Site-directed mutagenesis of *Thermus thermophilus* EF-Tu: the substitution of threonine-62 by serine or alanine

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Abstract The invariant threonine-62, which occurs in the effector region of all GTP/GDP-binding regulatory proteins, was substituted via site-directed mutagenesis by alanine and serine in the elongation factor Tu from *Thermus thermophilus*. The altered proteins were overproduced in *Escherichia coli*, purified and characterized. The EF-Tu T62S variant had similar properties with respect to thermostability, aminoacyl-tRNA binding, GTPase activity and in vitro translation as the wild-type EF-Tu. In contrast, EF-Tu T62A is severely impaired in its ability to sustain polypeptide synthesis and has only very low intrinsic and ribosome-induced GTPase activity. The affinity of aminoacyl-tRNA to the EF-Tu T62A·GTP complex is almost 40 times lower as compared to the native EF-Tu·GTP. These observations are in agreement with the tertiary structure of EF-Tu·GTP, in which threonine-62 is interacting with the Mg^{2+} ion, γ-phosphate of GTP and a water molecule, which is presumably involved in the GTP hydrolysis.

Key words: Elongation factor Tu; GTPase; GDP/GTP-binding protein

1. Introduction

The transition between the GTP- and GDP-bound states, driven by the hydrolysis of GTP, represents a molecular switch mechanism, which is utilized by the regulatory GTP/GDP-binding proteins. These enzymes are complexed to GTP in the active and to GDP in the inactive state. In their active conformation they interact with factors which transmit a signal or which stimulate the GTPase activity. The GTP/GDP-binding proteins are involved in a large number of cellular processes such as ribosomal protein biosynthesis, transmembrane signaling, differentiation and cell proliferation, translocation of nascent proteins and vesicle traffic (for review see [1]).

Among the GTP/GDP-binding proteins the bacterial elongation factor Tu (EF-Tu), $p21^{ras}$, which is the product of the *ras* proto-oncogene, and bovine rod transducin have been studied in the most detail. The crystal structures of EF-Tu·GDP, [2–4], EF-Tu·GTP [5,6], $p21^{ras}$ [7–10], the α-subunit of a heterotrimeric G-protein, $G_i\alpha 1$, [11] and the α-subunit of transducin [12,13] have been determined. Although their primary structures show only low sequence homology, the three-dimensional structures of the guanine nucleotide binding domains of these proteins are remarkably similar. The main differences between the GTP- and the GDP-liganded conformations of the nucleotide binding domains occur in the L_2-region (effector region, switch I region), connecting the first (from the N-terminus) α-helix with the unique antiparallel β-sheet, and in the second α-helix (helix B, switch II region), which follows this antiparallel β-sheet [6].

The low intrinsic GTPase activity of $p21^{ras}$ is enhanced by a GTPase activating protein, GAP [14]. Several point mutations in the 30–40 region of $p21^{ras}$ were reported to destroy the transforming activity of oncogenically activated forms of $p21^{ras}$, indicating that this region constitutes the GAP binding site (for review, see [15]). Therein a threonine at position 35 in $p21^{ras}$, corresponding to threonine-61 and threonine-62 in the EF-Tu from *Escherichia coli* and *Thermus thermophilus*, respectively, is invariant in all GTP/GDP-binding proteins [1]. This residue is involved in the coordination of the γ-phosphate of GTP, a Mg^{2+} ion, and a water molecule, which probably attacks the γ-phosphate during GTP hydrolysis. A similar coordination was described for the homologous threonine-177 in the transducin complex with guanosine-5′(8-thio)triphosphate [12]. In the $p21^{ras}$·GDP complex, however, threonine-35 is not coordinated to Mg^{2+} and points towards the solvent [7–9]. No reliable localization of the threonine-61 residue in the structure of EF-Tu·GDP from *E. coli* was possible, since only the crystal structure of a nicked molecule, lacking the amino acid residues 45 to 58 in close vicinity to threonine-61, was determined.

Upon the substitution of threonine-35 by alanine, the GTPase stimulation of $p21^{ras}$ [16–18] as well as of the related protein Rap1A [19,20] by GAP was abolished, while the substitution of threonine-35 by serine maintained the GAP sensitivity of $p21^{ras}$ [21]. However, the affinity for GTP and GDP of both $p21^{ras}$ and Rap1A carrying the amino acid substitution T35A were not affected [17,19]. In the case of the aminoacyl-tRNA·EF-Tu·GTP ternary complex, the GTPase stimulating factor corresponding to GAP is the programmed ribosome.

The aim of this investigation was to study the functional role of threonine-62 in EF-Tu. For this purpose we replaced threonine-62 by serine or alanine in *T. thermophilus* EF-Tu and overproduced the corresponding protein variants in *E. coli*. The biochemical properties of the purified EF-Tu variants were studied in partial reactions of the elongation cycle.

2. Materials and methods

Analytical grade chemicals were obtained from Merck. [^{3}H]GDP (10 Ci/mmol), [^{14}C]phenylalanine (475 Ci/mol) were from Amersham Buchler, [γ-^{32}P]GTP (5000 Ci/mol) was from DuPont.

Synthetic oligodeoxyribonucleotides, 5′-CCGTGTTGATGCTAATCCCCCG and 5′-GTGTTGATCGCAATCCCCCGC (altered nucleo-

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Abbreviations: DTT, dithiothreitol; EF-Ts, elongation factor Ts; EF-Tu, elongation factor Tu; GAP, GTPase activating protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; [IASNE-s^{2}C75]Tyr-tRNA^{Tyr}, Tyr-tRNA^{Tyr} alkylated with *N*-iodacetyl-*N*′-(5-sulfo-1-napthyl)-ethylendiamin on the 2-thiocytidine incorporated into position 75.

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tides compared to the wild-type sequence are underlined), were used as mutagenic primers for the T62S and T62A substitutions, respectively. DNA synthesis, site-directed mutagenesis, recombinant DNA techniques and bacterial transformation were performed as reported previously [22]. The presence of mutations was confirmed by DNA sequencing. The previously reported conditions [23] were applied for the overproduction of the EF-Tu variants in *E. coli* JM109 [24] containing either pEFTu-S62 or pEFTu-A62, for harvest of the bacteria grown in 10 l fermenters, and for the purification of the proteins, including a thermal denaturation step.

The nucleotide-free forms of the EF-Tu variants were prepared according to Limmer et al. [25], and the GTPase activity was determined as previously described [26]. The binding of aminoacyl-tRNA to EF-Tu·GTP was measured at equilibrium using a spectroscopic method [27] and by protection from hydrolysis of aminoacyl-tRNA by EF-Tu·GTP [28]. The EF-Ts-catalyzed GDP-release from EF-Tu·GDP was determined as previously described [29].

Nucleotide binding was achieved by incubation of (1.1 ml) 11 nM solution of nucleotide-free EF-Tu at 0°C for 5 minutes in 50 mM sodium borate, 50 mM NH_4Cl, 50 mM KCl, 10 mM $MgCl_2$, pH 7.5, with 44 nM [^{3}H]GDP (10 Ci/mmol) and [^{3}H]GTP (7 Ci/mmol), respectively. The exchange of radioactively labeled nucleotide for cold GDP was started, after adjusting the temperature to 37°C, by adding 220 μM GDP. At defined time intervals the residual amount of radioactively labeled, EF-Tu-bound nucleotide was determined in aliquots containing 10 pmoles EF-Tu by filtration of the reaction mixture through nitrocellulose filters [30].

In vitro EF-Tu dependent poly(Phe) synthesis was measured in homologous *T. thermophilus* systems as described recently [22].

3. Results

The replacement of threonine-62 by serine or alanine in the *T. thermophilus* EF-Tu was achieved by site-directed mutagenesis and analyzed by DNA-sequencing. The mutated genes were cloned in the expression vector pKK223–3, which has successfully been used in a previous study for the overexpression of the *T. thermophilus tufA* gene in *E. coli* [23]. The procedure, optimized for the overproduction and purification of the native EF-Tu, including a thermal denaturation step, could be applied without changes for the two EF-Tu variants with the amino acid substitutions. The overproduction yielded EF-Tu amounts comprising 25% of the total soluble cellular protein. From 40 g of cells 100 mg of homogeneous EF-Tu variants were obtained. Both proteins exhibited the same thermostability as reported for the unchanged *T. thermophilus* EF-Tu [31].

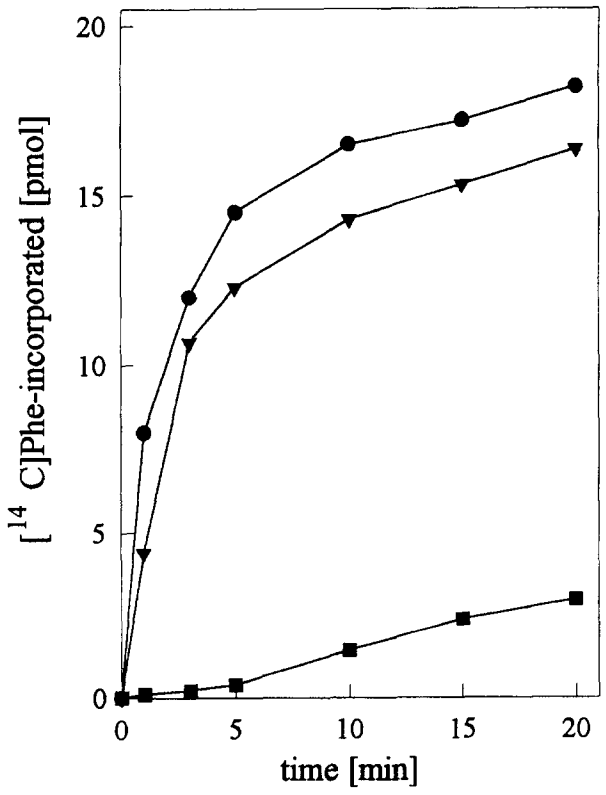

Fig. 1. In vitro poly(U)-dependent synthesis of poly(Phe) using native *T. thermophilus* EF-Tu (●), EF-Tu T62S (▼), and EF-Tu T62A (■).

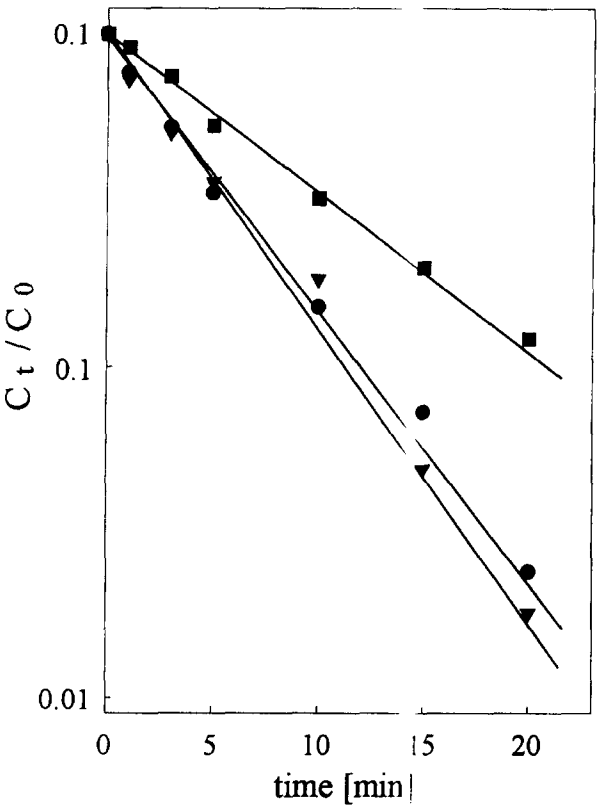

Fig. 2. Kinetics of the GDP dissociation from the native EF-Tu (●), EF-Tu T62S (▽), and EF-Tu T62A (■). C_t/C_0 is defined as the concentration of EF-Tu·[^{3}H]GDP at indicated time over the initial concentration of EF-Tu·[^{3}H]GDP.

While the poly(U)-dependent poly(Phe) synthesis with EF-Tu T62S was only slightly reduced as compared to that with the native EF-Tu, the poly(Phe) synthesis with EF-Tu T62A was very slow (Fig. 1). After 20 minutes only 16% poly(Phe) synthesis was reached with EF-Tu T62A as compared to wild-type EF-Tu.

The stoichiometry of GDP- or GTP-binding to EF-Tu remained unchanged by the replacement of threonine-62 with serine and alanine. To address more quantitatively the question of whether the reduced activity of EF-Tu T62A in poly (U)-dependent poly(Phe) synthesis was due to the altered EF-Tu interaction with nucleotides, we measured the dissociation rates for GTP and GDP of the EF-Tu variants. Radioactively labeled GDP and GTP were added to the nucleotide-free EF-Tu to form EF-Tu·[^{3}H]GDP and EF-Tu·[^{3}H]GTP complexes, respectively, and the decrease of the EF-Tu-associated radioactivity upon addition of unlabeled GDP was measured. As com-

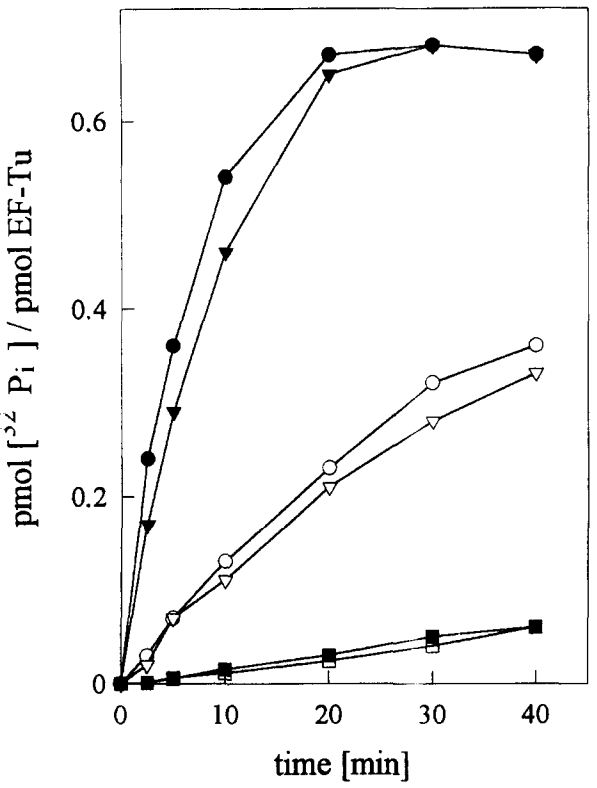

Fig. 3. Kinetics of the GTPase reaction of the native EF-Tu (○), EF-Tu T62S (▽), and EF-Tu T62A (□) in the absence (open symbols) and in the presence (filled symbols) of *T. thermophilus* 70S ribosomes.

pared to the native EF-Tu, no difference was found in the rate of GTP dissociation from both altered EF-Tu variants (data not shown) or in the rate of GDP dissociation from EF-Tu T62A. Only the dissociation rate of GDP from EF-Tu T62S was slightly slower than that of the wild-type EF-Tu (Fig. 2). There were also no differences observed for the rate of EF-Ts-mediated nucleotide dissociation, which was measured in the case of both EF-Tu variants at 0°C. These rates are identical to those observed with native EF-Tu (data not shown).

Since the reduced efficiency of EF-Tu T62S and, more pronounced, of EF-Tu T62A in poly(Phe) synthesis compared to the native EF-Tu cannot be attributed to altered nucleotide binding, we measured the apparent equilibrium dissociation constants, K_d, for the aminoacyl-tRNA·EF-Tu·GTP ternary complex formation. In a spectroscopic assay the binding of fluorescent (IASNEs^{2}C) Tyr-tRNA^{Tyr} to EF-Tu·GTP and its variants was measured at 4°C. The K_d for native EF-Tu, EF-Tu T62S and EF-Tu T62A were $16.8 \pm 3.1 \times 10^{-10}$ M, $31.7 \pm 12 \times 10^{-10}$ M and $618 \pm 99 \times 10^{-10}$ M, respectively. Thus the lack of interaction of threonine-62 with the γ-phosphate of GTP in the case of EF-Tu T62A decreases the affinity of aminoacyl-tRNA for EF-Tu·GTP about 40-fold. This change, although significant, is not dramatic and probably indicates a slight structural alteration of the protein in the vicinity of the aminoacyl-tRNA binding site upon the T62A substitution.

The intrinsic and ribosome-induced GTPase activity of EF-Tu T62S was slightly reduced compared to that of the wild-type EF-Tu (Fig. 3). The activity of EF-Tu T62S was stimulated by the addition of *T. thermophilus* 70S ribosomes. In contrast, the EF-Tu T62A showed only 10% residual intrinsic GTPase activity as compared to the native EF-Tu. Moreover this GTPase could not be stimulated by the addition of ribosomes. The results of these experiments are in accordance with results obtained with in vitro translation assays (Fig. 1), where a very low activity of EF-Tu T62A was observed.

With respect to intrinsic GTPase activity the EF-Tu T62A is similar to EF-Tu in which the histidine-85 is replaced by leucine (data not shown). The EF-Tu H85L variant has a very slow, but clearly detectable GTPase, but is, in contrast to EF-Tu T62A, completely void of activity in translation [22].

4. Discussion

The intrinsic GTPase activity of the EF-Tu from *T. thermo-*

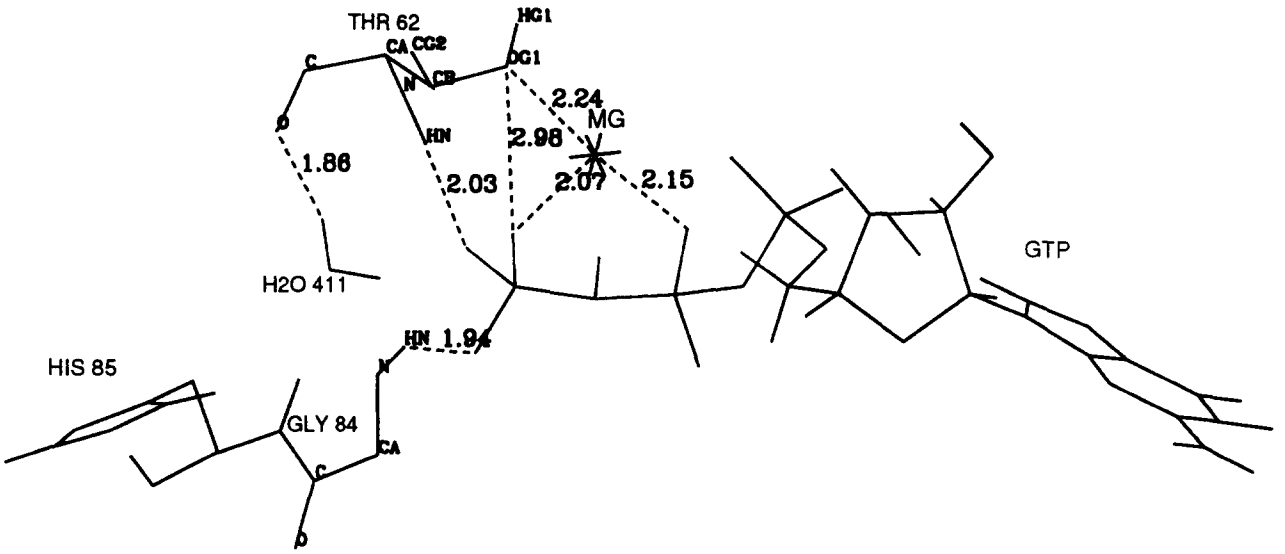

Fig. 4. Structure and interactions of threonine-62 in the *T. thermophilus* EF-Tu·GppNHp complex [6]. GppNHp, threonine-62, glycine-84, histidine-85, magnesium ion and water molecule 411 are shown. Solid lines represent covalent bonds, dashed linesdepict the non-covalent interactions and distances (given in angstroms).

philus was only slightly impaired when replacing threonine-62 by serine and almost fully abolished by its substitution with alanine.

As determined by X-ray structure analysis of *T. thermophilus* EF-Tu·ppNHp [6], the side-chain hydroxyl group of threonine-62 is coordinated to the γ-phosphate of the nucleotide and simultaneously to the magnesium ion. The other two oxygens of the γ-phosphate are coordinated to the main chain imino groups of threonine-62 and glycine-84. The main chain carbonyl group of threonine-62 is also hydrogen-bonded to a water molecule, which probably reacts with the γ-phosphate [6]. Thus, the threonine-62 residue plays a crucial role in the positioning of several atoms involved in the GTPase (Fig. 4).

The side chain of histidine-85 depicted in Fig. 4 was suggested to function as a catalytic residue, which initiates the GTP hydrolysis by accepting a proton from a reactive water molecule. The resulting hydroxyl ion then attacts the γ-phosphate of GTP to form a pentacovalent transition state, which is stabilized by the magnesium ion, threonine-62 and glycine-84 [32]. It was pointed out that the histidine-85 has to undergo a conformational change to be placed in the vicinity of the reactive water [6]. Such conformational change may be regulated by binding of the aminoacyl-tRNA·EF-Tu·GTP complex to the ribosomal A-site.

In EF-Tu T62A the hydroxyl group of threonine-62, which is a part of the GTP binding site and, at the same time, a catalytic element of the GTPase, is missing. This results in severe inhibition of the GTPase. It is remarkable that the dissociation rate of GDP and GTP from the EF-Tu T62A variant is the same as that of the native protein. This could indicate that the interaction of the OH group of threonine-62 does not contribute essentialy to the binding energy of the GTP and that the function of this OH group is apparently more related to the ribosome-regulated catalysis of the GTPase.

Very slow GTP hydrolysis can, however, take place even in the absence of this essential hydroxyl group (Fig. 3). This is similar to the very slow GTPase activity which was observed in the case of *E. coli* EF-Tu, in which the histidine-84 (85 in *T. thermophilus* EF-Tu) was replaced by alanine [33]. Apparently a slow intrinsic GTPase can be achieved by placing an isolated water molecule into a lipophilic pocket in the vicinity of the γ-phosphate of GTP. Since activation of this water cannot be achieved when the histidine-85 or threonine-62 are missing, no stimulation of the GTPase by ribosomes was observed with the corresponding EF-Tu variants.

The minor decrease of the GTPase activity with the substitution of threonine-62 by serine is not surprising. Evidently the hydroxyl group of serine can substitute well for the function of the threonine hydroxyl group. The slight reduction in the GTPase activity can be explained by the increased mobility of the serine hydroxyl group as compared to the hydroxyl group in threonine, where its rotation may be restricted due to the steric hindrance of the additional methyl group. The importance of this methyl group is also evident from the perfect invariance of the threonine at the homologous position in all GTP/GDP-binding proteins [1].

The EF-Tu·GTP displayed a 40-fold reduced aminoacyl-tRNA binding affinity upon the substitution of threonine-62 by alanine. This probably results in poor ribosome binding of the corresponding ternary complex. In accordance with this observation, the bacterial growth is not affected during the overproduction of the non-functional *T. thermophilus* EF-Tu T62A in *E. coli*. In contrast, the overproduction of EF-Tu H84G, which is probably also unable to hydrolyze GTP, leads to severe retardation of bacterial growth [22]. Since the overproduced EF-Tu from *T. thermophilus* participates in the protein biosynthesis of *E. coli* [34], the growth retardation in the latter case may be due to the permanent binding of the non-hydrolyzing EF-Tu variant to the ribosome resulting in a polysome block. This effect does not occur with EF-Tu T62A·GTP, which binds poorly to aminoacyl-tRNA and ribosomes.

Threonine-62 of *T. thermophilus* EF-Tu is located in the C-terminal end of the effector region (amino acid residues 39–66), which harbors a part of the aminoacyl-tRNA binding site. Several experiments suggest that the 3′-end of the aminoacyl-tRNA binds to the interface between domains I and II of EF-Tu (for references see [35]) in the vicinity of the effector region [36]. Functional studies with variants of $p21^{ras}$ indicated that the GAP interaction site of this regulatory protein is located in the effector loop. Provided the GTPase activities of EF-Tu and $p21^{ras}$ are regulated by a similar mechanism, the L_2-region of EF-Tu (effector region) should be involved in ribosomal interaction [26] and transmission of the signal for accelerated GTP hydrolysis. Threonine-62, being located at the C-terminal end of the effector loop, is catalytically involved in the GTPase and may fulfill the function of the main signal receptor transmitting a conformational change and, at the same time, modulating the GTPase activity.

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