

## Structure of a Mutant EF-G Reveals Domain III and Possibly the Fusidic Acid Binding Site

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The crystal structure of *Thermus thermophilus* elongation factor G (EF-G) carrying the point mutation His573Ala was determined at a resolution of 2.8 Å. The mutant has a more closed structure than that previously reported for wild-type EF-G. This is obtained by a 10° rigid rotation of domains III, IV and V with regard to domains I and II. This rotation results in a displacement of the tip of domain IV by approximately 9 Å. The structure of domain III is now fully visible and reveals the double split  $\beta$ - $\alpha$ - $\beta$  motif also observed for EF-G domain V and for several ribosomal proteins. A large number of fusidic acid resistant mutations found in domain III have now been possible to locate. Possible locations for the effector loop and a possible binding site for fusidic acid are discussed in relation to some of the fusidic acid resistant mutations.

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### Introduction

In the living cell the synthesis of new proteins is done on the ribosome. The mRNA is the template and its codons are sequentially presented in the ribosomal A-site (acceptor site). Here the codons form base-pairs to the anticodons of an incoming aminoacyl transfer RNA (aa-tRNA). The tRNA enters the ribosome in complex with elongation factor Tu (EF-Tu) and GTP. When the GTP is hydrolyzed to GDP, EF-Tu changes its conformation and loses its affinity for the tRNA as well as for the ribosome. This leads to the binding of the aa-tRNA into the A-site. Chain elongation takes place as transfer of the polypeptide chain attached to the tRNA in the P-site (peptidyl tRNA site) to the aminoacyl group of the A-site tRNA.

EF-G participates in the elongation phase of protein synthesis by translocating the peptidyl tRNA from the A-site to the P-site (Kaziro, 1978). EF-G in complex with GTP binds to the factor-binding site of ribosomes in the pre-translocation state. After hydrolysis of the bound GTP to GDP and translocation EF-G:GDP loses its affinity for the ribosome. As a result the tRNAs and the mRNA are moved on the ribosome exposing the next codon of the mRNA in the A-site. EF-G also has a role after the termination phase of translation. EF-G together with ribosomal recycling factor (RRF) facilitates the release of deacylated tRNA and mRNA from the ribosome and splitting of the ribosome into two subunits (Kaji *et al.*, 1998; Karimi *et al.*, 1999).

EF-G from *Thermus thermophilus* consists of 691 amino acid residues, including the initiator methionine (Yakhnin *et al.*, 1989). It is folded into five domains and is highly elongated (Ævarsson *et al.*, 1994; Czworkowsky *et al.*, 1994).

Several different GTP-hydrolyzing factors bind to the ribosome (Haselkorn & Rothman-Denes, 1973). Structural similarities between these have been identified (Ævarsson, 1995). In particular elongation factor Tu (EF-Tu) in complex with aa-tRNA is mimicked by EF-G (Nissen *et al.*, 1995). Thus, domains I and II of EF-G are structurally

Abbreviations used: EF-G, elongation factor G; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate; GDPNP, guanylyl imidodiphosphate; GDPCP, phosphomethylphosphonic acid guanylate ester; FA, fusidic acid; MIC, minimal inhibitory concentration; aa-tRNA, aminoacyl tRNA; EM, electron microscopy; RNP, ribonucleoprotein; RRM, RNA recognition; SRL, sarcin/ricin loop.

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similar to domains I and II of EF-Tu while domains III, IV and V of EF-G mimic the tRNA.

It is well known that EF-Tu undergoes significant structural rearrangements during its functional cycle (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993). In the case of EF-G we are less well informed (Czworkowski & Moore, 1997). Since the two proteins have significant structural similarities and bind to the same site on the ribosome one may ask whether the two proteins undergo similar or different conformational changes in their functional cycles.

In the presence of fusidic acid EF-G remains bound to the ribosome after GTP hydrolysis and translocation (Burns *et al.*, 1974; Willie *et al.*, 1975). Similarly kirromycin locks the ternary complex of EF-Tu:tRNA:GDP on the ribosome after GTP hydrolysis (Wolf *et al.*, 1977). Cryo-electron microscopy (EM) reconstructions of ribosomes in the post-translocational state with fusidic acid blocked EF-G:GDP (Agrawal *et al.*, 1998, 1999; Stark *et al.*, 2000) and of kirromycin blocked EF-Tu:GDP:tRNA on the ribosome (Stark *et al.*, 1997) show that EF-G and the ternary complex occupy closely the same space. Domains III, IV and V of EF-G are located where the tRNA of the ternary complex is situated in the decoding part of the ribosomal A-site on the 30 S subunit. The conformation of the elongation factors when bound to the ribosome is somewhat different from the crystal structures of the factors in isolation (Agrawal *et al.*, 1998; Stark *et al.*, 1997).

We have determined the crystal structure of *Thermus thermophilus* EF-G carrying the point mutation histidine 573 to alanine at 2.8 Å resolution. Histidine 573 is a surface residue situated at the tip of domain IV and part of the conserved YHEVDS motif. Even though several alterations of this region of the molecule prevent translocation, this activity is not affected by the His573Ala mutation (Martemyanov *et al.*, 1998). The crystal structure of this mutant has a more closed conformation than wild-type EF-G. In addition domain III which previously only has been poorly characterized can now be fully traced. These observations give further insight into the dynamics of EF-G during its functional cycle as well as insights into the locations of fusidic acid resistant mutations.

## Results

### Structural description

Crystallographic data of the mutant H573A of *T. thermophilus* EF-G complexed with GDP have been collected to a resolution of 2.8 Å at beamline I711 at the MAX laboratory (Table 1). In contrast to previous studies of EF-G we were able to shock freeze the crystals and collect data at 100 K. Molecular replacement with the whole molecule of EF-G from previous studies did not work, but the domains I/II and IV/V could be localized separately.

**Table 1.** Data collection and structural refinement statistics

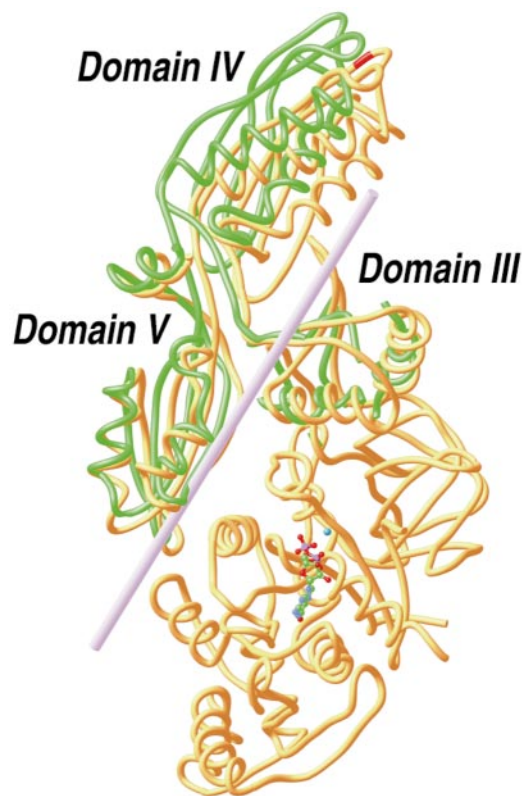
A. Data collection	
Space group	$P2_12_12_1$
Cell dimensions (Å)	$76.70 \times 85.97 \times 113.37$
Data resolution (Å)	2.8
Number of unique reflections	18,060
Completeness (overall/outer shell) (%)	95/96.8
$I/\sigma_I$ (overall/outer shell)	$12.5\sigma/2.2\sigma$
R-merge (%)	10.4
B. Refinement	
Refinement resolution (Å)	25-2.8
No. of molecules in the asymmetric unit	1
R-factor (%)	21.2
R-free (%)	29.5 (3.6% test set)

Model building and refinement led to structural interpretation of residues 6-39 and 68-688 of the 691 residues in *T. thermophilus* EF-G. It has not been possible to model residues 40-67 normally referred to as switch I in G proteins. The crystals have essentially the same packing as wild-type EF-G except that the *b*-axis is decreased by close to 20 Å. The main difference is that domain II moves into contact with the nucleotide binding site of a neighbouring molecule. The previously poorly observable domain III becomes locked by neighbour molecules.

The structures of domains G and II are essentially unaltered. However, the current structure reveals an interesting flexibility of EF-G. When compared to the wild-type structure, domains III, IV and V have rotated to a significant extent with respect to domains G and II. The rotation is 10° around an axis between domains G and V, as shown in Figure 1. The axis of rotation traverses through the solvent region between domains I and V. Residues 401-404 is a coil linking domains II and III and their flexibility facilitates the dynamics of the molecule. This region lacks hydrogen bonds to the rest of the molecule and contains solvent exposed Ile402 and Val404. Despite this rotation the interfaces between the domains are not changed significantly.

As a result of this change the tip of domain IV, where the mutation is located, has moved by about 9 Å. Similar structural changes have also been observed for wild-type EF-G in complex with GDP (J. Czworkowski & P. B. Moore, personal communication). The vicinity of residue 573 is unaltered by the mutation and it is neither directly involved in crystal contacts in the wild-type EF-G structures nor in the present structure.

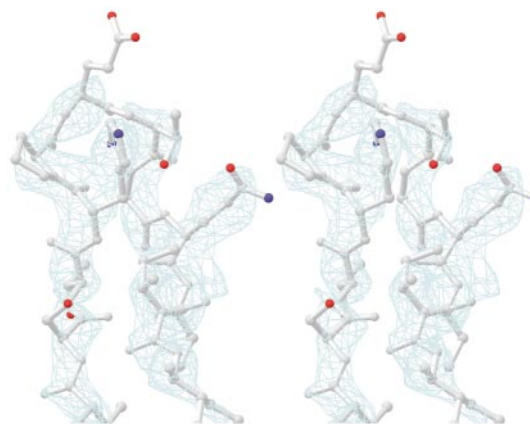
A shortcoming in previous crystallographic investigations of EF-G has been the inability to clearly define the structure of domain III. From the current data we are able to trace domain III entirely (Figure 2). The reason is probably that the previously observed flexibility of domain III in



**Figure 1.** Comparison of the conformations of wild-type EF-G (green) with EF-G His573Ala (orange). The axis of rotation is indicated. For clarity only domains III, IV and V of wild-type are shown. The location of the mutation is shown in red.

these crystals is limited by tighter packing in the current crystals.

The structural arrangement of domain III relative to domain IV and V is in fair agreement with the partial C $\alpha$  tracing in previous structures (Ævarsson *et al.*, 1994; Czworkowski *et al.*, 1994). Domain III of EF-G, comprising residues 400 to 482, folds into a double split  $\beta$ - $\alpha$ - $\beta$  motif analogous to EF-G domain V (Ævarsson *et al.*, 1994). The four-



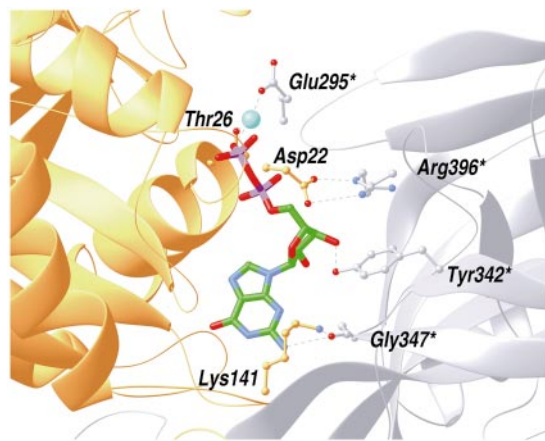
**Figure 2.** Electron density of domain III. A composite omit map of the  $\beta$ -hairpin strands 2 $_3$  and 3 $_3$  (residues 442-450) contoured at 1.0 $\sigma$ .

stranded right hand twisted antiparallel  $\beta$ -sheet is overlaid on one side by two  $\alpha$ -helices. In line with Ævarsson *et al.* (1994) helices are denoted by capital letters, strands by figures and domains by subscript in the following. Helix B $_3$  forms the interface to domain V (strand 4 $_5$ ) and the switch II region of the G-domain. Strands 2 $_3$  and 3 $_3$  extend beyond the domain III  $\beta$ -sheet into a  $\beta$ -hairpin. This extension is in contact with helix A $_4$  of domain IV and thereby supports domain IV in this conformation (Table 2).

The bound GDP nucleotide is well defined in the structure. An electron density with short coordination distances to its ligands and located in the classical Mg $^{2+}$  position is interpreted as a magnesium ion. It coordinates one oxygen atom of the GDP  $\beta$ -phosphate, the side-chain oxygen atom of Thr26 and the carboxyl group of Glu295 of a symmetry-related molecule. The ligand orientations suggest an octahedral coordination with water molecules as ligands in the three remaining positions (Figure 3). A magnesium ion has not been observed so far in EF-G structures, but is frequently observed in EF-Tu (Polekhina *et al.*, 1996).

**Table 2.** Interactions between domain III and other parts of EF-G

Domain III		Other domains	
Residue	Secondary structure	Residue	Structure element
L457	Helix B $_3$	D89	Loop 2 $_C$ -B $_C$ (Switch II)
E433	Helix A $_3$	I384	Strand 2 $_2$
E434	Helix A $_3$	A318	Strand 3 $_2$
E434	Helix A $_3$	I316	Strand 3 $_2$
P436	Loop A $_3$ -2 $_3$	I384	Strand 11 $_2$
P444	Strand 2 $_3$	Q551	Helix A $_4$
E406	Coil 13 $_2$ -1 $_3$	F673	Strand 4 $_5$
V408	Strand 1 $_3$	L649	Loop 3 $_5$ -B $_5$
V408	Strand 1 $_3$	F673	Strand 4 $_5$
I409	Strand 1 $_3$	L649	Loop 3 $_5$ -B $_5$
E456	Helix B $_3$	F653	Helix B $_5$
P479	Strand 4 $_3$	F653	Helix B $_5$



**Figure 3.** The GDP molecule with a  $Mg^{2+}$  (light blue). The orange molecule is the one binding the GDP. The gray molecule is symmetry-related in the current crystal. The magnesium ion is coordinated by Thr26, an oxygen molecule of the GDP  $\beta$ -phosphate moiety and Glu295 of the symmetry-related molecule. The crystal contacts in this area of the structure are not present in previous structures.

### Fusidic acid resistance

The antibiotic fusidic acid prevents EF-G from leaving the ribosome and is used clinically, primarily against *Staphylococcus aureus*. Mutants resistant to fusidic acid were selected from the wild-type *S. aureus* strain FDA486 (minimum inhibitory concentration (MIC) 0.25  $\mu\text{g}/\text{ml}$ ). Selections were made on LB solid media containing either 1 or 10  $\mu\text{g}/\text{ml}$  fusidic acid sodium salt. Eighty independent fusidic acid resistant mutants were selected, 52 at 1  $\mu\text{g}/\text{ml}$  and 28 at 10  $\mu\text{g}/\text{ml}$ . Sequencing of the *fusA* gene of each mutant (the wild-type sequence of *fusA* from FDA486 is deposited at GenBank as AJ237696) revealed that 62 had mutations altering EF-G. The locations of the 18 non-EF-G mutations have not been determined. Among the mutants with alterations in EF-G we identified 18 different single amino acid substitutions in EF-G, and one mutant with two amino acid substitutions in EF-G (Table 3). Most fusidic acid resistance mutations in EF-G alter amino acids in domain III (ten different amino acids, 16 mutations), with only a few affecting domain I (two mutations) or domain V (one mutation). Mutations to fusidic acid resistance in *Salmonella typhimurium* have previously been identified (Johanson & Hughes, 1994) and in contrast to *S. aureus*, all cause amino acid substitutions in EF-G. The fusidic acid resistance mutations in *S. typhimurium* are found in similar positions as in *S. aureus* but are more evenly distributed between domain I (nine mutations), domain III (seven mutations) and domain V (four mutations). The fusidic acid MIC values are much higher in *S. typhimurium* than in *S. aureus* (Table 3), reflecting poor uptake through the *Salmonella* cell

wall rather than differences in sensitivity of the ribosome EF-G complex. Relative differences in MIC within each species are, however, significant.

## Discussion

### Conformational states of elongation factors during their functional cycles

In the crystal studies of EF-G two main conformations have been observed. One is the now classical GDP conformation (Czworkowski *et al.*, 1994; Al-Karadaghi *et al.*, 1996), which is only slightly changed in the case of the nucleotide free factor (Ævarsson *et al.*, 1994). The other one is the current one which is slightly more bent. This has also been observed for the wild-type factor (P. Moore & J. Czworkowski, personal communication). In addition EF-G has been studied in complex with the non-hydrolyzable GTP analog GDP-PCP. Here the conformation observed does not differ significantly from the classical GDP conformation (P. Moore & J. Czworkowski, personal communication).

The two crystallographically observed conformations of EF-G are partly related to the packing in the crystals. Thus, the current bent conformation depends on the intermolecular contacts around the magnesium ion. Whether the structural conformations represent distinct functional states is unclear as the general crystal packing confines the bending of EF-G in the different crystals. However, the difference illustrates how the molecule can bend.

Looking into the details of the molecular mimicry of the crystal structures of EF-G:GDP and the ternary complex of EF-Tu and tRNA (Czworkowski *et al.*, 1994; Nissen *et al.*, 1995; Al-Karadaghi *et al.*, 1996) it is evident that EF-G has a more bent conformation (Liljas *et al.*, 2000). The mutant H573A of EF-G is even more bent. However, in the EM studies of these factors when locked on the ribosome in complex with fusidic acid and kirromycin, respectively, EF-G is more open (Agrawal *et al.*, 1998, 1999; Stark *et al.*, 2000) and EF-Tu becomes more closed (Stark *et al.*, 1997). Whether this is due to the binding of the antibiotics or the interactions with the ribosome is not fully understood. In the case of EF-G the fitting of the crystallographic model of EF-G:GDP to the experimental EM density requires a rotation of domains III, IV and V with regard to domains I or G and II. This rotation is opposite to the one presented here. Thus, in addition to the standard crystallographic GDP conformation there is a more open conformation of EF-G in complex with fusidic acid on the ribosome (Agrawal *et al.*, 1998) and a more bent conformation of EF-G with the mutation H573A. In EF-G domains I and II move as one unit, whereas in EF-Tu the conformational change occurs between domains I and II (Kjeldgaard *et al.*, 1993). Thus, these molecules

differ in their dynamic behavior as they go through their functional cycles. Although a complete comparison has not yet been made of the crystallographic and EM results it seems as if EF-Tu and EF-G may undergo different conformational changes off and on the ribosome.

### Domain III

Domains III and V of EF-G have the same fold, the double split  $\beta$ - $\alpha$ - $\beta$  fold. This fold is observed in a vast number of ribonucleotide binding proteins and is also referred to as the ribonucleoprotein (RNP) or RNA recognition (RRM) motif. For example, it is found in eukaryotic mRNA processing snRNPs such as U1A (Nagai *et al.*, 1990; Oubridge *et al.*, 1994). Several of the ribosomal proteins for which the structures are known (S6, S8, L1, L6, L9, L12, L22, L30) (reviewed by Al-Karadaghi *et al.*, 2000; Ramakrishnan & White, 1998) also have the split  $\beta$ - $\alpha$ - $\beta$  motif. This is by far the most frequently occurring motif in the translation system. A structural comparison of domains III and V shows that when the  $\beta$ -structures are aligned the  $\alpha$ -helices do not superimpose well. However, proteins S6 (Lindahl *et al.*, 1994) and U1A (Nagai *et al.*, 1990) superimpose nicely on domain V, while ribosomal protein L22 (Unge *et al.*, 1998), a protein with a single split  $\beta$ - $\alpha$ - $\beta$  fold, superimposes well on domain III. The extended loop in these proteins coincides with the loop between strands 2<sub>3</sub> and 3<sub>3</sub> that supports domain IV.

In U1A the conserved RNP1/2 motifs (Burd & Dreyfuss, 1994) in the central two  $\beta$ -strands expose aromatic side-chains to the solvent that are involved in stacking with the bases of a single-stranded region of U1RNA (Oubridge *et al.*, 1994). Many ribosomal proteins share this feature of exposing aromatic or hydrophobic groups to the solvent. They may be involved in intercalation or other interactions with the bases of rRNA (Draper, 1999). Extended loop regions of the ribosomal proteins may also be well suited to interact with rRNA (Liljas & Al-Karadaghi, 1997). In the case of S6 the central  $\beta$ -sheet region is part of the interface to S18 and strand 4, and helix B is in contact with the 16 S rRNA flanked by the extended  $\beta$ -hairpin (Agalarov *et al.*, 2000). In the case of domains III and V of EF-G it is less clear if and how they would interact with rRNA despite the structural similarity, and with hydrophobic residues suitably

exposed to the solvent and loop regions related to the ones in proteins S6 or L22.

Domain III is the only covalent connection between the N-terminal block of EF-G comprising domains G and II and the C-terminal region, domains IV and V. The flexibility initially observed for domain III may be of functional importance as is the case for the other flexible regions, switch I and II. Alternatively, domain III may act as a spacer that prevents a closer approach between domains II and IV.

The structure of domain III of EF-G extends the mimicry of the molecular envelopes of EF-G and the ternary complex of EF-Tu with aminoacyl tRNA. Thus, domain III corresponds to the acceptor-stem of Cys-tRNA positions 1 to 6 and positions 66 to 73 in the ternary complex (Nissen *et al.*, 1996). The newly discovered tRNA mimicry by the ribosomal release factor (RRF) is based on a totally different protein folding (Selmer *et al.*, 1999).

### The effector loop

Switch I (the effector loop) is a highly flexible region in all G-proteins. The effector loop of EF-G shows a high degree of sequence similarity to the one in EF-Tu. The residues of the most flexible part of the effector loop in EF-Tu (55-63 in *Thermus aquaticus*) are highly conserved in EF-G and changes occur only for solvent exposed residues in EF-Tu (Figure 4). The switch I region in EF-G, comprising residues 40-65, has not been possible to model into the electron density map even though substantial densities are present in this part of the map. In EF-Tu transitions in secondary structure between the GTP and the GDP bound forms have been characterized (Polekhina *et al.*, 1996). Because of the strong sequence similarity between these regions in EF-G and EF-Tu one may consider that similar conformations occur.

In EF-Tu the effector loop is constituted by a constant element, helix A', and a conditional region which can either fold into helix A'' or a  $\beta$ -hairpin (strands b and b'), depending on whether GTP or GDP is bound to the protein. Upon GTP hydrolysis and helix A'' unwinding the O' of Thr62 (EF-Tu, *T. aquaticus*), which coordinates the Mg<sup>2+</sup>, is shifted 13 Å away from its initial position and becomes solvent exposed in the GDP conformation (Polekhina *et al.*, 1996). Ile61 and Ile63, which are in van der Waals' contact with Tyr88 in the GTP conformation,



**Figure 4.** The sequence comparisons of the effector loops in EF-G from *T. thermophilus* and EF-Tu from *T. aquaticus*. The secondary structure elements on the two conformations of EF-Tu are indicated.

**Table 3.** Location of new fusidic acid (FA) resistance mutations in *S. aureus*<sup>1</sup> and previously found alterations in *S. typhimurium*<sup>2</sup> EF-G (Johanson & Hughes, 1994)

FusR mutation in EF-G	MIC (µg/ml)		Structural interpretation
	<i>S.aur.</i>	<i>S.typ.</i>	
T84A <sup>2</sup> (double mutant A66V,T88A)		1200	T84 is located in switch II which is known from EF-Tu to undergo conformational changes during GTP hydrolysis. Pro85 and the main-chain of the Mg <sup>2+</sup> coordinating T62 (switch I) occupy the same space in the EF-Tu:GDP and EF-Tu:GTP conformations, respectively. The T84A mutation may impair a similar alteration in EF-G
F90L <sup>1</sup>	96		Direct effector loop and/or FA interaction. F90 is in an equivalent position to EF-Tu:GTP Y88. I63 and I65 (both in the outer flexible part of the effector loop) probably pack against this residue in the EF-G:GTP conformation
A104E <sup>2</sup>		400	Steric hindrance will probably change the orientation of the switch II helix as in the case of EF-Tu:GDP pushing domain II away
F108S <sup>2</sup>		2400	Near the P-loop/Q117 interaction area
Q117L <sup>1</sup> , Q117R <sup>2</sup>	16	400	Impairing P-loop and switch II coupling. May lock switch II in a conformation when the nucleotide site is empty. L88 packs against hydrophobic part of R117
T120I <sup>2</sup>		2400	Close to L88 and Q117. The T120I mutation will furthermore abolish a bridging water molecule between domains G and V. The position of this water molecule in EF-Tu is occupied by the carboxyl group of E118. In EF-Tu:GTP E118 bridges to domain III and in EF-Tu:GDP E118 coordinates H85 (EF-G H87) of switch II. Steric hindrance of L88 will favour a nucleotide free conformation of EF-G
V121L <sup>2</sup>		400	Collision with V88 in EF-G:GDP but not in the nucleotide free conformation of EF-G
Q124H <sup>2</sup>		2400	The side-chain of Q124 is close to V88 of switch II
A134T <sup>2</sup>		1200	The mutation is in the core of the G domain. The structural surroundings in EF-G and in EF-Tu are stable and independent of nucleotide binding
L157P <sup>2</sup>		2400	L157 is the C-terminal residue of helix D <sub>G</sub> and the side-chain is in contact with the C-terminal part of helix C <sub>G</sub> . The strong mutant position Q117 is in this end of helix C <sub>G</sub> , but not in direct contact with L157. The mutation would probably provide larger orientational freedom of the C-terminal part of helix C <sub>G</sub> . Backbone is part of the binding face towards residues N639 and R666 of domain IV
P405L <sup>1</sup> , P405Q <sup>1</sup>	3, 1.5		FA interaction or changed flexibility of the domains II and III linker region. P405 is at the edge of a spatial cluster of mutations in domain III conferring FA resistance
P407L <sup>1, 2</sup>	6	2400	Residue making the final bend of the linker between domains II and III. A non-proline probably makes the linker even more flexible. Close to T437, G453, H458
V408F <sup>1</sup>	2		Mutations at positions 405, 407 and 408 are in the interface between domains III and V. FA resistance conferring mutations have larger side-chains probably affecting the relative orientation between domains III and V
A427D <sup>2</sup>		1600	Near the tentative effector loop locations
L431Q <sup>2</sup>		>3200	Possible effector loop interactions
D435N <sup>1</sup>	24		Possible effector loop interactions
P436Q <sup>2</sup>		2400	Direct FA steric hindrance and hindrance of an EF-Tu:GDP effector loop conformation. The side-chain may be located in the space occupied by T64 in EF-G:GDP and may form a H-bond with H458 as assumed for T64
T437I <sup>1</sup>	12		Positions 431, 435, 436 and 437 are all in the interface to the tentative effector loop EF-Tu:GDP location. Resistance by direct FA or effector loop interactions are possible
Q448H <sup>2</sup>		1600	Change in the conformation of the domain III β-strands 2 and 3 and thereby the relative orientation of domain IV
S452V <sup>1</sup>	3		Side-chain points towards P407. S452V will in analogy to mutations at positions 405, 407, 408 push domain IV away from domain III
G453S <sup>1</sup> , G453C <sup>1</sup> , G453V <sup>1</sup>	12, 4, 6		Serine side-chain will make hydrogen bond to side-chain of H458. All three mutations will push H458 into the interface of domains III and G
M454I <sup>2</sup>		2400	Interaction with tip of effector loop or direct FA interaction. Close to many strong fusidic acid resistant mutations
L457F <sup>1</sup>	3		Part of the signalling pathway between the P loop and domain III
H458Y <sup>1</sup>	32		Direct interaction with FA? The hydroxyl group addition to the side-chain ring may confer sterical hindrance of FA binding. May hydrogen bond to Thr64 in EF-G:GDP retained ensuring continued stabilization of the effector loop GDP conformation
R465L <sup>1, 2</sup> , R465C <sup>1</sup> , R465S <sup>1</sup> , R465H <sup>1</sup>	3, 3, 4, 4	2400	Possible effector loop interactions. A possible interaction partner is Q57 of the effector loop in its GTP binding conformation. FA resistance conferring mutations at position 465 probably weakens this interaction favouring a GDP binding conformation of the effector loop
G618C <sup>2</sup>		1600	On the outside of domain V pointing away from EF-G towards the solvent. Probably ribosome interaction
G629D <sup>2</sup>		1200	Local distortion of the backbone in domain V pushing R628 towards domain IV, forcing a more closed conformation of domain IV
P648Q <sup>2</sup>		800	Mediates the interaction between E603 (domain IV) and R628 (domain V) making the distance between the two longer, pushing domain IV towards a more closed conformation
R659L <sup>1</sup> (double mutant G465C, R659L)	4		This position is a leucine in EF-G <i>T. thermophilus</i>
M671I <sup>2</sup>		800	Closest part of domain V to the tip of the effector loop in EF-G:GDP, but the residue is pointing towards the centre of the domain. A mutation of F672, pointing towards the tip of the effector loop and situated at the interface between domains G, III, V should have a larger resistance potential

MIC values are listed in separate columns for *S. aureus* (wild-type = 0.25) and *S. typhimurium* (wild-type = 100). All numbering in EF-G is according to *T. thermophilus* with the initiator methionine being position 1. EF-Tu *T. aquaticus* structure references are to RCSB Protein Data Bank coordinates 1TUI and 1B23.

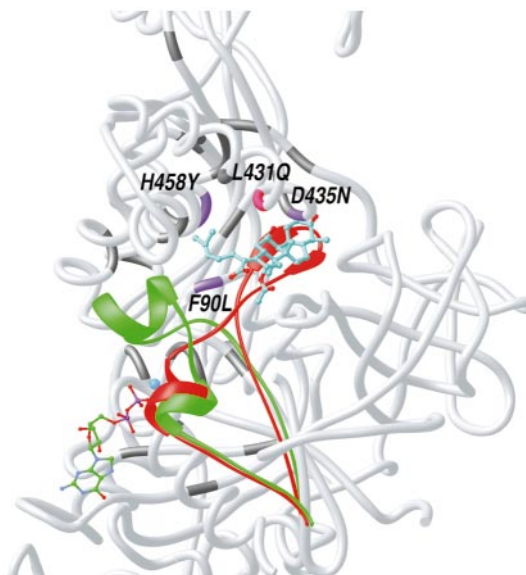
instead pack against the hydrophobic part of Lys90 while Tyr88 is shifted away by 6 Å. This shift also results in the loss of hydrogen bonding between the Thr62 main-chain nitrogen and an oxygen atom of the GTP  $\gamma$ -phosphate moiety. In solution without crystal packing constraints the effector loop of EF-G could adopt essentially the same conformations as in EF-Tu without spatial conflicts. Thus, the space occupied by the effector loop  $\beta$ -hairpin in the GDP binding form of EF-Tu is empty in EF-G (Figure 5). This would bring the EF-G side-chains of Ile63 and Ile65 into van der Waals' contacts with Leu 92. The GTP conformation of the effector loop would allow Thr64 in EF-G to form a ligand to the  $Mg^{2+}$  that increases the affinity for GTP. In this conformation Phe90 would be in contact with Ile63 and Ile65.

Our crystal structure shows that Glu295 from a symmetry-related molecule forms a ligand to the magnesium ion in a position equivalent to Asp51 in helix A' of EF-Tu, in both the GDP and the GTP form. This prevents EF-G in the present crystal structure from having an A' helix in either of the two EF-Tu conformations of the effector loop.

EF-Tu alternates with EF-G to bind to a factor binding site on the ribosome. This site is partly composed of the sarcin/ricin loop (SRL) of 23 S rRNA, which has been shown to be protected by EF-G or EF-Tu from chemical modifications at positions G2655, A2660, G2661 and A2665 (Moazed *et al.*, 1988). EF-G has specific affinity for a synthesized 27-mer oligoribonucleotide with the same sequence as the SRL, and the strength of this binding is comparable of that of EF-G to the ribosome (Munishkin & Wool, 1997). The binding of EF-G to the oligo-RNA is abolished by GDP and stabilized by GTP analogs. These observations, and the docking of EF-G onto the ribosomal 50 S structure (Ban *et al.*, 1999) based on EF-G linked hydroxyl radical probing to the tip and stem of the SRL (Wilson & Noller, 1998), strongly suggest that helix A'' of the effector loop (switch I) of EF-Tu/EF-G is in direct contact with the SRL mediating the signal for GTP hydrolysis to the ribosome. This may be the major interaction between the elongation factors and part of the SRL surface, in part explaining why the elongation factor loses affinity for the ribosome upon GTP hydrolysis as helix A'' unwinds. Reconstructions at 20 Å resolution of 70 S ribosomes stalled by EF-G in complex with fusidic acid are in agreement with the docking experiments (Stark *et al.*, 2000).

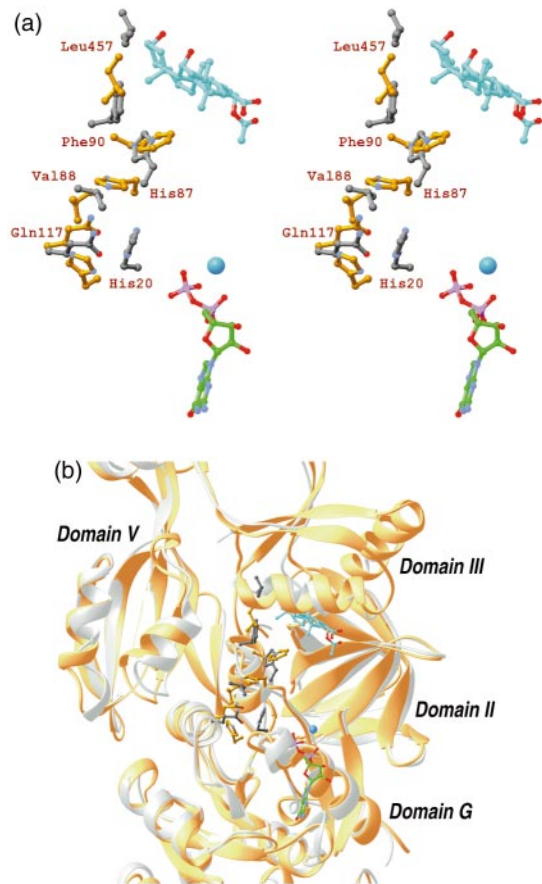
### The conformational changes

In the nucleotide free form of EF-G the P-loop has collapsed into the pocket otherwise occupied by the guanine nucleotide phosphate moieties. This conformational change links His20 of the P-loop via the side-chain of Gln117 to the switch II region (Al-Karadaghi *et al.*, 1996). In the present, bent, conformation of EF-G, Gln117 forms a hydrogen



**Figure 5.** EF-G point mutations in violet (*S. aureus*) or red (the strongest from *S. typhimurium*) conferring strong resistance towards fusidic acid visualized in the present structure (other resistance mutations are indicated in gray). Spontaneous mutations observed under fusidic acid selective pressure are mainly clustered in the helix C<sub>G</sub> and helix B<sub>3</sub>. These helices are at the interface where the domain mobility of the protein is observed, between domains G and V as well as G/II and III, respectively. The mutations are located near to the potential location of the effector loop of EF-Tu in the GDP conformation (red). The effector loop of EF-Tu:GDPNP:Cys-tRNA<sup>Cys</sup> (green) is also shown. The suggested location of fusidic acid at the tip of the effector loop in the GDP conformation is indicated (light blue). Mutations are only observed in the part of the structure shown.

bond to His87 of the switch II loop region (Figure 6(a)). This “domino effect” of side-chains originating from the nucleotide binding site pushes Phe90 away from the interface between domains I and III and towards domain III. This leads to the more open conformation of EF-G, as observed for the empty or classical GDP conformations (Figure 6(b)). The location of Phe90 in the current bent conformation is very close (about 3 Å) to the position occupied by the corresponding residue, Tyr88, in the GTP conformation of EF-Tu. This indicates that the GTP conformation of EF-G could also be bent, since Phe90 can not simultaneously push domain III away. Thus, the current structure has two elements of the GTP conformation or state: Phe90 in close proximity to its GTP location, and the presence of a magnesium ion at the nucleotide. The open conformation of EF-G may facilitate the movement of the effector loop out of the GDP conformation. In turn, this opening of the EF-G structure could simplify the transition between the EF-G:GDP and EF-G:GTP conformations of the



**Figure 6.** The “domino effect” of conformational changes around the P and switch II loops and the interface to domain III between the open nucleotide free (gray) and bent (orange) conformations of EF-G. The GDP molecule of the present structure and the potential location of fusidic acid are shown. (a) A stereo picture. (b) The residues of the “domino effect” shown in relation to the main-chain structure in the two main conformations.

effector loop, possibly explaining in part EF-G’s role as its own nucleotide exchange factor.

### Fusidic acid resistance

Fusidic acid traps EF-G on the ribosome after translocation and GTP hydrolysis (Burns *et al.*, 1974; Willie *et al.*, 1975), thus blocking further protein synthesis. It is not known where fusidic acid binds to the complex of EF-G and the ribosome. Three models can be proposed.

(1) Fusidic acid may bind to EF-G:GTP on the 70 S ribosome (and remain bound after GTP hydrolysis and translocation).

(2) Fusidic acid may bind to EF-G:GDP on the 70 S ribosome (after EF-G has performed translocation).

(3) Fusidic acid may bind to the 70 S ribosome at a location made accessible by the interaction of EF-G with the ribosome (either before or after translocation).

The identification of chromosomal mutations in bacterial strains resistant to fusidic acid shows a strong correlation between resistance and mutations in EF-G (Johanson & Hughes, 1994; this work). This suggests to us that EF-G and not the ribosome is the target for fusidic acid. In particular, mutations often occur in the interface between the two parts of the molecule that are mobile with regard to each other, domains G and II *versus* domains III, IV and V (Johansson *et al.*, 1996). Some of these mutations may facilitate the conformational changes in EF-G required for dissociation from the ribosome despite the presence of fusidic acid, whereas others may prevent fusidic acid from binding to its target site. The observed mutations are the result of *in vivo* selection pressure for both fusidic acid resistance and for EF-G functionality. Reasonable structural interpretations of the fusidic acid resistance mutations in EF-G and how they might mediate resistance are summarized in Table 3.

Amino acid substitutions in *S. aureus* EF-G conferring the highest levels of resistance towards fusidic acid are F90L, D435N and H458Y. These mutations flank the cavity in the present structure where the effector loop of EF-Tu:GDP would be situated (Figure 5). In addition Q117L is in the area between this cavity and the nucleotide binding site and participates in the “domino” effect of conformational changes originating from the nucleotide binding site. In contrast, there is no striking relationship between the fusidic acid resistance mutations and the location of the effector loop in the GTP conformation. One simple interpretation is that fusidic acid may binds to EF-G at the site for the tip of the effector loop in the GDP conformation of EF-G. In fact, the size and the shape of fusidic acid makes it fit nicely into this site. This binding would prevent EF-G from adopting the GDP conformation and thus prevent its dissociation from the ribosome. According to this interpretation at least some of these high level resistance mutations should act to prevent the binding of fusidic acid to the EF-G ribosome complex.

One of these mutated residues, F90L, has also a crucial role in the conformational change between the open and the bent conformation of the current structure. As stated above, it is a major cause in pushing domain III away from domain I, leading to the open conformation. In the bent conformation F90L occupies almost the same space as Tyr 88 in EF-Tu in the GTP conformation. At the moment we are unable to distinguish whether the resistance associated with the F90L mutation is caused by direct effects on fusidic acid binding, or by altering the possibilities for conformational transitions.

The fusidic acid resistance mutation P407L has been studied extensively *in vitro* and *in vivo* (Macvanin *et al.*, 2000). This mutation reduces the affinity of EF-G for GTP, thus favouring the EF-G:GDP form. In addition, this mutation enhances the interaction between EF-G and the transcrip-

tional regulator molecule ppGpp. Thus, the P407L mutation alters the binding and exchange of guanine nucleotides on EF-G. This residue is not in direct contact with the guanine nucleotide but its position at the end of the linker between domains II and III suggests that the mutation may make the link more flexible.

In conclusion, this paper describes a new structural conformation of EF-G. This new structure supports the view that EF-G is composed of two blocks (domains I and II, and domains III, IV and V) that move as units with respect to each other. The more bent conformation of EF-G presented here makes domain III more rigid and possible to trace completely. This domain has the same topology as domain V of EF-G. A number of fusidic acid resistant mutations, mostly in domain III, have now been located. Switch I (the effector loop) has not been possible to localize. If switch I adopts conformations similar to those in EF-Tu it would, in the GDP conformation, be close to many of the fusidic acid resistant mutations in domain III. This suggests a binding site for fusidic acid at the tip of the effector loop in the GDP conformation that would explain how EF-G gets trapped on the ribosome after GTP hydrolysis and translocation, since the effector loop cannot access its proper location needed for dissociation.

## Materials and Methods

Cloning and purification of *T. thermophilus* EF-G was performed as described (Martemyanov *et al.*, 1998). Crystals of the EF-G mutant His573Ala were grown from hanging drop vapour diffusion experiments at pH 7.4 against a reservoir of 100 mM Hepes, 50 mM Tris, 22% (w/v) polyethylene glycol 8000 (Fluka). 4  $\mu$ l of a 10 mg/ml protein solution, 0.5 mM GDP, 3 mM Mg<sup>2+</sup>, 5 mM Tris-HCl (pH 7.6) was mixed with 4  $\mu$ l reservoir solution, equilibrated for one day and streak seeded. Crystals were observed after a few hours and of suitable size for X-ray experiments three days later.

A crystal was transferred to a reservoir solution containing 0.5 mM GDP and 25% (v/v) glycerol as cryo protectant before freezing in a liquid nitrogen cryo stream. The speed of this step was crucial for the quality of the data.

Diffraction data from one crystal of dimensions 0.3 mm  $\times$  0.4 mm  $\times$  0.15 mm were collected to a resolution of 2.8 Å at liquid nitrogen temperature (Oxford Cryosystems) on a MAR 345 detector at beam line I711 at the MAX II synchrotron, Lund, Sweden. The wavelength was 0.935 Å. The crystal has space group *P*2<sub>1</sub>2<sub>1</sub> with cell axes of 76.70 Å  $\times$  85.97 Å  $\times$  113.37 Å and one molecule in the asymmetric unit.

The diffraction data was integrated in DENZO (Otwinowski & Minor, 1997), scaled in SCALEPACK, truncated in TRUNCATE (Collaborative Computational Project, 1994; French & Wilson, 1978) and a test set chosen using UNIQUE and FREERFLAG (Collaborative Computational Project, 1994).

The structure of H573A was solved by molecular replacement using AMoRe (Navaza, 1994) with PDB-entry 1DAR as search model (Al-Karadaghi *et al.*, 1996). The search was done using domains I/II and VI/V separately. The model was built using the program O (Jones *et al.*, 1991) and refined in CNS version 0.5 and version 0.9a (Brünger *et al.*, 1998). The current model comprises 658 (95%) of the 691 amino acid residues, one GDP molecule, one Mg<sup>2+</sup> and 20 water molecules. Grouped B-factor refinement was carried out in CNS.

Figures were prepared using the program Ribbons (Carson, 1997) and the sequence alignment with ALSRIPT (Barton, 1993).

The atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank with accession number 1FNM.

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