Elongation Factor Ts Can Act as a Steric Chaperone by Increasing the Solubility of Nucleotide Binding-Impaired Elongation Factor-Tu†

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ABSTRACT: Several elongation factor (EF) Tu mutants (T25A, H22Y/T25S, D80N, D138N) that have impaired nucleotide binding show decreased solubility on overexpression in the *E. coli* cell, an indication that they do not fold correctly. Moreover, EF-Tu[T25A] and EF-Tu[D80N] were shown to inhibit cell growth on expression, an effect attributed to their sequestration of EF-Ts [Krab, I. M., and Parmeggiani, A. (1999) *J. Biol. Chem. 274*, 11132−11138; Krab, I. M., and Parmeggiani, A. (1999) *Biochemistry 38*, 13035−13041]. We present here results showing that the co-overexpression of EF-Ts at a 1:1 ratio dramatically improves the solubility of mutant EF-Tu, although in the case of EF-Tu[D138N]—which cannot at all bind the nucleotides available in the cell—this is a slow process. Moreover, with co-overexpression of EF-Ts, the mentioned growth inhibition is relieved. We conclude that for the formation of a correct EF-Tu structure the nucleotide plays an important role as a "folding nucleus", and also that in its absence EF-Ts can act as a folding template or steric chaperone for the correct folding of EF-Tu.

Bacterial elongation factor (EF)[1] Tu is a GTPase that transports aminoacyl (aa-)tRNAs to the mRNA-programmed ribosome in the elongation cycle of protein synthesis [reviewed in (*1*)], accelerating dramatically the elongation process and contributing to its high selectivity. It binds guanosine di- and triphosphate—but not the monophosphate (*2*)—with high affinity and possesses an intrinsic ability to catalyze γ-phosphate hydrolysis of bound GTP. This catalytic activity is increased by approximately 5 orders of magnitude when the transported aa-tRNA has codon−anticodon interaction with the mRNA in the A site of the elongating ribosome. EF-Tu from *Escherichia coli* is only moderately temperature-stable in vitro (rapidly inactivated at temperatures above 45 °C) when bound to a GDP or GTP nucleotide (*3, 4*) and unstable in the absence of bound nucleotide (*5*). On the other hand, the complex with its guanine nucleotide exchange factor EF-Ts is stable in the absence of nucleotide for extended periods of time (*6*). EF-Tu with the substitution Asp138→Asn specifically binds xanthosine nucleotides (di- or triphosphate) instead of guanine nucleotides (*7*). It was used as a tool to investigate the energy consumption by EF-Tu during elongation (*8, 9*). Because it hydrolyzes XTP[1] specifically, the NTP consumption by EF-Tu becomes distinguishable from that of other protein synthesis factors. Unfortunately, the yield of soluble protein after overexpression of this mutant protein in *E. coli* is very low, making it laborious and time-consuming to isolate it in significant amounts. Noteworthy, although xanthosine and its monophosphate form are present in the cell as intermediates in the pathway of guanosine nucleotide synthesis, XDP and XTP are not. Although any NDP will be converted to NTP by nucleoside diphosphokinase, the formation of nucleoside diphosphates from the monophosphates is catalyzed by base-specific enzymes (adenylate kinase, cytidylate kinase, guanylate kinase, uridylate kinase, and deoxythymidylate kinase) (*10*). However, no xanthosylate kinase exists in *E. coli* (*10*), and the consequent lack of polyphosphorylated xanthosyl nucleotides in the cell makes EF-Ts the only possible source of stabilization of EF-Tu[D138N]. Indeed, the maximum level of this mutant found in the soluble fraction on overexpression never exceeds one-tenth that of wt EF-Tu in the cell, which corresponds closely to the level of cellular EF-Ts (Albert Weijland, private communication).

This observation prompted us to investigate if the solubility of EF-Tu[D138N] was somehow limited by the amount of EF-Ts available. For this purpose, we constructed a vector which allows simultaneous overexpression of glutathione-*S*-transferase (GST)-fused EF-Tu (*11*) and of EF-Ts. This vector was then used to not only express GST-EF-Tu-[D138N], but also express other EF-Tu mutants with strongly decreased affinity for guanine nucleotides, which limits their solubility on overexpression similarly to that of EF-Tu-[D138N].

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[1] Abbreviations: EF, elongation factor; NDP/NTP, nucleoside di-/triphosphate; ME, 2-mercaptoethanol; XDP/XTP, xanthosine di-/triphosphate.

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Table 1: Plasmids Used in This Study

plasmid name	expressed proteins	reference[a]
pGtAM-wt	GST-EF-Tu wt	(*12*)
pTuTs	GST-EF-Tu wt + EF-Ts	
pGtAp-T25A	GST-EF-Tu[T25A]	(*13*)
pTu[T25A]Ts	GST-EF-Tu[T25A] + EF-Ts	
pGtAp-H22Y/T25S	GST-EF-Tu[H22Y/T25S]	(*13*)
pTu[H22Y/T25S]Ts	GST-EF-Tu[H22Y/T25S] + EF-Ts	
pGtA-D80N	GST-EF-Tu[D80N]	(*12*)
pTu[D80N]Ts	GST-EF-Tu[D80N] + EF-Ts	
pGtA-D138N	GST-EF-Tu[D138N]	(*9*)
pTu[D138N]Ts	GST-EF-Tu[D138N] + EF-Ts	

[a] Plasmids for which no reference is given are new constructs of this work.

MATERIALS AND METHODS

Construction of the EF-Tu Mutants Coexpressing EF-Ts. The EF-Tu vector pTuTs was derived from pGtAM-wt, a pGEX-2T (*11*) derivative expressing GST-fused *E. coli* EF-TuA (*12*). First the entire *tufA* gene was PCR-amplified using an oligonucleotide that hybridizes within the GST coding sequence and one that hybridizes to the end of the *tufA* coding sequence, introducing a *Pst* site adjacent to the stop codon. This fragment was cloned *Bam*HI−*Pst*I into a pGEX-2T derivative that has a *Pst* site instead of the *Eco*RI site (A. Bernardi, unpublished). Then the *Bam*HI−*Nco*I fragment comprising the major part of the EF-Tu sequence was replaced by that from pGtAM-wt, to avoid amplification errors in this region. The remaining part of the EF-Tu open reading frame was verified by sequencing. Next, a 1.26 kb *Sma*I−*Pst*I fragment from pTS21 (a gift of Dr. Yu-Wen Hwang, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, N.Y. 10314) containing the *E. coli tsf* gene was inserted using a *Pst*I linker on the *Sma*I blunt end. A 200 bp spacer devoid of transcription stops separates the EF-Tu and EF-Ts open reading frames. pTu[D138N]Ts was derived from this vector by transferring the 240 bp *Acc*I−*Acc*I *tuf* gene-internal fragment from pGtA-D138N into pTuTs. pTu[T25A]Ts, pTu-[H22Y/T25S]Ts, and pTu[D80N]Ts were made by transferring the 760 bp *Bam*HI partial *Acc*I fragment from the respective GST-EF-Tu-expressing vectors (see Table 1).

Overexpression of the EF-Tu Mutants Coexpressing EF-Ts. For overexpression studies, cells were grown in LB medium with 100 μg/mL ampicillin at the indicated temperatures and induced with either 300 or 75 μM isopropylthiogalactoside. The development of the optical density at 600 nm was followed in time. At specified times, cells from samples of 20 mL culture per $OD_{600\ nm}$ were collected by centrifugation and resuspended in 1.3 mL of lysis buffer (50 mM Tris·HCl, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 0.5 mg/mL lysozyme), incubated on ice for 30−45 min, and then sonicated for 2 min total time. A sample of the soluble fraction was separated from insoluble debris by centrifugation at 13 000 rpm in a microfuge for 10 min. The pellet was resuspended in an equal volume of cracking buffer. Fifteen microliters of supernatant and an equivalent amount of resuspended pellet were analyzed on SDS−PAGE.

RESULTS

High-Level Simultaneous Overexpression of GST-EF-Tu and EF-Ts from pTuTs. To obtain simultaneous overexpression of GST-EF-Tu and EF-Ts in stoichiometric amounts, we constructed vector pTuTs (see Materials and Methods), which unites both genes in the same operon under the control of the ptac promoter. The induction of expression by isopropylthiogalactoside resulted in very high overexpression of both GST-EF-Tu and EF-Ts in the cell at approximately equal levels, in soluble amounts even somewhat better than the overexpression of EF-Tu alone (not illustrated). We subsequently made derivatives containing different EF-Tu mutations to see the effect of the coexpression of EF-Ts on their solubility (see Table 1).

Overexpression and Solubility of Xanthosine Nucleotide-Specific EF-Tu[D138N]. When we induced expression of pTu[D138N]Ts, we expected to see an increase in the fraction of EF-Tu[D138N] that is present in the supernatant of an S30 extract. However, at short incubation times (<6 h), we did not observe any significant increase in solubility compared to the overexpression of EF-Tu[D138N] alone. On the other hand, the overexpressed EF-Ts in that case was very soluble. Nevertheless, we were encouraged by the finding that the supernantant contained small but increasing amounts of soluble GST-EF-Tu[D138N] at longer induction periods (up to 22 h). In contrast, in the absence of coexpression of EF-Ts, no significant solubilization was found at any time. Combining the coexpression of EF-Ts with a decrease in the induction temperature (from 30 to 24 °C), which has in general a beneficial effect on the solubility of overexpressed proteins, we obtained the result shown in Figure 1A. After 7 h of induction with 0.3 mM IPTG, already high amounts of both EF-Tu and EF-Ts have been produced, but only part of the GST-EF-Tu[D138N] is found in the supernatant, unlike the EF-Ts, which is mostly soluble. However, after 32 h, well into the stationary growth phase, approximately 50% of the GST-EF-Tu is soluble. After 70 h, when the cells are hardly growing anymore, very high amounts of soluble mutant protein are obtained: approximately 20 mg of pure thrombin-cleaved EF-Tu/EF-Ts complex per liter of culture. These results emphasize the difficulty that the EF-Tu molecule has to achieve its native fold in the absence of a suitable nucleotide to serve as a "folding nucleus" and show that EF-Ts can, probably through a template action, assist in the folding process. To verify if the observed increase in soluble EF-Tu, in Figure 1A not observably accompanied by a decrease in the amount of insoluble factor, is just due to the high concentration of EF-Ts in the cytoplasm assisting in the folding of newly transcribed EF-Tu, or if insoluble factor can still refold and go into solution in the presence of EF-Ts, we did the control experiment in Figure 1B. After 8 h of induction, a sample was analyzed for solubility, and at the same time, further protein synthesis was stopped by the addition of 170 μg/mL chloramphenicol to the growth medium. The sample analyzed after 25 h shows that almost all of the EF-Tu that at first was insoluble, now is present in the supernatant, showing that, indeed, refolding of precipitated EF-Tu takes place.

Growth Inhibition by EF-Tu[T25A] and EF-Tu[D80N] Is Relieved by Overexpression of EF-Ts. We have recently shown that EF-Tu carrying the substitutions T25A or D80N displays an increased stability of the complex with EF-Ts which causes them to interfere in vitro with the dissociation of wt EF-Tu·GDP by EF-Ts (*12*, *13*). The new vector coexpressing EF-Ts allowed comparison of the in vivo effect

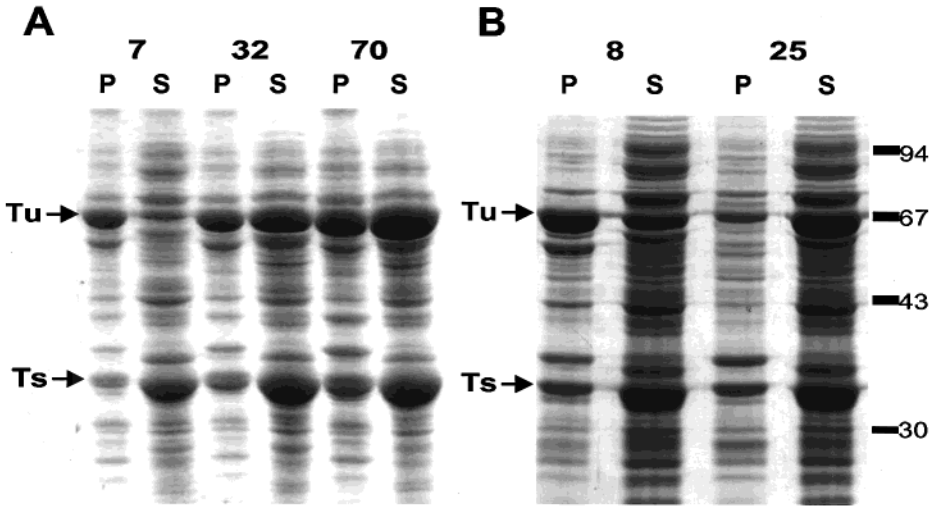

FIGURE 1: Long induction of pTu[D138N]Ts. Panel A: SDS−PAGE showing S30 supernatant (S) and pellet (P) fractions at the indicated time after induction of expression by isopropylthiogalactoside at 24 °C. The position of GST-EF-Tu[D138N] is indicated by an arrow marked Tu and that of EF-Ts by Ts. Panel B: idem for cells harvested after 8 h induction during normal growth, after which protein synthesis was arrested using chloramphenicol and another sample was analyzed after 25 h.

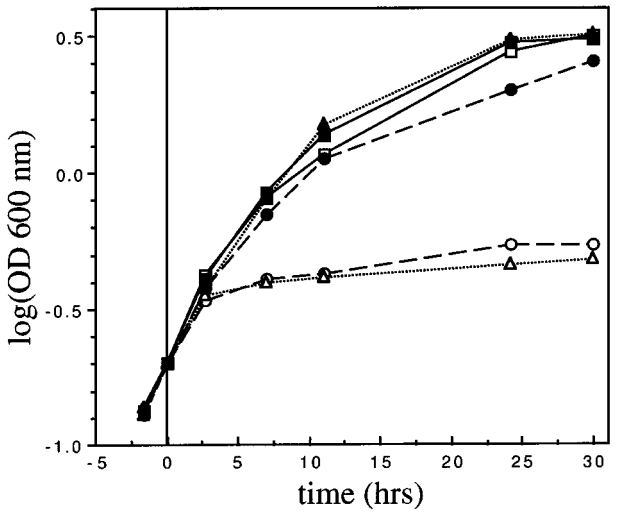

FIGURE 2: Increase of cell density during induction of mutant EF-Tu with and without coexpression of EF-Ts. Plot of $\log(OD_{600\ nm})$ of LB cultures at different times relative to the time of induction (at an $OD_{600\ nm}$ value of 0.2 and 24 °C) of the plasmids expressing GST-EF-Tu wt (□,■), GST-EF-Tu[T25A] (△,▲), and GST-EF-Tu[D80N] (○,●) without (open symbols) or with (filled symbols) coexpression of EF-Ts.

of these mutants in the presence of normal (limiting) amounts of EF-Ts and in the presence of equal amounts of the exchange factor. As illustrated in Figure 2, overexpression of GST-EF-Tu[T25A] or GST-EF-Tu[D80N] alone strongly decreases the growth rate, whereas 1:1 coexpression of EF-Ts completely relieves this inhibition. Thus, these mutated EF-Tu have a dominant negative behavior toward the wild-type EF-Tu in the cell in the presence of limiting amounts of EF-Ts, an effect that can be attributed to sequestering of the available EF-Ts by the mutants. In fact, since GST-fused EF-Tu can only to a small extent (~5%) participate in protein synthesis (*14*), it is unlikely that the growth-inhibiting effect is due to direct interference of the mutant GST-EF-Tu with the protein biosynthetic process. In line with this, the other mutants that did not show in vitro interference with EF-Ts stimulation of wt EF-Tu·GDP dissociation also did not inhibit growth (not shown). Even expression of GST-EF-Tu-[D138N]—whose complex with EF-Ts, once formed, cannot be dissociated in the cell for lack of XDP/XTP—did not retard growth. This is in agreement with the observed slow solubilization of EF-Tu[D138N] even in the presence of excess EF-Ts and implies that the correct folding of the factor is so slow that sequestering of EF-Ts does not occur while the cells are still actively growing.

Overexpression and Solubility of EF-Tu Mutants with Low Affinity for Guanine Nucleotides. The two mutants mentioned above, and a third one, the double mutant H22Y/T25S, also displayed a low yield of soluble protein on overexpression. All these mutant EF-Tu display a strongly decreased affinity for guanine nucleotides. For EF-Tu[T25A] and EF-Tu-[D80N], the low yield is in part attributable to their dominant-negative effect described above, causing inhibition of further protein synthesis. This is in part also due to lower solubility of the factors. Figure 3 illustrates this for mutant T25A and double mutant H22Y/T25S. At short times after the start of induction (<5 h), only a fraction of the GST-EF-Tu[T25A] is found in the supernatant (Figure 3A). At longer times, the solubility increases to more than 50%, but the total amount of protein does not further increase. The protein extracts show a characteristic fuzziness of the bands on SDS−PAGE at longer induction times, which coincides with the occurrence of spontaneous lysis in the cultures. The double EF-Tu mutant H22Y/T25S is expressed at a much higher level, but the amount of full-length product in the supernatant remains low (Figure 3C). Moreover, it suffers from a degradation problem: an approximately 30 kDa band increases in intensity in the supernatant (Figure 3C). At longer expression times (>20 h), this band increases strongly in intensity, while at the same time the band of full-length product disappears from the pellet (not shown). For both mutants, coexpression of EF-Ts dramatically improves the fraction of GST-EF-Tu that is present in the supernatant, although it takes several hours before the majority of the product is found in the soluble fraction (Figure 3B,D). Note that the ~30 kDa band due to degradation is not detectable in Figure 3D. Thus, EF-Ts is able to assist folding of EF-Tu also for mutants which do not bind nucleotides strongly enough to induce and maintain their native structure in the cell.

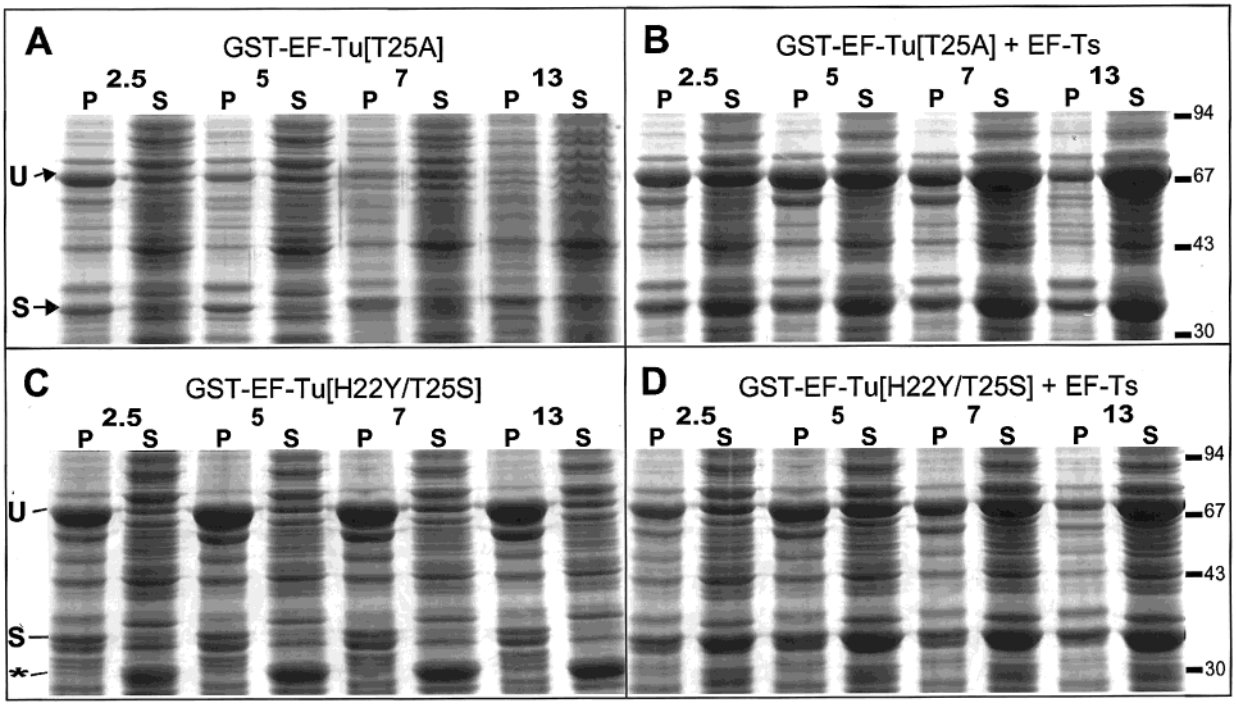

FIGURE 3: Improved production and solubility of mutant EF-Tu when co-overexpressing EF-Ts. SDS−PAGE showing S30 supernatant (S) and pellet (P) fractions at the indicated time after induction of expression by isopropyl thiogalactoside at 24 °C. The position of GST-EF-Tu is indicated by an arrow marked U and that of EF-Ts by S. The arrow marked with an asterisk indicates the position of the approximately 30 kDa soluble degradation product discussed in the text. The induction of GST-EF-Tu[T25A] gave rise to lysis phenomena at longer times coinciding with fuzziness of the protein bands on the gel.

DISCUSSION

We have observed recently that several mutants incapable of or defective in binding the nucleotides available in the cell are only partially soluble on overexpression. EF-Tu from *E. coli* is not a very stable protein. Temperature-induced inactivation occurs at relatively low temperatures: $\theta_{1/2}$, the temperature at which half of the EF-Tu is inactivated after 8 min, is ~52 °C for EF-Tu·GDP and ~47 °C for EF-Tu·GTP (*3*). Nucleotide-free EF-Tu from *E. coli* is even less stable in vitro and rapidly loses activity at 4 °C. This loss of activity, however, can in certain conditions be reversed up to a certain point by addition of GDP; this reactivation is accelerated by the antibiotic kirromycin (*5*). On the other hand, the nucleotide-free complex with EF-Ts is very stable on the long-term (*6*). Clearly, the interactions of EF-Tu with the nucleotide and the bound magnesium, or in their absence with EF-Ts, are essential for maintaining a correctly folded state of EF-Tu. We suspected, therefore, that the low solubility of these mutants and their reduced nucleotide binding are connected. Correct folding of EF-Tu in the cellular environment could be dependent on a nucleotide acting as a template for the (re)folding of the protein or at least stabilizing a spontaneously correctly folded protein. In the absence of a suitable nucleotide, the only other possible stabilizing factor would be EF-Ts, which is present at a lower concentration than EF-Tu in normal circumstances [approximately one-tenth of the EF-Tu concentration (*15*)].

In this study, we have investigated the effect of simultaneous overexpression of GST-fused EF-Tu and EF-Ts on the yield of soluble GST-EF-Tu in the postribosomal supernatant fraction. We observed a general beneficial effect on the solubility of the expressed proteins. Even the overexpression level of soluble wt EF-Tu seems somewhat improved (not shown), but the effect is more obvious for mutants T25A, H22Y/T25S, and D80N which have a reduced nucleotide affinity. Most striking is the effect for mutant D138N, which cannot bind any nucleotide available in the cell and is very little soluble in normal circumstances. Differently from the other mutants, however, significant formation of soluble product occurs only well into the stationary phase, while EF-Ts is immediately mostly soluble. In fact, even initially insoluble EF-Tu can go into solution on continued incubation when new protein synthesis has been arrested with chloramphenicol. This is indicative of a slow conformational rearrangement of initially misfolded and insoluble protein, which can form a stable, soluble complex with EF-Ts once the (near)-native fold is achieved. Thus, EF-Ts appears to act as a folding template, a steric molecular chaperone [as defined by Ellis (*16*)] for the folding of EF-Tu. For the other mutants, the residual nucleotide binding ability seems to allow the nucleotide to act as a folding template up to a certain point, but the lower affinity limits its effectiveness and/or decreases the in vivo stability of the resulting protein. Consequently, a 1:1 stoichiometry of EF-Ts is necessary for maximum solubility and long-term stability.

As a second important finding, we here show that mutants T25A and D80N display in vivo a dominant-negative phenotype, similar to that of its Ras p21 homologues that cause sequestration of the guanine nucleotide exchange factor, preventing the wild-type factor from being activated. This complements the in vitro observation of an interfering effect of both these mutants on the stimulation by EF-Ts of the dissociation of EF-Tu[wt]·GDP (*12*, *13*). Especially mutant D80N has a very strong growth-inhibiting effect, indicating that the available EF-Ts is efficiently sequestrated.

The slow residual growth is probably mostly sustained by the intrinsic nucleotide exchange rate on EF-Tu. Coexpression of EF-Ts at a 1:1 stoichiometric ratio completely alleviates this growth inhibition. The fact that growth inhibition takes place shortly after the mutant EF-Tu is expressed testifies to the relatively fast folding of mutants T25A and D80N assisted by the residual guanine nucleotide binding activity, in contrast to mutant D138N, for which the folding lags so far behind expression that no noticeable growth inhibition takes place.

Recently several papers have shown that EF-Tu shows some chaperone-like properties in vitro, binding to and stimulating the refolding of denatured proteins (*17*, *18*). This observation was subsequently extended also to the translation factors EF-G and IF-2 (*19*). Moreover, EF-Tu was shown to have quite reactive cysteines, imparting to it protein-disulfide isomerase activity, another important function in the process of establishing and maintaining correct protein structure (*20*). While the relevance of these findings for protein folding in vivo is not clear and requires further investigation, we establish here that in certain conditions exchange factor EF-Ts can act as a steric chaperone for the folding of EF-Tu in the cell. Thus, it is quite interesting that several protein factors that are essential for the protein production machinery in the cell may have an additional function in assisting the correct folding of the products as well.

In conclusion, one can say that in the cell, the ability of EF-Tu to rapidly fold into a native conformation greatly depends on its ability to bind nucleotide, acting as a template around which the correct structure of EF-Tu takes shape. When the affinity for nucleotide is strongly reduced, folding can still occur relatively rapidly, but the level of correctly folded and thus soluble protein in the cytoplasm is limited by the amount of available EF-Ts. When no cellular nucleotide can bind at all, the folding to a soluble native conformation hardly or only very slowly occurs. A native folding of EF-Tu can then only be achieved in the presence of equal amounts of EF-Ts. Thus, EF-Ts acts as a steric chaperone for the folding of EF-Tu.

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