THE FUNCTION OF PSEUDOURIDYLIC ACID IN TRANSFER RIBONUCLEIC ACID*. PHOTOCHEMICAL AND CHEMICAL MODIFICATION OF FORMYLMETHIONINE tRNA OF *E. COLI*

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1. Introduction

We have previously reported [1] that modification of the single pseudouridine (Ψrd) in the G-T-Ψ-C sequence of *E. coli* formylmethionine tRNA, by cyanoethylation with acrylonitrile, blocked acceptor activity but that cyanoethylation of the 4-thiouridine (4-Srd) residue had little or no effect. In that work, selective modification was achieved by first physically separating the two reactive nucleotides onto different fragments of $tRNA^{fMet}$ before chemical reaction (fig. 1). When recombined the unmodified fragments could regenerate acceptor activity [1, 3] but the cyanoethylated 3′-fragment could not restore activity when added to the unmodified 5′-fragment. We proposed that this effect was due to a distortion of tertiary structure caused by the introduction of the $-CH_2CH_2CN$ group at a sensitive region of the molecule, namely at the Ψrd locus, because of a previous study in which cyanoethylation of this Ψrd in intact $tRNA^{fMet}$ led to a gross change in the Mg^{2+}-dependent T_m profile [6]. We did not consider the possibility that the two fragments simply failed to associate. In order to study this question further, we have used as a probe the light-induced cross-linking reaction between the two non-adjacent bases, 4-Srd (position 8) and cytidine (position 13) described for several tRNAs by Yaniv and coworkers [4, 5] since this reaction depends on a certain degree of structural integrity of the tRNA molecule [4, 7].

Our results show first, that although the 5′-fragment contains both nucleotides involved in the cross-linking reaction, no reaction takes place unless the 3′-fragment is added, and second, that cyanoethylation of the Ψrd of the 3′-fragment does not affect its ability to stimulate the cross-linking reaction, although it does block methionine acceptance activity. This indicates that the structural alterations which follow from cyanoethylation do not extend to the 4-Srd-containing region of the molecule, suggesting that the G-T-Ψ-C region is spatially separate from the dihydrouridine loop-acceptor stem area.

* Part V of a series.

2. Experimental

$tRNA^{fMet}$ was purified from *E. coli* B tRNA by counter-current distribution and DEAE-Sephadex chromatography [8]. We thank Dr. B.P. Doctor of Walter Reed Army Research Institute, Washington, D.C., for his participation in this preparation and for the use of his facilities. The tRNA was stored in 5 mM tris-HCl, pH 7.4 containing 10 mM magnesium acetate at -170°. The specific activity of the sample used in these studies was 1244 pmoles of methionine accepted per A_{260} unit. 3′-Three-quarter (L) and 5′-one-quarter (N) molecules were prepared by partial digestion of $tRNA^{fMet}$ with T_1 RNase and chromatographically purified as described previously [1]. Purified L and N fragments were recovered by EtOH precipitation, dialyzed against H_2O to remove traces of urea and salts and stored at -170°.

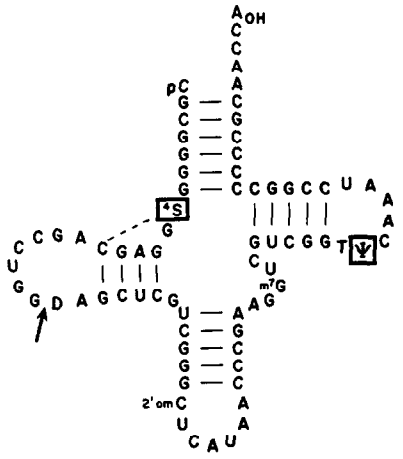

Fig. 1. The primary sequence of *E. coli* $tRNA^{fMet}$ as determined by Dube et al. [2]. The arrow shows the point of selective T_1 RNase cleavage [3] and the dashed line connects the 2 nucleotides involved in photoproduct formation [4, 5].

^{14}C-Methionine was purchased from New England Nuclear or Schwarz Radiochemicals, acrylonitrile (chromatoquality grade) from Matheson, Coleman and Bell, and propionitrile from Eastman Organic Chemicals.

Irradiation of $tRNA^{fMet}$ and fragments was done in 20 mM Hepes buffer, pH 8.0, 2 mM magnesium acetate and 2 mM potassium chloride at 2–4° in a Rayonet photochemical reactor (Southern New England Ultraviolet Co.) at 320–380 nm. For most of these experiments, irradiation of fragments was performed after a prior incubation of samples at 30° for 20 min in assay buffer (see legend of fig. 3) although this incubation later proved unnecessary (see fig. 5). Formation of the 4-Srd-cytidine cross-linked photoproduct was assayed by its fluorescence after reduction with sodium borohydride [4]. Two samples were taken for each point to verify the proportionality of fluorescence with concentration. Fluorescence emission at 440 nm was determined in an Aminco-Bowman spectrophotofluorometer at an exciting wavelength of 390 nm [9]. Since the fluorescence excitation spectrum corresponded to a major peak in the absorption spectrum of the irradiated and $NaBH_4$-reduced $tRNA^{fMet}$, and the unreduced irradiated molecule showed the characteristic spectrum described by Favre et al. [4] for irradiated $tRNA^{Val}$, it was assumed that this assay is a true measure of the cross-linked photoproduct described by these authors. In addition, appearance of fluorescence requires prior irradiation and does not appear in tRNAs or fragments which do not contain both 4-Srd at position 8 and cytidine at position 13 [4, 10]. Details of the assay procedure will be published elsewhere.

Cyanoethylation of fragments with acrylonitrile was performed as described previously [1] except that prior EDTA treatment to remove tightly bound Mg ions was omitted. Concentrations of L and N fragments were 0.7 A_{260} units and 0.45 A_{260} units per ml of the reaction mixture and incubation was for 75 and 60 min, respectively, at 60°. The treated fragments were recovered and freed of the reagent by repeated ethanol precipitation.

Methionine acceptor activity was determined as described previously [1] but at 30°.

3. Results

3.1. *Fragment complementation*

Reconstitution of acceptor activity from the 5′-one-quarter molecule (N) and the 3′-three-quarter molecule (L) is illustrated in fig. 2. The L fragment had no activity alone and there was a 30-fold stimulation of the activity of the N fragment when L fragment was added. At the equivalence point, the ratio of N/L was 0.5. Since the theoretical value is 0.35 [3], N was 70% as pure as L, which itself was rather pure since the overall efficiency of reconstitution in this system was 80% (see legend to fig. 2).

3.2. *Requirements for photoproduct formation*

Yaniv and coworkers [4, 5] have reported that irradiated $tRNA^{fMet}$ yields the same characteristic fluorescent product after $NaBH_4$ reduction as does $tRNA^{Val}$ and thus presumably forms the same cross-link between $4\text{-}Srd_8$ and Cyd_{13} that they found in $tRNA^{Val}$. We have confirmed that irradiated and reduced $tRNA^{Val}$ and $tRNA^{fMet}$ have the same fluorescence excitation spectrum. The kinetics of this cross-linking reaction at 2–4° in the presence of Mg ion is illustrated in fig. 3. The rate of reaction of intact $tRNA^{fMet}$ was extremely rapid, being complete in less than 15 min, and the product was quite

Table 1

Comparison of methionine acceptance and fluorescent intensity of $tRNA^{fMet}$ and fragments[a].

	Methionine acceptance		Fluorescent intensity
	Nonirrad.	Irrad.	Irradiated
	(pmoles/A_{260} of N)		(Units ($\times 10^3$)/A_{260} of N)
$tRNA^{fMet}$	4850 (100%)	4850 (100%)	17.2 (100%)
L + N	4134 (85%)	3786 (78%)	13.9 (81%)
L	0 (0%)	–	0 (0%)
N	66 (1.4%)	–	3.2 (19%)

[a] Methionine acceptance was measured as described in Experimental in duplicate for the irradiated and nonirradiated samples from the experiment of fig. 3. Values for fluorescent intensity were taken from the plateau portion of each curve of fig. 3. Under the conditions of this experiment (N/L = 0.34), N fragment is limiting.

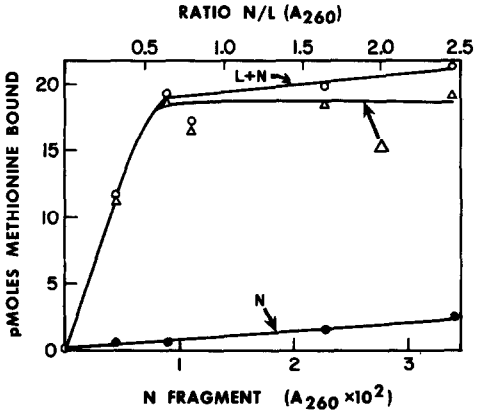

Fig. 2. Titration of fragments N and L for reconstitution of methionine acceptor activity. Increasing amounts of N fragment were added to 0.014 A_{260} units of L fragment and the mixture was assayed as described in Methods. •, N fragment alone; ○, L + N fragment; △, net activity. The plateau value is 1320 pmoles/A_{260} of L or 80% of theory based on the purity of the input $tRNA^{fMet}$.

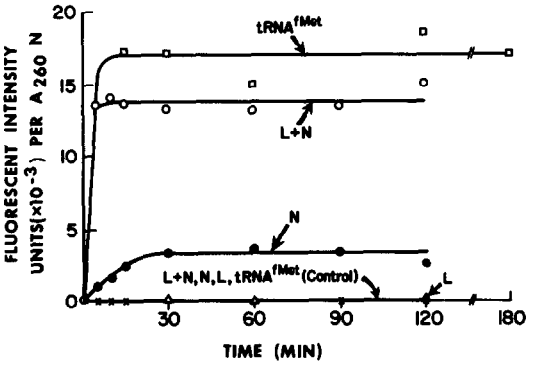

Fig. 3. Kinetics and fragment specificity of photoproduct formation. 1.05 A_{260} units of L and 0.36 A_{260} units of N fragment either together or separately were preincubated in a total volume of 0.25 ml containing 100 mM Hepes buffer, pH 8.0, 10 mM MgOAc and 10 mM KCl. After 20 min at 30° each sample was diluted five-fold with H_2O and irradiated as described under Methods. 0.1 ml samples were removed in duplicate at the times indicated and assayed for photoproduct formation. Fluorescent intensity was recorded as arbitrary units per A_{260} of N fragment. In the case of $tRNA^{fMet}$ preincubation was omitted and calculations were based on the N fragment content of the tRNA. No such calculation was needed for L fragment since all values were zero. •, irradiated N fragment alone; ×, irradiated L fragment alone; ○, irradiated L + N fragment; □, irradiated $tRNA^{fMet}$; △, non-irradiated $tRNA^{fMet}$, L + N, N or L fragments.

stable to excess irradiation, at least up to 180 min. When N and L fragments were combined under conditions such that N (the 4-Srd_8 and Cyd_{13}-containing fragment) was limiting, the rate was also extremely rapid, and the yield of product approached that for intact tRNA. On the other hand, irradiation of N alone yielded a product only at a slower rate and to a much lower extent than when complemented by L. As expected, L alone did not yield any product, nor did any of the unirradiated samples.

A comparison of acceptor activity with the cross-linking ability illustrated in fig. 3 is shown in table 1. From this table it is clear that prior cross-linking does not inactivate $tRNA^{fMet}$ or L + N complexes,

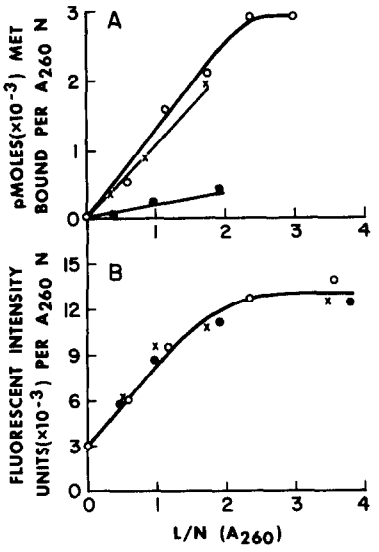

Fig. 4. Effect of cyanoethylation of L fragment on (A) reconstitution of methionine acceptor activity and (B) ability to stimulate photoproduct formation. To 0.057 A_{260} units of untreated N fragment increasing amounts of untreated L, propionitrile-treated L (L_{PN}), or acrylonitrile-treated L (L_{AN}) fragments were added to 0.5 ml incubation mixtures as described in the legend to fig. 2. After prior incubation, aliquots were removed for assay of acceptor activity and the samples were irradiated for 30 min at 1–4°. Photoproduct formation was determined by fluorescence as described in fig. 3. ○, Untreated L; ×, L_{PN}; •, L_{AN}.

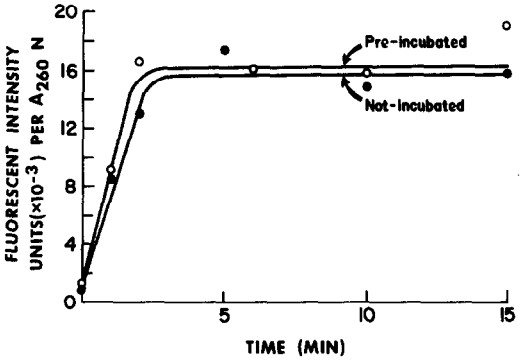

Fig. 5. Preincubation requirement for photoproduct formation. L and N fragments mixed in an A_{260} ratio of 3:1 (0.377 A_{260} of L and 0.114 A_{260} of N in 0.25 ml) were preincubated for 20 min at 30° and irradiated as described in the legend to fig. 2. For measurements of nonincubated samples, the fragments were mixed and immediately irradiated. Assays for fluorescent intensity were done on duplicate samples as before. ○, Preincubated; •, not preincubated.

and also that the ability of L to stimulate the cross-linking reaction is paralleled by its stimulation of acceptor activity. This point is illustrated more fully below (see fig. 4). Note also that irradiated N alone yields 19% of the maximum photoproduct but only 1.4% of the maximum acceptor activity. This suggests the presence in N of a contaminating fragment unable to accept methionine, but still capable of inducing the conformation needed for cross-linking. This point being further investigated.

3.3. *Effect of cyanoethylation*

Reaction of L fragment with acrylonitrile was previously shown to lead to inactivation of its ability to stimulate acceptor activity at a rate consistent only with cyanoethylation of the single Ψrd residue in this fragment [1]. These results are confirmed here and show in addition that such a cyanoethylated L fragment is still completely functional for stimulation of the cross-linking reaction (fig. 4). In this experiment, propionitrile-treated L was used as a control and both methionine acceptance and photoproduct formation was measured as a function of the amount of L added to a fixed amount of N. Panel A shows the expected loss of acceptor activity of L after cyanoethylation, while panel B illustrates the ability of modified L to induce the cross-linking reaction. The relatively high value observed for N alone in panel B is due to the fact that irradiation was carried out for 30 min so that even the slower blank reaction had reached completion.

3.4. *Requirement for preincubation*

Fragments L and N can combine almost instantly at 2–4° to provide the configuration needed to juxtapose 4-Srd_8 with Cyd_{13} for the cross-linking reaction. As seen in fig. 5, no prior incubation is needed to stimulate photoproduct formation, since the rate and extent of increase in fluorescence are almost the same in both preincubated and nonincubated samples. Note that under our conditions, maximum cross-linking occurs after 2 min of irradiation.

4. Discussion

In this paper we have studied the structural requirements for the light-induced cross-linking reaction between two non-adjacent bases, 4-Srd and Cyd, in $tRNA^{fMet}$ and using this reaction as a tool, have examined the extent of structural change induced by cyanoethylation of the Ψrd residue. The results lead to several important conclusions.

First, since the 5′-one-quarter molecule (N) does not yield photoproduct when irradiated even though both reactive residues are contained in the fragment, N fragment alone must be unable to orient itself into the proper configuration needed to juxtapose 4-Srd and Cyd for cross-linking. It instead requires the participation of the 3′-three-quarter fragment (L). The parallel between the titration curves for photo-reaction and for methionine acceptance shown in fig. 4 indicates that L fragment participates stoichiometrically in both reactions. Whether whole L fragment is needed to stimulate cross-linkage or only a portion of it can fulfil this requirement is not known. It would be interesting to determine the minimum size fragment which would still possess the ability to induce cross-linking in the N fragment. Such a study is in progress.

The fact that the reconstituted $tRNA^{fMet}$ (L + N) is active for both photoreaction and methionine acceptance indicates that the presence of a cleavage in the dihydrouridine loop has no effect upon the ability of the molecule to assume the conformation necessary for both reactions. On the other hand, cyanoethylation of the Ψrd of the G-T-Ψ-C loop in the L fragment blocks charging of the reconstituted tRNA but does not affect the ability of the L + N complex to make photoproduct. The modified L fragment must, therefore, not only be able to form a complex with N, but the complex must have a 'correct' configuration at least insofar as the region around the cross-link is concerned. The complex cannot be completely 'correct' however, since cyanoethyl-L + N complexes cannot be productively recognized by methionyl-tRNA synthetase.

We have previously discussed two alternative explanations for the loss of acceptor activity upon cyanoethylation [1]. Either, (a) the Ψrd residue is directly involved in the recognition site(s) for synthetase, or (b) introduction of a CH_2CH_2CN group at a sensitive locus in the tRNA disrupts its tertiary structure sufficiently so as to block the charging reaction. Alternative (b) is preferred because of reports that this Ψrd can be removed from tRNA with at least partial retention of acceptor activity (literature cited in ref. [1]) although a tRNA has been recently described in which the G-T-Ψ-C region has been excised [11] with attendant failure to accept amino acid. In addition, cyanoethylation of intact $tRNA^{fMet}$ does induce detectable conformational changes [6].

Since cyanoethylation of the G-T-Ψ-C loop does not perturb the photosensitive region sufficiently to block the cross-linkage reaction, the structural deformation that follows cannot be extensive in nature. Our results can be understood in a general way in terms of a model for tRNA like that proposed by Connors et al. [12] or by Danchin [13] in which the G-T-Ψ-C region is spatially separated from the dihydrouridine loop-acceptor stem area.

The association of L and N fragments to form a structurally correct complex takes place readily. Even at 1–4°, the association reaction is very rapid, as judged by the ability to make photoproduct. Using acceptor activity as an assay, a requirement for preincubation could not be shown previously [3], but it was possible that annealing of the two fragments took place during the charging reaction itself. However, since the photoproduct assay was done in the cold, this possibility has now been disproved. Such a rapid reconstitution is not a general phenomenon, however, as association of half molecules of $tRNA^{Val}$ requires extensive reannealing before either acceptor activity or the cross-linking reaction can be demonstrated [14]

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