On the Reactivity of Pyridoxal-5′-phosphate with Yeast tRNA^{Phe} and tRNA^{Tyr}

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Yeast tRNA^{Phe} and tRNA^{Tyr} were reacted with the fluorescent reagent pyridoxal-5′-phosphate and the modified tRNAs were analysed with respect to the number and position of modified nucleosides and with respect to aminoacylation.

a) Following the intrinsic fluorescence of pyridoxal-5′-phosphate, the treatment of tRNA^{Tyr} with increasing amounts of pyridoxal-5′-phosphate revealed about 50 mol of reagent or a even higher number bound per one mol of tRNA^{Tyr}. After borohydride reduction (in order to stabilize the linkage) of this modified tRNA^{Tyr} and purification with reverse phase chromatography a modified tRNA^{Tyr} was obtained carrying about 2 mol of the reagent.

b) Both tRNA^{Tyr} and tRNA^{Phe} treated with pyridoxal-5′-phosphate and reduced exhibited almost unchanged aminoacylation as compared to the unmodified tRNAs.

c) Pyridoxal-5′-phosphate treated and reduced tRNA^{Phe} and tRNA^{Tyr} were digested with ribonuclease T_1 and the resulting oligonucleotides were separated. However, no fluorescent oligonucleotide and no difference to an oligonucleotide pattern obtained from unmodified tRNA were observed.

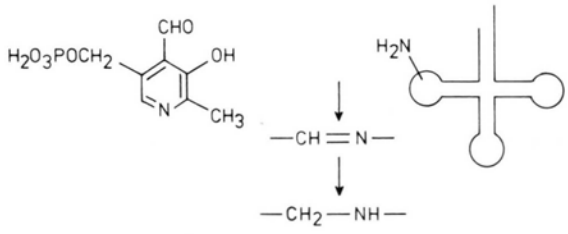
Thus, pyridoxal-5′-phosphate might have been bound to the highly purified yeast tRNA^{Phe} and tRNA^{Tyr} samples either via an unstable linkage or not covalently. This result is controversial with respect to the specific reaction of pyridoxal-5′-phosphate with unfractionated tRNAs from colon carcinoma and tRNAs from *E. coli* as reported in the literature.

Introduction

The aim of modification of tRNA by a fluorescent reagent is to obtain information on tRNA structure as well as on tRNA-protein interaction. The fluorescent dye has to bind specifically to one or a few nucleosides in the tRNA [for reviews see 1–4] and the reaction conditions have to be mild so that neither damage to the structure of tRNA nor incorrect recognition resulting in misaminoacylation occurs.

Recently Kopelovich and Wolfe [5] modified unfractionated tRNA from human colon carcinoma and some *Escherichia coli* tRNAs with pyridoxal-5′-phosphate. They suggested that the aldehyde group of the reagent might react with an amino group of the tRNA and the resulting Schiff base might be transformed in a stable covalent C-N linkage by borohydride reduction. In particular guanosine 20 in the dihydrouridine loop of the tRNA seemed to be reactive from inhibition studies with N-acetoxy-2-acetylaminofluorene [6] using unfractionated *E. coli* tRNA. This course of reaction was also suggested from the reaction of the related aldehydes kethoxal and glyoxal with tRNA^{Phe} as reported in the literature [7].

Since pyridoxal-5′-phosphate should react mildly [5], is fluorescent [8], and is not very large, we aimed to prepare yeast tRNA^{Phe} and tRNA^{Tyr} modified in the dihydrouridine loop with this reporter group.



Experimental

Materials

Pyridoxal-5′-phosphate was purchased from Boehringer (Mannheim), sodium borohydride, salts and buffer substances (ultrapure grade) from Merck (Darmstadt), RNase T_1, E.C. 3.1.4.8, from Sankyo (Tokyo, Japan), snake venom phosphodiesterase (1 mg/ml), E.C. 3.1.4.18, and alkaline phosphatase from *E. coli* (1 mg/ml), E.C. 3.1.3.1, from Boehringer (Mannheim), and [^{14}C]phenylalanine and [^{14}C]tyrosine (50 Ci/mol) from Schwarz Bioresearch (Orangeburg, USA). tRNA^{Phe} and phenylalanyl-tRNA synthetase, E.C. 6.1.1.20, from yeast were isolated according to [9, 10], tRNA^{Tyr} and tyrosyl-tRNA synthetase, E.C. 6.1.1.1, from yeast according to [11].

Spectroscopy

Ultraviolet absorbance measurements were performed with a Shimazu double beam spectropho-

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tometer UV-200 and a Zeiss PMQ-3 spectrophotometer. Relative fluorescence intensities were measured with a modified Farrand MK-1 spectrofluorometer equipped with a Varian-F-80 A X-Y-recorder.

Fluorescence titration

100 µl samples containing 1 A_{260} unit of tRNA and various amounts of reagent were incubated at 37 °C for 30 min in 20 mM borate buffer, pH 8.0, containing 5 mM $MgSO_4$. The reaction mixture was passed through a Sephadex G-25 column (1.7 × 38 cm) equilibrated with water at pH 6.0. The fluorescence intensity of the eluate was measured by using 37 µM pyridoxal-5′-phosphate in aqueous solution at pH 6.0 as a standard at 20 °C. The observed fluorescence intensity was then calculated as relative fluorescence intensity per A_{260} unit of tRNA.

Reaction of pyridoxal-5′-phosphate with tRNA

30–300 nmol tRNA (20–200 A_{260} units) were reacted with pyridoxal-5′-phosphate dissolved in water of pH 8.0, in 20 mM borate buffer, pH 8.0, containing 5 mM $MgSO_4$ in a total volume of 1–2 ml. The reaction was continued at 37 °C for 30–60 min. Then the mixture was cooled in the ice bath and tRNA was precipitated by adding three volumes of cold ethanol in 0.2 M KCl. After centrifugation, the precipitant was washed with cold ethanol followed by vacuum drying. Then it was dissolved in 1 ml of 0.2 M Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and reduced by adding a 3500–7000 fold molar excess of $NaBH_4$ dissolved in 500 µl of cold 0.2 M Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$. Reduction was continued for 15–30 min at 0 °C in the dark, and then free $NaBH_4$ was hydrolyzed by addition of 1 N acetic acid to pH 4 in the ice bath. tRNA was separated from the reagents on a Sephadex G-25 column (1.7 × 38 cm) equilibrated with water. The pooled tRNA fractions were concentrated and loaded on a RPC-5 column (0.7×90 cm) equilibrated with 50 mM phosphate buffer pH 7.0, containing 10 mM $MgSO_4$ and 0.4 M NaCl. After washing with the same buffer, the tRNA was eluted with a linear gradient of 2 × 250 ml, 0.4 M to 1.0 M NaCl in 50 mM phosphate buffer, pH 7.0, containing 10 mM $MgSO_4$ at 40 °C. The appropriate fractions were pooled and dialyzed against water in the cold room, then these samples were concentrated and reprecipitated with ethanol in 0.2 M KCl to remove any contamination. The precipitant was dissolved in a small volume of water and desalted on a Biogel P-2 column (1.5 × 30 cm) equilibrated with water. The pyridoxal-5′-phosphate treated tRNAs were stocked at −20 °C.

Aminoacylation

100 µl of the reaction mixture contained 150 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM ATP, 0.05 mM [^{14}C]phenylalanine or [^{14}C]tyrosine and about 0.2 A_{260} units of tRNA. It was started by adding 3.4 µg of phenylalanyl-tRNA synthetase or 10.5 µg of tyrosyl-tRNA synthetase. In 10 µl aliquots the acid-precipitable radioactivity was determined [9–11].

Ribonuclease T_1 cleavage of modified tRNA and oligonucleotide separation

3 A_{260} units of $tRNA^{Phe}$ treated with $NaBH_4$ and 15 A_{260} units of $tRNA^{Phe}$ treated with pyridoxal-5′-phosphate and $NaBH_4$ as described above were incubated with 100 and 250 units of RNase T_1 in 1.5 ml of 50 mM Tris-HCl, pH 7.5 at 37 °C for 16 h, respectively. The reaction mixture was adjusted to 20 mM Tris-HCl, pH 7.5 and 7 M urea and then loaded on a DEAE-cellulose column (0.7 × 85 cm) equilibrated with 20 mM Tris-HCl, pH 7.5. The column was eluted with a linear gradient of 2 × 200 ml 0 M to 0.3 M NaCl in 20 mM Tris-HCl, pH 7.5 at a flow rate of 16 ml/h.

34 A_{260} units of $tRNA^{Tyr}$ treated with pyridoxal-5′-phosphate and $NaBH_4$ were digested with 500 units of RNase T_1 and further dealt with as above.

Results

Reaction of $tRNA^{Tyr}$ and $tRNA^{Phe}$ with pyridoxal-5′-phosphate

$tRNA^{Tyr}$ was reacted with a 10–500 fold excess of pyridoxal-5′-phosphate in an aqueous borate buffer in the presence of Mg^{2+} at pH 8 and the excess of reagent was removed by gel filtration. This pyridoxal-5′-phosphate treated $tRNA^{Tyr}$ exhibited fluorescence excitation and emission maxima of 330 nm and 420 nm (Fig. 1), which correspond to the respective maxima of free pyridoxal-5′-phosphate in aqueous solution. The measured fluorescence was normalized to a standard solution of the reagent. The number of pyridoxal-5′-phosphate molecules bound to $tRNA^{Tyr}$ was estimated assuming that its

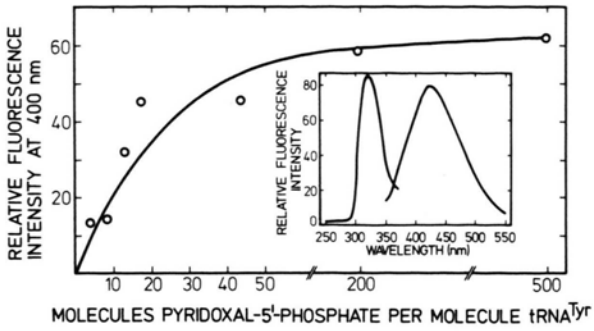

Fig. 1. Relative fluorescence intensity of pyridoxal-5′-phosphate treated $tRNA^{Tyr}$ at various initial molar ratios of reagent to $tRNA^{Tyr}$. Excitation was done at 330 nm, emission was measured at 420 nm. Insert: uncorrected fluorescence excitation and emission spectra of pyridoxal-5′-phosphate treated $tRNA^{Tyr}$ (in 10^{-4} M aqueous solution at pH 3.8 and 20 °C). Band widths of 5 and 10 nm at the excitation and emission side, respectively, were used.

fluorescence quantum yield is unchanged upon binding to tRNA as is the quantum yield of pyridoxamine-5′-phosphate upon complex formation with human or bovine serum albumin [8]. As is evident from Fig. 1 saturation of $tRNA^{Tyr}$ was observed at a molar ratio of pyridoxal-5′-phosphate over $tRNA^{Tyr}$ in the range of 50–200.

In contrast the number of pyridoxal-5′-phosphate molecules bound to $tRNA^{Tyr}$ considerably changed when borohydride reduction [12] was performed after the pyridoxal-5′-phosphate treatment and the $tRNA^{Tyr}$ was then purified by RPC-5 chromatography. It eluted in a major fraction corresponding to 95% of the applied $tRNA^{Tyr}$. The mean number of pyridoxal-5′-phosphate molecules bound to $tRNA^{Tyr}$ in this fraction is 2.3, irrespective of the initial molar ratio of the reagent to $tRNA^{Tyr}$. No absorbance change was observed at all with this modified yeast $tRNA^{Tyr}$ around 325 nm, whereas Kopelovich and Wolfe [5] with human and *E. coli* tRNAs reported appearance of an absorption peak at that wavelength.

With yeast $tRNA^{Phe}$ the estimation of the number of pyridoxal-5′-phosphate molecules bound per molecule of $tRNA^{Phe}$ by means of fluorescence was not possible, because of the overlapping emission spectra of pyridoxal-5′-phosphate and the Y base of $tRNA^{Phe}$. Nevertheless $tRNA^{Phe}$ was treated in the same way as $tRNA^{Tyr}$ and identified by oligonucleotide analysis.

Aminoacylation of pyridoxal-5′-phosphate treated $tRNA^{Phe}$ and $tRNA^{Tyr}$

The aminoacylation was investigated under standard aminoacylation conditions [9–11] and determined to be 1600 pmol tyrosine per A_{260} unit to $tRNA^{Tyr}$ and 1750 pmol phenylalanine per A_{260} unit of $tRNA^{Phe}$. Aminoacylation of the modified tRNAs as described above led to a small enhancement of 2% with $tRNA^{Tyr}$, whereas a reduction of 8% in aminoacylation was observed with $tRNA^{Phe}$. Thus the modification of $tRNA^{Phe}$ and $tRNA^{Tyr}$ with pyridoxal-5′-phosphate has only minor influence on the extent of aminoacylation compared to the native tRNAs.

Separation of oligonucleotides from ribonuclease T_1 digestion of pyridoxal-5′-phosphate treated $tRNA^{Phe}$ and $tRNA^{Tyr}$

Native $tRNA^{Phe}$ was treated with sodium borohydride, digested with RNase T_1, and the resulting oligonucleotides were separated on DEAE cellulose (Fig. 2a). Concomitantly $tRNA^{Phe}$ which was first

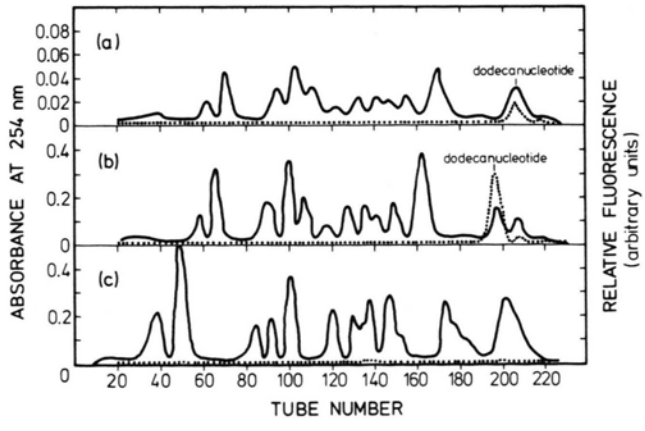

Fig. 2. Separation of oligonucleotides obtained by digestion with ribonuclease T_1 of a) $tRNA^{Phe}$ treated with sodium borohydride, b) $tRNA^{Phe}$ treated with pyridoxal-5′-phosphate followed by borohydroxide reduction and c) $tRNA^{Tyr}$ treated with pyridoxal-5′-phosphate followed by borohydride reduction. Relative fluorescence at 420 nm (· · ·), UV absorbance at 254 nm (———).

reacted with excess pyridoxal-5′-phosphate, subsequently reduced by sodium borohydride, was then digested with RNase T_1 and the resulting oligonucleotides were separated analogously (Fig. 2b). The composition of oligonucleotides and their respective elution volumes are nearly identical in both $tRNA^{Phe}$ samples. However, no fluorescent oligonucleotide, resulting from modification with pyridoxal-5′-phosphate, was detected. Only one fluorescent oligonucleotide was observed in both elution patterns and was identified by nucleoside analysis as being the Y base containing dodecanucleotide.

$tRNA^{Tyr}$ was treated analogously as $tRNA^{Phe}$, reduced with borohydride and digested in one case (data not shown), and reacted with pyridoxal-5′-phosphate prior to reduction and digested in the other case (Fig. 2c). Again, no fluorescent oligonucleotide, revealing the fluorescence characteristics of pyridoxal-5′-phosphate, could be detected.

Concluding Remarks

In the present investigation evidence is presented, that pyridoxal-5′-phosphate is not reacting with yeast $tRNA^{Phe}$ and $tRNA^{Tyr}$ with formation of a stable covalent linkage. Since linking via reduced Schiff base seems to be stable upon RNase T_1 digestion as reported in the case of proflavine and ethidium bromide linkage to $tRNA^{Phe}$ [12], we have to conclude that with yeast $tRNA^{Phe}$ and $tRNA^{Tyr}$ pyridoxal-5′-phosphate may have not so a specific reactivity towards guanosines as has been reported previously [5] for unfractionated human colon carcinoma tRNA and some *E. coli* tRNAs.

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