Eukaryotic selenocysteine tRNA has the 9/4 secondary structure

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Abstract There are two secondary structure models for the eukaryotic selenocysteine (Sec) tRNA^{Sec}. One model, the 9/4 structure, was experimentally tested and possesses acceptor and T-stems with 9 and 4 bp, respectively [Sturchler et al., 1993; Hubert et al., 1998]. The other one, the 7/5 secondary structure with a bulge in the T-stem, was derived from theoretical calculation [Ioudovitch and Steinberg, 1999]. In this report, we show more experimental results supporting the 9/4 secondary structure. Several tRNA^{Sec} mutants, whose secondary structure can adopt only the 9/4 structure, were active for serylation and selenylation. Some mutants that cannot base-pair between positions 26 and 44 to provide the 6 bp anticodon stem were still active, inconsistent with the model by Steinberg. We also show that the orientation of the V-arm directly or indirectly influences the selenylation activity, and that the rigid 6 bp D-stem is important. Finally, we conclude that all tRNAs^{Sec} possess the 13 bp domain II made by the stacking of the colinear AA and T-stems, whether they present the 9/4 structure in Eukarya and Archaea or the 8/5 structure in bacteria.

Key words: tRNA; Selenocysteine; Selenium; tRNA structure

1. Introduction

Selenocysteine (Sec) plays a key role in the redox function of glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases. Sec is formed on its tRNA^{Sec} from seryl-tRNA^{Sec} by Sec synthase. The selenocysteine tRNA^{Sec} has two characteristic features. It possesses the anticodon UCA complementary to the selenocysteine codon UGA. The other feature is the peculiar 9/4 secondary structure, differing from the general standard 7/5 tRNA structure; 9 and 7 stand for the lengths of the AA stem, 4 and 5 for those of the T-stem. The 9/4 secondary structure of the eukaryotic tRNA^{Sec} was suggested based on the secondary structure of the *Escherichia coli* tRNA^{Sec} having the 8/5 structure [1]. Experimental evidence arguing in favor of the 9/4 secondary structure of the eukaryotic tRNA^{Sec} has been shown [2,3]. This secondary structure was different from the 7/5 model which has an unexpected bulge in the T-stem [4]. Recently, it has been shown that the archaeal tRNA^{Sec} can adopt the 9/4 secondary structure as well [3,5]. Thus, it is considered that tRNA^{Sec} can fold into the 9/4 or 8/5 secondary structures. However, Ioudovitch and Steinberg [5] have maintained firmly their 7/5-bulge secondary structure model for the eukaryotic tRNA^{Sec}, based on theoretical considerations [6–8]. They however agreed for the existence of the 8/5 and 9/4 structures of eubacterial and archaeal tRNAs^{Sec}, respectively [5]. Their theory can mislead the readers that the eukaryotic tRNA^{Sec} adopts the 7/5-bulge secondary structure only. The purpose of this report is to clarify the situation by bringing new arguments in favor of the 9/4 secondary structure of the eukaryotic tRNA^{Sec}.

The selenocysteine synthesis and its incorporation into selenoproteins is well established in bacteria. The tRNA^{Sec} is first charged with Ser by the conventional Ser-tRNA synthetase (SerRS). The product Ser-tRNA^{Sec} is subsequently bound by selenocysteine synthase (SecS), an enzyme which converts the Ser-residue to Sec, using an activated phosphoselenoate compound as the Se-donor. Sec-tRNA^{Sec} is then brought to an in frame Sec-specifying UGA codon by a specific elongation factor different from EF-Tu.

The current state of research on the eukaryotic tRNA^{Sec} can be summarized as follows. The functional sites on tRNAs for the aminoacyl-tRNA synthetases have been termed the identity sites on tRNA [9]. We determined the recognition sites on for the selenocysteine synthase on the tRNA^{Sec} [10,11]. Eukaryotic tRNA^{Sec} exhibits an aminoacyl acceptor-stem with a unique length of 9 bp. None of the point mutations on the 9 bp AA-stem significantly modified the selenylation level. In contrast, reduction of the AA-stem length to 8 bp led the tRNA^{Sec} to lose or reduce its ability to efficiently support selenylation [12]. This result provided strong evidence that the length of the acceptor stem is of prime importance for the serine to selenocysteine conversion step.

The tRNA^{Sec} has a short 4 bp T-stem and an elongated 6 bp D-stem, as well as the long 9 bp AA-stem described above. We showed that the elongated 6 bp D-stem was another essential element on this tRNA, because tRNA^{Sec} mutants having a D-stem length decreased to 4 bp were inactive in selenylation. Therefore, the long 9 bp AA-stem and the elongated 6 bp D-stem are two essential recognition sites for selenylation. This was confirmed by an identity switch from tRNA^{Ser} to tRNA^{Sec}. In this experiment, the 7 bp AA-stem and 4 bp D-stem of tRNA^{Ser} were converted to the active mutants having the 9 bp AA-stem and 6 bp D-stem [13].

We succeeded in the conversion of tRNA^{Ser} to tRNA^{Sec}, but the selenylation activity was weak and about 1/20 of that of the native tRNA^{Sec} [14]. This suggests an influence of base specificity on the tRNA^{Sec} and/or the involvement of the orientation of the V-arm. We also showed that the 4 bp T-stem of the tRNA^{Sec} is not important for selenylation, because tRNA^{Sec} mutants having 3, 4 or 5 bp T-stems still possessed selenylation activity [14]. The length of the coaxial helix (domain II) formed by the stacking of the AA and T-stems is essential for serylation, because tRNA^{Sec} having 12 or 13 bp

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Abbreviations: Sec, selenocysteine; SecS, selenocysteine synthase; SerRS, Seryl-tRNA synthetase; AA, aminoacyl-acceptor; T, TψC; D, dihydrouridine; AC, anticodon; V, variable arm

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of domain II were still active, but not the mutants carrying 11 or 14 bp of domain II. This suggests that the SerRS measures the length between the discriminating base G73 and the V-arm [14,15].

The secondary structure model for the $tRNA^{Sec}$ was proposed based on enzymatic and chemical probing [2]. RNase T2 did not digest the bulge position in the 7/5-bulge model and modification with dimethyl sulfate showed the absence of this bulge in the 7/5-bulge structure of Steinberg et al. Despite this experimental evidence, Ioudovitch and Steinberg [8] concluded that our data fit the 7/5-bulge model better than the 9/4 secondary structure model. Their analysis revealed the ability to comply with their L-form compensatory rules within the 7/5 structure. After the publication by Ioudovitch and Steinberg [8], Krol and coworkers presented more experimental data supporting the eukaryotic 9/4 model, as well as a secondary structure model for the $tRNA^{Sec}$ of the Archaea *Methanococcus jannaschii* which can also fold into the 9/4 structure model [3]. This secondary structure model for the archaeal $tRNA^{Sec}$ has been also confirmed by Ioudovitch and Steinberg [5]. They

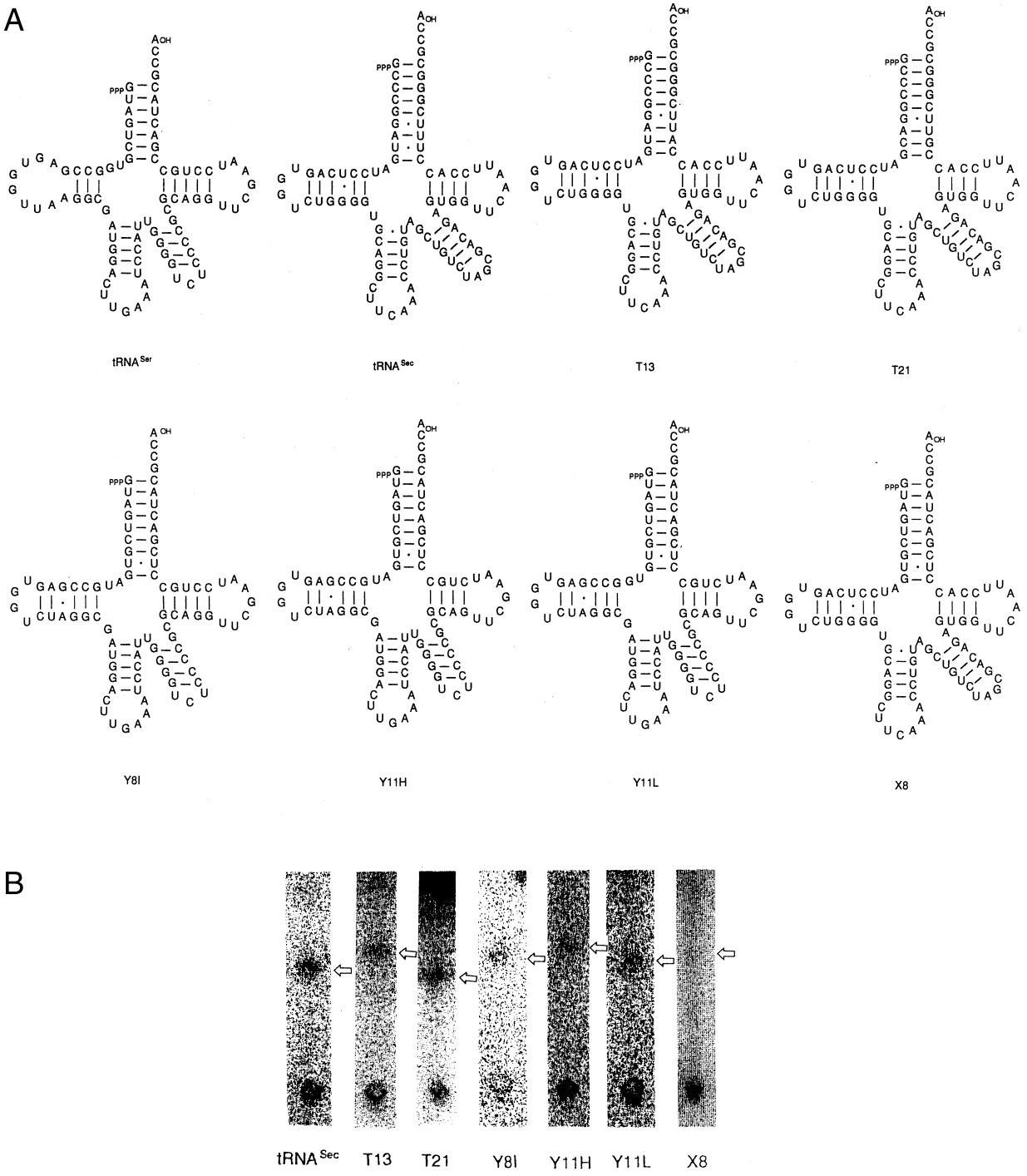

Fig. 1. Eukaryotic active selenocysteine tRNAs. A: Secondary structure models of various tRNAs. B: The TLC patterns of [^{75}Se]Sec produced on and liberated from those tRNAs.

showed that the archaeal tRNA^{Sec} adopts the 9/4 secondary structure only, because they failed to construct the 7/5 secondary structure model they proposed for the eukaryotic counterpart. From these circumstances, we show several active tRNA^{Sec} mutants that can fold only into the 9/4 secondary structure. It was impossible to construct the 7/5 secondary structure model with these mutants. They were active in solution, showing that they adopt the 9/4 secondary structure in the reaction mixture to prepare the [^{75}Se]Sec-tRNA. As a matter of fact, these mutants were active for serylation by the SerRS in the first step of the Sec-tRNA synthesis. As well as the 9/4 structure, we showed other important results, such as the rigid 6 bp D-stem and the orientation of the V-arm. Active tRNA^{Sec} mutants having only the 9/4 secondary structure.

2. Materials and methods

2.1. tRNA constructs and in vitro transcription by T7 polymerase

Synthetic mammalian wild-type (wt) and mutant Sec tRNAs were constructed by hybridizing six couples of 14–24mer oligonucleotides or a couple of 90mer oligonucleotides containing the desired sequences as described in [12,13]. In these constructs, the promoter of the T7 RNA polymerase is included immediately 5′ of the coding sequence. Conditions for transcription in vitro with T7 RNA polymerase were as described in [12,13]. The RNA products were purified by gel electrophoresis and electroeluted.

2.2. Serylation and selenylation of tRNAs

Prior to use, tRNA transcripts were renatured by heating to 65°C for 3 min and then at 25°C for 5 min. tRNA in 20 μl of 0.2 M HEPES–Na (pH 7.4), 20 mM $MgCl_2$, 20 mM KCl, 10 mM mercaptoethanol, 0.4 mM serine, 5 mM ATP, and 5 μg SerRS were mixed with 10 μl of [^{75}Se]HSe^- (2 Ci/mmol), 10 μl of selenocysteine synthase and 10 μl of selenide-activating protein, and incubated at 30°C for 2 h [11]. After ethanol precipitation, alkaline hydrolysis of the [^{75}Se]Sec-tRNA^{Sec} in 2 M NH_4OH, 2 M mercaptoethanol for 30 min at 37°C released [^{75}Se]Sec, which was separated by TLC on silicagel G plates in *n*-butanol:acetic acid:water (4:1:1). ^{75}Se radioactivity was measured with a Fuji Bioimage BAS 2500 analyzer. Cold Sec (a generous gift of Prof. K. Soda, Kyoto University, Kyoto, Japan) was cochromatographed as a control and revealed by ninhydrin reaction.

3. Results and discussion

3.1. Active tRNA^{Sec} mutants having only the 9/4 secondary structure

Fig. 1A shows the secondary structures of several tRNA^{Sec} mutants and the tRNA^{Ser}. Mutants X8,T13 and T21 derived from the tRNA^{Sec}, Y8I, Y11H and Y11L from tRNA^{Ser}. All tRNAs except the tRNA^{Ser} in Fig. 1 were active for selenylation, as shown in Fig. 1B. T13 and T21 are mutants converting the U6*U67 (* indicates non-base-pairing) base pair in the AA-stem of tRNA^{Sec} to U6-A67 and C6-G67, respectively (the numbering is according to the secondary structure model by Sturchler et al. [2]). These two mutants conserved full serylation and selenylation activities [12]. Other mutants having A6-U67 or G6-C67 were also fully active in serylation and selenylation. These tRNA^{Sec} mutants adopt only the 9/4 secondary structure, like the archaeal tRNA^{Sec}. The mutant having G6-C67 is the same as the wt archaeal tRNA^{Sec}, for which Ioudovitch and Steinberg did not propose the 7/5 secondary structure model and agreed with the 9/4 structure. X8 is an active mutant of the tRNA^{Sec} obtained by exchanging the AA-stem with that of tRNA^{Ser} [13]. This showed that SecS did not require base-specificity in the AA-stem but only the length of the 9 bp AA-stem. This conclusion is consistent with the results by [12].

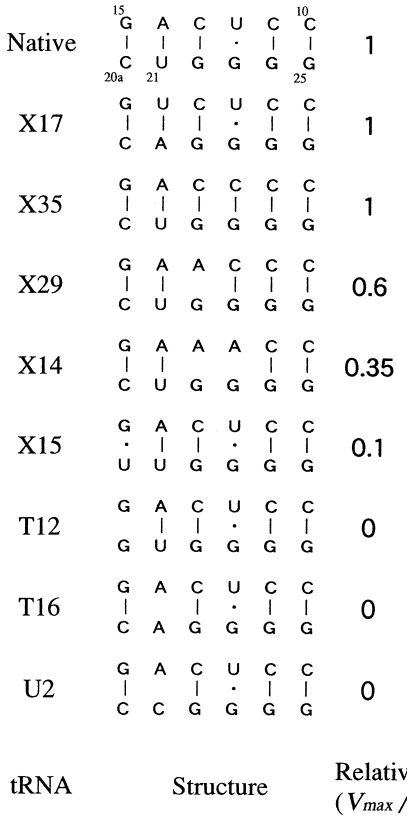

Fig. 2. Relationships between the structure of the D-stem and the selenylation activity.

The other three mutants Y8I, Y11H and Y11L in Fig. 1 are active derivatives of tRNA^{Ser} by an identity switch. Y8I is an active mutant with a 5 bp T-stem. This mutant having a 14 bp of domain II was weak in serylation but high in selenylation, like X33 in a previous paper [14]. Y11H and Y11L are active mutants having the 4 bp T-stem obtained by a 1 bp deletion from the original T-stem of tRNA^{Ser} and the 9 bp AA-stem obtained by addition of the 2 bp, U6*U67 and G7-C66. These two mutants have the V-arm of tRNA^{Ser}. In addition, C48, converted from A48, could not base-pair with U67, because original A48 base-paired with U67 in the 7/5-bulge model by Ioudovitch and Steinberg. This indicates that these two active mutants, Y11H and Y11L, can only adopt the 9/4 structure, not the 7/5-bulge secondary structure.

3.2. The rigid 6 bp D-arm lifts up domain II

In a previous report, the 6 bp D-stem was shown essential for selenylation activity [13]. However, not only the length of the D-stem, but also the sequence, revealed important to elevate the selenylation activity. Fig. 2 shows the relationship between structure of the D-stem and activity. The tRNAs having full activity are native Sec tRNA (U12-G23), X35(C12-G23) and X17(U14-A21). These D-stems possess a rigid structure. The activity of X29 having an A13*G22 mismatch was slightly less than that of the tRNA^{Sec} wt (about 60% of full activity) and that of X14 having the two A12*G23 and A13*G22 mismatches was 35%. The activity of X15 having the G15-U20a base pair dropped to 10%. T12 having G15*G20a and a 5 bp D-stem was inactive, showing that

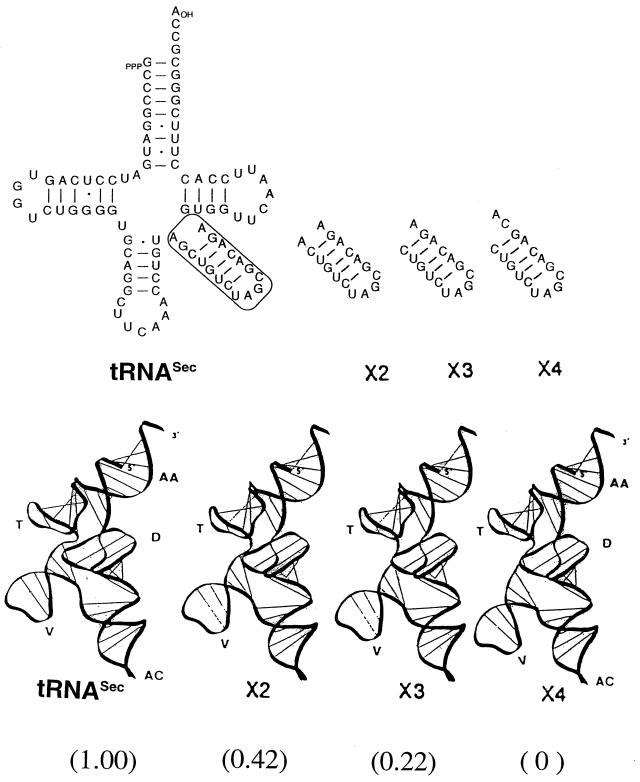

Fig. 3. Relationships between the orientation of V-arm and the selenylation activity. Upper panel: Secondary structure of the V-arm in the wt $tRNA^{Sec}$, mutants X2, X3 and X4. Lower panel: Proposed tertiary structure models for these mutants; parentheses indicate the relative selenylation activity (V_{max}/K_m of wt = 1).

the 6 bp D-stem is essential. T16 with A14*A21 and U2 with A14*C21 were inactive. Thus, the mutants having unpaired portions, such as X15, T16, and U2 were very weak or inactive. This shows that a rigid 6 bp D-stem is better for recognition by SecS. Base pair U12-G23 in the 6 bp D-stem may act in a base-specific manner. The 6 bp D-stem is completely different from the 3 bp D-stem in classical tRNAs. This rigid 6 bp D-stem lifts up domain II of $tRNA^{Sec}$ and the junction between domains I and II should be influenced by this 6 bp D-stem.

The model by Ioudovitch and Steinberg [5] proposed the existence of a 6 bp AC-stem. However, a 6 bp AC-stem is not essential, because the three active mutants Y20 Y23 and Y24 [13] have G26 and A44, which cannot base-pair. The active X3 mutant described in the next section has also an unpaired U26 and C44. They only have a 5 bp AC-stem, showing that a 6 bp AC-stem is not essential for serylation and selenylation.

3.3. The orientation of the V-arm influences the selenylation activity, not serylation

Fig. 3 (upper panel) shows the structure of the V-arms of a few $tRNA^{Sec}$ mutants. These mutants differ at positions 44-45 and 48, at the junction of the V-arm. Therefore, the orientation of the V-arm is different and proposed tertiary structure models for these mutants are shown in Fig. 3 (lower panel), derived from [2]. According to the number at position 48, the decrease of the position 44-45, the V-arm bends to the AC-arm and leaves from the T-loop. The selenylation activity, as shown in the parentheses of Fig. 3, decreased according to the bend of the V-arm. Mutants X2 and X3 were active, while mutant X4 was inactive. As a matter of fact, the native $tRNA^{Sec}$ mutants X2, X3 and X4 all displayed full serylation activity (data not shown). These results suggest that SecS directly or indirectly recognizes the orientation of the V-arm as well as the other two key elements, the 9 bp AA-stem and the 6 bp D-stem. SecS may bind inside the L-shape tRNA and may cover the V-arm. However, it is possible that the orientation of the V-arm indirectly influences the active tRNA structure for recognition by SecS and there may be some base-specificity for recognition of the V-arm by SerRS and SecS.

We suppose that Steinberg and coworkers insisted on the 7/5-bulge model in order to deduce a general 7/5 secondary structure for all tRNAs. However, the 5 bp T-stem+one bulge is not identical to the 5 bp-only in a tRNA structure. And their model of 7/5 secondary structure contains the four bases between AA-stem and D-stem, inconsistent with two bases of almost all tRNAs. Steinberg stands that all tRNAs must fit the L-type secondary structure model. Surprisingly, even mitochondrial tRNAs missing the D or T-arms fit the L-type [6]. However, one should take into account that biological systems can contain exceptions, such as the $tRNA^{Sec}$ with the 9/4 secondary structure. The 9/4 structure of the $tRNA^{Sec}$ is a true fact among Archaea and Eukarya. Mutant X33 having the 9/5 structure is well selenylated but scarcely serylated [14]. This means that SecS did not recognize the length of the T-stem but that SerRS measures the length of domain II. The tRNA mutants having 12 or 13 bp of domain II were acceptable by SerRS but mutants having 11 or 14 bp of domain II were not recognized by SerRS. Thus, SecS accepts mutants having 12, 13, and 14 bp of domain II.

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