On the Reactivity of Pyridoxal-5'-phosphate with Yeast tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Tyr}

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Yeast tRNA\textsuperscript{Phe}, Yeast tRNA\textsuperscript{Tyr}, Pyridoxal-5'-phosphate

Yeast tRNA\textsuperscript{Phe} and tRNA\textsuperscript{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 aminoaaclylation.

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

b) Both tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Phe} treated with pyridoxal-5'-phosphate and reduced exhibited almost unchanged aminoaacylation as compared to the unmodified tRNAs.

c) Pyridoxal-5'-phosphate treated and reduced tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Tyr} were digested with ribonuclease T\textsubscript{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\textsuperscript{Phe} and tRNA\textsuperscript{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 \textit{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 misaminoaacylation occurs.

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Recently Kopelovich and Wolfe [5] modified unfractionated tRNA from human colon carcinoma and some \textit{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 \textit{E. coli} tRNA. This course of reaction was also suggested from the reaction of the related aldehydes kethoxal and glyoxal with tRNA\textsuperscript{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\textsuperscript{Phe} and tRNA\textsuperscript{Tyr} modified in the dihydrouridine loop with this reporter group.

![Chemical structure of pyridoxal-5'-phosphate](image)

Experimental

Materials

Pyridoxal-5'-phosphate was purchased from Boehringer (Mannheim), sodium borohydride, salts and buffer substances (ultrapure grade) from Merck (Darmstadt), RNase T\textsubscript{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 \textit{E. coli} (1 mg/ml), E.C. 3.1.3.1, from Boehringer (Mannheim), and [\textsuperscript{14}C]phenylalanine and [\textsuperscript{14}C]tyrosine (50 Ci/mol) from Schwarz Bioresearch (Orangeburg, USA). tRNA\textsuperscript{Phe} and phenylalanyl-tRNA synthetase, E.C. 6.1.1.20, from yeast were isolated according to [9, 10], tRNA\textsuperscript{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-
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 x 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 x 38 cm) equilibrated with water. The pyridoxal-5’-phosphate treated tRNA was eluted with a linear gradient of 2 x 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 NaBH$_4$ and 3 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 x 85 cm) equilibrated with 20 mM Tris-HCl, pH 7.5. The column was eluted with a linear gradient of 2 x 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.

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
reacted with excess pyridoxal-5'-phosphate, subsequently reduced by sodium borohydride, was then digested with RNase T₁ 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 T₁ 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 unfraccionated human colon carcinoma tRNA and some E. coli tRNAs.

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