Chemical probes for tRNA tertiary structure

Comparative alkylation of tRNA with methylnitrosourea, ethylnitrosourea and dimethylsulfate

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The tertiary structure of tRNA in solution can be proved by chemical modification experiments. Three reagents, *N*-ethyl-*N*-nitrosourea, *N*-methyl-*N*-nitrosourea and dimethylsulfate which are known to alkylate nucleic acids at nucleophilic centers were compared. It is found that *N*-ethyl-*N*-nitrosourea and *N*-methyl-*N*-nitrosourea mainly react with phosphate residues and dimethylsulfate only with the bases. With dimethylsulfate the extent of alkylation of guanosines is about one order of magnitude higher than that of the phosphates by the nitrosocompounds.

Tertiary structure *tRNA* *Dimethylsulfate* N-*ethyl*-N-*nitrosourea* N-*methyl*-N-*nitrosourea*

1. INTRODUCTION

Chemical modification experiments have become a powerful tool for investigating the tertiary structure of nucleic acids. The experimental method to be employed is intrinsically simple and involves a statistical modification occurring less than once per nucleic acid molecule, followed by chain scission at the modified positions and analysis of the splitting products by rapid sequencing gel methodologies. Using this approach, it has been demonstrated recently that *N*-ethyl-*N*-nitrosourea (ENU) allows one to monitor the tertiary structure of several tRNA molecules in solution [1–3]. These studies took advantage of the property of ENU to react mainly with the free oxygens of the phosphodiester bonds in RNA [4–9]. But it is also known that ENU reacts, although to a lesser extent, with the nucleic acid bases [4–10], so that the possibility exists of probing them with this reagent. However, this would be difficult because of the very low yield of the modifications. We therefore introduced another *N*-nitroso derivative, *N*-methyl-*N*-nitrosourea (MNU), which modifies tRNAs at the level of the bases much more efficiently than ENU does [11]. Past studies have shown that one of the most reactive sites for *N*-nitroso compounds is nitrogen 7 in the guanosine residues. After MNU treatment m^7G should thus be formed in tRNA, a modification which allows an easy splitting of the tRNA chain and analysis of the modification pattern [12].

Here, we compare the reactivity of several tRNAs with ENU and MNU. Since dimethylsulfate (DMS), another alkylating reagent already used for probing the structure of tRNA [13,14], reacts with the same target as MNU should, we have introduced this reagent in a comparative study. We found that the extent of tRNA modification is much higher after DMS treatment than it is after ENU or MNU treatment. But the main observation of our work is that, unexpectedly, ENU and MNU gave

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the same modification patterns. MNU only allows the study of phosphate reactivities. Information about the accessibility of guanosine residues can only be extracted from DMS modification studies. Procedures for the application of alkylation reactions to tRNA studies are discussed.

2. MATERIALS AND METHODS

Plant tRNAs from yellow lupin seeds specific for methionine and phenylalanine [15] and yeast $tRNA^{Asp}$ [16,17] were purified by established procedures. Labelling at the 3′- and 5′-ends of tRNAs was carried out as in [18]. ENU was from Fluka and MNU and DMS were from Aldrich.

The treatment of $tRNA_i^{Met}$ with ENU was carried out as in [1,2]; i.e., in the presence of 140 mM ENU, 20% ethanol and 0.3 M sodium cacodylate buffer at pH 8.0 containing 20 mM $MgCl_2$, 100 mM NaCl and 2 mM EDTA, a condition which, at low temperatures, favours the native structure of the tRNA. The same procedure was used for the modification of $tRNA^{Phe}$ with MNU; in particular the reagent was introduced in the assays as an ethanolic solution. The modification of $tRNA^{Asp}$ with DMS was performed as in [13]; i.e., in the presence of 26 mM DMS and 50 mM sodium cacodylate buffer at pH 7.2, either under conditions favouring the native or the denatured form of the tRNA. Chain scission of the alkylated tRNAs at phosphodiester bonds or at alkylated bases was essentially as in [2,13]. The mixtures of radioactive tRNAs and oligonucleotides (50000 Cerenkov cpm) were analyzed by gel electrophoresis in 8 M urea as in [2,19]. Partial T_1 ribonuclease digests [20] and formamide ladders [21] were electrophoresed in parallel in order to assign the bands obtained after splitting of the alkylated tRNAs. The numbering of the bands on the gels follows that of the guanosine residues.

3. RESULTS

Figures 1 and 2 show two examples of alkylation experiments on plant tRNAs from lupin seeds, the initiator $tRNA_i^{Met}$ and the elongator $tRNA^{Phe}$. Alkylations with ENU were performed at different temperatures on $tRNA_i^{Met}$ labelled at its 5′-end (fig. 1) and those with MNU were done on 3′-end-labelled $tRNA^{Phe}$ (fig. 2). Strand splitting was obtained after alkaline treatment [2] on tRNA, treated either with ENU or MNU. This demonstrates that MNU methylates the free oxygens on the phosphodiester bonds. Furthermore, the extent

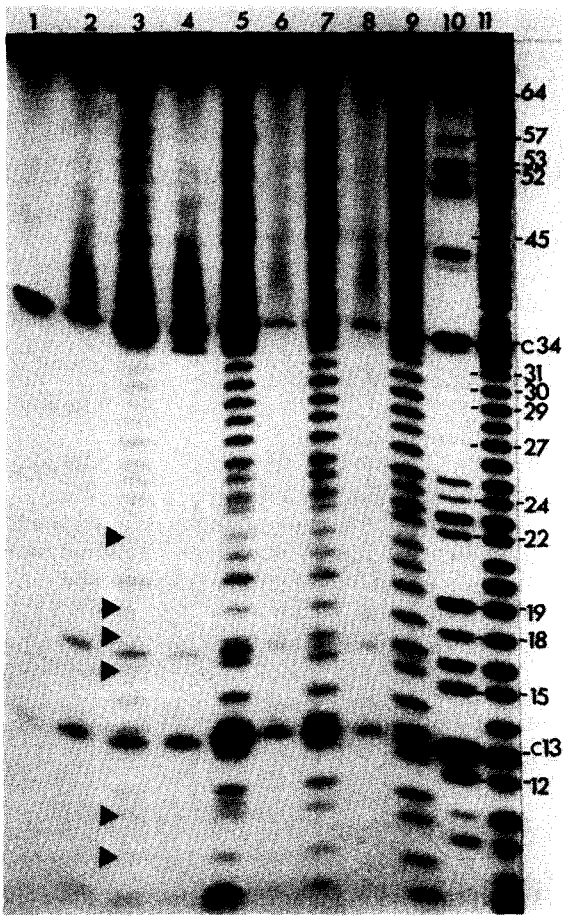

Fig. 1. Autoradiogram of a 15% acrylamide gel of an alkylation experiment with ENU on 5′-labelled $tRNA_i^{Met}$ from lupin: (1) control migration of tRNA submitted to any treatment; (2,4,6,8) control incubations in the absence of ENU at 20°C (180 min), 37°C (90 min), 50°C (15 min) and 80°C (2 min), respectively; (3,5,7,9) ENU treatment of tRNA under similar conditions; (10) partial ribonuclease T_1 digest; (11) formamide ladder. Phosphates protected against alkylation in the native structure of the tRNA are shown by triangles. Note the different migrations of the splitting products of ENU and formamide or ribonuclease T_1-treated tRNA [2], but the similar migration of the degradation products at C34 and especially at C13 (lane 9) and of the corresponding T_1 and formamide oligonucleotides (lanes 10,11).

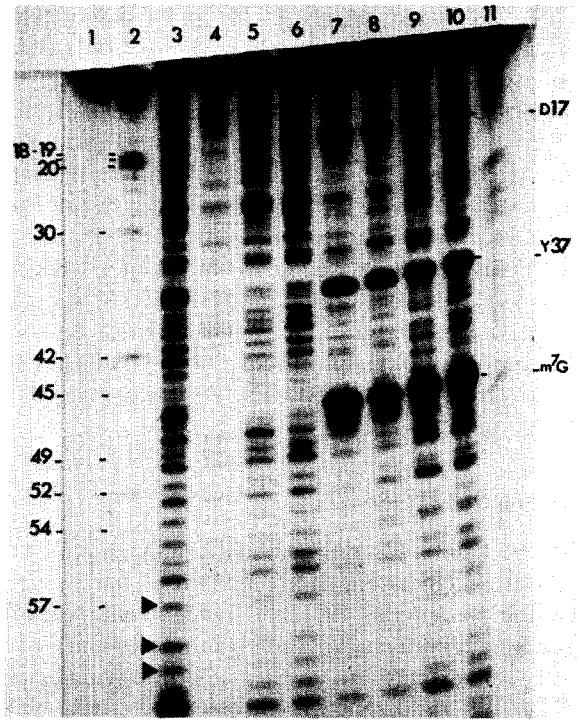

Fig. 2. Autoradiogram of a 15% acrylamide gel of an alkylation experiment with MNU on 3′-labelled $tRNA^{Phe}$ from lupin: (1) Control migration of tRNA submitted to any treatment; (2) partial ribonuclease T_1 digest; (3) formamide ladder; (4–6,8,9,10) MNU treatment of tRNA at 20°C for 180 min (4,8), at 37°C for 90 min (5,9) and at 80°C for 2 min (6,10); (7,11) control incubations in the absence of MNU. Chain scission was performed as in [2] for lanes (4–6,11) and as in [13] for lanes (7–10). The time for the autoradiography (about 4 days) was as in fig. 1. Protected phosphates are indicated by triangles.

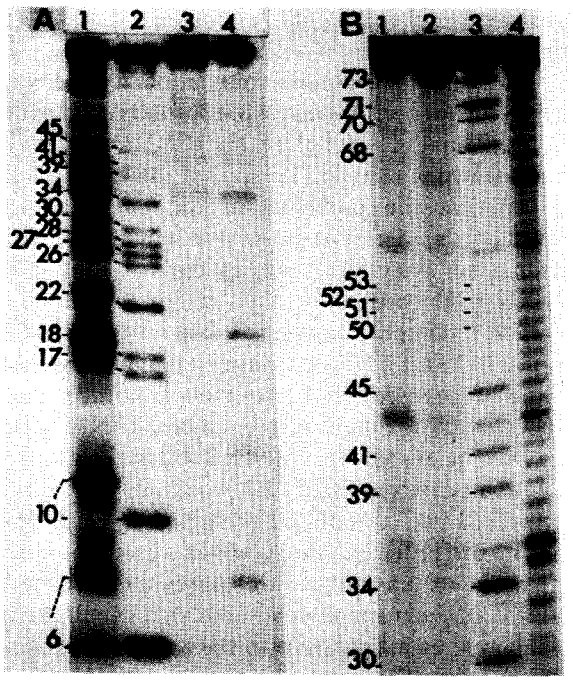

Fig. 3. Autoradiograms of 20% (A) and 15% (B) acrylamide gel of an alkylation experiment with DMS on 5′-labelled $tRNA^{Asp}$ from yeast. The alkylation was done under conditions where the tRNA is denatured; i.e., at 90°C for 1 min [2]; (A) (1) DMS treatment of tRNA as in [13], (2) partial ribonuclease T_1 digest, (3) formamide ladder, (4) control incubation in the absence of DMS, but other treatments as in [13]; (B) (1) control incubation in the absence of DMS and chain scission treatment as in [2]; (2) DMS treatment of tRNA as in [13] and chain scission as in [2], (3) partial ribonuclease T_1 digest; (4) formamide ladder. In (A) the difference of migration of the G oligonucleotides in lanes 1 and 2 is due to the difference between the chemical and enzymatic chain-splitting mechanisms. Whereas the radioactivity deposited on the gels was the same as in ENU or MNU experiments, the time for autoradiography was reduced by a factor 2–3 in this DMS experiment.

of the MNU reaction is comparable to that of the ethylation reaction since the intensity of the electrophoretic bands is similar (fig. 1 (5); fig. 2 (5).

The interpretation of the alkylation experiments and the correlation between chemical reactivity and solution structure of tRNA requires a low level of unspecific strand scission. We recall that the modifications with the *N*-nitroso-derivatives are carried out at pH 8.0, up to 80°C, and that the chain splitting at the alkylated phosphates involves an incubation at 50°C and pH 9.0 [2]. Therefore an important question arises concerning the stability of the tRNA molecules under such conditions, especially since it is known that RNA molecules can be partially degraded when exposed to higher temperatures, a property sometimes used for ladder preparations [22]. The control experiments reported in figures 1 and 2 show that the conditions in the chemical modification and strand scission treatments only slightly alter the integrity of the tRNA molecules. Some unspecific splits occurring during the handling of the tRNAs are nevertheless

observed, especially after residues C13 and C34 in the D- and anticodon-loops of $tRNA_i^{Met}$ (fig. 1).

An attempt to visualize alkylation reactions by MNU at the guanosine residues of tRNA is shown in fig. 2 (lanes 2–10). Here, the alkylated $tRNA^{Phe}$ has been treated as in [13] so that the chain would be split at the positions of the putative m^7G residues. It was surprising that no cleavages at guanosines were observed, except for residue 46 which is the constitutive m^7G of the extra-loop in $tRNA^{Phe}$. Other strong cuts arose at the modified bases Y37 and D17. Interestingly enough the splitting pattern resembles a phosphate splitting pattern and already appears on the control tRNA which was not incubated with MNU. To clarify this point we treated unmodified tRNA either with 0.5 M Tris–HCl buffer at pH 8.2 or with aniline buffer at pH 4.5 in order to check which reaction of the chemical scission procedure causes the damage in tRNA. The experiments showed that the degradations were essentially due to the aniline treatment.

Table 1

Mean reactivity in yeast $tRNA^{Asp}$ of phosphate residues with ENU and MNU and of guanosine residues with DMS

	ENU	MNU	DMS
Native $tRNA^{Asp}$ (20°C)	0.12 (10)	0.12 (10)	2.9 (54)
Denatured $tRNA^{Asp}$ (80°C)	0.33 (25)	0.35 (26)	3.7 (69)

Yeast $tRNA^{Asp}$ was alkylated with ENU, MNU or DMS either under conditions favouring the native or the denatured structure of the tRNA [2,13]. The modified tRNAs were split as in [2] for ENU and MNU alkylations, and as in [13] for DMS alkylation, and electrophoresed in the conventional manner. After autoradiography of the gels the Cerenkov radioactivity of the bands, corresponding to the splitting products and to intact tRNA, was counted in a scintillation counter. Results are expressed as the % of the mean radioactivity of one splitting product *vs* the total radioactivity deposited on the gel. Numbers between brackets correspond to the % of tRNA molecules which were alkylated. It is recalled that yeast $tRNA^{Asp}$ contains 75 phosphate and 24 guanosine residues [23]; in the DMS exp. it was found that only 11 guanosine residues were accessible to the reagent in the native tRNA (unpublished)

An alkylation experiment of yeast $tRNA^{Asp}$ by DMS is displayed in fig. 3. Sodium borohydride followed by aniline treatment [12,13] leads to a guanosine ladder with the modified tRNA, whereas alkaline treatment [2] did not degrade the alkylated tRNA. Control incubations of non-alkylated $tRNA^{Asp}$ by the two splitting procedures did not induce chain scission in the tRNA.

Finally, a comparison of the extent of alkylation in yeast $tRNA^{Asp}$ produced by ENU, MNU and DMS is given in table 1. It appears that guanosine residues are alkylated about 10-times more efficiently by DMS, than are the phosphate residues by ENU or MNU.

4. DISCUSSION

The *N*-nitroso compounds are a large group of chemical agents which are known to have potent biological activity [9]. The reaction of *N*-nitrosourea derivatives with nucleic acids occurs to a very low extent. The most studied derivatives are *N*-methyl- and *N*-ethyl-nitrosourea. They react with nucleic acids causing modifications of bases, sugars and phosphate residues, but to different extents [4–10]. The common feature of the reactions is the attack by these reagents of the nucleophilic centers in RNA or DNA; e.g., the oxygen and nitrogen positions. It has been reported that MNU alkylation of DNA at the phosphates yielded 20% of the total methylation but that ENU modified 80% of the phosphates [4–10]. Concerning the reactivity of the bases, one can say that m^7G is mostly observed with MNU. The most important differences between MNU and ENU are found in their ability to alkylate different sites in the base moieties, the ethylating reagent being the most efficient at oxygen atoms [7]. Furthermore it has been shown that MNU is much more efficient in alkylating sites in nucleic acids than ENU [4–11], and in the particular case of the tRNA the incorporation of alkyl groups is 13-times more for MNU than for ENU. The analysis in [11] suggests that the modification mostly takes place on the nitrogen atoms of guanosines at ~0.25 mol methyl groups incorporated/mol of tRNA.

These considerations support the idea that from such mild modification conditions one should obtain information on both phosphate and guanosine reactivities in tRNA using the nitroso compounds.

If so, one should be able to compare the guanosine accessibilities in tRNA under two different experimental conditions, namely in the presence of the nitroso reagents and of DMS, a methylating reagent already used in tRNA studies [13,14].

Here, two lines of information are brought together, refering to:

(1) The application range of the ENU, MNU and DMS modification reactions in the molecular biology of tRNA;

(2) The alkylation targets in tRNAs.

Concerning this second point, our studies showed that both ENU and MNU gave the same results with respect to the modification of phosphates and guanosines. The reactivity of ENU and MNU towards phosphates was similar and we did not see additional modifications of guanosines. After the chemical scission procedure specific for the alkylated bases, only 3 heavy bands appeared on the gels; they were due to the already existing modified bases in tRNA: m^7G, Y and D. These results contradict to those obtained under similar incubation conditions [11]. We cannot however, exclude the possibility of an alkylation of N7 positions in guanosines by MNU under our conditions, but if it exists it is very low and below the limits of the method used. Most probably the amount of bases alkylated has been overestimated in the earlier work, due for instance to inaccurate specific radioactivities of the hot reagents used. In that respect our methodology, although indirect, is more accurate since we are measuring the radioactivity of oligonucleotides originating from end-labelled tRNA specifically split at the alkylated positions.

As a first conclusion it appears that accessibilities of guanosine residues in tRNA can only be obtained from DMS modification studies, and that MNU, like ENU, allows one mainly to obtain information about phosphate accessibilities. This might be of interest since the size of the modifying group is smaller with MNU than with ENU.

The second striking fact which emerges from the present comparative alkylation study concerns the experimental restraints which should be overcome when probing the accessibility of the phosphates in tRNA, and more generally in RNA. They are much more severe than those encountered when probing the accessibility of the guanosines. Indeed, as seen in table 1, the reactivity of guanosines with DMS is much higher than that of phosphates with ENU or MNU. This was seen by measuring the radioactivity of the splitting products which reflects the chemical reactivity at those positions. This radioactivity is about 10-times higher in the denatured $tRNA^{Asp}$ for the DMS reaction than for the ENU or MNU reactions and even about 25-times higher in the case of native tRNA. Consequently, particular care must be taken during phosphate alkylation experiments, to verify that the controls do not show unspecific degradation patterns.

Such patterns can be evoked by the chemical handling of the tRNA; e.g., during the alkaline treatment used for splitting the tRNA at phosphate positions two strong cuts occur after C13 and C34 in $tRNA_i^{Met}$ (see fig. 1). This example is not unique. We have often observed low yield partial degradation in tRNAs. The location in the tRNA molecules and the mechanism of these degradation reactions will be described elsewhere (in preparation). During the procedure used to split at m^7G positions in tRNAs treated with DMS, unspecific degradations occur at all phosphate positions (see fig. 2). They are due essentially to the aniline treatment but the consequences for interpreting DMS experiments are not particularly far-reaching since the extent of the guanosine alkylation reactions is much higher than that of the degradations. This point is clearly illustrated in figures 2 and 3 in which degradation patterns appear after the prolonged autoradiography necessary to see the phosphate alkylations, but where they do not appear on the shorter autoradiography used for the DMS experiment (compare lane 7 in fig. 2 and lane 4 in fig. 3A). As a practical conclusion, it appears that careful controls, in which unmodified tRNA is submitted to the same treatments as modified tRNA, must be present in studies using end-labelled tRNAs, statistical modifications and gel procedures. Furthermore, due to the presence of m^7G in some tRNAs one should be careful when interpreting protection experiments in these tRNAs using DMS as a probe.

Finally, although the description of the phosphate residues protected in the native structure of the two plant tRNAs is not the principal matter studied in here, it can be noted that buried phosphates are found in the Tψ-loop of lupin $tRNA^{Phe}$ and in the D-stem and loop region of lupin $tRNA_i^{Met}$, as in [1,2].

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