THE FUNCTION OF PSEUDOURIDYLIC ACID IN TRANSFER RIBONUCLEIC ACID*. IRRADIATION AND CYANOETHYLATION OF *E. COLI* VALINE tRNA FRAGMENTS

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1. Introduction

In the previous paper [1], we have described some of the structural requirements for the light-induced cross-linking reaction between the two non-adjacent bases, 4-Srd$_8$ and Cyd$_{13}$, of tRNA^{fMet} and the use of this reaction as a tool to probe the extent of structural changes induced by cyanoethylation of the Ψrd residue of this molecule. In this report, we extend these results to tRNA$_I^{Val}$ of *E. coli*, a non-initiator tRNA species. It has already been shown by Yaniv and coworkers [2, 3] that irradiation of tRNA^{Val} at 335 nm induces a covalent cross-link between 4-Srd$_8$ and Cyd$_{13}$ and that endonuclease digestion of the tRNA prevents the reaction. Moreover, the molecule can be fragmented into two halves (fig. 1), which when recombined and reannealed, regenerate acceptor activity [6]. The 5′-half contains both of the residues involved in the cross-link and the 3′-half contains the only Ψrd residue.

By the use of these half molecules of tRNA, we have been able to show that tRNA^{fMet} [1], the 3′-fragment is necessary for the 5′-fragment to become intramolecularly cross-linked. On the other hand, unlike tRNA^{fMet}, cyanoethylation of the 3′-fragment blocks both acceptor activity and the ability to stimulate photoproduct formation.

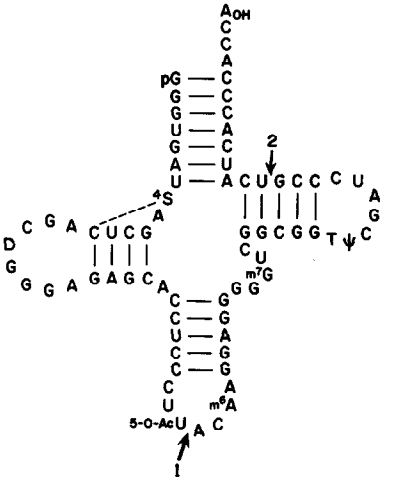

Fig. 1. The primary sequence of *E. coli* tRNA$_I^{Val}$ [4, 5]. Arrow 1 shows the point of cleavage by *B. subtilis* RNase [9] and arrow 2 shows the 3′-fragment arising from complete T$_1$ RNase digestion. The dashed line connects the two nucleotides involved in the cross-linking reaction [2,3].

Abbreviations:
Ψrd : pseudouridine
4-Srd: 4-thiouridine
Cyd : cytidine

* Part VI of a series. Part V, see ref. [1].
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2. Experimental

E. coli $tRNA_I^{Val}$ was purified by two cycles of chromatography on BD-cellulose columns [4]. The derivatization and stripping procedures were carried out as described previously [7]. The purified $tRNA^{Val}$ had an acceptance activity of 1538 pmoles valine per A_{260} unit before stripping. After stripping 25% of the charging activity was lost. All procedures were carried out in the absence of light in order to avoid formation of photoproduct [2, 3].

$tRNA^{Val}$ half molecules were prepared according to Oda et al. [6] by limited digestion with *Bacillus subtilis* ribonuclease. 307 A_{260} units of $tRNA^{Val}$ were dissolved in 12 ml of 80 mM tris-HCl pH 7.4, 20 mM MgOAc and treated with 38 units of *B. subtilis* ribonuclease for 10 min at 37°. Separation of the half molecules was accomplished by chromatography on a DEAE-cellulose column with a NaCl gradient in 20 mM tris-HCl, pH 8.0, 7 M urea. Subsequent purification of the 5′- and 3′-halves thus obtained was performed by DEAE-Sephadex A-25 column chromatography at pH 2.7. Since the chromatographic patterns obtained were very similar to those reported by Oda et al. [6] and the two fragments complemented each other in the expected proportion to reconstitute acceptor activity up to 83% of theory, the two fragments were assumed to correspond to the half molecules described by these workers. In addition, the putative 5′-fragment had the absorption spectrum characteristic of a 4-Srd containing polynucleotide while the 3′-fragment did not. A smaller oligonucleotide from the 3′-portion of of the molecule, $UpCpApUpCpApCpCpCpApCpCpA_{OH}$ was isolated by complete digestion of $tRNA^{Val}$ with T_1 ribonuclease and fractionation on a DEAE-Sephadex-urea column [9, 10].

Reannealing of the fragments was done either by preincubation in 20 mM tris-HCl pH 7.3, 200 mM NaCl, 10 mM $MgCl_2$ at 37° for 20 hr [6] or by heating the mixture to 65° and slowly cooling to 25° at 6°/hr for a total exposure of 12–16 hr.

Assays for valine acceptance activity were carried out as described by Weiss, Pearson and Kelmers [11] except that after trichloroacetic acid precipitation on nitrocellulose membranes, the filters were dissolved in Bray's solution [12] and counted at 90% efficiency.

Cyanoethylation of the Ψrd residue of the 3′-half molecule was done by reacting with acrylonitrile or propionitrile (as a control) for 70 min at 60° at a fragment concentration of 0.63 A_{260} units/ml, by the procedure described in the accompanying paper. [1].

Irradiation of $tRNA^{Val}$ and fragments was done in 20 mM cacodylate, pH 7.1, 10 mM Mg^{2+}, at 1–4° in a Rayonet photochemical reactor at 320–380 nm. In some experiments, 80 mM NaCl and 8 mM tris, pH 7.3 were also present. Formation of the 4-Srd-cytidine cross-linked photoproduct was measured by reduction with $NaBH_4$ to a new compound whose fluorescence at 440 nm was measured in an Aminco-Bowman spectrophotofluorometer [2, 13]. Since the absorption spectrum of the irradiated $tRNA^{Val}$, identical to that reported by Favre et al. [2], shifted after $NaBH_4$ reduction to a spectrum very similar to the fluorescence excitation spectrum (peak at 388 nm), it was assumed that the observed fluorescence was a true measure of the amount of the cross-linked photoproduct that was described by Yaniv and coworkers [2, 3]. Similar observations have also been made for $tRNA^{fMet}$ [1].

^{14}C-Valine (260 mCi/mole) was obtained from Amersham/Searle. *B. subtilis* ribonuclease was purified as described by Nishimura [8]. Acrylonitrile (chromatoquality grade) was from Matheson, Coleman and Bell, and propionitrile from Eastman Organic.

3. Results

3.1. *Fragment complementation*

The 3′- and 5′-halves of $tRNA^{Val}$ prepared as described in the Experimental section, were tested for their ability to accept valine. With these fragments, a reannealing preincubation is necessary before acceptor activity can be demonstrated [6], and this has been done in all cases except where indicated. The ability of the two halves to complement each other for restoration of acceptor activity is shown in fig. 2. In panel A the 5′-half was kept constant while the 3′-half was added in increasing amount. Panel B shows the reverse experiment. In both cases the restoration of activity increased proportionately upon the addition of the complemen-

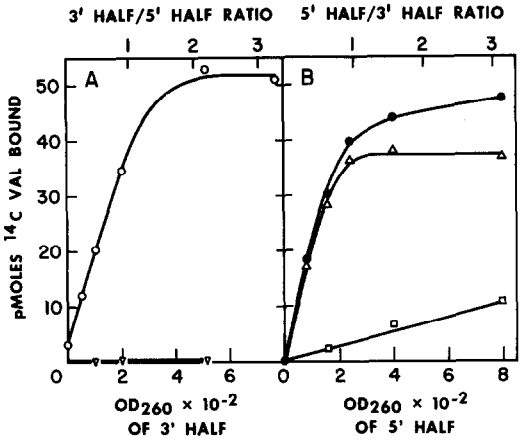

Fig. 2. Titration of the 3'- and 5'-half molecules for reconstitution of valine acceptor activity. Reannealing was done as described in Experimental for 20 hr at 37°. (A) To 0.095 A_{260} unit (4.75 A_{260} units/ml) of 5'-half, increasing amounts of 3'-half were added. (B) To 0.103 A_{260} unit (5.15 A_{260}/ml) of 3'-half, increasing amounts of 5'-half were added. For each point, 3 and 5 μl aliquots were assayed as in Experimental. ○, ●, combined fragments; ▽, 3'-half alone; □, 5'-half alone; △, activity corrected for that due to the 5'-half alone.

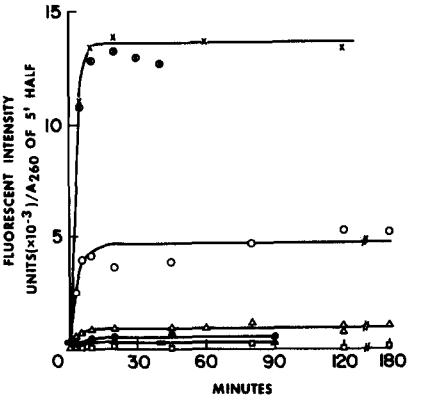

Fig. 3. Kinetics of photoproduct formation from $tRNA^{Val}$ and fragments. 3'- and 5'-half molecules singly or in combination were reannealed at 37° for 20 hr and then irradiated for the times indicated. Formation of photoproduct was detected by fluorescence and recorded as units per A_{260} of the 5'-half molecule. Reannealing was omitted for $tRNA^{Val}$ and calculation of fluorescent intensity was based on the 5'-half molecule content of the tRNA. ⊛, Irradiated $tRNA^{Val}$; ▬, $tRNA^{Val}$ protected from light; ×, 3'-half and 5'-half reannealed at a 3'/5' A_{260} ratio of 3.0 (5'-half 4.8 A_{260} units/ml) and irradiated; ○, 3'-half and 5'-half reannealed at a 3'/5' A_{260} ratio of 0.5 (5'-half 15.9 A_{260} units/ml) and irradiated; ●, same but without prior reannealing; △, 5'-half alone reannealed at 26.8 A_{260} units/ml and irradiated; ▲, same but without prior reannealing; □, 3'-half alone reannealed at 19.3 A_{260} units/ml and irradiated. The 3'-half and 5'-half separately or in combination was also tested with annealing but without irradiation and no fluorescence was detected. Each experimental point represents the average of two differently sized aliquots.

tary fragment, and a plateau level was attained when the added fragment was in excess. By extrapolation of the linear and plateau portions of the net curves, an equivalence ratio for the two fragments could be deduced. In this experiment the 3'/5' ratio at equivalence was 1.31 and 1.32 in terms of A_{260} units for panels A and B, respectively, compared to the calculated theoretical value of 1.28.

The 3'-half molecule was free of 5'-half since when tested alone, it did not accept valine, whereas the 5'-half was slightly contaminated (7%) with active material (fig. 2B). The fragments were rather pure since the overall efficiency of reconstitution in this system was 83% (see table 1).

3.2. *Requirements for cross-linking*

The kinetics and specificity of the Yaniv cross-linking reaction [2, 3] under our conditions are shown in fig. 3. There was a rapid formation of photoproduct when intact tRNA was irradiated, which was quite stable to further irradiation. Complete reaction was achieved in 10 min or less, and less than 1% photoproduct was detected in unirradiated samples.

In order to examine the structural requirements for the 4-Srd-Cyd cross-linking, 3'- and 5'-halves, separately or combined, were irradiated with and without previous reannealing. The results show clearly that the presence of the 3'-half is necessary for the activity of the 5'-half to be expressed and also that prior annealing of the two halves is necessary. Note that all of the unirradiated samples were completely inactive. With excess 3'-half (3'/5' ratio of 3) both the rate and yield of product from recombined halves were equal to that for intact tRNA. With a limiting amount of 3'-half (3'/5' ratio of 0.5), there was less product formed. The photoproduct levels reached in the two cases were in good agreement with the level of acceptor activity attained for the combinations (table 1).

Table 1
Comparison of acceptance and fluorescent intensity of $tRNA^{Val}$ and fragments.

	3′/5′ Ratio	Valine acceptance[a] (pmoles/A_{260} of 5′-half)	Fluorescent intensity[b] (Units × $10^3/A_{260}$ of 5′-half)
5′-half + 3′-half	3.0	2172 (100%)	13.6 (100%)
5′-half + 3′-half	0.5	886 (40.8%)	4.7 (34.6%)
5′-half alone	–	130 (6.0%)	1.0 (7.0%)
$tRNA^{Val}$	–	2616 (–)	13.0 (–)

[a] Values taken from fig. 2A.
[b] Values taken from the plateau region of fig. 3.

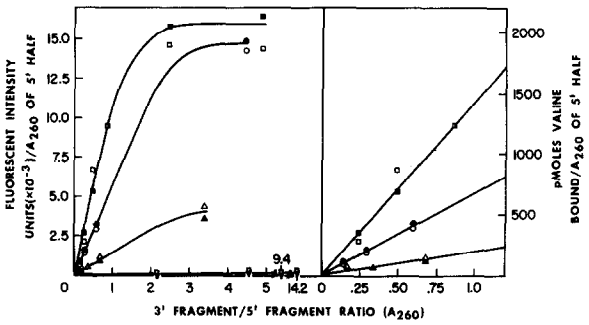

Fig. 4. Reconstitution of valine acceptor activity and photoproduct stimulatory activity with modified 3′-half molecules. A constant amount of 5′-half molecules, further purified by a second DEAE-Sephadex-urea chromatography, was mixed with increasing amounts of untreated or modified 3′-halves at a 5′-half concentration of 0.52 A_{260} units/ml. After reannealing at 65° as described in Experimental, aliquots were taken for assay of valine acceptor activity. The samples were then irradiated for 45 min at 1–4° and formation of the cross-linked photoproduct was assayed by fluorescence. Each point represents the average of two tubes assayed at different fragment concentrations. Blanks due to each component alone have been subtracted. The 3′-half blanks were the same as background, and the 5′-half blanks were 2% of the maximum values for acceptor activity or fluorescence intensity. The right hand panel shows in an expanded scale the linear portion of the curves. Untreated 3′-half molecules: ■, valine acceptance; □, fluorescent intensity. Propionitrile treated 3′-halves: ●, valine acceptance; ○, fluorescent intensity. Acrylonitrile treated 3′-halves: ▲, valine acceptance; △, fluorescent intensity. Untreated 3′-oligonucleotide UpCpApUpCpApCpCpCpApCpCpA; ▼, valine acceptance, ▽, fluorescence intensity (the 3′/5′ ratio in this case was expressed on a molar basis).

3.3. *Effect of cyanoethylation of the 3′-half molecule*

Experiments with mixed *E.coli* tRNA have shown that cyanoethylation with acrylonitrile at 60° leads to a rapid first order decay of valine acceptor activity with a $T_{1/2}$ of 32 min under the same conditions as used here [14]. This corresponds to a loss of 78% activity after 70 min of reaction. These kinetics are consistent only with reaction of the single Ψrd residue of the molecule [15] since the potentially reactive 4-Srd had been removed by prior I_2 oxidation [16] before cyanoethylation. This conclusion has now been confirmed by cyanoethylation of the 3′-half alone followed by subsequent reconstitution with untreated 5′-half. As was previously observed for $tRNA^{fMet}$ [1, 15], cyanoethylation of the tRNA fragment containing the G-T-Ψ-C sequence is sufficient to inactivate (fig. 4). The linear portion of the curves quantitatively measures the activity of the 3′-half molecules and shows that cyanoethylation has caused a loss of 70–80% of the valine acceptor activity, depending on whether comparison is made with the untreated or propionitrile-treated control. This level of inactivation is clearly within the range expected from the decay curves for intact tRNA, providing further indication that reaction has taken place with the Ψrd residue. Fig. 4 also illustrates the exact parallel between the stimulation of acceptor activity and of photoproduct formation on adding increasing quantities of 3′-half. Finally, unlike $tRNA^{fMet}$, cyanoethylation of the 3′-fragment inactivated the ability of the complex to stimulate photoproduct formation as well as the ability to accept valine.

A smaller fragment from the 3′-end of tRNA, the oligonucleotide UpCpApUpCpApCpCpCpApCpCpA$_{OH}$ was also tested for its ability to combine with the 5′-half in order to regenerate either acceptor activity or

the ability to form the cross-link. Clearly, under the annealing conditions used, this fragment was completely inactive. Other methods of annealing have not been tested.

4. Discussion

The original report by Favre et al. [2] stated that endonuclease digestion of $tRNA^{Val}$ prevented photoproduct formation, and more recently Pochon et al. [17] reported that a copolymer of cytidylic acid and 4-thiouridylic acid which was complexed with polyguanylic acid also could not form photoproduct. Our results confirm and extend these studies by demonstrating that although the 5′-half molecule contains both of the light-reactive nucleotides fixed in their normal relationship to each other with respect to primary structure, reaction cannot occur unless the complementary 3′-half is added and annealed into a 'correct' configuration. Clearly, the 5′-half alone must be unable to orient itself into the configuration needed to bring the two reactive residues into covalent bonding distance of each other. The exact parallel between the titration curves for cross-linking and for acceptance activity shows further that the 3′-half participates stoichiometrically in both reactions. Since the large oligonucleotide from the 3′-end was inactive, it is obvious that something more than a double-stranded acceptor arm is required, and experiments are in progress to determine what these requirements are.

Cyanoethylation of the 3′-half molecule blocked its ability to complement the 5′-half for reconstitution of valine acceptance at a rate which was consistent only with reaction of the Ψrd residue. The same result was also obtained for $tRNA^{fMet}$ [15] and is indicative of the importance of the G-T-Ψ-C region. It is not possible from these results, however, to distinguish between the effect on a 'recognition site' and an effect on the overall configuration of the tRNA. Unlike the reaction with $tRNA^{fMet}$, cyanoethylation of the 3′-half molecule of $tRNA^{Val}$ also blocked its ability to orient the 5′-half into its correct configuration for formation of the light induced cross-link. This result could be due to (a) failure of the cyanoethylated 3′-half to form a complex with the 5′-half, or (b) formation of a complex with the 5′-half which is so perturbed from the native structure by virtue of the derivatized Ψrd that the cross-linking reaction cannot take place. If (b) were true, it would place restrictions on allowable models for tRNA. Hypothesis (a) would be equally interesting, however, since it is not obvious how introduction of a CH_2CH_2CN group at the Ψrd locus could prevent association of two polynucleotides 34 and 42 units long with 12 base pairs between them. Moreover, cyanoethylation of the 3′-fragment of $tRNA^{fMet}$ does not block complex formation even though the 5′-piece is only 20 units long and there are only 10 base pairs between them. This aspect of the problem is currently under investigation.

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