REPLACEMENT OF Y BASE, DIHYDROURACIL, AND 7-METHYLGUANINE IN tRNA BY ARTIFICIAL ODD BASES

W. WINTERMEYER and H.G. ZACHAU
Institut für Physiologische Chemie und Physikalische Biochemie der Universität München, W. Germany

Received 2 September 1971

1. Introduction

Fluorescent dyes have been introduced into tRNA by reaction with the purine bases [1, 2], by coupling to the ribose moiety of the 3′-terminal adenosine [3, 4], by replacing this nucleoside by formycine [5], and by non-covalent association [6–10]. It was not possible by any of the four methods to attach a marker molecule with a high fluorescence quantum yield to a defined position without changes in the native structure of the tRNA and/or loss of biological activity. Such an attachment is highly desirable both for structural studies and for the investigation of tRNA-protein interactions [10]. We therefore developed a method to replace the Y base of yeast $tRNA^{Phe}$, which itself possesses a weak fluorescence, by the highly fluorescent compounds proflavine (PF) or ethidium bromide (EB). Some physical and biological properties of the modified tRNAs are described. The method is suitable also for the introduction of fluorescent bases into the positions of 7-methylguanine or dihydrouracil. The insertion of bases with other properties, e.g. metal binding, seems feasible.

2. Materials and methods

$tRNA^{Phe}$ and $tRNA^{Phe}$ (HCl) [11] were prepared from brewer's yeast tRNA (Boehringer, Mannheim) and accepted around 1100 pmoles Phe/A_{260} unit. PF (obtained as the sulphate from Fluka AG., Switzerland) was purified by repeated crystallizations of the free base from ethanol/water. EB (Serva Entwicklungslabor, Heidelberg) was pure in paperelectrophoresis. During all operations dye and tRNA-dye compounds were protected against light. Fluorescence was measured with a Hitachi MPF-2A fluorescence spectrophotometer.

Reaction with dyes: 13 A_{260} units $tRNA^{Phe}$ (HCl) were incubated with 2.6 μmoles PF (125-fold molar excess) in 1.0 ml 0.1 M sodium acetate, pH 4.3, at 37°. Tris-sulphate, pH 7.5, was added to a concentration of 0.1 M and the pH was adjusted. The mixture was extracted four times with phenol which is more efficient than ethanol precipitations to remove free and non-covalently bound dye. The aqueous layer was extracted with ether. In the case of EB, 11.5 A_{260} units $tRNA^{Phe}$ (HCl) were reacted with 7.7 μmoles dye as above (400-fold molar excess). The amount of the dye bound to tRNA was calculated from absorption measurements. With $\epsilon_{260} = 6.25 \times 10^5$ (M^{-1} cm^{-1}) for $tRNA^{Phe}$ (HCl) and the ε-values of table 1 one obtains for $tRNA^{Phe}$ – PF $A_{457}/A_{260} = 5.3 \times 10^{-2}$ and for $tRNA^{Phe}$ – EB $A_{510}/A_{260} = 6.3 \times 10^{-3}$. The absorption at 260 nm of labeled tRNA was always corrected for dye absorption.

Reduction with $NaBH_4$: 10–20 A_{260} units $tRNA^{Phe}$-dye were incubated with 20 mg $NaBH_4$ (or ^3H-$NaBH_4$) in 1.0 ml 0.2 M Tris-HCl, pH 7.5, 0.005 M $MgCl_2$ for 15 min at 0°. After addition of acetic acid to pH 4 the mixture was kept at 37° for 4 hr to split small amounts of unreduced tRNA-dye compounds. The solution was then dialysed and extracted with phenol and ether. The visible absorp-

 North-Holland Publishing Company – Amsterdam

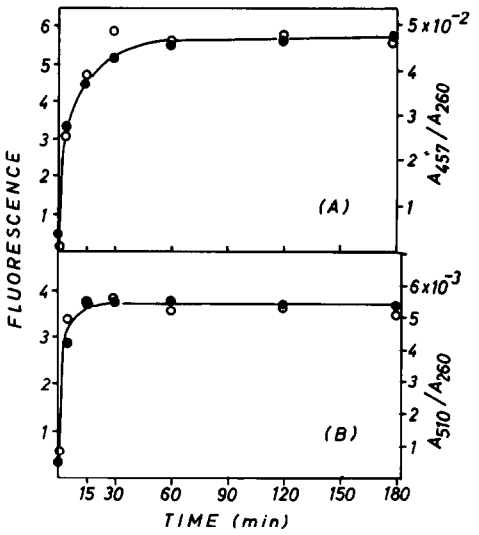

Fig. 1. Kinetics of the reaction of $tRNA^{Phe}$ (HCl) with dyes. (A) PF was incubated with 1 A_{260} unit of $tRNA^{Phe}$ (HCl) per point (see Methods). Fluorescence was measured at 507 nm upon excitation at 463 nm. (B) EB was incubated with 5 A_{260} units of $tRNA^{Phe}$ (HCl) per point (see Methods). Fluorescence was measured at 578 nm upon excitation at 300 nm. $tRNA^{Phe}$ bound only 5% dye compared to $tRNA^{Phe}$ (HCl). (–•–) dye absorption at visible λ_{max}/A_{260} of tRNA (–○–) fluorescence intensity in arbitrary units/A_{260} of tRNA.

tion of both PF and EB disappeared upon addition of $NaBH_4$. It reappeared during the incubation at acidic pH. If the reduction and reoxidation is done with free PF the spectrum is restored; the nature of the EB product(s) is not completely clear yet.

3. Results and discussion

3.1. *Preparation and analysis of $tRNA^{Phe}$-dye compounds*

When the Y base is excised from yeast $tRNA^{Phe}$ under acidic conditions, $tRNA^{Phe}$ (HCl) is formed which contains the ribose moiety of Y with a reactive aldehyde group [11]. Treatment with high concentrations of amine leads to an attachment of the amine and subsequent chain scission at the position of the ribose [12, 13], while, as was found now, treatment with amine at a hundredfold lower concentration results in a stable covalent attachment

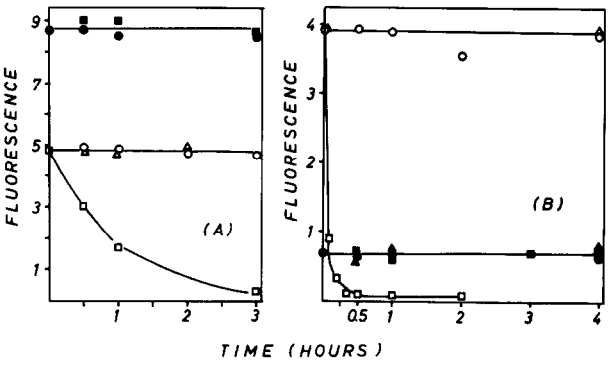

Fig. 2. Acid treatment of (A) $tRNA^{Phe}$-PF and $tRNA^{Phe}$-PF-red and (B) $tRNA^{Phe}$-EB and tRNA-EB-red. The labeled tRNAs were incubated at 37° at pH 7.5 (△, ▲), pH 7 (○, •), and pH 4 (□, ■). Full symbols, reduced; open symbols, not reduced. Aliquots were adjusted to pH 7.5, if necessary, and extracted with phenol and ether (see Methods). Ordinate as in fig. 1. The fluorescence of $tRNA^{Phe}$-PF-red was measured at 500 nm (excitation at 465 nm), for $tRNA^{Phe}$-EB-red, at 585 nm (excitation at 305 nm).

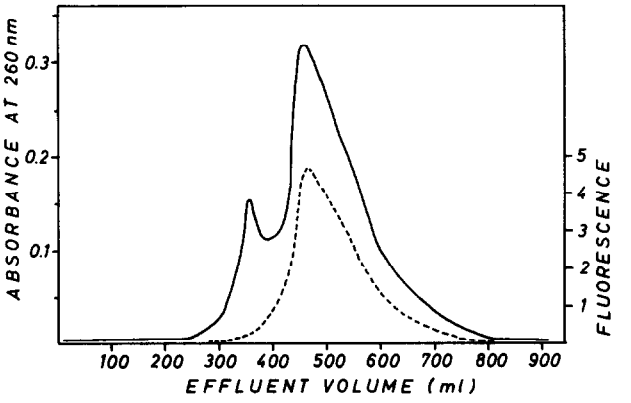

Fig. 3. Column chromatography of $tRNA^{Phe}$-PF. 70 A_{260} units of $tRNA^{Phe}$ (HCl) were reacted with PF for one hr (see Methods). The mixture was chromatographed on a column of BD cellulose (1.5 × 30 cm) with a gradient of 400 ml each of 0.4 and 0.8 M NaCl in 0.01 M Tris-HCl, pH 7.0, 0.005 M $MgCl_2$. —— absorbance; - - - - fluorescence intensity at 507 nm (excitation at 463 nm).

of the amine by N-glycoside or Schiff's base formation. The conditions for the attachment of PF and EB (see Methods) are similar to those used in related reactions [3, 4]. The time course of the reaction is shown in fig. 1. With lower dye/tRNA ratios

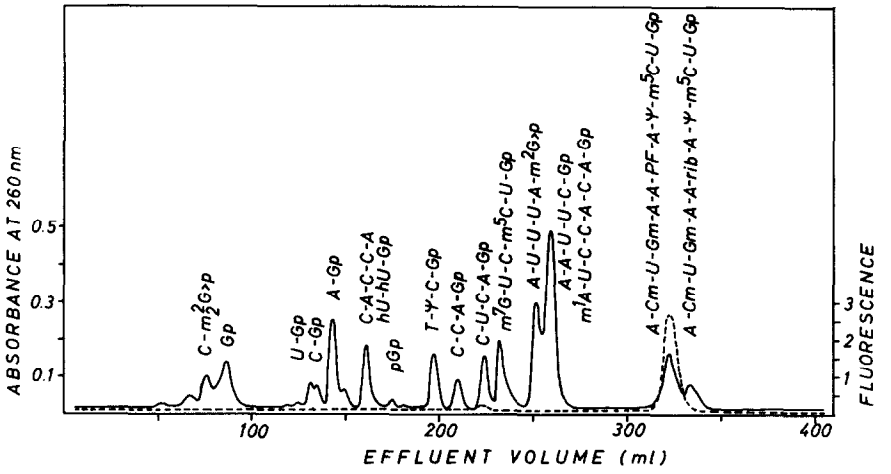

Fig. 4. Chromatography of a T1-RNase digest of tRNA^{Phe}-PF-red. 15 A_{260} units of tRNA^{Phe}-PF-red were digested with 100 units of T1-RNase and chromatographed on a DEAE-cellulose column as in [14]. —— absorbance, - - - - fluorescence intensity at 500 nm (excitation at 465 nm).

the reaction remained incomplete; in all cases dye and tRNA coprecipitated during the reaction. The tRNA-dye linkage is easily hydrolysed at pH 4.0 but is stable at pH 7.0 and 7.5. By reduction with $NaBH_4$ a secondary amine is formed at the tRNA-dye linkage which is stable also at the acidic pH (fig. 2). The relative fluorescence of reduced tRNA^{Phe}-PF (= tRNA^{Phe}-PF-red) is increased compared to the one of tRNA^{Phe}-PF while the fluorescence of tRNA^{Phe}-EB-red is decreased. The reduction of the tRNA-dye linkage was complete within 12– 15 min as measured by conversion to acid stability. Analysis of a T1-RNase digest of a ^3H-$NaBH_4$ reduced tRNA^{phe}-PF revealed that only about 5% of the dihydrouridine was reduced under these conditions.

On a preparative scale tRNA^{Phe}-PF (and tRNA^{Phe}-EB) can be easily separated from about 20% of unreacted tRNA^{Phe} (HCl) (fig. 3). With tRNA^{Phe}-PF-red the separation was not always complete. The oligonucleotide analysis of tRNA^{Phe}-PF-red (fig. 4) clearly shows that PF was inserted only in the former position of the Y base. The finding of about 70% fluorescent and 30% non-fluorescent dodecanucleotide agrees with the extent of labeling of this particular tRNA^{Phe}-PF-red preparation as determined by measuring the A_{466}/A_{260} ratio. Analogous oligonucleotide analyses were obtained with unreduced tRNA^{Phe}-PF and tRNA^{Phe}-EB. C-atom 1 of the ribose of tRNA^{Phe} (HCl), which formerly was attached to the Y base, can be removed by HIO_4 oxidation [13]. The resulting tetrose also reacts with PF under the described conditions. tRNA^{Phe}-PF with a tetrose instead of ribose seems to be an interesting compound for structural studies on the anticodon loop.

It was shown in a number of experiments that after excision of 7-methylguanine [14] from the CCA-half of tRNA^{Phe} and of dihydrouracil [15] from tRNA^{Phe} and tRNA^{Ser} the expected amounts of PF were incorporated.

3.2. *Spectral properties*

The spectral data of the covalently linked dyes (table 1) closely resemble those of the strong nucleic acid-dye complexes reported by several authors [6, 8, review 16]. The red shift may result from an interaction of the dye with the neighboring adenines. The additional red shift on reduction indicates a further improved overlap of the ring systems arising from decreased rigidity of the dye-ribose linkage. It cannot be excluded, however, that parts of the red shifts are due to the reactions at one of the amino groups of the PF, although the visible λ_{max} of alkaline hydrolysates of tRNA-PF-red is very close to the one of free PF.

Table 1
Spectral properties of $tRNA^{Phe}$-dye compounds.

	Absorption (visible λ_{max} nm)	ϵ at visible λ_{max} (M^{-1} cm^{-1})	Emission (λ_{max} nm)	Relative fluorescence intensity
PF	443 (443)	41×10^3	507 (507)	≡ 1.0
$tRNA^{Phe}$-PF	457 (453)	33×10^3	507 (507)	1.3
$tRNA^{Phe}$-PF-red	473 (466)	33×10^3	500 (500)	2.3
$tRNA^{Phe}$ (HIO_4)-PF	463			
Dodeca-PF	457		507	5.1
Dodeca-PF-red	463 (463)		500 (500)	3.9
EB	480	5.6×10^3	586	≪ 0.1
$tRNA^{Phe}$-EB	510	3.9×10^3	578	6.8
$tRNA^{Phe}$-EB-red	530	3.9×10^3	585	≡ 1.0

The spectra were measured in 0.01 M Tris-sulphate, pH 7.5; values in brackets after addition of 10 mM Mg^{2+}. The ϵ-values of PF and EB were taken from ref. [8] and [6], those of the bound dyes were assumed to be the same as for the strong tRNA complexes of PF [8] and EB [6]. Fluorescence data are uncorrected. PF emission was excited near the visible λ_{max} (cf. legend to fig. 1 and 2), EB emission at the main band around 300 nm. Fluorescence intensities are given relative to those of free PF and to $tRNA^{Phe}$-EB-red, respectively. $tRNA^{Phe}$ (HIO_4)-PF was prepared by treatment of $tRNA^{Phe}$ (HCl) with HIO_4, analine, and PF (see text).

Table 2
Biochemical assays.

	Aminoacylation (pmoles Phe/A_{260} unit)		Ribosome binding (% bound of input ^{14}C-Phe-tRNA)	
	synthetase from yeast	synthetase from *E. coli* K12	poly U	control
$tRNA^{Phe}$	1100	980	88	4
$tRNA^{Phe}$ (HCl)	1050	< 20	24	3
$tRNA^{Phe}$-PF	1025	210	53	4
$tRNA^{Phe}$-PF-red	880	< 20	57	7

Homologous aminoacylation was performed as in [11] with about 100 μunits [10] per assay of a 200-fold purified preparation of yeast Phe-tRNA synthetase (kindly donated by R. Hirsch); the heterologous one was as in [18] in the presence of 1.0 M $(NH_4)_2SO_4$ and over a period of 35 min. The ribosomes from *E. coli* K12 (E. Merck, Darmstadt) were washed 5 times with the buffer of [19] except that in the fourth cycle the buffer contained 0.5 M NH_4Cl. ^{14}C-Phe-tRNA was isolated on a Sephadex G-25-column (5 A_{260} units in 0.5 ml, 1 × 50 cm). The poly U directed binding was carried out as in [11], but the incubation was 45 min at 25°. The controls contained poly A instead of poly U.

The covalent tRNA-dye linkage forces the dye into a steric arrangement in the single stranded, probably stacked [17] chain of the anticodon loop which is similar to the arrangement in the intercalation model of Blake and Peacocke [16]. The similarities between the spectral properties of our compounds and those of the strong complexes support the model.

3.3. *Activity in biochemical assay systems*

$tRNA^{Phe}$-PF and $tRNA^{Phe}$-PF-red could be nearly fully charged by yeast phenylalanyl-tRNA synthetase (table 2). Also $tRNA^{Phe}$-EB was well chargeable under these conditions. The rates of charging of $tRNA^{Phe}$-PF and $tRNA^{Phe}$-PF-red were similar to those of $tRNA^{Phe}$ (HCl). The finding that the *E. coli* synthetase charged $tRNA^{Phe}$-PF to about 20% while it did not at all charge $tRNA^{Phe}$-PF-red (table 2) is in keeping with the notion that heterologous charging is a rather sensitive tool for the detection of small structural differences in tRNAs [18]. The plateau at 20% suggests the presence of a chargeable and a non-chargeable conformation of $tRNA^{Phe}$-PF, perhaps rotational isomers around the PF-ribose

linkage. The shoulder in the $tRNA^{Phe}$-PF peak of fig. 3, which in some experiments was even more pronounced, is also explained best by the presence of at least two conformations. The efficiencies of both $tRNA^{Phe}$-PF and $tRNA^{Phe}$-PF-red in the ribosome binding assay were intermediate between those of $tRNA^{Phe}$ and $tRNA^{Phe}$ (HCl) (table 2). The binding of $tRNA^{Phe}$ (HCl) which was not observed previously [11] (but see [20]) is probably due to the different ribosome preparation.

3.4. *Concluding remarks*

The covalent insertion of fluorescent dyes into the positions of various odd bases will allow interesting studies on the conformation of tRNAs, particularly if two different dyes are attached to the same tRNA molecule.

It seems justified to conclude from the physical and biological properties of the $tRNA^{Phe}$-dye compounds that their conformation is rather similar to the one of $tRNA^{Phe}$. This makes the compounds very suitable for the investigation of interactions between tRNA and aminoacyl tRNA synthetase or other components of the protein synthesizing machinery.

Acknowledgements

We thank Drs. R. Rigler and R. Thiebe for helpful discussions and Miss J. Patzschke for expert technical assistance. The work was supported by Deutsche Forschungsgemeinschaft, SFB 51, and by Fonds der Chemischen Industrie.

References

[1] L.M. Fink, S. Nishimura and I.B. Weinstein, Biochemistry 9 (1970) 496.
[2] D. Schneider, Thesis, Braunschweig 1971.
[3] J.E. Churchich, Biochim. Biophys. Acta 75 (1963) 274.
[4] K. Beardsley and C.R. Cantor, Proc. Natl. Acad. Sci. U.S. 65 (1970) 39.
[5] D.C. Ward, E. Reich and L. Stryer, J. Biol. Chem. 244 (1969) 1228.
[6] R. Bittmann, J. Mol. Biol. 46 (1969) 251.
[7] T. Tao, J.H. Nelson and C.R. Cantor, Biochemistry 9 (1970) 3514.
[8] H. Grosjean, J. Werenne and H. Chantrenne, Biochim. Biophys. Acta 166 (1968) 616.
[9] A. Surovaya, O. Borisova, T. Jilyaeva, V. Scheinker and L. Kisselev, FEBS Letters 8 (1970) 201.
[10] R. Rigler, E. Cronvall, M. Ehrenberg, U. Pachmann, R. Hirsch and H.G. Zachau, submitted to FEBS Letters.
[11] R. Thiebe and H.G. Zachau, European J. Biochem. 5 (1968) 546.
[12] P. Philippsen, R. Thiebe, W. Wintermeyer and H.G. Zachau, Biochem. Biophys. Res. Commun. 33 (1968) 922.
[13] W. Wintermeyer, Thesis, München 1971.
[14] W. Wintermeyer and H.G. Zachau, FEBS Letters 11 (1970) 160.
[15] B. Beltchev and M. Grunberg-Manago, FEBS Letters 12 (1970) 24.
[16] A. Blake and A.R. Peacocke, Biopolymers 6 (1968) 1225.
[17] W. Fuller and A. Hodgson, Nature 215 (1967) 817.
[18] R. Thiebe and H.G. Zachau, Biochim. Biophys. Acta 217 (1970) 294.
[19] M.V. Nirenberg, in: Methods in Enzymology, Vol. VI, eds. S.P. Colowick and N.O. Kaplan (Acad. Press, New York and London, 1963) p. 17.
[20] K. Ghosh and H.P. Ghosh, Biochem. Biophys. Res. Commun. 40 (1970) 135.