Interaction of a selenocysteine-incorporating tRNA with elongation factor Tu from *E.coli*

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ABSTRACT

Selenocysteine-incorporating $tRNA^{Sec}$(UCA), the product of *selC*, was isolated from *E.coli* and aminoacylated with serine. The equilibrium dissociation constant for the interaction of Ser- $tRNA^{Sec}$(UCA) with elongation factor Tu·GTP was determined to be $5.0 \pm 2.5 \times 10^{-8}$ M. Compared with the dissociation constants of the two elongator Ser-$tRNA^{Ser}$ species ($K_d = 7 \times 10^{-10}$ M), the selenocysteine-incorporating UGA suppressor tRNA has an almost hundred fold weaker affinity for EF-Tu·GTP. This suggests a mechanism by which the Ser-$tRNA^{Sec}$ is prevented in recognition of UGA codons. This tRNA is not bound to EF-Tu·GTP and is converted to selenocysteinyl-$tRNA^{Sec}$. We also demonstrate the lack of an efficient interaction of Sec-$tRNA^{Sec}$(UCA) with EF-Tu·GTP. The results of this work are in support of a mechanism by which the selenocysteine incorporation at UGA nonsense codons is mediated by an elongation factor other than EF-Tu·GTP.

INTRODUCTION

Selenoproteins are synthesized by cotranslational insertion of selenocysteine into the nascent polypeptide chain (1). An incorporation of selenocysteine at specific UGA nonsense codons was demonstrated for *E.coli*. This process depends on four genes: *selA*, *selB*, *selC* and *selD* (2). One of these genes, *selC*, codes for $tRNA^{Sec}$ having an UCA anticodon recognizing the nonsense UGA codon. This tRNA is first aminoacylated with serine and is later converted to selenocysteinyl-$tRNA^{Sec}$(UCA). The sequence of this tRNA (2) and of its gene (1) were determined. Gene products of *selA* and *selD* are necessary for the conversion of Ser-$tRNA^{Sec}$ to Sec-$tRNA^{Sec}$, but have not yet been closely characterized (4). The product of *selB*, SELB, is a GTP binding protein analogous to EF-Tu·GTP, but having an exclusive specificity only to Sec-$tRNA^{Sec}$ (5). The aim of this work was to study the interaction of EF-Tu·GTP with Ser-$tRNA^{Sec}$(UCA) and Sec-$tRNA^{Sec}$(UCA) in order to understand the mechanism of the aminoacyl-tRNA selection during the reading of UGA nonsense codons.

MATERIALS AND METHODS

Purification of $tRNA^{sec}$

Bulk tRNA was isolated from *E.coli* FM420(pMN81) cells according to Ehrenstein (6). From 10 g wet weight of bacteria 20 mg $tRNA^{bulk}$ were obtained. Purification of $tRNA^{Sec}$ to homogenity was achieved by chromatography on DEAE-Sephadex A50 (7) and a reversed salt-gradient chromatography on Sepharose 6B (8). The $tRNA^{Sec}$(UCA) in chromatographic profiles was assayed either by polyacrylamide gel electrophoresis (PAGE) (9) or aminoacylation with serine. The aminoacylation assay contained 50 mM Hepes pH 7.5, 50 mM NH_4Cl, 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 5 mM dithiothreitol, 1.5 to 15 μM tRNA and 30 to 300 μM [^{14}C]serine with a specific activity of 55 Ci/mol (Amersham-Buchler, Braunschweig, FRG). S100 supernatants were used as a source of Ser-tRNA synthetase (10). Interaction with EF-Tu·GTP was investigated by methods described in detail elsewhere. Immobilized EF-Tu·GTP from *Thermus thermophilus* was used for affinity chromatography (11). Quantitative determination of the dissociation constants was done as previously described (12). For hydrolysis protection assay (13) 1 μM EF-Tu·GTP was incubated in 50 mM K-phosphate pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM cysteine at 24°C in the presence of 0.2 μM $tRNA^{Sec}$(UCA) aminoacylated with [^{14}C]serine or [^{75}Se]selenocysteine. Homogenous *E.coli* EF-Tu was isolated according to (14) and had a specific activity of 22,000 units/mg protein. Sec-$tRNA^{Sec}$(UCA) was prepared *in vitro* under anaerobic conditions (Forchhammer, Böck, manuscript in preparation).

Two dimensional polyacrylamide gel electrophoresis of tRNA in 7 and 4 M urea was performed according to (9). Chemicals purchased from Merck (Darmstadt, FRG) were of highest available purity. DEAE-Sephadex A50 and Sepharose 6B were obtained from Pharmacia (Uppsala, Sweden).

RESULTS

Overexpression of the *selC* gene product in *E.coli*

$tRNA^{bulk}$ was isolated from aerobically grown *E.coli* FM420 cells containing the plasmid pMN81. The overexpression of the product of the *selC* gene, $tRNA^{Sec}$(UCA), is evident by comparison of two-dimensional PAGE obtained from this strain

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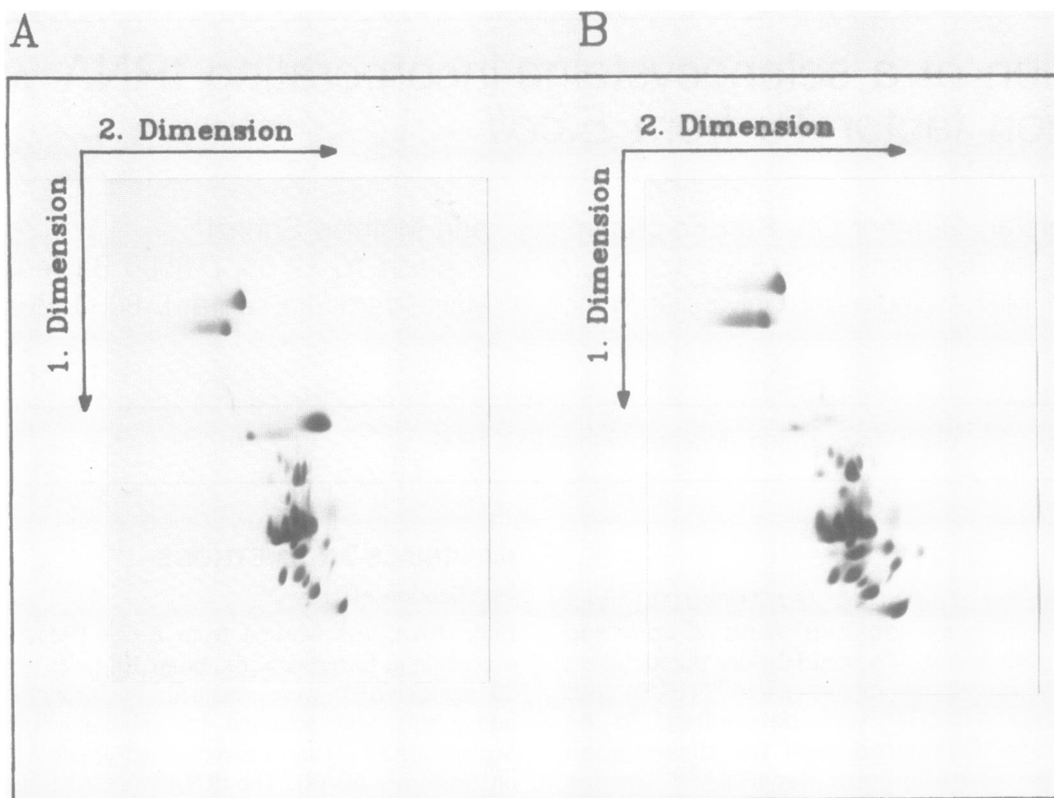

Fig.1: Two-dimensional gel electrophoresis pattern of $tRNA^{bulk}$ from (A) *E.coli* FM420(pMN81) and (B) *E.coli* JM101(pUC18).

and from a control *E.coli* strain, JM101, which contains a pUC18 plasmid lacking the *selC* gene (Fig.1). The two-dimensional gel electrophoresis patterns are very similar with the exception of a new prominent spot in the PAGE derived from FM420(pMN81) cells. This tRNA runs slowly in the first dimension (10% polyacrylamide) as expected for a species having an extremly long extra arm and a length of 95 nucleotides. Expression of $tRNA^{Sec}$(UCA) became weaker and finally diminished when FM420(pMN81) cells were stored at 4°C for several weeks. We do not have an explanation for this observation. In order to obtain satisfactory yields of $tRNA^{Sec}$, the FM420 cells had to be freshly transformed with pMN81. In the position on two-dimensional PAGE where the spot of $tRNA^{Sec}$(UCA) is visible in $tRNA^{bulk}$ from FM420(pMN81) strain no tRNA could be detected in *E.coli* JM101(pUC18). It is, however, possible that when $tRNA^{Sec}$(UCA) is not overexpressed the content of modified bases changes, giving rise to a different electrophoretic mobility. $tRNA^{Sec}$ was isolated on preparative scale using a combination of standard chromatographic methods: DEAE-Sephadex A50 chromatography and a reversed-salt gradient chromatography on Sepharose 6B. The aminoacylation activity of $tRNA^{Sec}$ was 1425 – 1500 pmoles serine/A_{260} units tRNA and was homogenous as judjed by PAGE. The $tRNA^{Sec}$(UCA) sequence and its extent of modification was checked by sequencing gels and HPLC. The results correspond with the sequence of the *selC* gene (1) and to that of $tRNA^{Sec}$(UCA) (3).

Interaction of aminoacyl-$tRNA^{Sec}$(UCA) with EF-TU·GTP

Affinity chromatography of aminoacyl-tRNAs on immobilized EF-Tu·GTP is a good method to screen for the formation of the ternary complex and allows a fast comparison of the affinities of different aminoacyl-tRNA species for this complex (11). In the experiment shown in Fig.2 we used this method to compare the binding efficiency of *E.coli* [^{14}C]Ser-tRNA isoacceptors from JM101(pUC18), which does not overproduce $tRNA^{Sec}$(UCA), with those isolated from strain FM420(pMN81). Ser-$tRNA^{Ser}$ isoacceptors (Fig.2A) bind efficiently to the immobilized EF-Tu·GTP and are eluted from the column only with a buffer of high ionic strength. The small amount of radioactivity in the void volume is due to free [^{14}C]serine. In Fig.2B the $tRNA^{bulk}$ from FM420(pMN81) was aminoacylated with [^{14}C]serine and passed through an EF-Tu·GTP affinity column. The difference to the profile in Fig.2A is obvious. Here part of the [^{14}C]Ser-tRNA elutes with a buffer of low ionic strength and the other part is eluted with a buffer of high ionic strength. The comparison of the relative amount of $tRNA^{Sec}$(UCA) in $tRNA^{bulk}$ from *E.coli* FM420 (pMN81) with the low ionic strength fraction (insert Fig. 2B) shows that Ser-$tRNA^{Sec}$(UCA) is not efficiently bound to the immobilized EF-Tu·GTP. The affinity chromatography demonstrates a weak binding of [^{14}C]Ser-$tRNA^{Sec}$(UCA) to immobilized EF-Tu·GTP and a strong interaction of the regular [^{14}C]Ser-$tRNA^{Ser}$ isoacceptors with the affinity matrix.

The results of the affinity chromatography experiments were substantiated by the determination of equilibrium dissociation constants of *E.coli* Ser-$tRNA^{Ser}$(V_1GA) and *E.coli* Ser-$tRNA^{Sec}$(UCA) on their interaction with *E.coli* EF-Tu·GTP. For this purpose we used a fluorescence titration assay in which Ser-$tRNA^{Sec}$(UCA) or Ser-$tRNA^{Ser}$(V_1GA) compete with [AEDANS-s^2C]Tyr-$tRNA^{Tyr}$ (12). The change in the fluorescence amplitute upon complex formation with EF-Tu·GTP (F/F_o) allows to calculate the respective dissociation constant. Typical titration curves are shown in Fig.3 and the dissociation

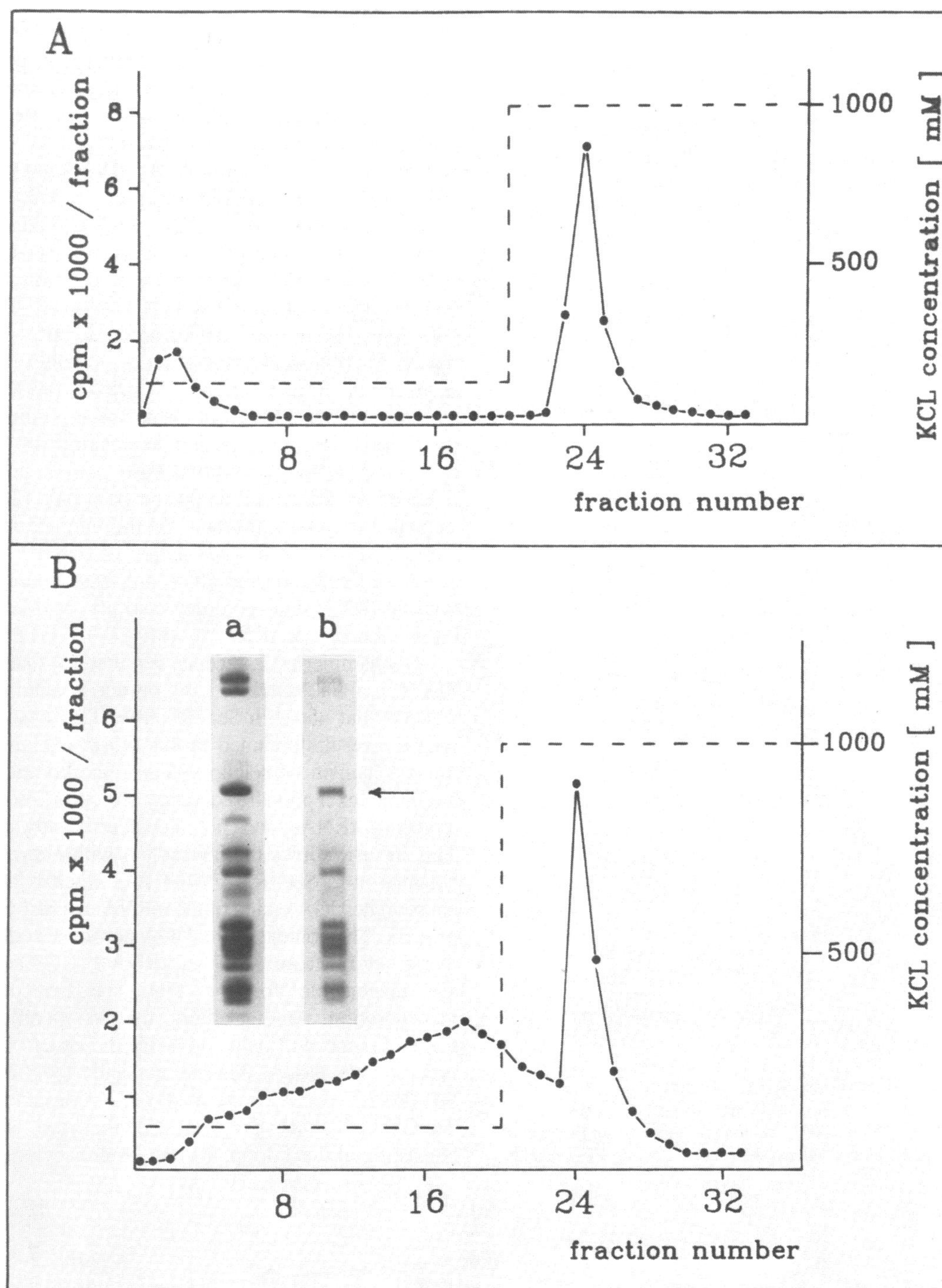

Fig.2: Affinity chromatography of [^{14}C]Ser-tRNA isoacceptors from (A) *E.coli* JM101(pUC18) and (B) *E.coli* FM420(pMN81) on EF-Tu·GTP. (●—●) [^{14}C]Ser radioactivity in the collected fractions, (– – –) gradient from low to high ionic strength in the elution buffer. The insert in B shows the one-dimensional PAGE of $tRNA^{bulk}$ from *E.coli* FM420(pMN81) (a) and (b) of the pooled low ionic strength fractions of the affinity chromatography shown in (B). The position of $tRNA^{Sec}$ is indicated by an arrow.

constants calculated by non-linear least square analysis of the titration data according to the following equation

$$R_o = K_d\,(F/F_o-1)/(F_\infty/F_o-F/F_o) + W_o(F/F_o-1)/(F_\infty/F_o-1) + I_o/[1+(K_i/K_d)\cdot(F_\infty/F_o-F/F_o)/(F/F_o-1)]$$

are summarized in Table 1.

In the equation, K_d and W_o are the apparent dissociation constant and the concentration of [AEDANS-s^2C]Tyr-$tRNA^{Tyr}$, respectively, K_i and I_o are the apparent dissociation constant and the concentration, respectively, of the competing aminoacyl-tRNA. R_o is the concentration of the elongation factor and F_∞/F_o is the maximal relative fluorescence change. With this method we could show that the affinity of Ser-$tRNA^{Sec}$(UCA) to EF-Tu·GTP is almost 100 fold lower than the affinity of the serine-inserting Ser-$tRNA^{Ser}$(V_1GA).

Since Ser-$tRNA^{Sec}$(UCA) does not interact efficiently with EF-Tu·GTP we tested the possibility of ternary complex

formation with Sec-tRNA^{Sec}(UCA). Selenocysteinylated tRNA^{Sec}(UCA) was prepared by *in vitro* conversion of Ser-tRNA^{Sec} under anaerobic conditions. A hydrolysis protection assay (13,15) was then used to test the interaction of [^{14}C]Sec-tRNA^{Sec}(UCA) with *E.coli* EF-Tu·GTP. The aminoacyl residue which normally spontanously hydrolyses from the tRNA is protected when the aminoacyl-tRNA is complexed with EF-Tu·GTP. Fig.4A demonstrates a lack of such interaction between Sec-tRNA^{Sec}(UCA) and the elongation factor. Under the same conditions the [^{14}C]Ser-tRNA^{Ser} isoacceptor is efficiently protected from hydrolysis by EF-Tu·GTP (Fig.4B). We conclude that both Sec-tRNA^{Sec}(UCA) and Ser-tRNA^{Sec}(UCA) do not interact with EF-Tu·GTP.

Table 1: Equilibrium dissociation constants for *E.coli* tRNAs charged with serine

aa-tRNA	anticodon	K_d[M]
Ser-tRNA^{Sec}	UCA	$5.0\pm2.5\times10^{-8}$
Ser-tRNA^{Ser}	GCU	$6.5\pm1.9\times10^{-10}$
Ser-tRNA^{Ser}	V_1GA	$7.2\pm0.1\times10^{-10}$

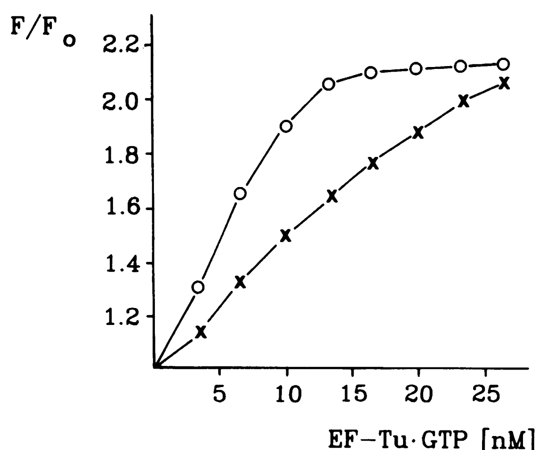

Fig.3: Competition of 40 nM *E.coli* Ser-tRNA^{Ser} (V_1GA) (×—×) and of 40 nM Ser-tRNA^{Sec} (UCA) (○—○) with 10 nM [AEDANS-s^{2}C]Tyr-tRNA^{Tyr} for their interaction with *E.coli* EF-Tu·GTP. The relative fluorescence increase of the AEDANS group (F/F_0) is plotted against the concentration of EF-Tu·GTP.

DISCUSSION

In *E.coli*, selenocysteine is incorporated in polypeptide chains by a tRNA recognizing UGA nonsense codons. This tRNA is first aminoacylated with serine to Ser-tRNA^{Sec} and then converted to selenocysteinyl-tRNA^{Sec} (2). If Ser-tRNA^{Sec} is an intermediate in incorporation of selenocysteine into the polypeptide chain at the UGA nonsense codon, then a mechanism must exist which prevents both the incorporation of serine at this position and the suppressor action of Ser-tRNA^{Sec} or Sec-tRNA^{Sec} at normal UGA stop codons. In this work we showed that the affinity of Ser-tRNA^{Sec}(UCA) to EF-Tu·GTP is nearly 100 times lower than that of other Ser-tRNA^{Ser} species. The reason for this weak interaction is probably the non-canonical sequence of tRNA^{Sec}(UCA). This tRNA has 8 base pairs in the aminoacyl-stem, an unusual guanosine at position 8, a pyrimidine in position 14 and a purine in position 48. In addition, the conserved purine-pyrimidine base pair at position 11–24 is replaced by a pyrimidine-purine base pair (2,3). The unusual sequence may be important for the interaction with ribosomal components or for the interaction of tRNA^{Sec} with the mRNA at a specifically located UGA nonsense codon. However, this unusual tRNA sequence may also play a role in excluding the interaction of Sec-tRNA^{Sec} with EF-Tu·GTP.

Forchhammer et al. recently reported (5) that the *selB* product, SELB, is a GTP/GDP binding protein which interacts with Sec-tRNA^{Sec} but not with Ser-tRNA^{Sec}. This observation, together with the results obtained in this work, explain, at least partly, the mechanism of selenocysteine incorporation. Ser-tRNA^{Sec}, having an exceptional secondary structure, different from other aminoacyl-tRNAs, does not interact efficiently with EF-Tu·GTP. This in turn allows the interaction with enzymes catalysing its conversion to Sec-tRNA^{Sec}(UCA), which is then bound to the appropriate UGA codon of the mRNA in a SELB·GTP dependent process. The context of the UGA nonsense codon, together with the structural features of Sec-tRNA^{Sec}(UCA) and SELB·GTP, are responsible for the high specifity of selenocysteine incorporation. Among others, the site specific mutagenesis of the *selC* gene will help to clarify the exact mechanism of this process. We assume that the unusually long aminoacyl-stem of Sec-tRNA^{Sec}(UCA) is the main reason for its low affinity to EF-Tu·GTP. It was demonstrated that the aminoacyl-domain consisting of the T-loop, T-stem, aminoacyl-stem and the CCA-

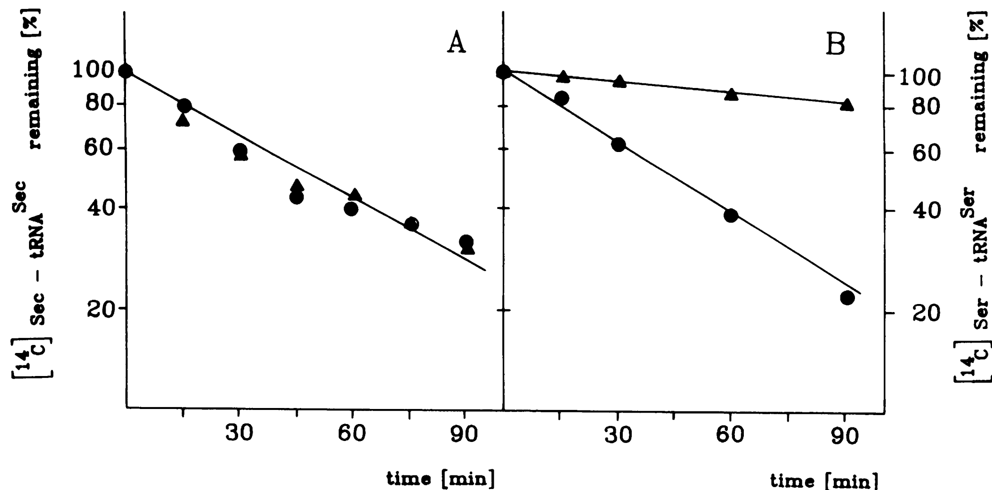

Fig.4: (A) Protection of Sec-tRNA^{Sec}(UCA) or (B) Ser-tRNA^{Ser}(V_1GA) against alkaline hydrolysis in the presence (▲—▲) and absence (●—●) of *E.coli* EF-Tu·GTP.

end of the tRNA is sufficient for the interaction with EF-Tu·GTP (16). However the exact length of the domain from the attached amino acid to the T-loop, rather than the number or the sequence of base pairs seems to be the important feature. His-$tRNA^{His}$ species have 8 + 5 base pairs in this domain (17), with an additional base at position −1, base-pairing with residue 72 of the tRNA 3′-terminus. Obviously His-$tRNA^{His}$ interacts with EF-Tu·GTP (18). Sec-$tRNA^{Sec}$ also has 8 + 5 base pairs in the aminoacyl-domain but a normal 4 nucleotide-long 3′-end and does not interact with EF-Tu·GTP.

The lack of this interaction is obviously not due to the amino acid attached to the 3′-end of this tRNA since neither Ser-$tRNA^{Sec}$ nor Sec-$tRNA^{Sec}$ form stable ternary complexes. This is in accordance with the postulated lack of amino acid specifity in the aminoacyl-tRNA·EF-Tu·GTP ternary complex formation (19). The SELB complex, on the other hand, has an amino acid specifity since it interacts with Sec-$tRNA^{Sec}$ but not with Ser-$tRNA^{Sec}$ (5).

It is interesting to note the absence of an inhibitory effect on *E.coli* growth when $tRNA^{Sec}$ is overexpressed. Under *in vivo* conditions neither the aminoacylation of $tRNA^{Sec}$(UCA) to Ser-$tRNA^{Sec}$ is suppressed nor does an other mechanism exist which prevents the supression of UGA codons by this tRNA. The results of this work provide one possible explanation for this discrepancy. Because Ser-$tRNA^{Sec}$ does not bind to EF-Tu·GTP it is available for rapid conversion to Sec-$tRNA^{Sec}$. In turn, the high affinity of Sec-$tRNA^{Sec}$ for SELB·GTP warrants its specific binding at the UGA codon for selenocysteine. The codon context is probabely important for the differenciation between UGA stop codons and the UGA selenocysteine codons.

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