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Chemical conversion of cytidine residues into 4-thiouridines in yeast $tRNA^{Phe}$. Determination of the modified cytidines

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ABSTRACT

Treatment of yeast phenylalanine tRNA with pressurized hydrogen sulfide results in conversion of cytidine residues into 4-thiouridine residues. Under conditions leading to an average modification of one cytidine per tRNA molecule 9 positions are thiolated. The 4-thiouridine residues are distributed along the tRNA molecule. Four of the reactive cytidines are located in single-stranded regions: Cm32, C60, C74 and C75. The five others are located in base pairs: C2, C27, C56, C61 and C63. Importance of replacement of an amino group by a thiol group on hydrogen bonding and on biological activity of the modified tRNA is discussed.

INTRODUCTION

Chemical modification of nucleotides has been widely used to investigate the secondary and tertiary structures of RNA molecules. Most common reagents available for this purpose are single-strand specific (e.g. kethoxal, bisulfite, dimethylsulfate), (for review see ref. 1). Other reagents have been used to probe the phosphate groups involved in tertiary structure folding (e.g. ethylnitrosourea) (2).

Nucleic acid modification has also been used to introduce chemically or photochemically reactive probes in order to study the topography of multimolecular complexes like ribonucleoproteins.

Chemical conversion of cytidine residues to 4-thiouridines (S^4U) seemed to be a valuable tool for both mentioned studies. It has been claimed that thiolation of cytidines by hydrogen sulfide treatment essentially occurred in exposed single-stranded regions (5). These authors mentioned that the higher-order structure of the RNA molecule was retained in the hydrogen sulfide-pyridine-water system used for this thiolation procedure (6). This chemical approach was also used for an examination of the secondary structure of 5S rRNA and U_6 snRNA (6,7).

One characteristic feature of 4-thiouridine residues is that they can be photoactivated by specific UV irradiation at 335 nm and are able to form

covalent bonds with nearby nucleophilic groups (8). In a previous paper we described the preparation of a $tRNA^{Phe}$ molecule containing cytidines converted into 4-thiouridines concomitantly with its application to a structural investigation of the ribosomal binding site of peptidyl-tRNA (9).

The present paper deals with the localization of the modified cytidines. Using the RNA sequencing procedure developed by Stanley and Vassilenko (10) combined with the modifications afforded by Nishimura and coworkers (11), we could characterize the positions of the S^4U residues. Results show that nine cytidines distributed along the tRNA molecule can be modified. These residues are located in single-stranded and base-paired regions as well.The effects of thiolation on the aminoacylation reaction are shown.

MATERIALS AND METHODS

Materials

Pure yeast $tRNA^{Phe}$ was prepared from crude brewer's yeast tRNA (Boehringer, Mannheim) by counter-current distribution (12) followed by chromatography on benzoylated DEAE-cellulose. [γ-^{32}P] ATP (3000 ci/mmole) was obtained from Amersham International ; T_4 polynucleotide kinase was purchased from Boehringer and P_1 nuclease was from P.L. Biochemicals. Thin-layer cellulose-coated plates were products of Schleicher and Schüll.

Preparation of $tRNA^{Phe}$ lacking 3'-terminal CCA_{OH}.

The 3'-terminal CCA_{OH} trinucleotide was removed from the tRNA molecule according to Uziel and Khym (13). The extent of the reaction was verified as described (13).

Thiolation of $tRNA^{Phe}$

Thiolation of tRNA was performed in a 50% pyridine containing 0.01 M phosphate buffer pH 7.0 by pressurized H_2S as earlier described (9). After 7h at 37°C tRNA was purified by chromatography on DEAE-cellulose. The S^4U content was determined spectrophotometrically according to Riehl et al (9).

Reincorporation of the 3'-terminal CCA_{OH} sequence

After thiolation the tRNA was renaturated as described in (14), and the CCA_{OH} terminus was reincorporated into the modified tRNA according to ref. 15.

Aminoacylation of [S^4U] $tRNA^{Phe}$

Aminoacylation of [S^4U] $tRNA^{Phe}$ was performed as described by Fasiolo et al (16).

Purification of [S^4U] $tRNA^{Phe}$

Thiolated tRNA was purified by electrophoresis on a 15% polyacrylamide-

urea containing gel (200 x 400 x 0,5 mm). After electrophoresis, the position of the tRNA was localized by UV shadowing on a fluorescent sheet (17); tRNA was eluted from the gel by the technique of Maxam and Gilbert (18) without carrier tRNA.

Limited alkaline hydrolysis of [S^4U] $tRNA^{Phe}$

The sequencing procedure of Stanley and Vassilenko (10) was applied with the modifications of Kuchino et al (11). 5 µg [S^4U] $tRNA^{Phe}$ were incubated for 15 min at 100°C in 40 µl 50% deionised formamide. The reaction was stopped by addition of 1 µl 3 M sodium acetate and 3 volumes ethanol.

5'-end labeling of tRNA fragments

The fragments were dissolved in 20 µl 0.1 M Tris-HCl pH 8 buffer containing 0.01 M $MgCl_2$, 0.002 M spermidine and 0.03 M 2-mercaptoethanol. Oligonucleotides were labeled at their 5'-end by incubation with 4 units of T_4 polynucleotide kinase and 200 µCi [γ-^{32}P]ATP. After 30 min at 37°C the reaction was stopped with 5 µl 10 M urea, and the labeled tRNA fragments were fractionated by electrophoresis on 10% and 20% polyacrylamide-urea containing slab gels (300 x 400 x 0,5 mm). The 5'-labeled fragments were visualized on the gel by autoradiography, cut out, eluted and finally precipitated with 3 volumes ethanol in presence of 10 µg carrier tRNA.

Total P_1 digestion and two-dimensional thin-layer chromatography

P_1 hydrolysis was carried out according to Silberklang et al (19). Each oligonucleotide was dissolved in 5 µl 0.05 M ammonium acetate pH 5.3, and digested to 5'-terminal nucleotide with 1 µg P_1 nuclease for 2.5 h at 37°C. These [^{32}P]-labeled nucleotides were identified by two-dimensional thin-layer chromatography (TLC) on cellulose-coated 10 x 10 cm plates using the solvent system described by Kuchino et al (11). 0.25 A_{260nm} unit standard 5'-phosphate S^4UMP was co-chromatographed with the [^{32}P]-labeled nucleotide digest to allow precise positioning of this residue.

RESULTS

Thiolation of $tRNA^{Phe}$

Treatment of RNA molecules with pressurized H_2S leads to a highly specific conversion of cytidines into 4-thiouridines (3-4). The conditions adopted here for the thiolation procedure were described in a previous paper (9). A 7h incubation at 37°C led to an average conversion of one cytidine residue per tRNA molecule. The thiolation reaction was then stopped and the [S^4U] tRNA was purified for subsequent analysis.

The integrity of this thiolated tRNA was verified by electrophoresis on

TABLE 1. AMINOACYLATION EXTENT OF THIOLATED $tRNA^{Phe}$

Sample	number of S^4U residues per molecule	CCA reincorporation	Aminoacylation %
Thiolated native tRNA	1.2	-	30
tRNA lacking 3'-CCA_{OH} during thiolation	1.3	95	90
Unthiolated tRNA	0	-	100

a polyacrylamide-urea containing gel (9).

Charging capacity of thiolated $tRNA^{Phe}$

Thiolation of native $tRNA^{Phe}$ molecule dramatically affects its aminoacid accepting capacity. As shown on table I, hydrogen sulfide treatment modifying one cytidine per molecule affects 70% of the charging capacity of the thiolated tRNA.

Nevertheless by removal of the 3'-terminal-CCA_{OH} prior to thiolation, followed by reincorporation of this terminal sequence after modification, 90% of the charging capacity was retained as compared to that of unthiolated tRNA. This result strongly suggests that, in the limited thiolation conditions adopted here, the two 3'-terminal cytidines are the primary targets. Modification of one of them leads to an important decrease in the aminoacylation capacity.

Localization of the 4-thiouridine residues in the thiolated tRNA

In order to determine the cytidine residues converted to 4-thiouridines, [S^4U] $tRNA^{Phe}$ was subjected to the limited formamide hydrolysis described by Stanley and Vassilenko (10) in conditions leading to one cut per molecule. The oligonucleotide mixture was then submitted to 5'-end labeling and fractionated by polyacrylamide-urea gel electrophoresis (Fig. 1). Each oligonucleotide was cut out of the gel and eluted. The 5'-terminal nucleotide of each fragment was determined by total P_1 nuclease digestion which hydrolyses nucleotides by leaving a 5'-terminal phosphate, followed by two-dimensional TLC as described in Materials and Methods. Figure 2 shows an autoradiogram of an oligonucleotide containing a S^4U residue at its 5'-terminus. The identification of a pS^4U spot was done by comparing its position to that of the four major nucleotides and standard S^4UMP.

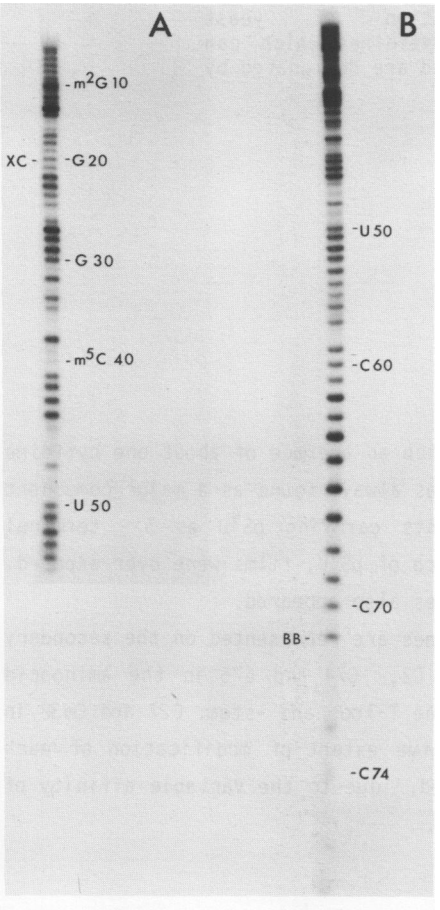

Figure 1 : Autoradiogram of 5'-labeled oligonucleotides obtained by limited formamide hydrolysis and fractionated by electrophoresis on 10%, (A) and 20% (B) polyacrylamide-urea gels

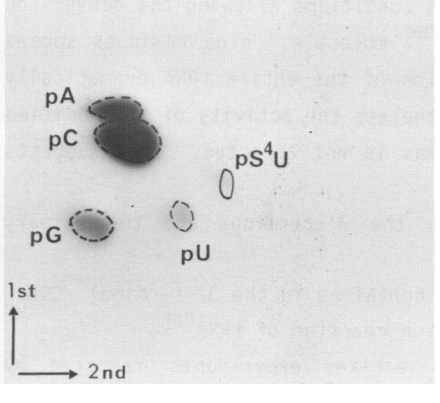

Figure 2 : Autoradiogram of a 2-dimensional chromatography of the [^{32}P] labeled 5'-terminal nucleotide digest.

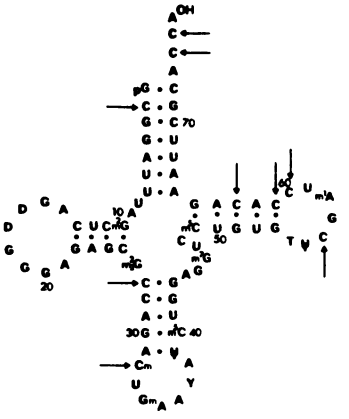

Figure 3 : Clover-leaf representation of yeast $tRNA^{Phe}$. Cytidines which can be modified are designated by an arrow.

Since conditions were selected in which an average of about one cytidine per molecule is converted, unreacted pC was always found as a major component in P_1 nuclease digests of those fragments carrying pS^4U as 5'- terminal nucleotide. In order to ensure the presence of pS^4U, films were over-exposed. As a consequence, contaminating nucleotides also appeared.

The positions of the modified cytidines are represented on the secondary structure model of $tRNA^{Phe}$ (fig. 3) : C2, C74 and C75 in the aminoacid acceptor stem; C56, C60, C61 and C63 in the T-loop and -stem; C27 and Cm32 in the anticodon-stem and -loop. The relative extent of modification of each cytidine can unfortunately not be measured, due to the variable affinity of kinase for the different oligonucleotides.

DISCUSSION AND CONCLUSION

Hydrogen sulfide treatment is a valuable method to chemically modify cytidines into 4 thiouridine residues. In conditions allowing the conversion of an average of one cytidine per $tRNA^{Phe}$ molecule, nine residues spread along the tRNA can be modified. Thiolation of the entire tRNA dramatically affect its aminoacylation capacity. Nevertheless the activity of the modified tRNA containing an intact 3'-CCA_{OH} terminus is not affected. This suggests that:

i) the single-stranded cytidines in the 3'-terminus are the primary targets in the thiolation;

ii) the N4 position of the cytidines contained in the 3'-terminal -CCA_{OH} sequence is essential for the aminoacylation reaction of $tRNA^{Phe}$.

This result is in agreement with earlier experiments reported by

Schulman et al (20,21). By treatment of formylmethionine tRNA with sodium bisulfite, they converted several exposed cytidine residues to uridine residues. These authors showed that the conversion of the cytidine closest to the 3'-terminus leads to a complete loss of the methionine accepting capacity, while modification of the adjacent cytidine has no effect on its aminoacid acceptance. Since thiolation of $tRNA^{Phe}$ leads to a 70% decrease of the phenylalanine acceptance when entire $tRNA^{Phe}$ is used (see table I), and to almost no decrease after removal of the 3'-terminal $-CCA_{OH}$ prior to this modification, we may estimate that about 70% of $tRNA^{Phe}$ molecules are modified at position C75. Experiments are in progress to separate active and inactive molecules following thiolation in order to specify which modification is responsible for the dramatic loss of the charging capacity of the $tRNA^{Phe}$.

Besides C74 and C75 located in the 3'-terminal sequence, 7 other cytidines could be modified along the tRNA molecule : C2, C27, Cm32, C56, C60, C61 and C63. As shown on table 1, these modifications do not seriously affect the charging capacity of the tRNA since 90% of the global activity is maintained. In order to check the possibility of inactivation due to modification at other than $-CCA_{OH}$ end, tRNA minus 3'-CCA_{OH} was reacted 2 days at 37°C to obtain full thiolation (see ref. 9). Unfortunately this modified molecule lost about 80% of its ability to be repaired at the $-CCA_{OH}$ terminus and did not allow to test valuably its charging activity. This could be the reflect of a modification of the biological active conformation. Using conditions in which only an average of one cytidine per molecule is modified, one cannot exclude that a single modification can lead to partial or total inactivation.

According to the secondary and tertiary structure models of $tRNA^{Phe}$ (22), only two modified cytidines (Cm32 and C60) are located in single-stranded regions. C2, C27, C61, and C63 are located in double-stranded regions and are involved in Watson-Crick base pairs. The other ones are involved in maintaining the tertiary stucture : C56 stabilizes the corner of the L-shaped molecule by forming a Watson-Crick tertiary base pair with G19 in the D-loop (22). The N4 position of C61 is participating in an other hydrogen bond with phosphate 60. Finally the stability of the anticodon loop is improved by an hydrogen bond between Cm32 and A38. It has to be noticed that in this case the hydrogen bond involves the oxygen in position 2 of the base, leaving the N4 group free (22).

It has been reported that chemical conversion of cytidines into 4-

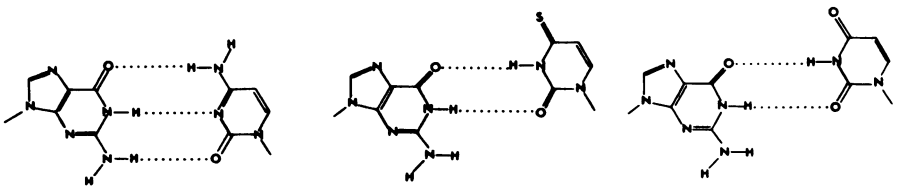

Figure 4 : Hydrogen bonding in guanosine-cytidine (A), guanosine - 4-thiouridine (B) and guanosine - uridine (C) base pairs.

thiouridines occurred only in single-stranded C residues (5). This assumption is in agreement with the observed dramatic decrease of charging ability of [S^4U] $tRNA^{Phe}$ containing the CCA_{OH} 3'-terminus during thiolation. Nevertheless our results show that other cytidines involved in secondary and tertiary structure folding are thiolated as well. This could be the result of a partial denaturation of the tRNA molecule during incubation in the hydrogen sulfide-pyridine-water system used here. However one cannot explain why in the acceptor stem the second base pair C2-G71 could be opened allowing thiolation of C2, whereas the first base pair of this stem pG1-C72 is maintained. The lack of detection of C72 modification could be due to the low yield of thiolation and also to the low labeling of the corresponding oligonucleotide, as attested by the very weak band of C72 in Figure 1. Anyway these results suggest that thiolation is not an ideal method to localize single-stranded cytidine residues in a RNA molecule.

It was reported that the presence of a thiol group at position 4 in poly C does not prevent base pairing with poly G (8). S^4U can exist in two forms, the 4-thiol (C-SH) form and the 4-thiono (C=S) one. Studies of the ultraviolet and infrared spectra of 4-thiouracil in various solvents and at different pHs showed that its neutral form has a 2-keto-4-thione structure (23). In the light of these structural features, guanine-4-thiouracil base pairing would contain only two hydrogen bonds (figure 4). This base pairing would resemble the guanine-uracil interaction as it is found between G4 and U69 in yeast $tRNA^{Phe}$. The substitution of the classical G-C base pair involving three hydrogen bonds by the G-S^4U one would lead to a minor structural distortion (22). As shown here, such changes of base pairing in the tRNA do not affect its biological activity. This is supported by crystallographic studies performed on yeast $tRNA^{Asp}$ which contains two G-U base pairs in the D-stem. Their presence does not fundamentally perturbe the helical conformation of this stem (24).

In conclusion thiolation of $tRNA^{Phe}$ leads to the conversion into 4-thiouridines of cytidine residues located both in single-stranded and base paired regions. The two 3'-terminal C74 and C75 cytidines are the primary targets of the thiolation reaction. This modification causes an important decrease of the aminoacylation capacity. However replacement of an amino group by a thiono one at internal positions within the tRNA does not affect its aminoacid charging activity, suggesting that the overall biologically active conformation of the tRNA is maintained.

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