THE RESPONSE OF THE DOUBLE STRAND-SPECIFIC NUCLEASE V₁ TO Y-BASE REMOVAL IN YEAST tRNA<Phe>*

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The patterns of limited hydrolysis of yeast tRNA<sup>Phe</sup> and tRNA<sub>Y</sub><sup>Phe</sup> by double strand-specific ribonuclease V₁ show some differences in cleavage of both the acceptor stem and the anticodon stem. These regions are considerably better substrates for RNase V₁ in tRNA<sub>Y</sub><sup>Phe</sup> than in tRNA<sup>Phe</sup>. The results are interpreted in favour of conformational changes taking place in yeast tRNA<sup>Phe</sup> upon the Y-base<sup>1</sup> removal.

The limited number of tRNA species which give well-diffracting crystals, includes yeast tRNA<sup>Phe</sup>. Tertiary structure of tRNA<sup>Phe</sup>, solved in great detail, proved very useful in analysing tRNA conformational dynamics in solution [reviewed in 1, 2].

A variety of biophysical and biochemical methods have been applied to characterize the conformational changes occurring in yeast tRNA<sup>Phe</sup> under various solvent conditions and in contact with low- and high molecular weight ligands. Limited enzymatic digestion of tRNA with endonucleases differing in specificity has been successfully used. One of those is RNase V₁ isolated from cobra Naja naja oxiana venom [3]. Using this enzyme similar cleavage sites and intensities were observed in several tRNAs, suggesting that nuclease V₁ is sensitive to RNA conformation rather than to specific sequences [4-9]. Most of the cuts were found in cloverleaf

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1 Abbreviations: Y-base, α-(carboxyamino)-4,9-dihydro-4,6-dimethyl-9-oxo-1H-imidazolo [1,2-a] purine-7-butryic acid dimethyl ester; h.p.l.c., high pressure liquid chromatography; enzymes: T₄ polynucleotide kinase (EC 2.7.1.78); alkaline phosphatase (EC 3.1.3.1); ribonuclease T₁ (EC 3.1.27.3);
helices, nevertheless, in some tRNA species, cutting was also observed in the structured single-stranded regions [5, 7, 9].

To learn more about the sensitivity of RNase V₁ to conformational changes in tRNA, we have used this probe to analyse the structural effect of Y-base excision from the anticodon loop of yeast tRNA^Phe. This covalent point-modification was shown previously to trigger conformational changes in the adjoining anticodon stem [10-13], as well as in three remote single-stranded regions [13-16].

MATERIALS AND METHODS

tRNAs: Yeast tRNA^Phe of specific phenylalanine acceptance about 1400 pmol/A₂₆₀ unit was prepared from crude brewer's yeast tRNA by the standard column chromatography procedures including benzyolated DEAE-cellulose and Sepharose 4B. Final purification was achieved by h.p.l.c. on the TSK-gel DEAE 2SW (Toyo-Soda) column. The tRNA deprived of the Y-base was prepared according to the procedure described by Thiebe & Zachau [17].

Enzymes: RNase V₁ was purchased from Pharmacia and RNase S₁ from PL Biochemicals. T₄ polynucleotide kinase, snake venom phosphodiesterase and RNase T₁ were from Boehringer. Calf intestine alkaline phosphatase was from Sigma, tRNA nucleotidyltransferase was a gift from Dr. R. Giege.

End labeling of tRNAs: The 3'-end labeling was done on a tRNA deprived of its 3'-terminal sequence by snake venom phosphodiesterase treatment. Reconstruction of the natural CCA-end was achieved with CTP, [α-³²P]ATP (400 Ci/mmol, Amersham) and tRNA-nucleotidyltransferase. The 5'-end labeling was done with [γ-³²P]ATP (3000 Ci/mmol, Amersham) and T₄ polynucleotide kinase on tRNAs dephosphorylated with calf intestine alkaline phosphatase. The dephosphorylated tRNAs were gel-purified on 15% polyacrylamide gel prior to the reaction with kinase. Labeled tRNAs were purified by polyacrylamide gel electrophoresis (15% gel, 8 M urea), eluted from the gel, and precipitated with ethanol. In typical experiments about 1 x 10⁶ c.p.m./g tRNA was obtained for both 5'- and 3'-end labeling.

Enzymatic digestions: Prior to enzymatic digestion the labeled tRNAs, supplemented with carrier tRNA of the same structure, were subjected to the standard denaturation-renaturation procedure in Tris/HCl buffer of pH 7.2 in the presence of NaCl and MgCl₂, as described previously [16]. The tRNA samples were preincubated for 5 min at 25°C and treated with RNase V₁. The course of tRNA hydrolysis was analysed after selected periods of time specified in legends to Figures. The reactions were quenched by mixing the aliquots with equal volumes of urea/tracking dyes/EDTA solution.
Electrophoresis and autoradiography: Electrophoresis was performed on 15% acrylamide/0.75% bis-acrylamide/7 M urea/50 mM Tris-borate, pH 8.3, 1 mM EDTA gels (40 x 30 x 0.04 cm) at 1000 - 1500 V for 3 - 4 h in various experiments. Autoradiography was at -20°C and the radioactivity present in tRNA and its fragments was measured in gel slices by Cerenkov counting.

RESULTS

Enzymatic digestions of labeled tRNAs were carried out under conditions that preserved tRNA tertiary structure (temperature 25°C, 10 mM Tris/HCl pH 7.2, 40 mM NaCl, 10 mM MgCl₂, 8 µM tRNA concentration). The tRNA-enzyme ratio was carefully controlled to ensure a low level of hydrolysis, i.e. statistically less than one cut per molecule (for all primary and secondary cuts taken together). Control samples were incubated in parallel, under the same conditions, but in the absence of the enzyme. The tRNA fragments were sized by electrophoresis on denaturing polyacrylamide gels and located by autoradiography. The cleavage sites were determined by running on the same gel either S₁ nuclease ladders, composed of tRNA fragments having the same 3'-OH end groups as the V₁ hydrolysis products.

![Fig. 1. Hydrolysis of 5'-end-labeled yeast tRNA^{Phe} (panel A) and tRNA^{Pro} (panel B) with V₁ nuclease. Autoradiography of tRNA fragments separated by denaturing polyacrylamide gel electrophoresis. Reaction conditions: 10 mM Tris/HCl pH 7.2, 40 mM NaCl, 10 mM MgCl₂, 0.2 mg/ml RNA, nuclease V₁ - 250 U/ml. Lanes A-D represent 2, 5, 10, 20 min hydrolysates; lane E, control; lane F, limited T₁ hydrolysate; lane G, formamide ladder](image-url)
(not shown), or standard alkaline hydrolysis and T₄ nuclease ladders containing tRNA fragments with 3′-phosphates. The tRNAs labeled either at 5′- or 3′-end were used in these studies to visualize better the cleavages taking place in the vicinity of each terminus.

All double-stranded cloverleaf stems are cleaved in yeast tRNAₚhe by endonuclease V₁. The representative hydrolysis pattern obtained for 5′-end-labeled tRNA is shown in Fig. 1A The amino-acid acceptor stem is hydrolysed extensively at its 3′-side, at phosphates 68, 69, 71 and 72. The tRNA fragments generated by cutting at these positions are, however, very poorly resolved (Fig. 1) as they migrate only a little faster than intact tRNA. At the opposite 5′-side of the acceptor stem the V₁ cleavages are considerably less efficient and they comprise phosphates 3, 4, 5, 7 and 8 with the strongest cut at phosphate 5. Contrary to the acceptor stem, the much shorter Ω-stem, containing four base pairs, is cleaved exclusively at its 5′-side. The most prominent cut occurs at phosphate 13 and is accompanied by two weak cleavages at neighboring phosphates 12 and 14. The anticodon arm double helix is hydrolysed symmetrically in yeast tRNAₚhe at phosphates 29 and 42. The former cut is considerably stronger, and, as in the case of the major Ω-stem cleavage, it is surrounded by two satellite cuts at phosphates 28 and 30. At the opposite side of the stem, besides a strong cut at phosphate 42, a weak cleavage appears at phosphate 43. Finally, very specific hydrolysis is observed at the 5′-side of the TΨC stem at phosphate 51. Its intensity is similar to that of the cut at phosphate 42 in the anticodon stem. At the 3′-side of the TΨC stem three weak cuts occur at phosphates 63-65. They are easily detectable in reactions with 3′-end-labeled tRNA at high enzyme/tRNA ratio (not shown). The V₁ nuclease cleavage pattern described above is basically in good agreement with those found earlier in yeast tRNAₚhe [5, 7]. However, some differences exist. These include the lack of weak hydrolysis in the Ω-stem at phosphates 22-25, detected by other authors [5, 7]. The remaining differences concern the distribution of cuts in the anticodon and acceptor stems.

The results shown in Fig. 2A, were obtained by hydrolysis of 3′-end labeled tRNAₚhe with RNase V₁ at lower enzyme/tRNA ratio. Hydrolysis is most efficient at the 3′-side of the acceptor stem at phosphates 68, 69, 71 and 72. The cleavages at phosphates 29 and 42 are barely discernible in these conditions, whereas practically no hydrolysis is observed in the D and TΨC stems or at the 5′-side of the acceptor stem. This reflects the highest affinity of nuclease V₁ to the longest acceptor stem containing seven base pairs, extended at its 3′-side by four single-stranded nucleotides in stacked conformation.

The nuclease V₁ hydrolysis of 5′- and 3′-end-labeled tRNAₚhe is shown
Fig. 2. Hydrolysis of 3'-end-labeled yeast tRNA\textsuperscript{Phe} (panel A) and tRNA\textsuperscript{Phe} (panel B) with V\textsubscript{1} nuclease. Reaction conditions and lane assignment were the same as specified in the legend to Fig. 1, except for lower V\textsubscript{1} nuclease concentration used — 100 U/ml.

for comparison in Fig. 1B and Fig. 2B, respectively. The reactions were performed in parallel with those of tRNA\textsuperscript{Phe}, under identical conditions, and the samples were run on the same gels. It is apparent that the Y-base removal from yeast tRNA\textsuperscript{Phe} does not change the cleavage pattern dramatically, nevertheless some qualitative and quantitative differences are noticeable in two double-stranded regions. The acceptor stem hydrolysis is more pronounced at its both sides in tRNA\textsuperscript{Phe}, and the differences in the cleavage efficiencies at phosphates 4, 5, 68, 69, 71, 72 reach, on the average, factor 2 in various experiments. There is also one minor qualitative difference in the acceptor stem hydrolysis manifested by the appearance of a very weak cut at phosphate 6, in tRNA\textsuperscript{Phe}. The other cut that occurs exclusively in tRNA\textsuperscript{Phe} is a faint cleavage at phosphate 31 in the anticodon stem. Besides that, the neighboring phosphates 29 and 30 are cleaved more effectively in tRNA\textsuperscript{Phe}. Also at the 3'-side of the anticodon stem two cuts at phosphates 42 and 43 are considerably stronger in tRNA\textsuperscript{Phe} than in tRNA\textsuperscript{Phe}.

DISCUSSION

There are probably three distinct double-strand-specific nuclease, isolated from cobra Naja naja oxiana venom, used in tRNA structure probing. The enzyme described originally by Russian scientists [3] has an $M_c$ of
15,900, the one reported later [5, 7] is twice as large, while the preparation purified recently [8] is of \( M_r \) 29,700. Except for the largest enzyme, they exhibit similar specificity, cleaving yeast tRNA\textsuperscript{Phe} exclusively in the acceptor and anticodon stems. The commercially available enzyme used in our studies is, as it was shown above, less specific and it behaves like nuclease \( V_1 \) described by Lockard & Kumar [5] and later by Rich \textit{et al.} [7].

The summary of cleavage data obtained in our work concerning yeast tRNA\textsuperscript{Phe} secondary and tertiary structures is shown in Fig. 3. To assign

![Fig. 3. Cleavage sites in yeast tRNA\textsuperscript{Phe} hydrolysed with double strand-specific nuclease \( V_1 \) presented on the cloverleaf secondary structure and simplified tertiary structure of the tRNA molecule.](image)

the observed variations in the efficiency of \( V_1 \) hydrolysis to the specific structural alterations taking place at the cleavage sites, a detailed structure of nuclease \( V_1 \) recognition site would be required. This, however, has not been yet satisfactorily resolved. It was suggested earlier [7] that it is the tRNA minor groove that is recognized by RNase \( V_1 \). According to more recent studies [9] the enzyme recognizes the specific stacked conformation of one strand that is frequently present in the base-paired helical regions. Besides the conformation of the sugar-phosphate backbone, at least four successive phosphate residues are required to form a suitable \( V_1 \) nuclease recognition size.

Whatever the detailed structure of the specific \( V_1 \) nuclease recognition site, it is considerably altered in two helical regions of yeast tRNA\textsuperscript{Phe}, upon the Y-base removal. This applies to both sides of the anticodon and
acceptor stems. As concerns the anticodon stem, double helix is a better substrate for V₁ nuclease in tRNA^Phe_. The change in the cleavage pattern observed in that region is consistent with some earlier n.m.r. results showing that the Y-base excision from yeast tRNA^Phe_ affects the helical conformation of the anticodon stem. The reported changes comprise the resonances of hydrogen-bonded protons of base pairs from that region [10, 12] as well as a methyl group resonance of m^3C40 [13].

More striking, however, is the enhanced reactivity of the acceptor stem in tRNA^Phe_. This indicates that the structural perturbation induced in the anticodon loop is propagated along the tRNA molecule, and besides the adjoining stem and more distant single stranded regions [13-16] also the amino-acid acceptor stem becomes affected. Thus, the question arises, how the local conformational change induced by the Y-base removal can be transmitted to the remote parts of the molecule. It is known from solution studies that RNA helix is a fluctuating dynamic structure, in which transient opening of individual base pairs occurs without the disruption of neighboring interactions [18, 19]. Moreover, besides such motions involving a wide range of time scales and maintaining individual torsions in the proximity of the local energy minimum, tRNA is known to undergo conformational interconversions comprising whole structural domains. Transition between two or even three conformations of the anticodon loop, occurring at low temperature either in the presence or in the absence of magnesium ions, is an example [20-24].

It seems that the above motions maintaining the tRNA structure in solution in continuous “breathing” could constitute the mechanism capable of transmitting the conformational perturbation throughout the molecule.

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REFERENCES


