Temperature dependent chemical and enzymatic probing of the tRNA-like structure of TYMV RNA

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Received December 17, 1987; Revised and Accepted February 10, 1988

ABSTRACT

In this paper we report on the thermal unfolding of the tRNA-like structure present at the 3' end of turnip yellow mosaic virus (TYMV) RNA. Diethyl pyrocarbonate (DEP), sodium bisulphite, nuclease S₁ and ribonuclease T₁ were used as structure probes at a broad range of temperatures. In this way most of the nucleotides present in the tRNA-like moiety were analysed. The melting behaviour of both secondary and tertiary interactions could be followed on the basis of the temperature dependent accessibility of the individual nucleotides or bases towards the various probes. The three-dimensional model of the tRNA-like domain (Dumas et al., *J. Biomol. Struct. and Dyn.* 4, 707 (1987)) was supported by the results to a large extent. The interactions occurring between the T- and D-loop appear to be more complex than proposed in the latter model. Additional evidence for the presence of the RNA pseudoknot (Rietveld et al., *Nucleic Acids Res.* 10, 1929 (1982)) was derived from the fact that the three coaxially stacked helical segments in the aminoacyl acceptor arm displayed different melting transitions under certain experimental conditions. Aspects of melting behaviour and thermal stability of double helical regions within the tRNA-like structure are discussed, as well as the applicability of nucleases and modifying reagents at various temperatures in the analysis of RNA structure.

INTRODUCTION

A model for the secondary structure of the tRNA-like 3' terminus of turnip yellow mosaic virus (TYMV) RNA has been reported earlier by Rietveld et al. (1) and Florentz et al. (2) (see also Fig. 1A). The proposed model was based upon chemical modification and enzymatic digestion studies and sequence comparisons. Although no standard cloverleaf like in tRNA was found, an aminoacyl acceptor arm could be constructed by the application of a new type of RNA folding (1,4). Basepairing between the triple-G sequence in loop I with a triple-C sequence at the 3' side of stem II, gives rise to a small double helical segment which can be stacked coaxially between hairpin stem I and II. The result of this special type of interaction, referred to as pseudoknotting, is an aminoacyl acceptor arm comprising twelve basepairs like in standard tRNA (see Fig. 1B). Recently, this model was refined by Dumas et al. (3), using modeling with molecular

graphics. The outcome of the latter study is depicted in Fig. 1C. The tertiary structure illustrates the overall resemblance in shape between classical tRNA and this tRNA-like structure.

Chemical and enzymatic probes have been used for the determination of secondary and tertiary structure in RNA (for a review see 5). The probes can also be used for obtaining information about the stability and melting behaviour of various structural elements in a RNA molecule. Up till now, this has been performed for three RNA species. Firstly, De Bruin and Klug (6) deduced the tertiary structure of a mammalian mitochondrial tRNA, lacking the dihydrouridine (DHU) stem and loop, by the use of diethyl pyrocarbonate (DEP) and dimethylsulphate (DMS) at various temperatures between 0° and 90°C. Secondly, 5S ribosomal RNA from various sources was analysed by Kjems et al. (7) and Pieler et al. (8). The use of DEP, DMS, sodium bisulphite and nuclease S₁ not only provided insight into the secondary and tertiary structure of the ribosomal RNA itself but also yielded information on RNA unfolding in general. Finally, it was shown that results obtained with chemical modification with DEP at various temperatures were in agreement with biophysical data collected for the 3' terminal 49 nucleotides colicin fragment of 16S rRNA (9).

In this paper a detailed analysis of the structure and stability of the tRNA-like domain at the 3' end of TYMV RNA is presented. A 112 nucleotides long, specifically end-labelled 3' terminal RNA fragment (see Fig. 1A) was submitted to probing studies at various temperatures. The use of DEP, sodium bisulphite, nuclease S₁ and RNase T₁ allowed us to distinguish double stranded from single stranded regions. RNase T₁ and nuclease S₁ are particularly useful for the localisation of single stranded RNA. It was found that RNase T₁ could not only be used under various buffer conditions but also at various temperatures up till 80°C. DEP is used for the detection of basepaired or stacked adenosine residues (10). Sodium bisulphite, which is used to convert cytidines into uridines (11), proved to be particularly suitable for the detection of tertiary interactions. The merits of the various probes used will be discussed. From the data obtained suggestions for a further refinement of the 3D model of the tRNA-like structure of TYMV RNA are given. A detailed discussion of the conformation and dynamics of the pseudoknot in the aminoacyl acceptor arm is also given.

MATERIALS AND METHODS

TYMV RNA: A 3' terminal fragment of TYMV RNA, 112 nucleotides in length, was isolated and end-labelled with [α -³²P]ATP (Amersham Int.) and ATP, CTP; tRNA nucleotidyl transferase as described by Rietveld et al. (1).

Sodium bisulphite modification: The conversion of cytidines to uridines was performed essentially as described by Pieler et al. (12). Solutions of 400 μ l each, containing 1.5 M NaHSO_3 (Merck), 10 mM MgCl_2 , 50 ng/ μ l carrier tRNA and about 300.000 Cerenkov counts of the 112 n fragment of TYMV RNA were incubated for 16 hours at various temperatures (5–60°C), followed by gel filtration on Sephadex G50. The column was equilibrated in 50 mM Tris.HCl pH 9.9. Radioactive fractions were pooled and incubated at room temperature for another 16 hours and subsequently the RNA was precipitated with ethanol and lyophilised. Detection of uridines present in unmodified RNA and cytidines converted to uridines was performed by chemical sequencing according to Peattie (13). After the U-specific hydrazine reaction and anilin treatment the radioactive samples were dissolved in a buffer containing 20 mM sodium citrate pH 5.0, 1 mM EDTA, 9 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol. Electrophoresis was on 10 or 15% polyacrylamide, 8M urea slabgels followed by autoradiography.

Diethyl pyrocarbonate modification: Carbethoxylation was carried out in a mixture containing 50 mM sodium cacodylate pH 7.0 with either 1 mM EDTA (semidenaturing conditions) or 1 mM MgCl_2 (native conditions), 50 ng/ μ l carrier tRNA and 150.000 Cerenkov counts of the 112 n fragment of TYMV RNA. The concentrations of DEP, needed for a sufficient degree of modification at each separate temperature and the optimal incubation times are summarised in Table 1. Note that the values deviate from those reported initially by De Bruin and Klug (6). Modification reactions were stopped by the addition of half a volume of 1.5 M sodium acetate pH 6.0 and two volumes of ethanol. The RNA was subsequently precipitated by centrifugation, treated with anilin, lyophilised and analysed by electrophoresis on 10 or 20% polyacrylamide, 8 M urea slabgels.

Nuclease S_1 digestion: Cleavage reactions were performed in 35 μ l incubation mixtures containing 30 mM sodium acetate pH 5.2, 5 mM ZnCl_2 , 100 mM NaCl, 5% glycerol, 150 ng/ μ l carrier tRNA and nuclease S_1 (Sigma). The optimal time of incubation and enzyme concentration for each temperature is given in Table 2. The amounts of nuclease S_1 used deviate appreciably from those reported by Pieler et al. (8). Digestion was stopped by the addition of 80 μ l cold ethanol followed by centrifugation. Pellets were dissolved in sample buffer containing urea and analysed on 15% polyacrylamide, 8 M urea slabgels.

RNase T_1 digestion: The digestion with RNase T_1 (Sankyo) was carried out in 50 μ l mixtures containing 50 mM sodium cacodylate pH 7.0, either 10 mM EDTA (semidenaturing) or 10 mM MgCl_2 (native), 200 ng/ μ l carrier tRNA and 300.000 Cerenkov counts of the 112 n fragment of TYMV RNA. The optimal incubation time and RNase T_1 concentration belonging to the various temperatures are given in

Table 3. The reactions were stopped by phenol extraction and the cleavage products were analysed on 10 or 15% polyacrylamide, 8 M urea slabgels.

Densitometry: The intensity of the bands on the autoradiographs was assessed visually. Visual quantification of melting behaviour of residues in critical regions, however, was confirmed by densitometry. Individual lanes of an autoradiograph were scanned at a wavelength of 600 nm with the aid of a Gilford 3000 spectrophotometer. Melting curves for the individual residues were constructed from the relative intensities of bands as quantified by the surface of peaks obtained from densitometry. In general, the visual interpretation was in good agreement with densitometric analysis.

RESULTS

Sodium bisulphite modification: The bisulphite ion preferentially reacts with cytidine residues in a single stranded region of RNA (8,12). Cytidines with the bisulphite adduct can be converted to uridines by alkaline treatment. These uridines are easily traced by RNA sequencing. In Fig. 2 the results are given of the modification of the 112 n fragment of TYMV RNA. Apparently, at low temperatures little modification takes place. The presence of bands in the region of the 3' terminal CCA-sequence (C₂ and C₃) indicates that this region is single stranded, although at 5°C this is not very clear. In the latter case it might have been appropriate to extend the time of incubation or to increase the nuclease S₁ concentration. Between 5 and 15°C the cytidines in the anticodon loop (C₅₅-C₅₉) and C₁₁ become accessible to an appreciable extent. When the temperature is raised to 20-25°C the cytidines at position 33 and 36 and those involved in pseudoknotting (C₂₅-C₂₇) become modified by the bisulphite. These residues, supposed to be involved in tertiary interactions, behave differently from cytidines in hairpin stems (see below). As far as the pseudoknot is concerned it is noteworthy that C₂₄ appears to become accessible

Fig.1. Secondary and tertiary structure of the 3' terminus of TYMV RNA comprising the tRNA-like structure.

A. Secondary structure of the 112 nucleotides long fragment used in this study. The 3' terminal four hairpins (I to IV) were proposed by Rietveld et al. (1) and Florentz et al. (2). The latter authors also proposed stem V. Numbering starts from the 3' end and the dashed line at the 5' end represents the genomic RNA of approximately 6000 nucleotides.

B. L-Arrangement of the tRNA-like structure of TYMV RNA as proposed by Rietveld et al. (1). The aminoacyl acceptor arm contains the RNA pseudoknot.

C. Stereo view of a computer graphics model of the 3' terminal 86 nucleotides of TYMV RNA according to Dumas et al. (3).

TABLE 1
Reaction conditions for DEP modification

10 mM MgCl ₂			10 mM EDTA		
incubation temperature (°C)	time (min)	amount of DEP (ul)	incubation temperature (°C)	time (min)	amount of DEP (ul)
4	240	20	0	240	20
12	170	20	15	150	20
21	110	20	30	50	20
37	30	20	45	15	20
42	20	20	50	10	10
50	25	10	55	7	10
55	15	10	60	4	10
60	10	10	65	3	10
65	5	10	70	2.5	10
70	35	5	75	2	10
80	25	5			
90	15	5			

For experimental details see Materials and Methods

at lower temperatures than C₂₅-C₂₇. Finally, cytidines in hairpin stems I, II and III (see Fig. 1A) are modified. It appears that stem II is the most stable one in the tRNA-like structure, having a T_m which is some 5 °C higher than that of stem I and III, which unfold around 35 °C. Cytidines upstream of hairpin III were not analysed, due to the poor resolution of this region on the

TABLE 2
Reaction conditions for nuclease S₁ digestion

incubation temperature (°C)	time (min)	amount of nuclease S ₁ (units)
0	60	2
15	45	2
20	40	2
25	35	2
30	30	2
35	30	1
40	30	0.5
45	15	0.5
50	15	0.2
55	8	0.2
60	4	0.2
65	3	0.2
70	2	0.2
80	1	0.2

For experimental details see Materials and Methods

TABLE 3

Reaction conditions for RNase T₁ digestion

10 mM MgCl ₂			1 mM EDTA		
incubation temperature (°C)	time (min)	amount of RNase T ₁ (units)	incubation temperature (°C)	time (min)	amount of RNase T ₁ (units)
0	240	0.0125	0	120	0.0125
15	120	0.0125	15	15	0.0125
20	100	0.0125	20	10	0.0125
25	60	0.00625	25	15	0.00625
30	45	0.00625	30	30	0.0025
35	30	0.00625	40	12	0.0025
40	22	0.00625	45	10	0.0025
45	15	0.00625	50	10	0.00625
50	10	0.0625			
60	5	0.125			
70	15	0.25			
80	15	0.50			

For experimental details see Materials and Methods.

polyacrylamide gels used. Densitometry confirmed the observations mentioned above. The big advantage of the bisulphite reaction is that upon densitometry the original, endogenous uridines can serve as internal standards. This implies that the extent of cytidine modification can be easily and unambiguously quantified (see Discussion).

Diethyl pyrocarbonate modification: Treatment with DEP was carried out in two types of buffer. When Mg²⁺ is included, the RNA is supposed to adopt a native conformation, whereas replacement of Mg²⁺ by EDTA results in a so-called semi-denaturated state of the RNA (10). Fig. 3A illustrates the results obtained under native conditions. The most remarkable feature is the behaviour of A₁₀. Even at the lowest temperature tested (0°C) it is already heavily modified and is far more reactive than residues in other single stranded regions like the anticodon loop (A₅₄ and A₅₆). This means that A₁₀ is neither stacked nor basepaired. The intense band at the A₁₀ position in the autoradiographs therefore serves as a convenient internal standard during the densitometric analysis. Beside the adenines from the anticodon loop and the ACCA 3' end also the A residues in the variable loop (A₄₄-A₄₅) and in loop IV (A₇₂ and A₇₆, which are not visible in Fig. 3A) are modified at relatively low temperatures. The adenines in loop II behave as if they are involved in tertiary interactions (see also 1). A₄ and A₅ are moderately accessible over the entire temperature range, although A₅ is slightly less modified compared to A₄. Above 60°C this difference levels off and modification of both residues slightly increases.

Fig.2. Temperature dependence of sodium bisulphite modification of cytidines in the tRNA-like structure of TYMV RNA. Control: RNA incubated in aqueous solution for 16 hours at room temperature; U: uridine specific sequence ladder. The numbering on the left and right indicate the position of uridines and cytidines, respectively (see also Fig.1A). Samples were analysed on 15 % polyacrylamide , 8M urea slabgels.

Residue A₁₆ at the top of stem I, behaves like a basepaired residue: modification only occurs at temperatures above 60°C. Unfortunately, stem II could not be analysed with DEP modification, due to the absence of adenines. Three adenines are present in stem III: A₄₇, A₄₉ and A₅₁. As can be seen in

Fig. 3A stem III starts melting from both ends simultaneously. At 65°C, A₄₇ and A₅₁ are carbethoxylated, whereas A₄₉ is still shielded. The helix-to-coil transition is completed at 70°C.

When Mg²⁺ is replaced by EDTA, both secondary and tertiary interactions are destabilised. As can be seen in Fig. 3B the melting temperatures of stem I, III and IV generally decrease by 10-15°C. In the case of tertiary interactions this effect is even more pronounced. Adenines 34 and 35 for instance are already modified at 21°C, whereas in the presence of Mg²⁺, modification takes place above 50°C only. When stem regions are compared it appears that stem V is the most labile one (T_m: 37-42°C). The unfolding of this stem, as monitored by the reactivity of A₉₉, seems to occur simultaneously with the destacking of the adenine residues in hairpin loop V (A₉₀-A₉₃). Note that A₉₃, which is on top of the supposed stack, becomes slightly modified even at 4°C. The next stem to melt is the anticodon stem III. Stem I and IV are more stable and melt above 60°C. Finally, A₁₀ which is not visible in Fig. 3B behaves as in the presence of Mg²⁺, although its accessibility is less pronounced (result not shown).

Nuclease S₁ digestion: The results are given in Fig. 4. Although the temperature ranged from 0 to 80°C the results obtained above 60°C are questionable, for reasons outlined below. From the fact that the CCA-end is frequently cut at 0°C it can be inferred that the concentration of enzyme used under these conditions was sufficiently high. In the same lane (0°C) a number of bands is observed below A₁₆, which disappear at higher temperatures. This might be an indication for the existence of structural variation in this part of the RNA (see Discussion). Between 10 and 40°C the aminoacyl acceptor arm is protected from nuclease digestion. The only sites that are weakly sensitive to nuclease S₁ are located in loop II (C₃₃-C₃₆) and in the stretch of RNA comprising U₂₁ to C₂₄. Other sites are found in loop IV (U₇₃-U₇₄) and within the 5' proximal 30 nucleotides (poorly resolved in Fig. 4). Especially above 40°C this latter region is heavily cleaved. Above 45°C bands appear between positions 4 and 13 from the 3' end. The analysis with nuclease S₁ could not be extended above 60°C for two reasons. UV melting studies revealed that, in the presence of Zn²⁺, RNA tends to precipitate at higher temperatures. At these higher temperatures we also observed random degradation of the RNA (results not shown). A correct interpretation of results obtained at temperatures above 60°C is therefore not possible.

RNase T₁ digestion: RNase T₁ was used earlier by us and others (see for instance 1,2 and 14) at 37°C under native (10 mM MgCl₂) and, more recently (15), under semi-denaturing (10 mM EDTA) conditions. We here describe a new

extension of the use of RNase T₁, by performing the digestions at a broad temperature range. Fig. 5A shows that in the native state, only a limited set of guanine residues are recognized by the RNase. The 112 n fragment of TYMV RNA is cut at G₂₈ and in stem and loop IV, which is in agreement with the results obtained by Rietveld et al. (1). The results obtained in the region around and within loop IV are not easily interpretable. The presence of two intrinsically weak pyrimidine-A sequences in the loop (U₇₃-A₇₂ and C₇₇-A₇₆) and a possible

Fig.3. Temperature dependence of the diethyl pyrocarbonate modification of adenines in the tRNA-like structure of TYMV RNA.

A. Experiments performed under native conditions (10 mM $MgCl_2$). Temperatures at which the modification took place are indicated above each lane. Control: RNA incubated in buffer for two hours at room temperature; T_1 RNase: RNase T_1 digestion under denaturing conditions (9 M urea, 1 mM EDTA, 55°C). The positions of some of the A- and G-residues are indicated. Samples were analysed on 20 % polyacrylamide, 9 M urea slabgels. This autoradiograph was also presented in (3).

B. Experiments performed under semi-denaturing conditions (10 mM EDTA). Samples were analysed on 10 % polyacrylamide slabgels. For further details see Materials and Methods.

Fig.4. Temperature dependence of nuclease S_1 digestion of the tRNA-like structure of TYMV RNA. Temperatures at which the enzymatic treatment was performed are indicated above each lane. Control: RNA incubated in nuclease S_1 buffer for 1 hour at 0°C ; A or U: adenine and uridine specific sequence ladders. The thick band at the bottom of the autoradiograph represents degradation occurring at the 3' terminal CCA-end. Samples were analysed on 15 % polyacrylamide, 8M urea slabgels.

heterogeneity in the primary structure makes proper assignments difficult. When the temperature is raised to 80°C , G_{48} and G_{52} are cleaved by the RNase, while all other guanines are still inaccessible. This also holds true for G_{62} which is located in the centre of stem III, thus being part of the same hairpin stem where also G_{48} and G_{52} are found. This is in agreement with the results of DEP modification. Apparently, in contrast to nuclease S_1 , RNase T_1 remains active at temperatures around 80°C .

When the RNA is in the semi-denatured state (10 mM EDTA) it is clear that the three guanines (G₁₃-G₁₅) involved in the formation of the pseudoknot are no longer shielded (see Fig. 5B). Especially the phosphodiester bond belonging to G₁₃ is frequently cut. Like in the presence of Mg²⁺, G₂₈ and G₃₇ are accessible over the entire temperature range, although the cleavage at G₂₈ seems to be less pronounced. Also, G₇₅ and the guanines present in stem IV are recognized by RNase T₁ even at low temperatures. The melting behaviour of G₁₇, G₃₉, G₄₈ and G₅₂ illustrates the fact that replacement of Mg²⁺ leads to an overall destabilisation of the secondary structure.

DISCUSSION

A spatial model for the tRNA-like structure of TYMV RNA was presented by Rietveld et al. (1,16) on the basis of chemical modification and enzymatic digestion studies. A special type of RNA folding was introduced to construct an aminoacyl acceptor arm which strongly resembled that of canonical tRNA. Although the similarity of this 3D model with the crystal structure of tRNA^{Phe} (17,18) was striking, no detailed structure model was available. A further refinement of the model was made possible by a variety of approaches. Sequencing of the 3' terminus of a number of related tymoviral RNAs not only confirmed the model for TYMV RNA but also emphasized the importance of some structural features (19). An important next step in the refinement was the computer graphics modeling study of Dumas et al. (3) which was not only based on the experimental data available but also made use of parameters of the RNA-A double helix (20) and the crystal structures of tRNA^{Phe} (17,18) and tRNA^{Asp} from yeast (21,22).

In this paper we describe detailed structure mapping studies using three base-specific probes and one single strand specific enzyme at a variety of temperatures and buffer conditions. This enabled us to monitor the behaviour of almost all nucleotides of the tRNA-like structure of TYMV RNA over a range of temperatures. This method not only gives insight into the conformation of the RNA but also highlights the structural dynamics. This powerful method was described for the first time by De Bruin and Klug (6) who solved the 3D structure of a mammalian mitochondrial tRNA. The information which can be obtained by the use of this method surpasses that of many other techniques. This approach provides the method of choice especially for the structural analysis of larger RNA molecules for which X-ray diffraction or NMR studies are not practicable or even impossible. Nevertheless, chemical and enzymatic probing at various temperatures has a few drawbacks. Firstly, to determine the

melting transition of individual base residues, the extent of modification or nuclease cutting has to be determined unambiguously. The latter demands for an internal reference which can be provided by a nucleotide which is known to be fully accessible over the complete temperature range. In some cases such a residue is not easy to find (see also 6). Secondly, different temperatures during the probing reactions requires different experimental conditions regarding reaction time and probe concentration. These must be optimised to ensure an average of less than 1 cut per molecule. The optimal reaction conditions described in this paper (Table 1-3) differ from those reported previously. Thirdly, enzymatic digestions have their own problems because of the temperature and pH optima of the enzyme concerned. Also, the necessary presence of divalent cations like Zn^{2+} or Mg^{2+} might create conditions which hamper a direct comparison of the results obtained with different probes. Nuclease S_1 which cleaves single stranded RNA optimally at pH 5.0 in the presence of Zn^{2+} illustrates the latter problems. Use of this nuclease above 60°C is not meaningful due to precipitation and degradation of the RNA, which is probably Zn^{2+} catalysed. This is illustrated by the fact that at

Fig.5. Temperature dependence of RNase T_1 digestion of the tRNA-like structure of TYMV RNA.

A. Experiments performed under native conditions (10 mM $MgCl_2$). The panels displayed result from two separate experiments.

B. Experiments performed under semi-denaturing conditions (10 mM EDTA). Alkaline: alkaline digest of the 112 n long fragment; T_1 RNase: RNase T_1 digestion under denaturing (9M urea, 1 mM EDTA, 55°C) conditions. Samples were analysed on 15% polyacrylamide, 8 M urea slabgels.

temperatures above 60°C enzymatic cleavage drastically diminishes (see Fig. 4). Furthermore, the low pH can lead to a cutting pattern which is different from that expected at physiological pH (see also reference 23, concerning stem V).

A disadvantage of the sodium bisulphite treatment is that the first reaction

Fig. 6. Melting curves for the individual adenines present in the anticodon stem of the tRNA-like structure. The curves were calculated from data obtained from densitometry of the lanes of the autoradiograph depicted in Fig. 3A. The optical density (OD) is given in arbitrary units.

takes place at rather non-physiological conditions (1.5 M NaHSO₃). However, the fact that detection of converted C-residues is performed by sequencing for U-residues is a major advantage, because the original U residues can serve as internal references. Another notable feature is the gradual suppression of band compression effects upon increasing cytidine-to-uridine conversion (see Fig. 1 and ref. 24). To find an internal reference in the case of DEP modification is more problematic. However, A₁₀ seems to be a very good choice because of its peculiar steric properties which involve a strong exposure into the solvent (3).

To our knowledge this paper describes for the first time the use of RNase T₁ as a structure probe at various temperatures. Furthermore, replacement of Mg²⁺ by EDTA does not inhibit the activity of this guanine-specific structure probe. Since the results obtained with RNase T₁ are in good agreement with the model

for the tRNA-like structure of TYMV RNA (see below) it would be very interesting to investigate the applicability of other probes at various temperatures. In this respect, the use of ethylnitrosourea (25) or the double strand specific RNase from cobra venom (26,27) might be worth studying.

The results presented in this paper (Fig. 2-5) are in excellent agreement with the data presented earlier (1,2). A notable exception is the extreme accessibility of A₁₀ towards DEP not observed in our previous studies (1). Furthermore, Florentz et al. (2) do not observe the same behaviour of G₂₈ and G₃₇ under native conditions as we find reproducibly (see Fig. 5A).

As far as the pseudoknot is concerned it is clear that the so-called connecting loops (A₁₀-U₁₂ and U₂₁-C₂₄), which are both single stranded stretches of RNA spanning the shallow and deep groove respectively (4), show different accessibilities towards the various probes. A₁₀ and C₁₁ are readily modified by DEP and sodium bisulphite respectively, which suggest that residues in the loop spanning the shallow groove are highly exposed (see Fig. 1C). We conclude therefore that the RNA stretch spanning the shallow groove has its nucleotides pointing into solution thereby rendering them reactive towards chemical probes, but on the other hand, shielding the phosphodiester bonds from enzymatic cleavage. This conclusion is in agreement with the 3D model in Fig. 1C (see also ref.3). On the other hand, C₂₄ reacts with bisulphite at elevated temperatures only, while nuclease S₁ cleaves U₂₁-C₂₄ but not A₁₀-U₁₂. This implies that the nuclease does not merely recognise single strandedness in itself but also needs a special conformation of the RNA.

The two basepaired regions in the pseudoknot, i.e. stem I and the tertiary interaction between can G₁₃₋₁₅ and C₂₅₋₂₇ can be easily distinguished using bisulphite modification (see Fig. 2). The autoradiograph shows that the tertiary interaction melts at a somewhat lower temperature than stem I and II. This difference in melting temperature is a reflection of the difference in stability between hairpin stems and tertiary interactions in a pseudoknot under these particular conditions (1.5 M NaHSO₃). This makes the sodium bisulphite modification a favourable method for the detection of pseudoknots. It must be emphasized, however, that under more physiological conditions, melting of pseudoknots appears to proceed more cooperatively (unpublished results). Although stacking and basepairing interactions were proposed earlier for A₄ and A₅, these residues are slightly modified with DEP even at low temperatures. A₄ is modified to a larger extent than A₅, a phenomenon which disappears at temperatures above 60°C. The carbethoxylation seen at low temperatures is probably due to breathing of the hairpin terminus. The latter explanation is

corroborated by NMR analyses of an RNA fragment resembling the aminoacyl acceptor arm of TYMV RNA (to be published).

In canonical tRNA the L-shape is maintained by a number of interactions between the T- and D-loop. Similar interactions were supposed to stabilise the putative L-shape of the tRNA-like terminus of TYMV RNA. The fact that the equivalent of the T-loop in TYMV RNA (loop II Fig. 1A) has a similar conformation as its tRNA counterpart was derived from ENU modification studies (28). The presence of a conserved G·C pair between the T- and D-like loops in all valine specific tRNA-like structures provided another similarity to tRNA (19). Computer graphics modeling learned that in the case of TYMV RNA, the interaction between phosphate-32 and A₃₄ and C₃₁ is stereochemically feasible. Moreover, adenine 76 could be interdigitated between A₃₄ and A₃₅, while the earlier mentioned G·C basepair between G₇₅ and C₃₆ could be formed. Taken together this creates a structural arrangement similar to that found in tRNA. However, the A₅₄-T₅₈ reversed Hoogsteen basepair, found in tRNA, has no equivalent in the tRNA-like structure. This results from the presence of a G-residue in loop II at position 37, which prevents the formation of a U-turn, necessary for the formation of the reversed Hoogsteen basepair.

Fig. 2 and Fig. 3A clearly show that C₃₃, C₃₆, A₃₄ and A₃₅ are involved in tertiary interactions, which is consistent with the model of Dumas et al (ref. 3 and Fig.1C). As these authors already pointed out, the interaction between loop IV and loop II in the tRNA-like structure is not equivalent to that between T- and D-loop in canonical tRNA. From the present results it is suggested that the computer graphics model for the tRNA-like structure needs some adjustment in this particular region. For instance, the accessibility of A₇₂ towards DEP at low temperatures under native conditions might not be in agreement with interdigitation of this residue between A₃₄ and A₃₅. Furthermore, A₇₆ and G₃₇ display accessibilities which are not easily inferred from the model. Also, at present we have no explanation for the cleavage at G₂₈ by RNase T₁ under native conditions. We note that this residue is on the border of stem II and the tertiary interaction. The inconsistencies mentioned above might be resolved by future biophysical studies.

Densitometric analysis of the autoradiographs presented in this paper generally leads to sigmoidal melting curves for individual base residues. In some cases subtle differences in the melting proces within a stem region can be observed. This is illustrated in Fig.6 which shows the melting curves for the adenine residues in the anticodon stem. A₅₁ and A₄₇ located at the top and bottom, respectively, become accessible to DEP at somewhat lower temperatures

than A₄₉ which is located in the central part of the hairpin. This suggests that the anticodon stem starts melting from both helix termini (see also Fig.3A). The results of the RNase T₁ digestion also point to a stable core of stem III (see Fig.5A).

It is interesting to see that the order in which hairpin I to V melt, corresponds with the free energy values calculated for each hairpin using the thermodynamic parameters given by Freier et al. and Sugimoto et al. (29-31). Stem V which melts around 50°C under native conditions clearly has the lowest $-\Delta G$ value (1.5 kcal/mole). It is followed by stem I ($-\Delta G = 6.3$ kcal/mole), while stem III and IV melt at a similar temperature. Stem II appears to melt at least above 80°C.

An important question raised upon the detection of pseudoknots is the one concerning their stability (4). From our present study it is clear that the pseudoknot in the aminoacyl acceptor arm melts cooperatively with stem I under native conditions. Only in 1.5 M sodium bisulphite the tertiary interaction seems to be disrupted at a somewhat lower temperature. The significance of this finding is limited however, because the entire tRNA-like structure appears to melt at much lower temperatures than under native conditions. A solid analysis of the thermodynamic properties of pseudoknotted structures probably demands for smaller RNA fragments, harbouring the two separate hairpin structures corresponding to the stem and loop regions of the pseudoknot structure. The study of these smaller RNA fragments may be helpful in the calculation of the thermodynamic parameters for the contribution to pseudoknot stability by the two single stranded connecting loops and the effect of stacking of the two helical segments.

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