthamide is amidated in an ATP-dependent manner by a yet unidentified enzyme. In yeast eEF2, the diphthamide is formed by modification of His$^{699}$. Recently, we modelled the diphthamide in a Fo–Fo electron density map from the 2.6 Å crystal structure of ADP-ribosylated yeast eEF2 [20] (Figure 3). Its structure was in agreement with the NMR analysis of the diphthamide, but furthermore suggested the chiral centre of the third carbon atom to be an R enantiomer.

The function of the diphthamide is still unknown. Interestingly, mouse and human DPH1 genes were identified previously as the tumour-suppressor gene, OVCA1 [21]. Knockout of one allele of DPH1 in mice results in increased tumour development, whereas loss of both alleles leads to death during embryonic development [22]. It is possible that the effects of Dph1 deficiency result from the lack of diphthamide on eEF2, and therefore the diphthamide may have an
important function in translation. On the other hand, several mutagenesis experiments resulting in strains that lacked the ability to form diphthamide or mutations of the diphthamide indicate that it is not strictly required for eEF2 function [16, 19, 23–25]. The cryo-EM studies of eEF2 in complex with the yeast 80S ribosome show that the area around the diphthamide at the tip of domain IV is close enough to interact with the ribosomal decoding site and, in particular, two universally conserved adenines in helix 44 of 18S rRNA [7], which serve to distinguish cognate from near-cognate codon–anticodon duplexes. This suggests that this area of eEF2, including the diphthamide, may somehow stabilize the codon–anticodon pairing during translocation, thereby preventing frame shifts. A cytoplasmic ADP-ribosyltransferase capable of ADP-ribosylating eEF2 in a wide variety of eukaryotic cell types has been identified [26–29], suggesting a possible regulatory mechanism of eEF2. The in vivo significance of this transferase activity, however, is still unclear, and the protein responsible for ribosylation is yet to be identified.

**Inactivation of eEF2 by kinases and bacterial toxins**

Both mammalian [30] and yeast [31] eEF2 can be phosphorylated by endogenous kinases which leads to translational down-regulation owing to a reversible inactivation of the factor. In rabbit eEF2, two threonine residues in the G2 region (also known as switch I) are modified [32], which correlates well with the lower affinity of phosphorylated rat eEF2 for GTP, while the affinity for GDP is unaltered [33]. The G2 region is likely to play an important role for binding/hydrolysis of GTP in the ribosomal context, but the region is disordered/unfolded and solvent-exposed in all known structures of eEF2 [2, 20] and thereby likely to be an excellent substrate for the kinase. In mammals, the eEF2 kinase belongs to the α-kinase family, and the phosphorylation of eEF2 is regulated by insulin, glutamate and β-adrenergic agonists (reviewed in [34]). Interestingly, phosphorylation of eEF2 is important in hibernating ground squirrels as part of their systematic down-regulation of energy-consuming cellular activities [35]. In yeast, phosphorylation of eEF2 is mediated by the Rck2 kinase and can be induced by osmotic shock [36].

The diphthamide is the exclusive cellular substrate for irreversible inactivation by DT (diphtheria toxin) from *Corynebacterium diphtheriae* and ETA (exotoxin A) from the otherwise unrelated *Pseudomonas aeruginosa* [37, 38]. Besides sharing the eEF2 diphthamide as substrate, the two catalytic domains of toxins can be aligned to a root mean square deviation of 2.1 Å and, in particular, have a common core fold around the NAD-binding site [39]. Both catalyse the transfer of ADP-ribose from NAD⁺ to the NE2 atom of the diphthamide imidazole ring in eEF2 [11, 12]. ETA is one of several *P. aeruginosa* virulence factors, and infections with *P. aeruginosa* are often observed in immunocompromised patients, where pronounced multidrug resistance makes antibiotic treatment difficult (reviewed in [40]). *C. diphtheriae* was one of the first toxin-producing bacteria to be discovered, and infection was previously considered as one of the most serious childhood diseases. Although most industrialized countries today have widespread immunization against diphtheria, some developing countries still experience outbreaks [41].

The mechanism for the ADP-ribosylation of the diphthamide by the toxin is still unclear. Several studies have shown that mutations of either the diphthamide or the proteins that synthesize the diphthamide confer resistance to both DT and ETA [16, 19, 23–25]. Kinetic data suggest that the ADP-ribosylation of eEF2 by ETA or DT is likely to be a SN1 substitution reaction involving an oxocarbenium cation [42, 43] and results in an inversion of configuration at the C1’ of the ADP-ribose [20]. This indicates that C1’ of NAD⁺ can only be attacked from one side by the nucleophilic NE2 atom of the diphthamide imidazole. The inversion is probably caused by the anomic carbon of the nicotinamide ribose being susceptible to a backside nucleophilic attack from the NE2 atom in diphthamide after cleavage of the nicotinamide ribose bond [42]. In addition, kinetic isotope effect experiments suggest that the complex reaches an early transition state either by activation of the leaving nicotinamide group or by increased stabilization of the oxocarbenium ion, which is not due to an increase in the nucleophilic character or participation from NE2 of the diphthamide. The relatively low nucleophilicity of the bulky diphthamide nucleophile at the proposed transition state could be a result of steric crowding and activation of transition state formation could result from eEF2 binding, which then increases the reaction rate [43]. Interestingly, the crystal structure of the ADP-ribosylated eEF2 showed that the interaction between Glu³⁵³ in ETA (or the corresponding Glu¹⁴⁶ in DT) and the nicotinamide ribose previously determined to be important for catalytic activity [44, 45] is replaced by the highly conserved Asp⁹⁶ in eEF2 after ADP-ribosylation (Figure 3) [20]. Near the end of the catalytic reaction, this interaction of Asp⁹⁶ in eEF2 with the 2’-OH of the N-ribose may orient the incoming cation until the covalent bond with the diphthamide imidazole is formed. In the structure of the ADP-ribosylated eEF2, the trimethylammonium group of the diphthamide seems to interact with the β-phosphate of the ADP-ribose (Figure 3) and may therefore have an important role in the reaction mechanism of ADP-ribosylation [20]. This is supported by previous work showing that the methylation of the diphthamide is very important for the ADP-ribosylating activity of the toxins. The precursor, diphthione, lacking the amide group, can still be ADP-ribosylated, although at a lower rate [15, 17].

Exactly how ADP-ribosylation of the diphthamide inhibits eEF2 function remains to be determined. Binding experiments of ADP-ribosylated eEF2 to the ribosome show a reduction of affinity for the pre-translocational ribosome, but no changes for the post-translocational ribosome [46]. Other competition and co-sedimentation experiments have shown that the ADP-ribosylated eEF2 is indeed able to form
a stable complex with the ribosome [20,47–49]. Another striking observation was that, while binding of GDP to wheat germ eEF2 was unaffected by ADP-ribosylation, GTP binding was significantly reduced, despite being approx. 60 Å away from the diphthamide [46,50]. These results disagree, however, with recent fluorescence spectroscopy experiments of nucleotide binding showing that ADP-ribosylation of yeast eEF2 has very little, if any, effect in binding both GTP and GDP [20]. In addition, other binding experiments have shown that ADP-ribosylated eEF2 still has ribosome-dependent GTPase activity and can dissociate from the ribosome [49].

Future perspectives

Although we have increased our understanding of eEF2 function, the exact mechanism of translocation remains to be determined, in particular how the energy derived from GTP hydrolysis is utilized for translocation. If the mechanism of translocation in eukaryotes and bacteria is conserved, hydrolysis of GTP by eEF2 is a quite early event and is used to greatly accelerate translocation. We do not know what triggers the GTP hydrolysis, but the sarcin–ricin loop is perhaps the only ribosomal element that comes close enough to interact with the nucleotide-binding pocket in eEF2 on the ribosome and therefore could be responsible for triggering the activity. Advanced kinetic studies similar to those conducted with great success for bacterial translocation are required urgently for a thorough mechanistic understanding of the translocation reaction in eukaryotes. In particular, such experiments may be required to elucidate the exact function of the diphthamide, which, because of its complicated synthetic pathway, surely must have an important function in the cell. The fact that the DPH1 gene has been shown to be a tumour-suppressor gene, playing an important role in cell proliferation, indicates that there could be a link between diphthamide and the control of cell growth.

To obtain a better understanding of how ETA and DT inactivate the elongation factor, it will be necessary to carry out further kinetic and structural studies of the interaction between eEF2 and the ribosylating toxins. Finally, a detailed understanding of how ADP-ribosylated eEF2 inhibits translation may require cryo-EM reconstructions of the modified factor in complex with the 80 S ribosome.

After submission of this paper, the structure of a mutant bacterial EF2 in complex with GDPNP has been published, and shows only very limited conformational changes compared with the structure of the EF2–GDP complex [51]. The recent structure of eEF2 in complex with exotoxin A explains the requirement of the diphthamide for ADP-ribosylation of eEF2 [52].

References


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