PROTECTION OF PHOSPHODIESTER BONDS IN YEAST $tRNA^{Val}$ BY ITS COGNATE AMINOACYL-tRNA SYNTHETASE AGAINST ALKYLATION BY ETHYLNITROSOUREA

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1. Introduction

Ethylnitrosourea is an alkylating reagent which reacts with the phosphates of nucleic acids [1,2]. We have found that it attacks, to almost similar extents, all phosphates in the unfolded $tRNA^{Phe}$ under mild conditions [3]. In the native $tRNA^{Phe}$, however, some phosphates were modified to a lower extent, suggesting that these residues are protected by the tRNA structure against the chemical attack. We could show that these low reactive phosphates are buried in the three-dimensional structure of $tRNA^{Phe}$. Ethylnitrosourea can thus probe the solvent accessibility of the phosphate groups in an RNA, and similarly to other nucleic acid base reagents used [4], can bring information about the tertiary structure of the nucleic acid in solution. Taking advantage of this property one might use ethylnitrosourea to probe the reactivity of the phosphates of tRNAs engaged in complexes with proteins. Since the reagent is small, it might allow the precise mapping of the tRNA areas in contact with the proteins in the complexes.

Here we present the results of an attempt to investigate the chemical modification by ethylnitrosourea of yeast $tRNA^{Val}$ bound to valyl-tRNA synthetase. We found that the synthetase protects phosphodiester bonds in the anticodon arm and in the variable loop of the cognate $tRNA^{Val}$. In these areas, the phosphates in positions 39–43 and 46–51 were protected the most efficiently. Under identical synthetase-tRNA ratios, non-cognate enzymes, such as phenylalanyl- and aspartyl-tRNA synthetases, influence only slightly the alkylation of the phosphates in the $tRNA^{Val}$.

2. Materials and methods

Pure $tRNA^{Val}$ (major species) was purified from crude brewer's yeast tRNA by counter-current distribution [5] followed by conventional chromatography techniques. Valyl-, aspartyl- and phenylalanyl-tRNA synthetases were purified according to [6–8]. Ethylnitrosourea was synthesized as in [9]. 3′-Labelling of $tRNA^{Val}$ was performed according to [10].

Alkylation of $tRNA^{Val}$, free or engaged in the complex with the synthetases, was performed at 20°C for 3.5 h in a 0.15 M sodium cacodylate buffer (pH 8.0) containing 5×10^{-3} M $MgCl_2$ and 3×10^{-4} M EDTA. Sodium cacodylate was chosen as the buffer because the pure tRNA was prepared as a sodium salt by dialysis against this salt and because we wanted to keep the ionic conditions of the nucleic acids unmodified. Ethylnitrosourea was added as a concentrated ethanol solution (2.5 μl) to 22.5 μl of the aqueous buffer containing either $tRNA^{Val}$ alone or both $tRNA^{Val}$ and the enzyme. The reagent was 70 mM in the reaction mixtures and 3′-labelled $tRNA^{Val}$ and cold carrier $tRNA^{Val}$ was 1.5 μM. The enzymes were 5 μM. In control experiments, ethanol was substituted for the reagent solution. Reaction time and temperature must be controlled carefully, since the extent (or the rate) of modification of the phosphates by the alkylating agent must be the same in both free and complexed tRNA in those regions which do not interact with the synthetase.

After modification all reaction mixtures were treated identically. The solutions were extracted with

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15 μl phenol in Eppendorf tubes and tRNA was precipitated by the addition of 3 μl carrier ribosomal RNA solution (5 mg/ml), 3 μl 3 M sodium acetate (pH 6.0) and 100 μl ethanol. The tRNA was then redissolved in 10 μl 0.3 M sodium acetate (pH 6.0) containing 10^{-2} M EDTA and precipitated again with ethanol. The modified tRNA was then dissolved in 10 μl 0.1 M Tris–HCl (pH 9.0) containing 10^{-3} M EDTA and incubated for 5 min at 50°C, conditions which are necessary to split the ribophosphate backbone at the alkylated positions, precipitated by the addition of 7 μl 3 M sodium acetate (pH 6.0) and 60 μl ethanol, rinsed with ethanol, and dried in vacuum. Under these conditions the alkylation of $tRNA^{Val}$ proceeded to a limited extent: <0.1 mol ethyl groups was incorporated/1 mol $tRNA^{Val}$. As these phosphate alkylations are scattered along the tRNA molecule, fragments of different sizes will be generated in amounts reflecting the extent of alkylation at the various phosphate positions. This low extent of modification guarantees also that the observed patterns of modification reflect the accessibility of tRNA phosphates in complexes partially undamaged by the modification. The analysis of the tRNA fragments produced by the modification was done by electrophoresis on acrylamide/8 M urea gels, essentially as in [11]. X-ray films were scanned with the Transidyne 2955 scanning densitometer using the linear scale of the instrument. For more experimental details and for a discussion of the methods see [3].

3. Results and discussion

As a prerequisite to the study of the complex formed between $tRNA^{Val}$ and aminoacyl-tRNA synthetases, we first investigated the alkylation of the free $tRNA^{Val}$. As we found for $tRNA^{Phe}$ [3] it appears that most of the phosphates of the free $tRNA^{Val}$ react with ethylnitrosourea (fig.1,2). It can be seen in fig.1 that the electrophoretic bands corresponding to the split products of $tRNA^{Val}$ at phosphates starting from position 30–66 are well resolved and that there is practically no degradation of the $tRNA^{Val}$ in this region as seen in the control experiments. No confident information about the rest of the molecule could be obtained in the present experiments (see legend to fig.1). The quantitation of the intensities of the electrophoretic bands, which are the reflection of the electrophoretic bands, which

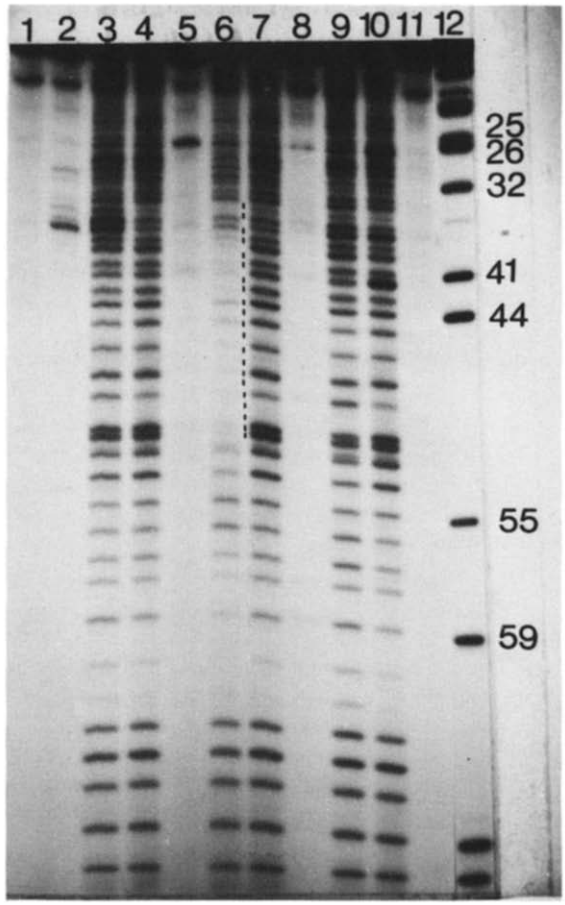

Fig.1. Autoradiogram of a 20% acrylamide gel of an alkylation experiment of 3'-labelled $tRNA^{Val}$ with ethylnitrosourea in the presence of aminoacyl-tRNA synthetases: (3,6,9) alkylation in the presence of phenylalanyl-, valyl- or aspartyl-tRNA synthetases, respectively. The protected area in track (6) is indicated by a dashed line; (4,7,10) alkylation in the absence of enzymes; (1,5,11) control incubations in the absence of both enzymes and reagent; (2,8) control incubations in the absence of reagent and in the presence of phenylalanyl- or valyl-tRNA synthetase, respectively; (12) incubation in the presence of RNase T_1 allowing the assignments of the electrophoretic bands. The numbering of the bands corresponds to that of the alkylated phosphates; for instance band 44 corresponds to phosphate 44 and to G43. As the treatment of alkylated $tRNA^{Val}$ includes ethanol precipitation, the short labelled oligonucleotides produced by splitting at phosphates 67–77 were partially lost. Confident ...ta-tion of phosphate reactivities within the 3'-part of ...A^{Val} was not possible due to the low resolution in this part of the gel and also to some unspecific degradation of the tRNA (this is visible on the upper part of the gel in the control experiments). It is seen however, that there is no strong protection of phosphates in positions 20–30, as the electrophoretic patterns are similar in the presence and absence of enzyme in that region (compare tracks 6 and 7). The weakness of bands 60 and 61 and the absence of bands between positions 44–55 is indicative of low reactive phosphates in the native $tRNA^{Val}$.

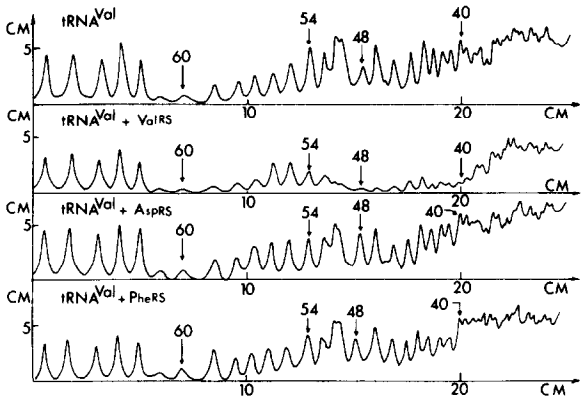

Fig.2. Densitometric tracing of patterns of phosphate alkylation in $tRNA^{Val}$ either free or in the presence of cognate or non-cognate aminoacyl-tRNA synthetases. Tracings were done for tracks (7,6,9,3); assignments of peaks were done using track (12) as a marker (see fig.1).

are the reflection of the reactivity of the phosphates, was done by densitometric tracing. The results presented in fig.1,2 show that these reactivities are not completely uniform under the conditions used. For instance, the bands corresponding to products split at phosphates 60 and 61 are practically absent on the electrophoretic pattern. Furthermore between position 44 and 55 there are two low reactive phosphates in the native $tRNA^{Val}$. This situation which is similar to that described for $tRNA^{Phe}$ [3] will be discussed in more detail in the case of $tRNA^{Val}$ and of other tRNAs in a forthcoming paper. Interestingly, these phosphate positions in $tRNA^{Val}$ correspond to positions of low reactive phosphates in $tRNA^{Phe}$ [3]. Consequently it can be concluded that the structural organisation in both tRNAs is similar.

The modification of $tRNA^{Val}$ engaged in the complex with its cognate synthetase was carried out under conditions providing the stability of the interaction, i.e., at 20°C and in the presence of Mg^{2+}. The enzyme was taken in 2.5-fold excess over tRNA, so that only the specific one-to-one complex is formed in the incubation mixture [12]. It was also verified by means of the nitrocellulose filter binding assay [13] that the complex remains stable until the end of the alkylation reaction. Taking into account these facts and also the results obtained with free $tRNA^{Val}$, it was found that the alkylation pattern of the tRNA phosphates was drastically changed in the complex (fig.1,2). The most important changes occur in the sequence starting from the 3′-side of the anticodon to the beginning of the T-stem. The effect of protection by the synthetase is variable along this sequence being maximal for phosphates 39–43 and 46–51. It is specific since in the presence of non-cognate synthetases (phenylalanyl- or aspartyl-tRNA synthetase) no pronounced alterations in the alkylation pattern of the phosphates in $tRNA^{Val}$ were observed. This does not mean that no interaction takes place between $tRNA^{Val}$ and non-cognate enzymes. Such interactions have actually be shown to occur, particularly with $tRNA^{Val}$ [14], but under the experimental conditions used in this work, the unspecific interactions are unfavoured.

The experimental procedure used in this work allowed only to probe the possible involvement of 39 out of the 77 phosphates, in the interaction of $tRNA^{Val}$ with its cognate synthetase (see fig.3). Of these 39 phosphates, 11 were found to be strongly protected by the enzyme and 6 others only partly. This number substantially exceeds that of the electrostatic contacts calculated for the interaction of $tRNA^{Val}$ with its synthetase [15] which obviously involve phosphates. This means that not all of these

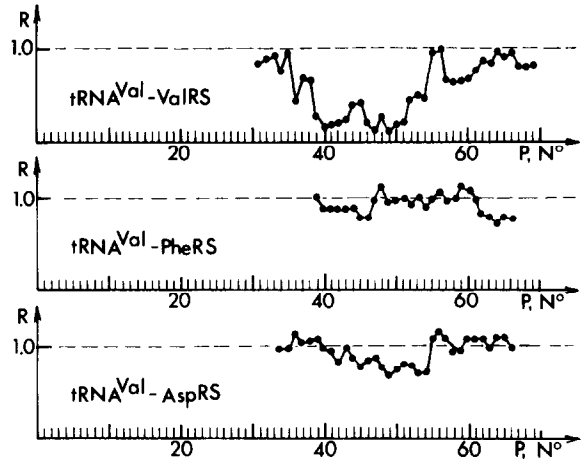

Fig.3. Summary of the effects of aminoacyl-tRNA synthetases on the reactivities of phosphates in $tRNA^{Val}$. *R*-values are the ratios between the intensities of the corresponding electrophoretic bands of the $tRNA^{Val}$ alkylation patterns in the presence of the enzyme and of free $tRNA^{Val}$. The intensities were measured as the peak heights of the densitometric tracings shown in fig.2. Phosphates 39,40,41,42,43,46,47,48, 49,50 and 51 are considered as strongly protected;phosphates 36,44,45,52,53 and 54 being only partly protected. The changes in phosphates reactivities were taken as significant if the deviation of *R*-values from 1 was >30% which was estimated as the standard error in our experiments.

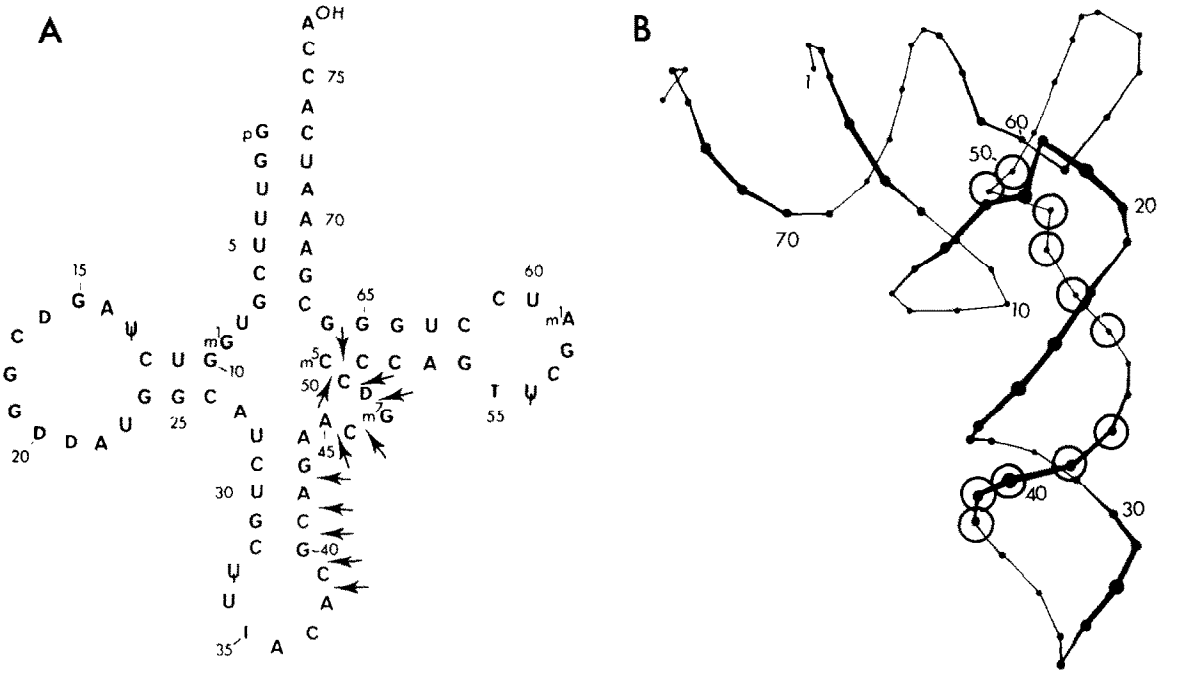

Fig.4. Positions of the phosphates strongly protected by valyl-tRNA synthetase in the $tRNA^{Val}$ structure: (A) in the cloverleaf structure [21] (→); (B) in the three-dimensional model (ORTEP drawing of the structure of yeast $tRNA^{Phe}$ calculated according to coordinates in [22]) (○).

phosphates participate in the interaction with cationic groups of the enzyme.

The methodology used here allowed us to approach the problem of conformational change in tRNA upon binding to the synthetase. Although our results do not allow to give a firm answer with regard to the whole tRNA molecule, they demonstrate the absence of unfolding of the central part of the $tRNA^{Val}$ molecule upon binding with valyl-tRNA synthetase as substantiated by the low reactivities of the phosphates 60 and 61 in both free and complexed $tRNA^{Val}$.

The results and conclusions of this work are summarised in fig.4 which shows the cloverleaf and three-dimensional model of $tRNA^{Val}$ with the positions of the most protected phosphates by valyl-tRNA synthetase. The number and the localisation of these phosphates clearly indicate the existence of close contacts between valyl-tRNA synthetase and the ribose–phosphate backbone of $tRNA^{Val}$ within its anticodon arm and variable loop. The importance of the variable loop in $tRNA^{Val}$ for the interaction with valyl-tRNA synthetase was already suggested by tRNA mischarging experiments [16] and that of the anticodon arm by UV crosslinking and nuclease digestion experiments [17–19]. The involvement of these regions in the interaction with the enzyme is in agreement with the general binding scheme proposed for tRNA-synthetase interactions [20]. It must be emphasised that in contrast with the crosslinking approach, where bases juxtaposed to the enzyme were probed, we present here results demonstrating for the first time the close contact of tRNA phosphates with a synthetase. This means probably that the synthetase does not interact with tRNA by discrete contacts but more likely that a large surface of $tRNA^{Val}$ including the different chemical groups of nucleotides is in contact with the protein. This is in good agreement with neutron small angle scattering studies which showed that the $tRNA^{Val}$ is buried in the enzyme, lying close to its centre of mass [12].

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