THE FUNCTION OF PSEUDOURIDYLIC ACID IN TRANSFER RNA. III. INACTIVATION OF FORMYLMETHIONINE TRANSFER RNA OF E. COLI BY CYANOETHYLATION WITH ACRYLONITRILE[‡]

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Summary

Cyanoethylation of formylmethionine tRNA at 60° resulted in a rapid pseudo-first order inactivation of acceptor activity ($T_{1/2}$, 28 min). Under the conditions chosen, only the ψrd in the T-ψ-C-G loop should have reacted. Absorbancy-temperature profiles of 94% inactivated tRNA revealed a marked decrease in ordered structure which is probably responsible for the loss of activity.

Previous work in several laboratories has shown that cyanoethylation of particular minor nucleotides in tRNA leads to the loss of specific functional activities. Cyanoethylation of the inosinic acid residues located in the anticodon "wobble" position in yeast $tRNA^{ala}$[1] (2) and $tRNA^{val}$ (3) blocks the ability to bind to ribosomes in response to messenger RNA triplets but does not affect amino acid acceptor activity. Similar observations have been made in our laboratory with the $tRNA^{arg}$ of E. coli (4). The loss of acceptor activity after cyanoethylation of "all" of the ψrd residues in yeast tRNA has also been described (5). We have previously reported a direct correlation between the degree of cyanoethylation of ψrd and the loss of acceptor activity for most amino acids in a mixed E. coli tRNA preparation (6, 7). As the average number of ψrd residues in E. coli tRNA is close to two (8), it was possible that the loss of activity was due to cyanoethylation of ψrd in either the T-ψ-C-G loop or the other region of ψrd occurrence, the anticodon loop and/or stem.

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[1]Abbreviations used are: $tRNA^{\text{amino acid}}$, tRNA species specific for the named amino acid; ψrd, pseudouridine; 4-Srd, 4-thiouridine; 7-MeGuo, 7-methyl guanosine; Hepes, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

In order to resolve this question, we have initiated experiments on purified $tRNA^{Fmet}$ from E. coli which contains ΨrdOnly in the T-Ψ-C-G sequence (9). Our results show that (a) there is a rapid first order inactivation of acceptor activity when the tRNA is reacted with acrylonitrile (CH_2:CHCN) but not with the control, propionitrile (CH_3CH_2CN), under conditions such that the only other minor nucleotides known to be present, 4-Srd and 7-MeGuo (9), do not react, and (b) as a result of cyanoethylation the modified tRNA has undergone a deformation in its structure which is probably responsible for its lack of biological activity. These results are similar to our previous findings with unfractionated E. coli tRNA (7).

Experimental

Materials: Purified $tRNA^{Fmet}$ from E. coli B (10) (in our hands, sp. act. 907 pmoles met/A_{260} unit or approximately 57% pure) was obtained from Oak Ridge. Unfractionated E. coli B tRNA was either purchased from Schwarz BioResearch or prepared by the method of Muench and Berg (11). ^{14}C-methionine was obtained from Amersham/Searle. The true specific activity was 61.3 mc/mmole by isotope dilution analysis, corresponding to 106 cpm/pmole of methionine under the counting conditions used (79% efficiency). ^{12}C-acrylonitrile, chromatoquality grade, was obtained from Matheson, Coleman and Bell and propionitrile from Eastman. ^{14}C-acrylonitrile (sp. act. 1.16×10^8 dpm/mmole) was obtained from Mallinkrodt Nuclear and freed from the hydroquinone stabilizer by high vacuum distillation. The specific activity was determined by counting a known weight of material. 7-MeGuo was obtained from Cyclo Chemical Company. The isomeric alkaline cleavage products of 7-MeGuo were prepared according to Townsend and Robins (12). Analysis by chromatography on cellulose layers in isobutyric acid-NH_3-H_2O (86:5:14) showed complete cleavage of 7-MeGuo (R_f 0.76) to the open-ring isomers (R_f 0.48 and 0.40).

Methods: $tRNA^{Fmet}$ was desalted on a Sephadex G-25 column equilibrated with 10^{-3} M EDTA, pH 8.0, concentrated by lyophilization, and dialyzed against water to remove EDTA. Mixed disulfide formation between the 4-Srd residue and mer-

captoethanol was carried out in a reaction mixture consisting of desalted tRNA, 8 A_{260} units/ml, 7 mM Tris buffer pH 7.0, 1.7 mM mercaptoethanol, 3.2 mM I_2, and 1.6% KI (13). After incubation for 30 min at 0° C, the tRNA was isolated and washed free of I_2 by three ethanol precipitations, and finally dissolved in water. Reaction of the oxidized tRNA with either acrylonitrile or propionitrile was carried out in a solution of 1 M reagent, 50 mM Na_2CO_3 buffer, 2.3 mM EDTA, and 7.5 A_{260} units/ml of tRNA, final pH 9.3 at 60° C, for the indicated times in tightly sealed vessels. The reaction was stopped by neutralization with HCl, and the tRNA isolated and washed by ethanol precipitation. Regeneration of the 4-Srd residue was accomplished by reduction with 0.1 M $Na_2S_2O_3$ for 30-45 min at 23°. The extent of oxidation and reduction was monitored spectrophotometrically (Fig. 1).

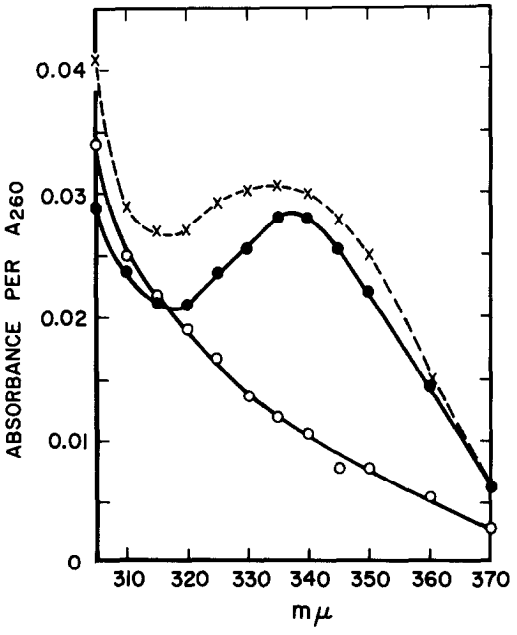

Fig. 1. Absorption spectrum of $tRNA^{Fmet}$. Untreated tRNA, •; tRNA oxidized with I_2 in the presence of mercaptoethanol, o; oxidized tRNA reduced with $Na_2S_2O_3$, X. tRNA samples were prepared as described in Experimental, and then placed in 3 mM Tris, 10 mM $Mg(OAc)_2$ (pH 7.4), for spectral measurements. The curves have been adjusted to an A_{260} value of 1.0 for each sample.

Reaction of 7-MeGuo and its open-ring derivatives with acrylonitrile was carried out in a mixture containing 0.13 M $NaBO_4$ buffer (pH 9.3 at 60°), 0.02 M nucleoside, and 1 M ^{14}C-acrylonitrile in tightly sealed tubes at 60° for 30 and 60 min. Reactions were stopped by neutralization with HCl. Most contaminant radioactivity was removed by repeated lyophilization. Final purification was achieved by TLC chromatography in isobutyric acid-NH_3-H_2O (86:5:14) followed in the same dimension by n-butanol-H_2O (86:14) two times. Radioactive bands were located by autoradiography and those coincident with UV-absorbing bands were scraped from the plate and counted. The number of moles of cyanoethyl groups bound per mole of total nucleoside were then calculated.

Results

Protection of the 4-Srd residue. The sequence studies by Dube, et al. (9) on $tRNA^{Fmet}$ showed that the only minor nucleosides are one each of Ψrd, 4-Srd, and 7-MeGuo. Since 4-Srd residues react readily with acrylonitrile, it was first necessary to reversibly block this residue in the tRNA before attempting cyanoethylation of the Ψrd residue. This was accomplished by forming a mixed disulfide with mercaptoethanol in the presence of I_2 as described by Carbon and David (13). The absorption spectrum in the 300-370 mμ region (Fig. 1) was used as a measure of the completeness of the reaction. A distinction between mercaptoethanol-4-Srd mixed disulfide and $(4\text{-Srd})_2$ disulfide cannot be made by spectral measurements alone (13). However, since mercaptoethanol was present in 200-fold excess over the 4-Srd residues and excess I_2 was maintained throughout the reaction as shown by the brown iodine color of the reaction solution, it is highly unlikely that any $(4\text{-Srd})_2$ was formed. More direct evidence for the formation of mixed disulfide is the fact that 50% of the methionine acceptor activity of unfractionated E. coli tRNA was lost after simple iodine oxidation, while the activity was unaffected after oxidation in the presence of mercaptoethanol although the characteristic 4-Srd spectrum disappeared in both cases. It is clear from Fig. 1 that oxidation could be readily accomplished, and subsequent full regeneration was possible by $Na_2S_2O_3$ treatment. Previous

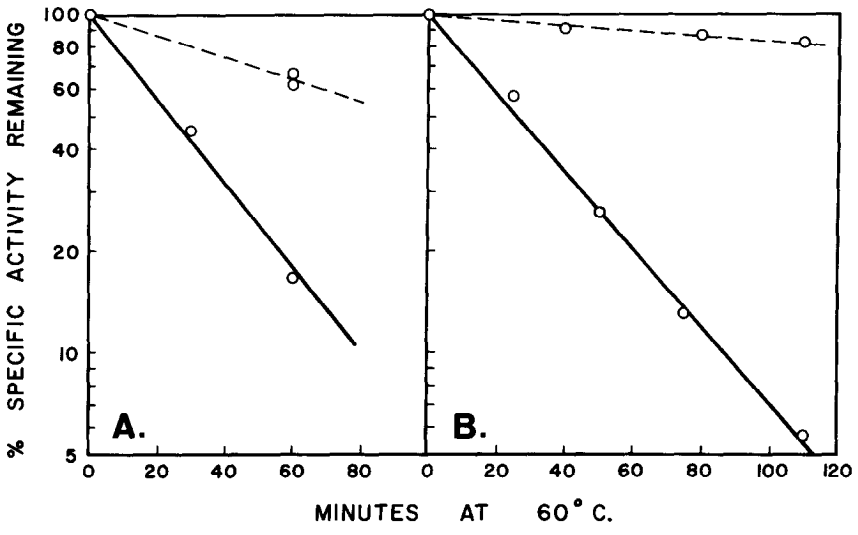

Fig. 2. Loss of methionine acceptor activity after cyanoethylation of $tRNA^{Fmet}$ and $tRNA^{met}$. Acceptor activity was determined using the Hepes assay mixture described by Weiss, et al. (10) in a volume of 0.25 ml, and the general procedure previously described (14). Each experimental point represents the average of two tubes assayed at different tRNA concentrations to insure the proportionality of methionine accepted with tRNA added. — — —, propionitrile treated; ————, acrylonitrile treated. A. Unfractionated E. coli tRNA (Schwarz) containing both methionine tRNA species was reacted as described in Experimental except that oxidation at 4 mM nucleotide was carried out before the tRNA was desalted, the tRNA concentration during cyanoethylation was 4 mM in nucleotide and the acrylonitrile concentration was 0.89 M. B. Purified $tRNA^{Fmet}$ was desalted, oxidized, reacted with acrylonitrile or propionitrile, and reduced as described in Experimental.

studies have directly shown that I_2 oxidation of unfractionated tRNA protects 4-Srd residues from cyanoethylation by acrylonitrile (15), and it is assumed that the same was true for $tRNA^{Fmet}$ in the present case.

Inactivation of $tRNA^{Fmet}$. The effect of cyanoethylation on the methionine acceptance activity of unfractionated tRNA (containing both $tRNA^{Fmet}$ and $tRNA^{met}$) and the purified $tRNA^{Fmet}$ is shown in Fig. 2A and 2B, respectively, as a function of the time of treatment with the reagent. It is clear that the reaction is pseudo-first order, as expected from our earlier findings (6, 7), and that substitution of propionitrile for acrylonitrile abolishes the effect. The rate constant for the inactivation, corrected for the loss of activity in the propionitrile controls and normalized to 1 M acrylonitrile was 0.0248 min^{-1} for

the unfractionated tRNA and 0.0245 min^{-1} for the purified $tRNA^{Fmet}$. This corresponds to a $T_{1/2}$ of 27.9 and 28.3 minutes respectively, which is very similar to the value of 20 min found previously for the average of all amino acid acceptor activities. It should be emphasized that these assays were done with an excess of charging enzyme and in all cases methionine acceptance was directly proportional to the amount of tRNA tested.

It is known that certain tRNA species can be converted to an inactive form by first unfolding the molecule and then allowing it to re-fold in the absence of Mg^{++} (16). Since the cyanoethylation reaction was performed under such conditions, it was possible that the tRNA was inactive for this reason. To test this possibility, the tRNA samples were heated at 60° and then cooled in the presence of Mg^{++}. These conditions are known to reactivate other tRNAs (16), but did not activate any of the treated tRNA (Table I). We conclude that the loss of activity must be a true consequence of the chemical modification reaction.

Non-reactivity of the 7-MeGuo residue. 7-MeGuo is known to undergo ring cleavage under mildly alkaline conditions to the isomeric N^6-ribosyl-$N^{5(6)}$-formyl-5,6-diaminoisocytosine derivatives (17, 18) although the reaction is markedly depressed when 7-MeGuo is in an RNA chain (19, 20) probably because of

TABLE I

Attempted Heat Renaturation of Methionine Acceptor Activity of Cyanoethylated $tRNA^{Fmet}$

Previous treatment of tRNA sample	Specific acceptor activity	
	Before heating	After heating
	pmoles met/A_{260} unit	
Propionitrile for 0 min	820	830
Propionitrile for 110 min	637	653
Acrylonitrile for 110 min	37.7	39.6

tRNA samples prepared as described above were heated to 60° for 2 min in 0.23 ml of 110 mM Hepes buffer, pH 8.0, 11 mM $Mg(OAc)_2$, and 11 mM KCl. After chilling to 0°, ATP, mercaptoethanol, enzyme, and ^{14}C-methionine were added and the samples assayed in the standard way.

the ionic repulsion effect of the negatively charged phosphate groups on the attacking hydroxide ion. Potentially, inactivation could occur as a result of (a) the splitting of the 7-MeGuo residue by the conditions used for cyanoethylation, (b) reaction of 7-MeGuo with acrylonitrile, or (c) reaction of the open-ring derivative produced at pH 9.3 and 60° with acrylonitrile. The loss of activity observed in the presence of propionitrile is a measure of the maximum effect that can be attributed to (a). Either (b) or (c) was a possibility since our previous studies (21) did not examine the reactivity of these compounds with acrylonitrile. For example, alkaline cleavage of 7-MeGuo might not affect acceptor activity but subsequent cyanoethylation might do so.

To examine this point, 7-MeGuo and its open-ring derivative were reacted with ^{14}C-acrylonitrile at pH 9.3 and 60° for 30 and 60 min, and subsequently purified from contaminating radioactivity. Under the conditions used, about half of the 7-MeGuo is cleaved to the open-ring forms after 60 min, and an unidentified UV-absorbing compound is produced from the open-ring compounds. From the amount of radioactivity associated with the several UV-absorbing compounds found in the reaction mixture (the same compounds were found in the propionitrile control), an upper limit to the extent of reaction of $< 8\%$ could be set for either compound after 60 min reaction at 60°. Moreover, such reactions would be expected to be further slowed when 7-MeGuo is in tRNA. Clearly the small extent of the reactions cannot account for the 80% loss of activity observed for tRNA after 60 min incubation.

Effect on tertiary structure. The distortion of tertiary structure of the tRNA due to cyanoethylation was measured by examining the absorbancy-temperature profiles. Fig. 3 compares the T_m profiles for control, propionitrile-treated, and acrylonitrile-treated $tRNA^{Fmet}$ (panel B) and unfractionated tRNA (panel A). It is clear that cyanoethylation has in both cases caused a marked decrease in the sharpness of melting that is indicative of a decrease in the ordered structure of the molecule. Very similar changes in T_m profiles of yeast tRNA have also been reported by others (5, 22, 23) after cyanoethylation of "all" of the Ψrd residues.

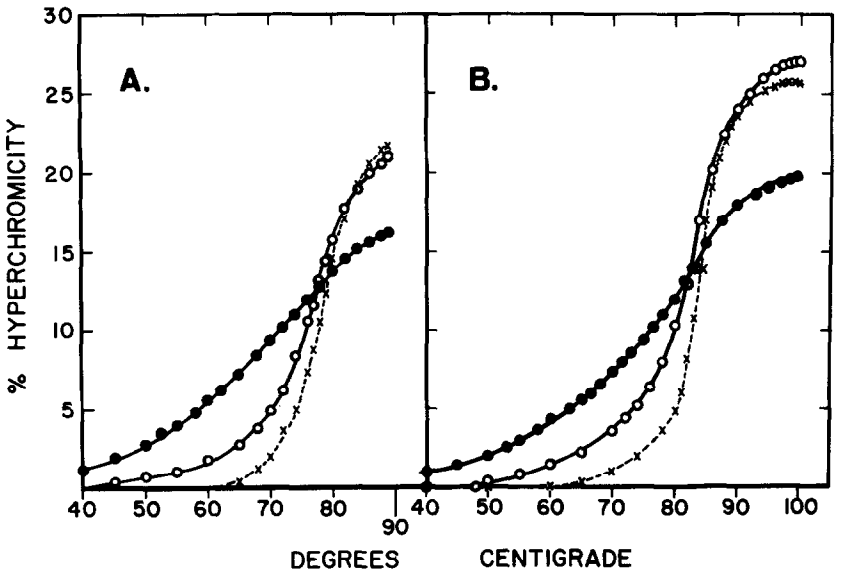

Fig. 3. Absorbancy-temperature profiles of treated tRNAs. The data shown have been normalized to the same A_{260} value at 23° C and replotted from the original continuous recording. The solvent used was 10 mM Tris, 5 mM EDTA and 7 mM $Mg(OAc)_2$ at pH 7.4. A. Unfractionated, desalted E. coli tRNA (11) was reacted with acrylonitrile without prior I_2 oxidation. X, untreated tRNA; o , tRNA treated at 60° for 95 min without acrylonitrile; ●, tRNA reacted with acrylonitrile for 92 min at 60°. B. Purified E. coli $tRNA^{Fmet}$ was desalted, oxidized, reacted with propionitrile or acrylonitrile, and then reduced with thiosulfate as described in Experimental. X, untreated $tRNA^{Fmet}$, o, tRNA treated with propionitrile for 110 min at 60°; ●, tRNA reacted with acrylonitrile for 110 min at 60°.

Discussion

The results presented here show that cyanoethylation of $tRNA^{Fmet}$ produces two major changes in properties: (1) a marked decrease in Mg-stabilized tertiary structure, and (2) a loss of ability to be recognized by methionyl-tRNA synthetase. The effect of cyanoethylation was assumed to be on the Ψrd of the T-Ψ-C-G region since (a) of the three minor nucleotides in this tRNA it was the only one capable of reacting under the conditions chosen, and (b) of the major nucleotides, only uridine is known to be reactive, but at only 4% of the rate of Ψrd (21).

Several recent three-dimensional models of tRNA structure (24-26) have used interaction between the T-Ψ-C-G loop and other areas of the molecule as a means of stabilizing the proposed structure. Such models receive support from

the accumulated evidence showing that this loop is not exposed in native tRNA.[2] In this context it is reasonable to suppose that addition of a CH_2CH_2CN group to the N_1 of the Ψrd in that loop while the tRNA is denatured at 60° prevents reformation of the native structure upon cooling. The distortion of tertiary structure would then be the primary event due to cyanoethylation, and the loss of biological activity follows from this.

Experiments using ^{14}C-acrylonitrile are now in progress to demonstrate unequivocally that the inactivating event in $tRNA^{Fmet}$ is cyanoethylation of the Ψrd and not of any other nucleotide. Further studies of the structure-function relationships in this molecule are also in progress.

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