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*This paper is dedicated to Professor Maciej Wiewiórowski*
***Review***

# Possible evolution of factors involved in protein biosynthesis[o]

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**The elongation factors of protein biosynthesis are well preserved through out evolution. They catalyze the elongation phase of protein biosynthesis, where on the ribosome amino acids are added one at a time to a growing peptide according to the genetic information transcribed into mRNA. Elongation factor Tu (EF-Tu) provides the binding of aminoacylated tRNA to the ribosome and protects the aminoester bond against hydrolysis until a correct match between the codon on mRNA and the anticodon on tRNA can be achieved. Elongation factor G (EF-G) supports the translocation of tRNAs and of mRNA on the ribosome so that a new codon can be exposed for decoding. Both these factors are GTP binding proteins, and as such exist in an active form with GTP and an inactive form with GDP bound to the nucleotide binding domain. Elongation factor Ts (EF-Ts) will catalyze the exchange of nucleotide on EF-Tu.**

**This review describes structural work on EF-Tu performed in our laboratory over the last eight years. The structural results provide a rather complete picture of the major structural forms of EF-Tu, including the so called ternary complex of aa-tRNA:EF-Tu:GTP. The structural comparison of this ternary complex with the structure of EF-G:GDP displays an unexpected macromolecular mimicry, where three domains of EF-G mimick the shape of the tRNA in the ternary complex. This observation has initiated much speculation on the evolution of all factors involved in protein synthesis, as well as on the details of the ribosomal function in one part of elongation.**

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**Abbreviations:** A site, aminoacyl-tRNA site; cryo-EM, cryo electron microscopy; EF, elongation factor; IF, initiation factor; RF, release factor; aa-tRNA, aminoacylated tRNA; GDPNP, guanosine-5'-($\beta$,$\gamma$-imido)triphosphate; G-protein, GTP-binding protein; G-domain, GTP-binding domain; P loop, phosphate-binding loop; P site, peptidyl-tRNA site.

The transformation of the genetic information on DNA into actively functioning proteins in a living cell is a very complicated process. The information for a specific gene is first transcribed into a working copy on the mRNA. The ribosome together with this mRNA then performs the chemical production of the polypeptide with the correct amino-acid sequence coded for in the mRNA. This translation of genetic information is traditionally seen as happening in three separate phases: initiation, elongation and termination. At all three phases accessory protein factors are helping the ribosme to perform its function both fast and with high accuracy. Some of these protein factors are GTP binding proteins (G-proteins), which are active when bound to GTP (Bourne *et al.*, 1990; 1991).

In the bacterial system three initiation factors (IFs) are involved in correctly assembling the two ribosomal subunits at the start codon of the mRNA. One of these, the G-protein IF-2, binds to the specific initiator tRNA, fMet-$tRNA_i^{fMet}$, which by its anticodon recognizes the start codon. IF-2 also brings the initiator tRNA to the peptidyl-tRNA site (P site) on the ribosome. In the eukaryotic system the intiation phase is much more complicated and presumably under much more control than in the simpler system of prokaryotes (Merrick, 1994).

The termination phase is less well understood than the other two phases. It is controlled by release factors (RFs), of which two (RF1 and RF2) in the bacterial system recognize the stop codons. In this sense both RF1 and RF2 must be simulating the anticodon of tRNAs and it is well known that termination can be suppressed by special suppressor tRNAs which do recognize stop codons by codon-anticodon interaction. The function of RF1 and RF2 is stimulated by the G-protein RF3 and results in the release of the fully synthesized protein and separation of the ribosomal subunits and mRNA. In the eukaryotic system only one stop codon recognizing factor (eRF1) has been found (Zhouravleva *et al.*, 1995).

The elongation phase has been extensively studied over many years (Kaziro, 1978; Miller & Weissbach, 1977). It is also relatively well conserved through out evolution. Thus, mainly two elongation factors are involved in the process of sequential adding of amino acids to the growing polypeptide. Elongation factor Tu (EF-Tu; in eukaryotes EF-1$\alpha$) binds aa-tRNA and protects the aminoester bond from hydrolysis in the ternary complex aa-tRNA:EF-Tu:GTP. In the first part of elongation the ternary complexes will try to decode the mRNA codon in the ribosomal aminoacyl-tRNA site (A site) in very rapid competition with each other. Upon correct codon-anticodon interaction in the A site the ribosome will greatly stimulate the GTP hydrolysis on EF-Tu. The complex EF-Tu:GDP subsequently leaves the ribosome and the aa-tRNA is moved to the peptidyl transferase center of the ribosome. During the second part of elongation the elongation factor G (EF-G; EF-2) stimulates the translocation of tRNAs in the A and P sites together with mRNA so that the next codon is exposed in the A site. The peptidyl-tRNA is left in the P site, while deacylated tRNA is moved into the exit site (E site). EF-Tu is recycled into its active aa-tRNA binding form by the nucleotide exchange factor elongation factor Ts (EF-Ts; EF-1$\beta$).

The literature on the function and biochemistry of elongation factors (especially from *Escherichia coli*) is extensive but has been reviewed several times. Some useful reviews can be found in the References (Clark *et al.*, 1995; Faulhammer & Joshi, 1987). Structural studies on G-proteins have recently been reviewed, with special emphasis on the similarities and differences in the GTP binding domains (Kjeldgaard *et al.*, 1996). The present review will describe in some detail the structural studies from our laboratory on bacterial EF-Tu. Similar studies have been performed also in the laboratories of Dr. Reuben Leberman (MPI Heidelberg and EMBL Grenoble),

Prof. Mathias Sprinzl (University of Bayreuth) and Prof. Frances Jurnak (University of California at Riverside).

## RESULTS AND DISCUSSION

Structural studies on EF-Tu during the last decade have revealed models for the major functional states of this protein. The inactive form of EF-Tu:GDP has been determined from *Thermus aquaticus* (Polekhina *et al.*, 1996) and from *Escherichia coli* (Abel *et al.*, 1996; Polekhina *et al.*, 1996). The structure displays a three domain protein of about 400 amino acids (Fig. 1). The N-terminal domain (domain 1 or G-domain; about 200 amino acids) is a classical nucleotide binding domain in the sense that it contains a central six-stranded $\beta$-sheet, which is mainly parallel, surrounded by six $\alpha$-helices. The nucleotide binding site is found at the C-terminal edge of the $\beta$-sheet, where also a phosphate binding loop (P loop) and an $\alpha$-helix dipole is stabilizing the binding of the $\beta$-phosphate. Signature sequences typical of all G-proteins are found in this domain, and are for most parts directly involved in binding or specific recognition of the nucleotide. The structure of domain 1 of EF-Tu is thus typical of the G-domain of all G-proteins (Kjeldgaard *et al.*, 1996). Two regions of the G-domain called switch I (or effector loop) and switch II show structural variability according to the nature of the bound nucleotide. These regions thus function as external signals to other macromolecules. Domains 2 and 3 (both about 100 amino acids long) are $\beta$-barrels which are held together by strong interactions (Berchtold *et al.*, 1993). In all known structures they are found together as one structural unit.

When amino-acid sequences of EF-Tu (EF-1$\alpha$) from many organisms are compared it is seen that they are surprisingly well preserved through evolution (Fig. 2). The sequences tend to be a little longer for the higher organisms due to insertions in certain of the loops of the structure. One major insertion in archaebacteria and in eukaryotes is found in the switch I region (effector loop), most likely reflecting the likelihood that this region is the part interacting with the ribosome, which is larger in these kingdoms. Some variability is also found just before the final $\alpha$-helix of domain 1 probably because this part interacts with the nucleotide exchange factors, EF-Ts or EF-1$\beta$, which have no known sequence similarity. Some sequences have also a C-terminal extension. The high degree of conservation of sequence makes it very likely that the basic structure of EF-Tu and EF-1$\alpha$ is the same in all organisms. This points to the fact that protein biosynthesis and especially the elongation phase is a very fundamental biological process and that proteins and RNAs in translation represent very ancient macromolecules.

The structure of the active form of EF-Tu:GDPNP, where GDPNP is a nonhydrolysable analog, has been determined both from *T. thermophilus* (Berchtold *et al.*, 1993) and from *T. aquaticus* (Kjeldgaard *et al.*, 1993). The structure reveals how the introduction of a $\gamma$-phosphate at the nucleotide binding site, first induces a small local structural change where a peptide bond near the conserved Gly84 is flipped (Kjeldgaard *et al.*, 1996), but secondly produces a somewhat larger structural change of the two switch regions (Fig. 1). As the $\alpha$-helix in switch region II is part of the interface between domains 1 and 3, the final result is a very large change of the conformation of domain 1 relative to domains 2 and 3 (Fig. 1). This conformational change, corresponding to an approximately 90° rotation of domain 1 on the surface of domains 2 and 3, creates a completely new interface between the two parts of the molecule. One has to assume that during the transformation from the inactive to the active form of EF-Tu, domain 1 has to temporarily dissociate from domains 2 and 3, and then reassociate on the new interface (Kjeldgaard *et al.*, 1993). This is reminiscent of what happens in the hetero-

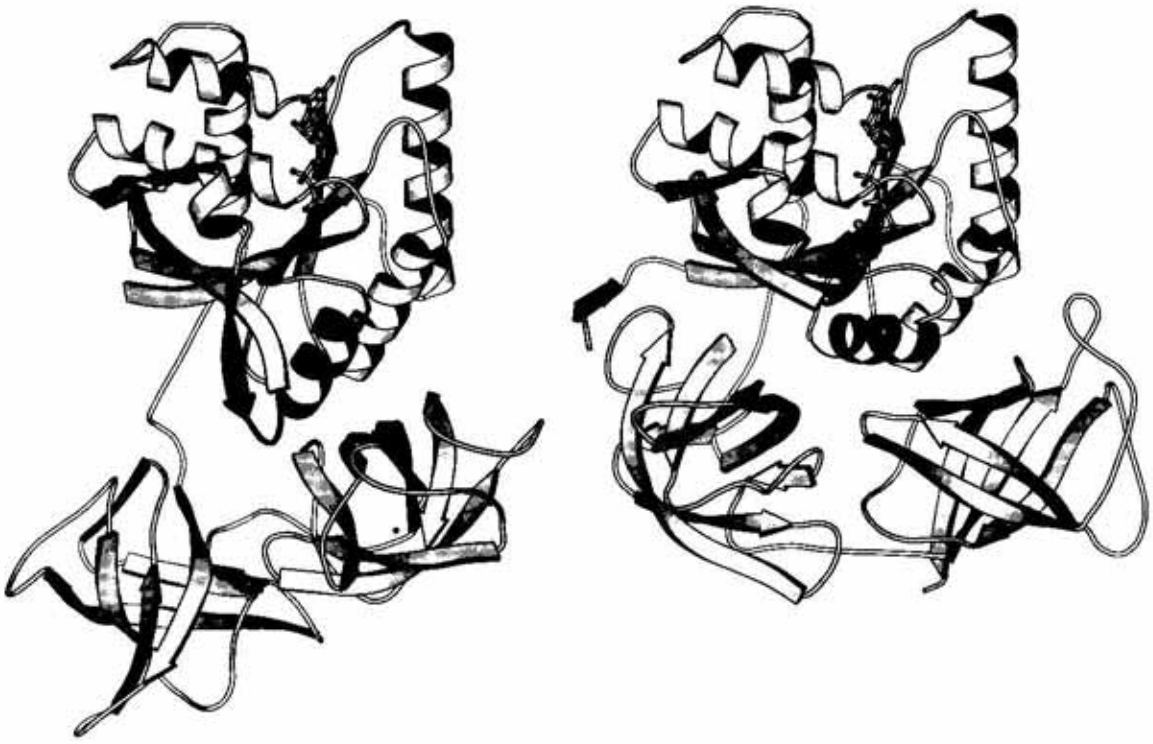

**Figure 1. Structures of *T. aquaticus* EF-Tu:GDP and EF-Tu:GDPNP.**

EF-Tu:GDP is to the left, with domain 1 at the top, domain 2 at the bottom left and domain 3 at the bottom right. Switch region I is light grey and switch region II is dark grey. EF-Tu:GDPNP is to the right. Bound nucleotides are shown in ball-and-stick model. The orientation of domain 1 of each molecule is kept the same in both structures. Notice the large rearrangement of domains 2 and 3. The Figure has been drawn using MOLSCRIPT (Kraulis, 1991).

trimeric G-proteins (Bourne *et al.*, 1990; 1991). These consist of three domains G$\alpha$, G$\beta$ and G$\gamma$, which are found in a complex when GDP is bound to G$\alpha$. The activated transmembrane receptor acts as a nucleotide exchange factor, and in the case of these G-proteins the G$\alpha$ subunit with bound GTP is released from G$\beta\gamma$. From the structure of the complex EF-Tu:EF-Ts from *E. coli* (Kawashima *et al.*, 1996) it can be seen, that part of the function of EF-Ts is to physically separate domain 1 from domain 3 during nucleotide exchange (S. Thirup, personal communication).

The structure of the ternary complex of yeast Phe-tRNA and *T. aquaticus* EF-Tu:GDPNP was determined a couple of years ago (Nissen *et al.*, 1995). It shows a very elongated complex in which the anticodon of the tRNA is pointing away from EF-Tu (Fig. 3). When compared to the structures of free Phe-tRNA (Jack *et al.*, 1976) and free EF-Tu:GDPNP (Kjeldgaard *et al.*, 1993) the components are very little altered in the ternary complex. The major difference is that the CCA-Phe end of tRNA is tucked away into a narrow cleft between domains 1 and 2. There is a major contact area between EF-Tu and tRNA involving the surface of domain 3 and one side of the T-stem helix. This does not make use of conserved residues of EF-Tu and only involves the backbone of the tRNA. It thus seems to be a rather unspecific interaction. It is interesting that this contact includes the ribose at position 64, which is phosphoribosylated in eukaryotic initiator tRNA (Basavappa & Sigler, 1991). Such a modification would prevent interaction with EF-1$\alpha$. The 5′-phosphate is specifically recognized in a small depression where all three domains come together. Conserved residues from all three domains are involved in this recognition. The single-stranded RNA at the 3′ end

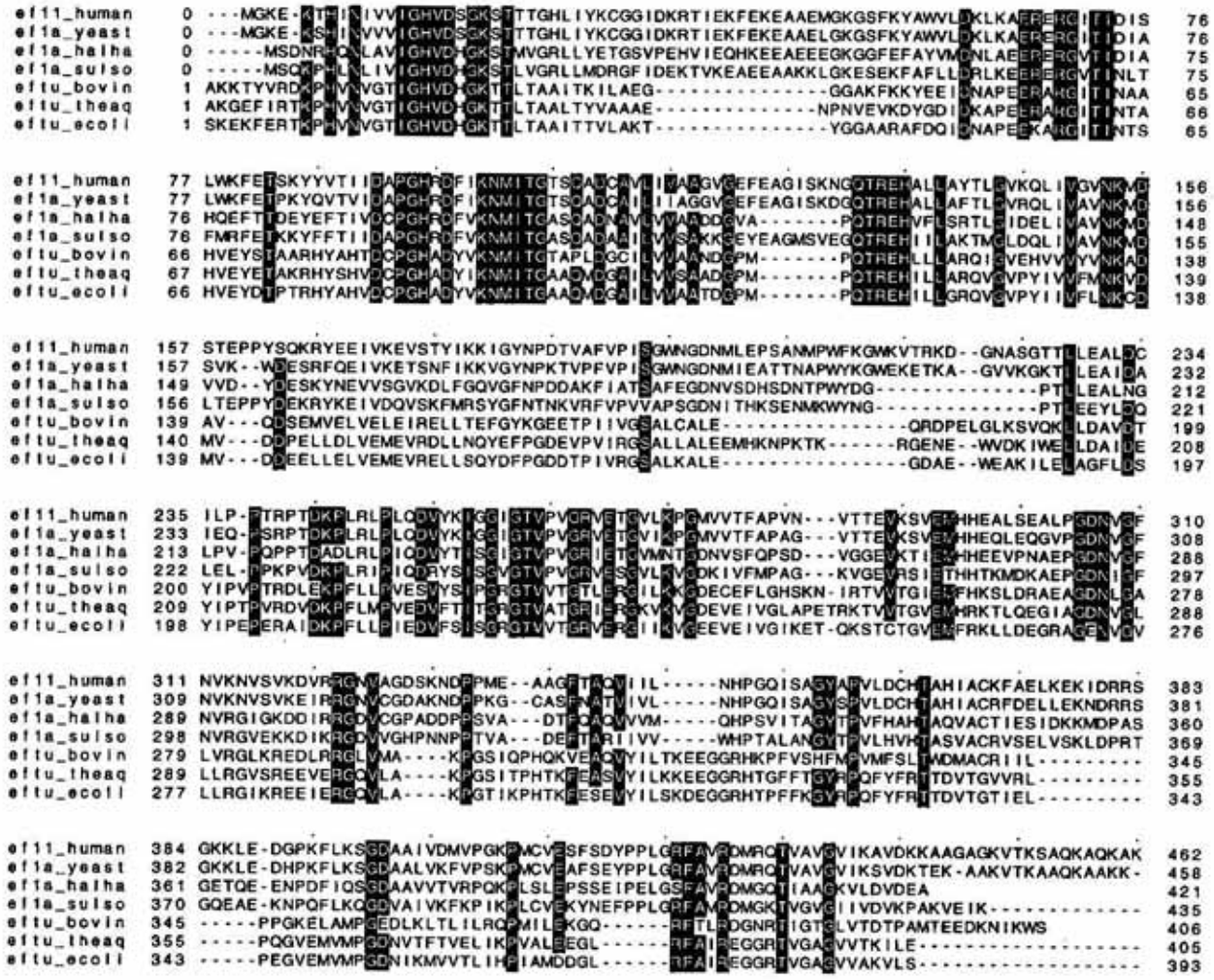

**Figure 2. Amino-acid sequence alignment of selected sequences of EF-Tu and EF-1$\alpha$.**

The top two sequences are from eukaryotes, the next two from archaebacteria, the next one from a mitochondrion, and the last two from prokaryotes. The alignment is performed by the sequence editor ALMA (Thirup & Larsen, 1990). The column at the left contains SWISS-PROT codes (Bairoch & Apweiler, 1998). These refer to: ef11_human, human EF-1$\alpha$ (Brands *et al.*, 1986); ef1a_yeast, baker's yeast EF-1$\alpha$ (Cottrelle *et al.*, 1985); ef1a_halha, *Halobacterium halobium* EF-1$\alpha$ (Fujita & Itoh, 1995); ef1a_sulso, *Sulfolobus solfataricus* EF-1$\alpha$, (Arcari *et al.*, 1993); eftu_bovin, bovine mitochondrial EF-Tu (Woriax *et al.*, 1995); eftu_theaq, *Thermus aquaticus* EF-Tu (Voss *et al.*, 1992); eftu_ecoli, *Escherichia coli* EF-Tu, (Jones *et al.*, 1980).

continues the curvature of the double helix of the acceptor stem, and the bases are stacked until the terminal A base, which is unstacked and bound to a specific binding pocket on the surface of domain 2 (Kjeldgaard *et al.*, 1993). This pocket has conserved (or conservatively substituted) hydrophobic residues on one side and a conserved Glu271 stacking on the A base on the other side (Fig. 4). This Glu271 also makes a hydrogen bond to the 2'OH of the ribose. Arg274 is contacting the terminal phosphate. The amino group of the attached amino-acid is held in a tight grip by backbone hydrogen bond donors and acceptors. There is no room for a formyl group, so that fMet-tRNA will not bind to EF-Tu. The amino-acid side chain is found in a pocket near His67, which was earlier crosslinked to aa-tRNA (Duffy *et al.*, 1981). The pocket is large enough to accommodate all amino acids. The relative positions of the pockets for the aminoester and the A base forces the pucker of

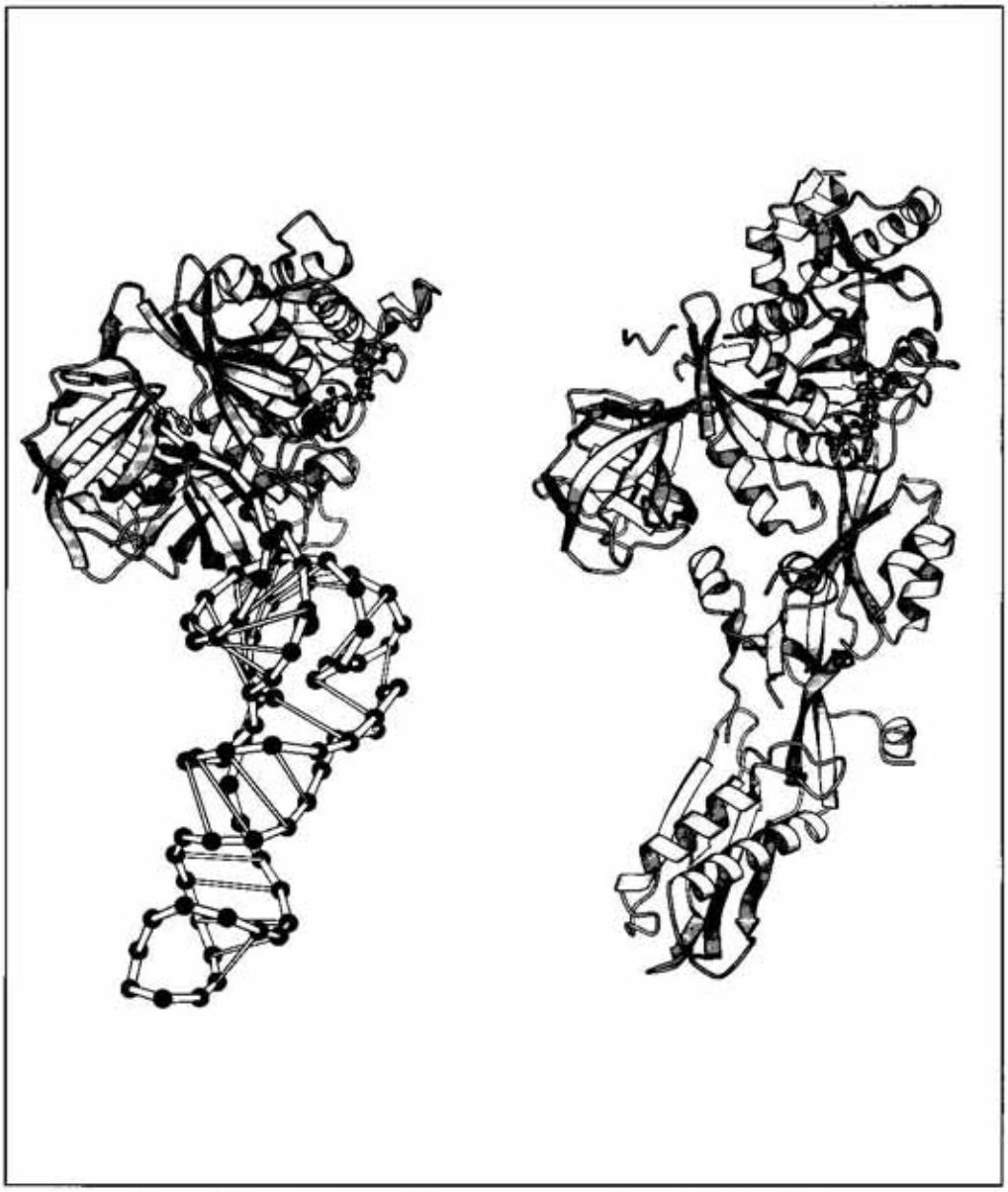

**Figure 3. Comparison of the structure of the ternary complex of EF-Tu with the structure of EF-G:GDP.**

The ternary complex is to the left with EF-Tu in approximately the same orientation as in Fig. 1. The tRNA backbone is shown as ball-and-stick model linking the phosphates. EF-G is to the right, with an uncomplete domain 3 in the middle, the elongated domain 4 pointing downwards, and domain 5 at the middle right. The Figure has been drawn using MOLSCRIPT (Kraulis, 1991).

the ribose to be 2′-endo. It is very likely that the interaction between the charge of the carboxylate of Glu271 and the charge distribution on the A base contributes to the stabilisation of the aa-tRNA binding to EF-Tu.

Although the structure of this ternary complex is based on components from different kingdoms it is believed that it does represent a canonical structure of all ternary complexes as the structures of both EF-Tu and tRNA are so well preserved (Nissen *et al.*, 1995). We have recently determined the structure of the ternary complex of *E. coli* Cys-tRNA and *T. aquaticus* EF-Tu:GDPNP (P. Nissen, personal communication). This structure is very similar to the first determined structure, although the Cys-tRNA is two nucleotides shorter and reveals some variation in the structure in the elbow of the tRNA. In fact the ternary complex seems to be a convenient way of deter-


**Figure 4. Details of the binding of CCA-Phe in the ternary complex of EF-Tu.**

The orientation is approximately the same as in Fig. 1. The CCA-Phe and selected amino-acid residues of EF-Tu are shown in ball-and-stick model. The Figure has been drawn using MOLSCRIPT (Kraulis, 1991).

mining structures of tRNAs. In our laboratory we have thus succeeded in obtaining crystals of several different ternary complexes (P. Nissen & O. Kristensen, personal communications).

When the structure of the ternary complex was compared to that of EF-G:GDP (Al-Karadaghi *et al.*, 1996; Czworkowski *et al.*, 1994) an unexpected similarity of the two structures was observed (Nissen *et al.*, 1995). EF-G has five domains of which domains 1 and 2 are very similar to the same domains in EF-Tu (Fig. 3). However, these two domains in the inactive form of EF-G have the same relative orientation as the domains of the active form of EF-Tu. Of the remaining three domains in EF-G, which all have folds similar to those of some known ribosomal proteins, the

domain 4 is very elongated and has an unusual lefthanded cross-over between two β-strands. Together the domains 3, 4 and 5 mimic the shape of a tRNA molecule (Nyborg *et al.*, 1996). This macromolecular mimicry points to the possibility that at some point in evolution, where protein biosynthesis could have been based to a larger extent on RNA, an ancestral version of both EF-Tu and EF-G existed, and that the ancestral EF-G recruited ribosomal proteins in the attempt to mimic tRNA, providing at the same time more flexibility in its function. The structure of EF-G:GDPNP is unfortunately still unknown, so that the conformational change of EF-G during its activation remains speculative. However, it is tempting to suggest that such a change does exist and that it helps the ribosome to go through the translocation step.

The high similarity of EF-G:GDP and the ternary complex provides a glimpse of the ribosomal function in one part of the elongation phase. EF-G:GDP is the form which leaves the ribosome after translocation has occurred. The next functional step after this is the binding of a new ternary complex with an anticodon corresponding to the newly exposed codon in the A site. It is thus conceivable that the last function of EF-G on the ribosome is to leave an imprint for a ternary complex on the surface of the ribosome (Liljas, 1996). Recently, cryo electron microscopy (cryo-EM) has provided a picture of the binding of the ternary complex on the ribosome (Stark *et al.*, 1997). The complex is trapped by the binding of the antibiotic kirromycin in a state just after GTP hydrolysis has occurred but before the aa-tRNA is moved into the peptidyl transferase center. In the cryo-EM picture it can be seen that domain 1 of EF-Tu is contacting ribosomal subunit 50S at the base of the L7/12 protrusion. Likewise domain 2 is in contact with the 30S subunit. This is interesting as it has been shown that all G-proteins active in protein biosynthesis are likely to have domains 1 and 2 in common (Ævarsson, 1995). It is thus conceivable that all these G-proteins have their GTPase activity stimulated by the same center on the ribosome. Furthermore, it is very likely that some translation factors, especially IF-2, RF1 and RF2, contain parts that mimic the structure of tRNA (Nissen *et al.*, 1995).

Although the recent structural results described above have provided an increase in the knowledge of the structural states of elongation factors, many structural details still remain an enigma. We have at present the knowledge of only one structural state of the function of EF-Ts while it is exchanging the nucleotide of EF-Tu. On the other hand, two structural models exist for this one state, namely the complex of EF-Tu:EF-Ts from *E. coli* (Kawashima *et al.*, 1996) and from *T. thermophillus* (Wang *et al.*, 1997). As a first assumption one would guess that these two structures would be very similar. But they are not! The sequence of *T. thermophilus* EF-Ts is about half the length of that from *E. coli*. From the structure of *E. coli* EF-Ts it can be seen that it consists of an N-terminal helical domain, a central domain of two three stranded β-sheets which are related by a pseudo twofold symmetry, and a C-terminal extension. In the C-terminal half of the central domain is inserted a helical hairpin. In the crystal structure this helical hairpin is involved in a dimerisation such that two molecules of the pseudosymmetric EF-Ts make a symmetric dimer. Both monomer binds one molecule of EF-Tu. The structure of *T. thermophilus* EF-Ts is composed of the N-terminal helical domain and the C-terminal half of the central domain including the helical hairpin. It is seen in the structure and it is known from biochemical studies that this EF-Ts only functions as a symmetrical dimer, which is about the size of the monomer of *E. coli* EF-Ts. The dimer of EF-Ts from *T. thermophilus* which is the size of one monomer from *E. coli* exposes two binding sites for EF-Tu, but now in a very different structure compared to the *E. coli* complex.

Why are these two structures so different? And do they really represent the true functional entities? The nucleotide free complex of EF-Tu:EF-Ts contains a conformation EF-Tu which mostly resembles that of EF-Tu:GDP, apart from the fact that domain 3 is removed from domain 1 by the interaction with EF-Ts. Is it likely that the introduction of GTP into this complex will change the conformations of switch regions I and II, and that EF-Ts will then catalyse the very large structural rearrangement of EF-Tu? Another strange observation is that the C-terminal extension only found in the *E. coli* EF-Ts is structurally simulating and partly replacing the structure of the switch I region albeit with the chain running in the opposite direction (S. Thirup, personal communication). Is this because the nucleotide free *E. coli* EF-Tu is not stable as it is known from biochemical studies and that it therefore needs to be stabilised until a nucleotide can be bound? Obviously, structural studies of intermediate states of the nucleotide exchange reaction of EF-Ts are needed. Such are also structural or functional studies establishing the stoichiometry of the biologically active EF-Tu:EF-Ts complex.

Another major puzzle is the very large structural rearrangement of EF-Tu during its activation or during GTP hydrolysis. On the ribosome EF-Tu has to release aa-tRNA to the peptidyl transferase center. This is happening after correct codon-anticodon interaction and after GTP hydrolysis induced by the ribosomal GTPase activating center. The structural change is much too large to be explained solely by the need for dissociation of EF-Tu from aa-tRNA. A much smaller structural change could easily destroy the high affinity binding pocket for aa-tRNA on the surface of EF-Tu. For instance, it is not necessary to assume a similar large structural change of EF-G:GTP during GTP hydrolysis. In fact, one possible model for the structure of EF-G:GTP is that the tRNA mimicking part is displaced by about 10 Å at the tip of domain 4 relative to the known structure of EF-G:GDP. The purpose of the GTP hydrolysis on EF-G would then be to physically force the anticodon helix of tRNA out of the A site of the ribosome (Abel & Jurnak, 1996). Is it possible that the large change seen in EF-Tu has to happen in order to physically force aa-tRNA into the peptidyl transferase center, which has to be a fair distance from the position of the CCA-aa end as seen in the cryo-EM picture?

There is no reason to doubt that the structural rearrangements of both EF-Tu and EF-G are functionally very important events during the elongation phase. There are two well known antibiotics whose function is to block this rearrangement. These are kirromycin acting on EF-Tu and fusidic acid acting on EF-G. Studies of antibiotic resistance mutants in both systems (Abdulkarim *et al.*, 1994; Johanson *et al.*, 1996) have shown that these antibiotics are found in the interface between domain 1 and 3 for EF-Tu and between domain 1 and domain 5 of EF-G. These are similar areas of the two proteins, and it is generally accepted that they are also the binding sites for the antibiotics. It has been claimed that the antibiotics function by inhibiting the release of the two factors, but they are equally well blocking essential events of physical movements of parts and components of the synthesizing machinery. Very recently we succeeded in obtaining well diffracting crystals of a quaternary complex of Phe-tRNA:kirromycin:EF-Tu:GDPNP (Kristensen *et al.*, 1996). The structure determination is well under way, and it is to be hoped that the final structure will reveal important details of the binding of kirromycin to EF-Tu.

Very significant advances in the structural knowledge of the elongation phase have been achieved during the last few years. There is no doubt that the next years will bring even more details of this important part of protein biosynthesis. In the same period significant advances are foreseen also for structural studies of both the initiation phase and the termination phase. Considerable advances in studies of ribosomal proteins and in ribo-

somal RNA are also within reach. Finally, detailed structural information about ribosomal subunits and of full ribosomal particles will be at hand in the very near future. All of this will most certainly transform our views on the functioning of protein biosynthesis.

I want to thank Professor B.F.C. Clark for his continued interest in the structural work on EF-Tu over many years. I also want to thank P. Nissen, O. Kristensen and S. Thirup for letting me mention their unpublished work. Finally, I appreciate the help from S. Thirup in setting up Fig. 2.

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