Analysis and crystallization of a 25 kDa C-terminal fragment of cloned elongation factor Ts from *Escherichia coli*

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Abstract A 25 kDa C-terminal tryptic fragment of elongation factor Ts has been purified to homogeneity. Experimental evidence suggests that the 25 kDa C-terminal and the 5.3 kDa N-terminal fragments are structurally independent domains. The N-terminal fragment is shown to be essential for the nucleotide exchange activity. Crystals of the C-terminal fragment belong to space group P2 or $P2_1$. The diffraction pattern shows a pronounced pseudo-C2 symmetry at low resolution. This pseudo symmetry increases when the crystals are irradiated with X-rays for a few hours.

Key words: Protein biosynthesis; Nucleotide exchange factor; Elongation factor Ts; Trypsin fragments; Crystallization; Pseudo symmetry; *Escherichia coli*

1. Introduction

The reaction cycle of elongation factor Tu (EF-Tu) is an integral part of protein biosynthesis in procaryotic organisms (for a review see [1]). EF-Tu forms a ternary complex with guanosine-triphosphate (GTP) and aminoacyl-tRNA (aa-tRNA). This complex delivers the activated amino acid to the A-site of the ribosome [2–3]. After GTP-hydrolysis EF-Tu leaves the ribosome as a binary complex with guanosine-diphosphate (GDP) [3–5]. In *Escherichia coli* (*E. coli*) the biosynthesis of peptide bonds is kept at a rate of approx. 10–20 bonds/s/ribosome [6–7]. In order to maintain the rate of formation of peptide bonds, the conversion of EF-Tu:GDP to EF-Tu:GTP:aa-tRNA must proceed with at least the same rate.

Different groups [8–11] have determined values of the rate constant for dissociation of GDP from EF-Tu in the range 10^{-2} to 10^{-3} s^{-1}. These values are much too low to account for the in vivo rate of formation of peptide bonds. In the presence of the 30.3 kDa cytoplasmic protein elongation factor Ts (EF-Ts) an EF-Tu:EF-Ts:GDP intermediate is rapidly produced. The nucleotide GDP dissociates from this intermediate with a rate constant of 10^1 to 10^3 s^{-1} [10–11] resulting in the stable EF-Tu:EF-Ts complex. Binding of GTP to EF-Tu:EF-Ts is very fast, while the dissociation of EF-Ts from EF-Tu:EF-Ts:GTP proceeds with a rate constant of 13 s^{-1} [12]. The exchange reaction is summarized in Fig. 1. The overall increase of the nucleotide exchange rate in the presence of EF-Ts is of the order 10^3 to 10^4, in agreement with the rate of peptide bond formation (for a review on the rate-constants in the exchange reaction see [12]).

Hwang and Miller [13] have shown that the exchange reaction follows the substituted enzyme pathway as illustrated in Fig. 1 and several reports suggest the presence of a structural isomerization-step in the EF-Tu:EF-Ts:GDP complex [14–17]. It has been demonstrated [18] that the site of interaction with EF-Ts is partly located in domain II/III of EF-Tu which is in good agreement with the previously proposed EF-Ts binding site in domain III [19–20]. However, a recent study by Hwang et al. [21] indicates that also residues 154–199 located in domain I of EF-Tu are involved in EF-Ts binding. Very little structural information about EF-Ts is available, other than the nucleotide sequence of the EF-Ts encoding gene *tsf* [22]. More information concerning the EF-Tu:EF-Ts interaction could come from X-ray analysis of EF-Ts and the EF-Tu:EF-Ts complex. Though crystals of this complex have been obtained [23–24] no structure has been reported.

In this work we report a possible two-domain-structure of EF-Ts and the successful crystallization and preliminary X-ray investigation of a 25 kDa C-terminal fragment obtained by tryptic digestion of cloned and overexpressed intact EF-Ts from *E. coli*.

2. Materials and methods

2.1. Materials

E. coli JM105 [25] and N4830-1, which is a P1 transductant of N4830 [26], were used for cloning and expression. Restriction endonucleases and modifying enzymes were purchased from Boehringer-Mannheim, Germany. Vent polymerase for PCR was from New England Biolab, USA. Sequenase version 2.0 was from USB, USA, and [α-^{35}S]dATP (>1000 Ci/mmol) was from Amersham International, UK. Q- and S-Sepharoses were from Pharmacia, Sweden, and AcA54 from IBF, France. DEAE-Separon was from TESSEK, Denmark and Centricon concentrators were from AMICON, USA. The pGEX-1 vector [27] was kindly provided by Naomi Halachmi, Technicon, Haifa. T_4 ligase and factor X_a were gifts of H.C. Thøgersen, Laboratory of Gene Expression, Aarhus University. L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin, elastase, chymotrypsin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), DL-dithiothreitol (DTT) and Ficoll were from Sigma, USA. OGP was from Boehringer-Mannheim, Germany and [^{3}H]GDP (14 Ci/mmol) used in activity assays was from Amersham International, UK. Filters with pore size 0.45 μm were from Gelman Sciences, USA. Chemicals not described above were of analytical grade.

2.2. Cloning in expression vectors

Prior to cloning, the *tsf* gene, which encodes *E. coli* EF-Ts, was PCR amplified according to the manufactors instruction (New England Biolabs). For cloning in the inducible runaway expression vector pCP40 [28] the following two primers were used: 5'-AGGACGAATTCCAAGGAATATAGCCATGGCTGAAATTACCGC and 5'-AGGACGAAGCTTTTAAGACTGCTTGGACAT. Embedded restriction sites made possible a directional cloning in the EcoRI/HindIII sites of pCP40. For cloning in the pGEX system the following primers were used: 5'-AGGACGGATCCCGGTTCCATCGAGGGTCGTGCTGAAATTACCGCA and 5'-AGGACGGATCCTCATTAAGACTGCTTGGACATC. EF-Ts is in this system expressed as part of a fusion

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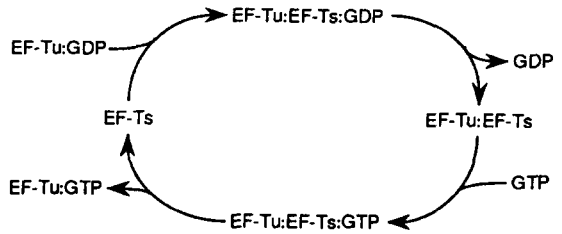

Fig. 1. Overview of the EF-Ts cycle in *E. coli*.

protein, however, the primer and cloning design makes possible a release of authentic EF-Ts subsequent to cleavage with the blood clotting factor X_a. Upon cloning the *tsf* gene sequences were confirmed by sequencing using Sangers dideoxy chain-termination method [29].

2.3. Expression and purification of EF-Ts

Upon propagation in the strain N4830-1, which is a λ lysogen for the temperature sensitive repressor cI857, expression was induced at A_{450} = 0.8 by increasing the temperature to 42°C for a few hours. The non-ribosomal fraction of crude *E. coli* cell extract was chromatographed on Q-Sepharose using a linear 10–250 mM KCl gradient in 50 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.5 mM PMSF, 15 μM GDP. Active fractions were desalted and acidified to pH 5 and further chromatographed on S-Sepharose using a linear 10–500 mM NaCl gradient in 50 mM sodium acetate, pH 5.0, 1 mM DTT, 0.5 mM PMSF, 15 μM GDP. Finally EF-Ts was purified by gel filtration on Ultrogel AcA54 in a buffer containing 50 mM Tris-HCl, pH 7.6, 80 mM KCl, 1 mM DTT, 0.5 mM PMSF and 15 μM GDP.

Expression, purification and release of authentic EF-Ts, using the pGEX gene fusion system, was done as described previously for recombinant EF-Tu [30].

Protein obtained from either of the two expression systems was further purified by anion exchange HPLC on DEAE-Separon using a 0–500 mM KCl gradient in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF. Buffer-exchange to storage buffer (20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF) and concentration was done with Centricon-C10 concentrators. Protein concentration was measured using the modified method of Bradford [31]. The protein was frozen in liquid nitrogen and stored at −80°C.

2.4. Electrospray mass spectrometry

The electrospray mass spectra of the intact protein were recorded on a Vestec electrospray mass spectrometer (Vestec Corp., Houston, TX). The samples were dissolved in 2% acetic acid/50% methanol to a concentration of approximately 0.2 μg/μl and introduced by a syringe pump at a flow rate of 1 μl/min. The spectra were recorded with a scan rate of 10 s/scan using a mass window of *m/z* 500–1500 [32]. The molecular weight of the protein was calculated by wieghted averaging as described [33].

2.5. Purification of the C-terminal fragment

Purified EF-Ts at a concentration of 3.4 mg/ml in a buffer containing 6.7 mM Tris-HCl, pH 7.0, 3.3 mM $MgCl_2$, 25% (v/v) glycerol was digested with TPCK treated trypsin for 17 h at 4°C. The weight ratio of trypsin to EF-Ts was 1 : 1000 and the reaction was stopped by adding PMSF to a concentration of 0.5 mM. The digest was subjected to anion exchange HPLC using a 0–500 mM KCl gradient in 30 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF). Prior to freezing in storage buffer soybean trypsin inhibitor was added in a weight ratio of 3 : 1 relative to the originally added amount of trypsin.

2.6. Analysis of the C-terminal fragment

N-Terminal sequencing was performed on an Applied Biosystems 477A Protein Sequencer (Applied Biosystems, USA) using the standard Edman degradation method. Analysis of amino acid composition was done as described by Sottrup-Jensen [34]. Nucleotide exchange activity measurements on both intact EF-Ts and the C-terminal fragment were performed as described by Miller and Weissbach [35].

2.7. Crystallization and X-ray analysis of the C-terminal fragment

Crystallization of the C-terminal fragment was done by vapour diffusion in 24-well Cryschem-trays (Charles Supper Co., USA). Sitting drops of 4–10 μl composed of equal amounts of protein in storage buffer and reservoir were equilibrated against a reservoir of 800 μl. All reservoirs contained 1 mM DTT and 0.5 mM PMSF. Since the reservoir controls the pH when using ammonium sulphate [36] all reservoirs contained 10 mM MES or HEPES and were adjusted to the desired pH with Tris. All crystallizations were set up using a modified Gilson crystallization robot [37]. All solutions used for crystallization were filtered through 0.45 μm pore size filters prior to use. Crystal density measurements were done using the Ficoll gradient method [38]. Crystals were crosslinked by adding 1% (w/v) glutardialdehyde to the reservoir and leaving overnight prior to density measurements. X-Ray diffraction studies were performed on a FR571 rotating anode (Enraf-Nonius, Holland) operated at 45 kV, 85 mA and equipped with a FAST area detector (Enraf-Nonius, Delft) or an oscillation camera. Data processing was done using the MADNES software package [39] and the program PROCOR [40].

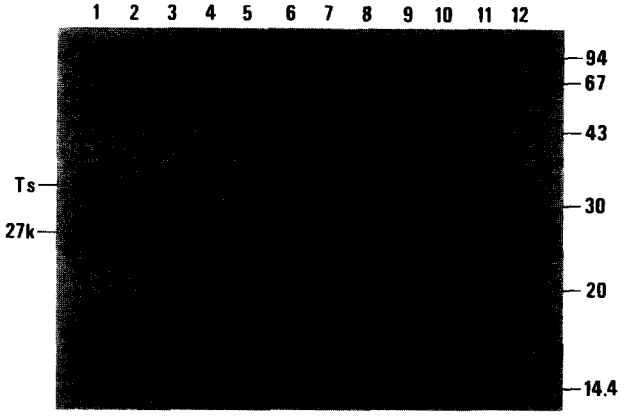

Fig. 2. Trypsin treatment of intact EF-Ts analysed with SDS-PAGE. SDS-PAGE was performed as described in [44]. Lane 1–4 = intact EF-Ts was incubated with 1% (w/w) trypsin under the conditions described in section 2 but glycerol was omitted. Samples of 0.7 μg were withdrawn after 5, 15, 30 and 60 min. Lane 5 = molecular weight markers (Pharmacia, Sweden). Lane 6–9 = same as lane 1–4 but with 25% (v/v) glycerol in the incubation mixture. Lane 10 = 0.7 μg intact EF-Ts. Lane 11 = 0.7 μg C-terminal fragment. Lane 12 = washed and redissolved crystal. The panel on the right shows the position and M_r (kDa) of the molecular weight markers. The panel on the left shows positions of intact EF-Ts and the major tryptic fragment with an apparent molecular weight of 27 kDa (27k).

3. Results

Plasmid encoded EF-Ts was analyzed by mass spectrometry. The mass of pCP40*tsf* encoded EF-Ts was determined to be 30294 ± 6 Da and when expressed as a fusion protein in the pGEX system the mass of released EF-Ts was 30296 ± 6. As the theoretical mass of EF-Ts without the N-terminal methionine is 30292 the results strongly indicate that the N-terminal

Table 1
N-Terminal sequencing of the C-terminal fragment

Cycle #	Amino acid	Amount
1	Ala/Lys	112/95
2	Gly/Ala	126/195
3	Asn/Gly	84/208
4	Val/Asn	112/181

The amino acids and the corresponding amounts in pmol were determined for the first 4 cycles in the automated Edman degradation.

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1   MAEITASLVKELRERTGAGMMDCKKALTEANGDIELAIENMRKSGAIKAAKKAGNVAADG  60
61  VIKTKIDGNYGIILEVNCQTDFVAKDAGFQAFADKVLDAAVAGKITDVEVLKAQFEEERV 120
121 ALVAKIGENINIRRVAALEGDVLGSYQHGARIGVLVAAKGADEELVKHIAMHVAASKPEF 180
181 IKPEDVSAEVVEKEYQVQLDIAMQSGKPKEIAEKMVEGRMKKFTGEVSLTGQPFVMEPSK 240
241 TVGQLLKEHNAEVTGFIRFEVGEGIEKVETDFAAEVAAMSKQS                  283
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Fig. 3. Amino acid sequence of EF-Ts as derived from the gene sequence [22]. Trypsin cleavage occurs on the C-terminal side of the residues marked with asterisks.

methionine of authentic EF-Ts is lacking and that posttranslational modifications are not present. A minor peak in the spectrum of pCP40*tsf* encoded EF-Ts indicated that a 5–10% fraction of overexpressed EF-Ts possibly possess an N-terminal formylated methionine, i.e. the mass being 150 Da higher. The yield of purified EF-Ts expressed from pCP40 was in the range of 1 mg/g wet cell paste and the yield of purified EF-Ts fusion protein about 10 mg/g wet cell paste. The following results were obtained using EF-Ts purified from the pGEX system.

Trypsin treatment performed as described in section 2 gave a major fragment with a molecular weight of 27 kDa as estimated from SDS-PAGE (Fig. 2). When glycerol was omitted two additional fragments of 19 and 18 kDa were produced (Fig. 2). Digestion of intact EF-Ts with elastase, and to a lesser extent chymotrypsin, revealed the presence of a fragment of approximately the same size as the major tryptic fragment (not shown). In anion exchange HPLC the major fragment always eluted in two very poorly resolved peaks. When present the two additional fragments of 19 and 18 kDa coeluted with the major fragment. Remaining intact EF-Ts was separating well from the major fragment (purification results are not shown). The purity of the major fragment after purification is shown in Fig. 2.

N-Terminal sequencing of the major fragment revealed a double sequence in which the two sequences were occurring in approximately the same amounts (Table 1). This pattern could be fitted perfectly into the sequence of EF-Ts revealing two adjacent cleavage sites at the carboxy-side of lysine-51 and lysine-52 (Fig. 3). The true molecular weight of the major fragment (in the following refered to as the C-terminal fragment) was calculated from the sequence to be 25 kDa. Analysis of the amino acid composition of the C-terminal fragment as well as of intact EF-Ts was performed. Different molar ratios of amino acids were predicted from the sequence for both intact EF-Ts and the C-terminal fragment and the same ratios were calculated from the experimental data. For both the C-terminal fragment and intact EF-Ts the experimental values were found to be consistent with the values predicted from the sequence. The most prominent of these ratios are shown in Table 2. We

Table 2
Analysis of the amino acid composition of the C-terminal fragment and intact EF-Ts

Amino acid ratio	Theoretical ratio intact EF-Ts	Observed ratio intact EF-Ts	Theoretical ratio C-terminal fragment	Observed ratio C-terminal fragment
Val/Ala	0.744	0.736	0.933	0.905
Pro/Leu	0.313	0.301	0.417	0.407
Met/Phe	0.900	0.880	0.600	0.592

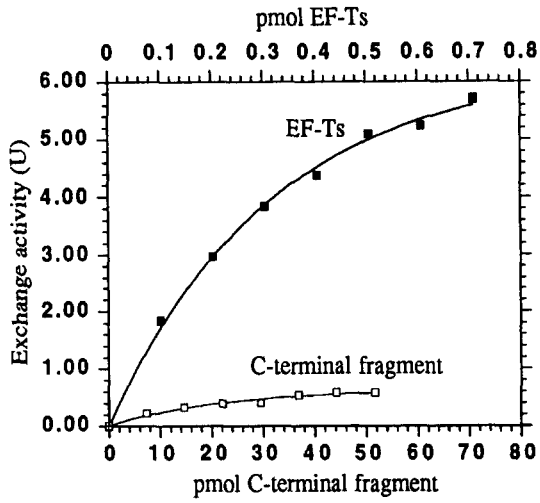

Fig. 4. Nucleotide exchange measurements on intact EF-Ts and the C-terminal fragment. Exchange activity is plotted against the amount of added EF-Ts (abscissa in top of figure) or C-terminal fragment (abscissa in bottom of figure).

therefore conclude that the two tryptic fragments dissociate after the hydrolysis.

Nucleotide exchange activity measurements on intact EF-Ts showed a specific activity of intact EF-Ts of 19.7 U/pmol. The specific activity of the C-terminal fragment was estimated to be 0.03 U/pmol (Fig. 4). One U is defined as described in [35].

For preliminary crystallization trials partly trypsinized EF-Ts was used. This was obtained by adding 1% (w/w) trypsin to 10 mg/ml intact EF-Ts in storage buffer. After incubation for 15 min on ice the reaction was stopped by adding 3% (w/w) soybean trypsin inhibitor. Spontaneous nucleation and growth of many very small crystals occurred under the conditions shown in Table 3. For unknown reasons nucleation of this crystal form did not take place with purified material using the same conditions. Instead massive nucleation of very thin needles occurred. However, streakseeding [41] using the crystals from partly trypsinized EF-Ts produced small single crystals from the purified C-terminal fragment. The best of these with a size of approx. 0.05 mm were used in macroseeding [41] with purified material to obtain crystals suitable for X-ray studies

Fig. 5. Crystal of the C-terminal fragment obtained by macroseeding. The dimensions of the crystal is $0.2 \times 0.2 \times 0.12$ mm^3.

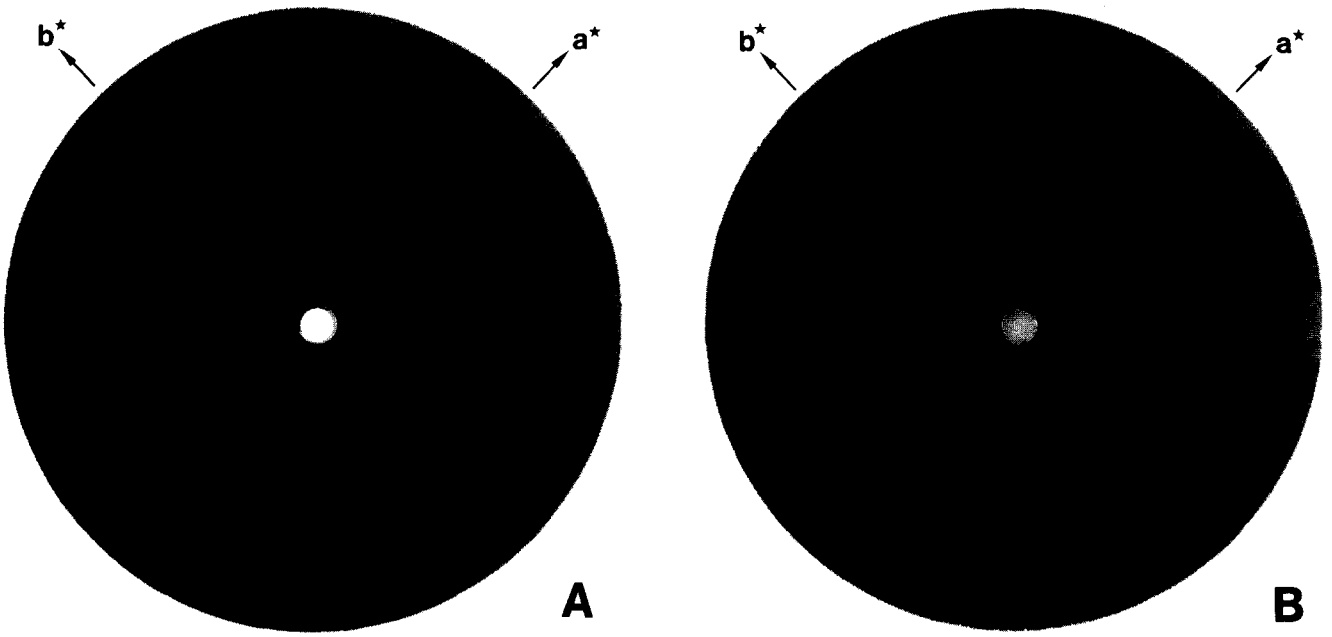

Fig. 6. 2° oscillation photographs showing C2 symmetry increasing with time. (A) The crystal was exposed for 5 h at a crystal to film distance of 86 mm. The photograph shows the slightly misoriented a*, b* plane with the two axes oriented as shown. The resolution on the edge of the circular area is 2.6 Å. (B) Oscillation photograph obtained from the same crystal using the same conditions and covering the same oscillation range but in direct continuation of (A).

(Fig. 5). SDS-PAGE of washed and redissolved crystals gave a single band of the same molecular weight as the C-terminal fragment (Fig. 2). Crystallization was found to be absolutely dependent on the presence of the non-ionic detergent *n*-octyl-β-D-glycopyranoside (OGP). Glycerol helped to reduce the nucleation of the thin needles described above. It also increased the rate of crystal growth by a factor of 3. Crystallization conditions are listed in Table 3.

Crystals diffracted X-rays to a maximum resolution of 3.0 Å on an oscillation camera (Fig. 6). Several partial datasets were collected from which the cell dimensions were determined to be $a = 111.0$ Å, $b = 96.0$ Å, $c = 84.3$ Å, $\beta = 100.2°$ on average with less than 1% deviation between different crystals. The spacegroup was found to be P2 or $P2_1$ but exhibiting pseudo C2 symmetry as illustrated by the intensity statistics of one of the partial datasets (Table 4). Similar statistics were found for all the other partial datasets as well. During X-ray exposure the pseudo C2 symmetry of the diffraction pattern increased to an extent that a complete transformation to spacegroup C2 can be assumed (Fig. 6). The cell parameters remained constant during this transformation. The possibility of spacegroup $P2_1$ could not be eliminated because of the pseudo C2 symmetry giving absences overlapping with absences in spacegroup $P2_1$. The datasets collected had more than 50% reflections with $I/\mathrm{sig}I > 3$ at a resolution of 3.6 Å. R_{sym} was generally very high for data with a resolution better than 6 Å in all the datasets (Table 4). However these *R*-factors could be diminished considerably by integrating the same data in spacegroup C2 instead of P2 (Table IV). The crystals were stable in the beam for at least 5 h showing beginning decay of the high resolution reflections after this period of exposure.

Crystals were not stable in the Ficoll gradient used for density measurements if crosslinking with glutardialdehyde was omitted. Crosslinking did not affect the morphology of the crystals. A density of 1.190 was determined giving a protein content in the crystals of 54.1%. A number of 8 monomers per

Table 3
Crystallization conditions for the C-terminal fragment

	Spontaneous nucleation[a]	Streakseeding	Macroseeding
Precipitant:	52–54% $(NH_4)_2SO_4$[b]	48–49% $(NH_4)_2SO_4$	48–49% $(NH_4)_2SO_4$
pH:	7.2–8.0	6.3–7.0	6.3–7.0
Buffer:	Tris/HEPES	Tris/HEPES or Tris/MES	Tris/HEPES or Tris/MES
Additives:	2% (w/v) PEG6000	0.5% (w/v) OGP	0.5% (w/v) OGP
	0.5% (w/v) OGP	5% (v/v) glycerol	5% (v/v) glycerol
Crystal size:	<0.03 mm	0.05–0.1 mm	$0.2 \cdot 0.2 \cdot 0.12$ mm^3
Temperature:	20°C	20°C	20°C
[Protein]:	8–10 mg/ml	3 mg/ml	3 mg/ml
Growth time:	5 days	3–4 days	1 week

All specified values are for the conditions in the droplet after equilibration. [a]The conditions for spontanous nucleation were determined with partly trypsinized EF-Ts. [b]Percentage (v/v) in the reservoir of a saturated $(NH_4)_2SO_4$ solution.

Table 4
Statistics of a single 20° dataset as output from PROCOR

Resolution (Å)	$<I/\sigma I>$ h + k even[a]	$<I/\sigma I>$ h + k odd[a]	R_{sym} (%) P2	R_{sym} (%) C2	Completeness (%)
∞–6.33	20.22	4.76	8.4	4.4	17.9
6.33–4.47	11.25	4.69	13.0	8.0	18.7
4.47–3.65	5.50	4.30	24.2	18.1	16.4

Data were collected on one crystal during a period of 17 h. The same data were integrated by MADNES in both spacegroup P2 and C2. [a]$<I/\sigma I>$ was calculated for data integrated in spacegroup P2.

asymmetric unit in spacegroup P2 gives a theoretical density of 1.195 using the model of Westbrook [38] while 7 or 9 monomers would result in theoretical densities of 1.171 and 1.220, respectively. Furthermore an odd number of monomers per asymmetric unit would be inconsistent with the pseudo-C2 symmetry of the primitive spacegroup. With 8 monomers per asymmetric unit in spacegroup P2 the V_m = 2.21 Å^3/Da which is well within the values usually observed [42].

4. Discussion

Expression in the pGEX system proved to be a very efficient way of obtaining large amounts of intact EF-Ts. Since the purification is essentially a one-step procedure, the time needed is also reduced dramatically thereby minimizing loss of catalytic activity during purification. EF-Ts produced in this way was shown to be in a catalytically active conformation. EF-Tu has previously been expressed in the same system with good results [30] and it might therefore be generally applicable for purification of functionally active procaryotic proteins.

A very rapid trypsin digestion of EF-Ts leading to the production of the C-terminal fragment indicates that the cleavage site is located in a highly accessible region of the protein. The effect of glycerol on the specificity of the reaction indicates that glycerol either protects the region containing the additional cleavage site(s) or stabilizes the protein in a conformation where this region is inaccessible. A stabilization of one particular conformation is supported by the positive effect of glycerol on crystallization. The decrease of speed of the reaction upon addition of glycerol can be explained simply by the increased viscosity of the solution.

Determination of the amino acid composition of the purified fragment revealed that the remaining N-terminal fragment of 5.3 kDa dissociates from the C-terminal fragment after trypsin digestion. This indicates that the N-terminal fragment constitutes a structurally independent domain with a loose association with the rest of the molecule.

The activity of the C-terminal fragment was estimated to be 0.15% of the activity of intact EF-Ts and it is therefore concluded that the N-terminal part of EF-Ts is essential for the nucleotide exchange activity. Since the C-terminal fragment is able to crystallize the possibility that the decrease in catalytic activity is a result of destabilization leading to random rearrangements or denaturation of the fragment is ruled out. The very low activity of the isolated C-terminal fragment might suggest that the N-terminal part of the protein binds to EF-Tu during the exchange reaction. Two studies report that EF-Ts from both *Thermus thermophilus* [17] and *E. coli* [43] is protected against tryptic cleavage when bound to EF-Tu, indicating that lysine-51 and lysine-52 of *E. coli* EF-Ts are protected by this interaction. This makes it likely that the two lysines and possibly the N-terminal fragment are located close to the site of interaction with EF-Tu, thereby supporting the suggestion that the N-terminal fragment is involved in EF-Tu binding. Most likely the C-terminal part of EF-Ts also binds to EF-Tu since previous studies (see Introduction) have shown that EF-Ts binds to all three domains of EF-Tu.

Preliminary investigations of the crystals indicate that collection of data to a resolution of at least 3.6 Å is possible using conventional sources. The above mentioned high R-factors of the small datasets is most likely a consequence of the X-ray induced C2 symmetry of the crystal lattice. Crystal decay as well as spacegroup transformation in the crystals can probably be eliminated by collecting data at cryogenic temperature. Crystallization and resolution limit of the data can possibly be improved by cloning one of the two C-terminal fragments, thereby increasing the homogeneity of the protein.

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