ROLE OF EXPOSED CYTOSINE RESIDUES IN AMINOACYLATION ACTIVITY OF $tRNA^{Trp}$

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1. Introduction

Selective chemical modification of tRNA is a conventional procedure used to detect bases which are essential for the interaction of tRNA molecules with synthetases (see [1–3]). Bisulfite is a suitable reagent in such studies since it permits conversion of cytosine residues in an RNA molecule into uridine residues (see [4]). This reaction has been employed in studying a number of tRNA–synthetase complexes [1–5]. Here, we have detected cytosine residues in bovine $tRNA^{Trp}$ whose conversion into uracil residues makes the tRNA lose its ability to be aminoacylated with tryptophan in the presence of homologous tryptophanyl-tRNA synthetase. The conversion of two cytosine residues in the anticodon loop (C_{31} and C_{33}) inactivates the tRNA whereas the C → U conversion of the second base in the anticodon (C_{34}), as well a similar conversion of cytidines at the CCA-terminus of the molecule, does not inactivate tRNA. A possible effect produced by modifying the anticodon loop on anticodon stem structure is discussed.

2. Materials and methods

Beef $tRNA^{Trp}$ was a generous gift of Dr S. Litvak (France), and some of its preparations were isolated as in [6]. Bovine tryptophanyl-tRNA synthetase was prepared as in [7] by M. Nurbekov from this laboratory. RNases T_1, T_2 and U_2 were purchased from Sankyo (Japan). RNase from *Physarum polycephalum* and polynucleotide kinase from *Escherichia coli* infected with T_4 phage were kind gifts of T. Avdonina and A. Puzyrev from this laboratory. The following reagents were commercially obtained: *E. coli* alkaline phosphatase (Sigma); Sephadex G-50 (fine) (Pharmacia Fine Chemicals); [γ-^{32}P]ATP, spec. act. 3000 Ci/mmol (Radiochemical Centre, Amersham); sodium bisulfite (granular) (Mallincrodt Chemical Works).

$tRNA^{Trp}$ (8 A_{260} units) was modified with bisulfite in 0.15 ml 1.5 M $Na_2S_2O_5$ (pH 5.8) containing 10 mM $MgCl_2$ at 20°C for 2 h. $tRNA^{Trp}$ lost 50% of its activity within this time. The reaction was stopped by gel filtration of the solution on a column packed with Sephadex G-50 (fine). The uridine bisulfite adducts were demodified by incubating tRNA in 0.2 ml 0.1 M Tris–HCl (pH 9.0) for 24 h.

The tRNA inactivated by 50% with bisulfite was aminoacylated in a total volume of 0.5 ml containing 0.02 mM $tRNA^{Trp}$, 0.4 mM L-tryptophan, 5 mM Na-ATP (pH 7.0), 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl, and 0.3 μM tryptophanyl-tRNA synthetase. The mixture was incubated at 37°C for 10 min, the reaction was stopped by adding 50 μl 2 M Na-acetate (pH 4.5), the solution was cooled in an ice bath and loaded onto a column (0.5 ml) packed with benzoylated DEAE-cellulose. $tRNA^{Trp}$ and $Trp\text{-}tRNA^{Trp}$ were separated as in [8]. Nearly identical quantities of free and charged tRNAs were obtained thus suggesting the 50% activity of modified tRNA.

The modification of $tRNA^{Trp}$ with a mixture of CH_3ONH_2 and $Na_2S_2O_5$, the subsequent demodification of uridine adducts, the 5′-labeling of tRNA preparations, and the limited hydrolysis of $tRNA^{Trp}$ with RNases T_1, T_2 and U_2 were performed as in [9]. The hydrolysis with RNase from *Physarum polycephalum* was conducted in 5 μl buffer containing 50 mM Na-acetate (pH 4.5), 2 mM EDTA, and 3 units RNase/μg tRNA.

3. Results and discussion

When beef $tRNA^{Trp}$ is modified with bisulfite under conditions favouring preservation of the spatial

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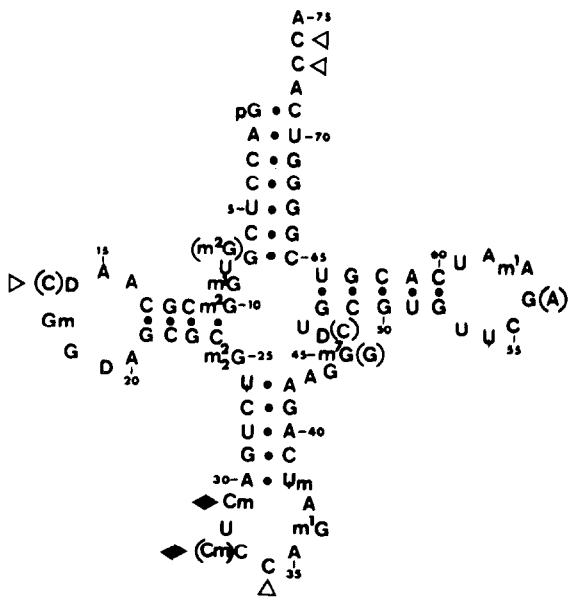


Fig.1. The primary structure of $tRNA^{Trp}$ (beef) [21]. Symbols designate cytidine residues that are converted into uridine by modification with bisulfite under the conditions when the 3-dimensional structure of the tRNA is preserved [9]. C residues whose conversion into U inactivates tRNA (◀) or has no effect on aminoacylation capacity (▷).

structure, 5 cytidine residues (fig.1), as well as one more cytidine substituting for dihydrouridine (D_{15}) in some tRNA molecules, are converted into uridine [9]. The residual acceptor activity of such a $tRNA^{Trp}$ preparation does not exceed 5% of the initial activity.

In order to locate the cytosine residues whose conversion causes a loss of the activity, we used a strategy employed while studying $tRNA^{Tyr}$ [10]: $tRNA^{Trp}$ was modified until its activity was halved, then aminoacylated with tryptophan in the presence of tryptophanyl-tRNA synthetase, after which the aminoacylated and non-charged tRNA molecules were separated by chromatography on benzoylated DEAE-cellulose. Thereupon we compared the primary structures of 4 $tRNA^{Trp}$ preparations:

(A) $tRNA^{Trp}$ that had not been modified with bisulfite;

(B) $tRNA^{Trp}$ capable of being aminoacylated after the reaction with bisulfite;

(C) $tRNA^{Trp}$ inactivated by 50%, without fractionation on benzoylated DEAE-cellulose;

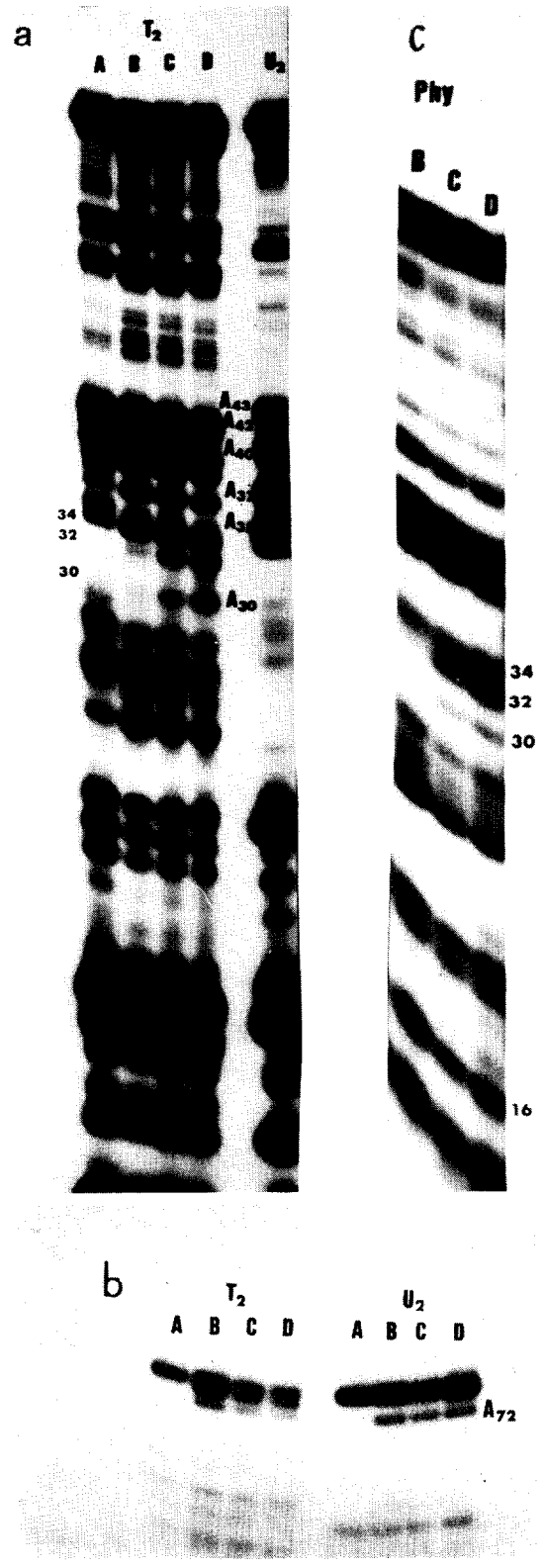
(D) $tRNA^{Trp}$ inactivated after the reaction with bisulfite.

To detect C → U conversions in the above 4 preparations, we used a technique [9,11] based on the exhaustive chemical modification of tRNA with a mixture of $Na_2S_2O_5$ and NH_2OCH_3. The phosphodiester bond thus formed between the resulting cytidine derivative [12] and its 3′-neighbour is resistent to the action of nucleases [13], which makes it possible to detect easily C → U conversions in the RNA molecule. Indeed, as can be seen in fig.2, the phosphodiester bonds at positions 34,73 and 74 in preparation (A) are almost fully resistant (the slight hydrolysis might be attributed to incomplete modification of cytidine residues with the CH_3ONH_2–$Na_2S_2O_5$ mixture). Cytidines C_{31} and C_{33} are methylated at the ribose residues; their phosphodiester bond with their 3′-neighbours is not cleaved by nucleases at all; however, as shown in [9,11], when these cytidines (as well as cytidine C_{73}) are converted into uridine, hydrolysis of the phosphodiester bonds between these residues and their 5′-neighbours accelerates abruptly. This is shown in fig.2 for preparations (C) and (D) in the case of both RNases, T_2 and from *Physarum*. It follows therefore that at least some of the C_{31}, C_{33}, C_{34}, C_{73} and C_{74} residues in these preparations have been converted into uridine residues.

In preparation (B), no bands corresponding to positions 30 and 32 can be observed, indicating that C_{31} and C_{33} are not converted into U in $tRNA^{Trp}$ which retains its acceptor ability after modification with bisulfite. Bands corresponding to breaks in the tRNA molecule at positions 34,73 and 74 have nearly the same intensity in preparation (B) as those in preparations (C) and (D). Hence the degree of C → U conversions at these positions is identical for the inactivated and active tRNAs.

Therefore, C → U conversions at positions 34,73 and 74 do not result in $tRNA^{Trp}$ inactivation whereas $tRNA^{Trp}$ containing U_{31} and/or U_{33} is incapable of being aminoacylated. Our data do not allow us to unambiguously conclude whether tRNA molecules in which C_{34}, C_{73} and C_{74} were converted into uridines at the same time remain active, or whether only those molecules which comprise simultaneously merely one or two transformed bases in different combinations retain their activity.

We have shown earlier that a C → U conversion in the anticodon loop can influence the conformation of the anticodon stem [14]. Since C_{31} is adjacent to the stem, one may assume that such an influence is caused, first of all, by this nucleotide. It should be taken into



consideration that a new complementary base pair $U_{31} \cdot A_{37}$ may be formed when C_{31} is converted into U_{31}, which makes the anticodon stem one base pair longer and causes contraction of the anticodon loop. We believe that $tRNA^{Trp}$ inactivation upon the conversion of C_{31} into U_{31} should be attributed to the indirect effect of this change on the conformation of adjacent regions rather than to its direct involvement in the interaction with the synthetase.

The $C_{33} \rightarrow U_{33}$ conversion apparently produces a lesser effect on the structure of the anticodon stem, and the loss of tRNA activity here seems to indicate that C_{33} is directly involved in the interaction with the synthetase. Since, in contrast to most of tRNAs, the first position of the anticodon (C_{33}) in $tRNA^{Trp}$ does not 'wobble', this nucleotide may in principle be used by the synthetase to discriminate between tRNAs. The necessity of C_{33} for the acceptor function to be preserved is not inconsistent with the Rich–Schimmel hypothesis [15] according to which the contact between tRNA and the synthetase occurs at the inner side of the L-shaped tRNA molecule where C_{33} resides.

As shown above, the acceptor activity of tRNA is not abolished when C_{34}, the central nucleotide of the anticodon, is converted into U_{34}. It is known that, in *E. coli* $tRNA^{Trp}$, the $C_{34} \rightarrow U_{34}$ conversion impairs the interaction with the homologous synthetase (the K_m increases 60-times and the V_{max} decreases 6-fold) [16]; moreover the tRNA acquires the capacity to interact with glutaminyl-tRNA synthetase [16–19]). Here we have not determined the parameters of tRNA aminoacylation since we dealt with a complex mixture of molecules modified at different sites. What follows from our results is that the $C_{34} \rightarrow U_{34}$ conversion produces no dramatic effect on the capacity of beef $tRNA^{Trp}$ for aminoacylation.

As shown in [20], a modification of the nucleotide composition of the anticodon loop makes $tRNA^{Trp}$ lose its capacity to induce conformational changes in the synthetase. Taking the results of this work into account, the participation in the induction of conformational changes should be ascribed to the C_{31} and/or C_{33} bases of the anticodon loop.

Fig.2. Autoradiogram of different $tRNA^{Trp}$ preparations after limited hydrolysis: (a) hydrolysis with RNases T_2 and U_2; (b) the same gel after a shorter exposure time; (c) hydrolysis with RNase from *Physarum polycephalum*. For description of preparations (A)–(D), see text.

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