TEMPERATURE-DEPENDENT INACTIVATION OF $tRNA^{Tyr}_{E.coli}$ ACCEPTOR FUNCTION WITH IODINE: INFLUENCE OF THE 3'-TERMINAL pCpA SEQUENCE*

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1. Introduction

Tyrosine transfer RNA from *E. coli* contains two neighbouring 4-thiouridines in position 8 and 9 from the 5'-end [1]. These minor nucleosides of yet unknown function [2, 3] have been oxidized with iodine (potassium triiodide) to the intramolecular disulfide without loss of tyrosine acceptor activity [4, 5]. The temperature-dependent iodine inactivation of $tRNA^{Tyr}$ presented here needs elevated temperature and higher iodine concentrations than needed for disulfide formation.

2. Experimental

Pure $tRNA^{Tyr}_2$ was isolated after fractionation of crude *E. coli* tRNA on DEAE-Sephadex A-50 [6], followed by chromatography on benzoylated DEAE-cellulose [7]. $tRNA^{Tyr}_{pC\text{-}3'}$, (i.e. $tRNA^{Tyr}_2$ ending with 3'-terminal pC and lacking the terminal pCpA sequence) was prepared as described by Zubay [8]. A control experiment had shown that this procedure gives $tRNA^{Tyr}_{pC\text{-}3'}$, which is fully repaired by tRNA-adenylyl(cytidylyl)transferase (table 1). Aminoacyl-tRNA synthetase (*E. coli*) free from tRNA-adenylyl(cytidylyl)transferase was prepared as described recently [9].

Table 1

tRNA adenylyl (cytidylyl) transferase-catalyzed incorporation of [^{14}C]CMP into $tRNA^{Tyr}_{pC\text{-}3'}$, after iodine treatment at 60°.

Time of iodine treatment at 60° (min)	Incorporation of [^{14}C]CMP ([^{14}C]AMP) into $tRNA^{Tyr}_{pC\text{-}3'}$, moles CMP (AMP)/mole $tRNA^{Tyr}_{pC\text{-}3'}$	
0	1.02	(1.00)
2	1.06	
15	1.08	
60	1.04	(1.00)

The same result was obtained upon [^{14}C]AMP incorporation (in the presence of CTP, in parentheses).

tRNA adenylyl(cytidylyl)transferase (EC 2.7.7.25) was prepared as follows: the first purification step (chromatography of a ribosome free *E. coli* supernatant on DEAE-cellulose) and the assay for this enzyme were performed as described earlier [10]. The fractions containing the enzyme were pooled and brought to 42% saturation with ammonium sulfate (1 hr at 0°). The precipitate was dissolved in a small volume of buffer and passed through a Biogel P-200 column (2 × 40 cm) in 0.05 M potassium phosphate pH 8.0–0.001 M $MgCl_2$–0.01 M mercaptoethanol–5% glycerol. The enzyme fractions were pooled and placed directly on a 2.3 × 9 cm hydroxyapatite (Bio-Gel HTP) column equilibrated with the same buffer. Elution of the enzyme was achieved with a linear gradient from 0.05 to 0.2 M potassium phosphate

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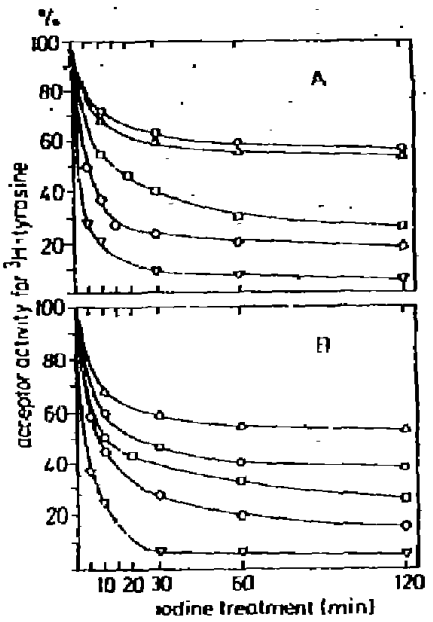

Fig. 1. Tyrosine acceptor activity after iodine treatment of $tRNA_2^{Tyr}$ (A) and $tRNA_{pC-3}^{Tyr}$, (B) at 20° (Δ—Δ), 30° (○—○), 40° (□—□), 50° (◇—◇) and 60° (▽—▽). Addition of further iodine (430 μM) after 60 min had no influence on the degree of $tRNA^{Tyr}$ inactivation. In the absence of iodine no inactivation of tyrosine acceptor function was observed under the conditions stated above. Mercaptoethanol did not reactivate inactivated $tRNA_2^{Tyr}$.

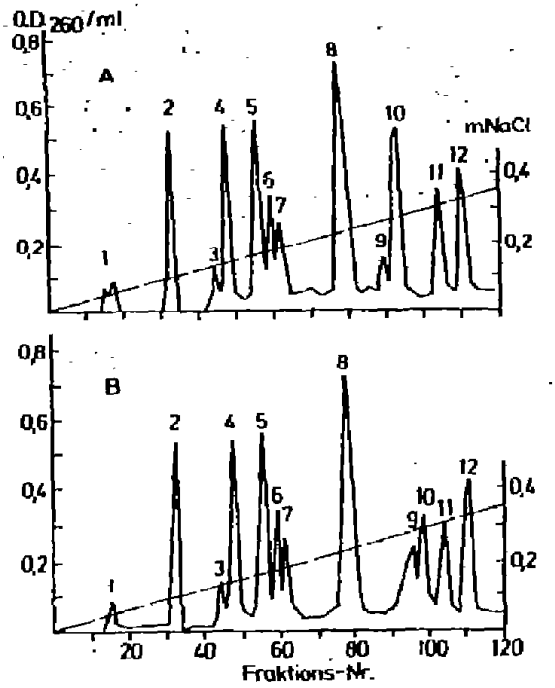

Fig. 2. Fragments produced by RNAase T_1 from $tRNA_2^{Tyr}$ (A) and iodine treated (60 min/60°) $tRNA_2^{Tyr}$ (B). After desalting, the corresponding fragments from fig. 2A and 2B were compared by co-chromatography on cellulose thin-layer plates using the solvents t-butanol/HCOOH (1:1) pH 3.8 or isobutyric acid/conc. NH_3/H_2O (577:38:385 – v:v:v) pH 4.3. Fragments in fig. 2A [17]: 1 = Gp; 2 = Gp; 3,4 = dinucleotides; 5,6 = trinucleotides; 7 = TψCGp; 8 = hexanucleotides; 9 = SSCCCGp (S = s^4U, details on the modification of this material to UUCCCGp will be described later); 10 = anticodon-containing dodecanucleotide $ACUG^+UAA^+A\psi CUGp$ (G^+ = G derivative of unknown structure; A^+ = ms^2i^6A); 11 = 3'-terminal nonadecanucleotide; 12 = bihelical oligonucleotide containing a 5'-terminal heptanucleotide and the 3'-terminal nonadecanucleotide [1]. Fragments 1 to 8, 11 and 12 were identical in fig. 2A and 2B. Fragments 9 and 10 in fig. 2B are products of fragment 10 from fig. 2A.

($MgCl_2$, mercaptoethanol and glycerol as above). The resulting tRNA adenylyl(cytidylyl)transferase was 700-fold purified.

Iodine treatment of *E. coli* $tRNA_2^{Tyr}$: 2.4 A_{260} units (4 nM) of $tRNA^{Tyr}$ or $tRNA_{pC-3}^{Tyr}$, were incubated in 500 μl 0.04 M potassium phosphate, pH 8.0, at various temperatures (legend to fig. 1). A 50 μl aliquot was removed and 430 nmoles iodine in 2 μl 0.3 M potassium iodide in 50% aqueous ethanol were added to the rest. Further 50 μl aliquots were removed at different times and their excess iodine was removed by addition of 1 ml water-saturated ether followed by vigorous mixing and phase separation. The mixture was dipped into dry ice/ethanol and the ether decanted from the frozen aqueous phase. This extraction was repeated and the remaining aqueous phase was used for tyrosine acceptor assay. The first aliquot to which iodine had not been added, was treated in the same way. Aliquots of iodine treated $tRNA_{pC-3}^{Tyr}$ were aminoacylated in the presence of tRNAadenylyl(cytidylyl)transferase (40 μg) under conditions which allowed rapid and complete repair of the 3'-terminal CCA sequence (table 1 and [10]).

Amino acid acceptor function was assayed as described by Hoskinson and Khorana [11] and measured by means of the filter technique of Mans and Novelli [12].

Oxidation with iodine of the two 4-thiouridines in $tRNA^{Tyr}$ to the intramolecular disulfide was followed by measuring the optical density at 330 nm. The 4-thiouridines (λ_{max} 330 nm) in $tRNA^{Tyr}$ reacted to form the disulfide (no λ_{max} at 330 nm) in less than 1 min under our conditions (see above). ^{3}H-Activity was measured as described earlier [13].

Iodine treatment of nucleosides or nucleotides: the four common nucleosides and nucleotides, pseudouridylic acid, ribothymidine, 4-thiouridine (s^4U) and 4-thiouridine-disulfide were treated with iodine as described and iodine was removed as above. The products were investigated by thin-layer chromatography on cellulose using several solvent systems.

Digestion of $tRNA_2^{Tyr}$ before and after iodine treatment as above (60 min/60°) was performed with RNAase T_1 (Sankyo) and the resulting fragments were separated on a DEAE-Sephadex A-25 column in 7 M urea, using a linear NaCl gradient (fig. 2A and 2B).

3. Results and discussion

In the presence of iodine, the two 4-thiouridines in $tRNA^{Tyr}_{E.coli}$ react very rapidly to form an intramolecular disulfide without loss of tyrosine acceptor activity [4, 5]. Under our conditions (fig. 1) we observed complete disulfide formation in less than 1 min, whereas acceptor activity, as described by other authors [4, 5], was still not significantly affected. The different rates of disulfide formation (very fast) and inactivation of tyrosine acceptor function (slow, fig. 1) indicate that the two processes are independent of each other.

Finally we found that 4-thiouridine and 4-thiouridine-disulfide were modified on the nucleoside, oligonucleotide and tRNA level to uridine under our conditions of iodine treatment, a reaction which does not affect the aminoacceptor function of $tRNA_2^{Tyr}$ ([2, 3], details on the modification of 4-thiouridine to uridine on the nucleoside, oligonucleotide and tRNA level will be published later). The four common nucleosides and nucleotides [15] as well as pseudouridylic acid and ribo-thymidine were not modified by iodine.

The results of $tRNA^{Tyr}$ inactivation experiments with excess iodine at different temperatures are summarized in fig. 1. Two facts should be pointed out:
i) the iodine inactivation of tyrosine acceptor activity is *independent* of the iodine concentration at high iodine concentrations and proceeds to *temperature dependent* plateaux of acceptor activity. This reaction to plateaux has some similarity to the temperature-dependent degradation of tRNA with polynucleotide phosphorylase [14], which is also not fully understood. As in that case, depending on the temperature, a fraction of the tRNA molecules is resistant to inactivation. Hence, iodine inactivation may have something to do with tRNA conformation and tertiary structure.
ii) The inactivation curves for 40, 50 and 60° for $tRNA^{Tyr}$ (fig. 1A) and $tRNA^{Tyr}_{pC\text{-}3}$, (fig. 1B) are nearly identical but there is a significant and reproducible difference at 20 and 30°. The degree of inactivation of $tRNA^{Tyr}$ (fig. 1A) at these temperatures is identical and if one plots % inactivation after 120 min against temperature, one gets a curve of similar shape as the melting curve of this tRNA [6]. For $tRNA^{Tyr}_{pC\text{-}3}$ inactivation we find differing inactivation plateaux at 20 and 30°, and hence one gets a straight line if % inactivation after 120 min are plotted against the corresponding temperature. This means – if one assumes that iodine inactivation has something to do with tRNA conformation – that the 3′-terminal pCpA sequence, at least of $tRNA^{Tyr}_{E.coli}$, takes part in, or influences, this tRNA's tertiary structure as has been postulated in several tRNA models.

There is the following evidence for the molecular mechanism of the tRNA inactivation described here:
i) the fact that AMP and CMP acceptor function is unaffected (table 1) means, that the tRNA's basic structure, the elements necessary for tRNA – tRNA-adenylyl(cytidylyl)transferase recognition, remains intact.
ii) Since different chromatographic and electrophoretic techniques had failed to demonstrate any degradation or dimer formation of $tRNA_2^{Tyr}$ upon iodine treatment, we compared the fragments of untreated (fig. 2A) and iodine treated (fig. 2B) tRNA produced by RNAase T_1. The main difference was found for the anticodon-containing dodecanucleotide (fig. 2A, peak 10), which, if isolated from iodine-treated $tRNA_2^{Tyr}$, yields two major products (fig. 2B, peaks 9 and 10). Peak 10 in fig. 2B is eluted significantly later than the unmodified oligonucleotide (fig. 2A, peak 10). This would mean that most of the minor nucleotide G^+ [16] has lost its positive charge [17] upon iodine treatment. The structure of G^+, which is the first letter in the anticodon of $tRNA^{Tyr}$, is yet unknown, but modifications causing loss of this nucleotide's positive charge have already been observed after cyanogen bromide treatment [2,3] and

upon periodate oxidation (H.J. Gross, unpublished). Finally it is known that 2-methylthio-N^6-(Δ^2-isopentenyl) adenosine [18], the nucleoside next to the anticodon, forms several products of yet unknown structure even under mild conditions [5]. Hence, it may be concluded that the observed iodine- and temperature-dependent inactivation of $tRNA_2^{Tyr}$ acceptor function may be due to a modification in (G^+) and/or next to the anticodon (ms^2i^6A) and that this modification depends on the tRNA's tertiary structure at the given temperature.

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