Covalent attachment of fluorescent probes to the X-base of *Escherichia coli* phenylalanine transfer ribonucleic acid

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ABSTRACT

tRNA^{Phe}_{E. coli} was labeled with the N-hydroxysuccinimide esters of 1-dimethylaminonaphthalene-5-sulfonyl glycine and N-methylantranilic acid through reaction with the amino acid moiety of its X-base, whereby yields of 66% and 24%, respectively, were obtained. The purified dimethylaminonaphthalene-sulfonate derivative could not be aminoacylated and was found to be a strong competitive inhibitor of phenylalanine-tRNA synthetase [$K_i = 8 \times 10^{-7}$ M]. The N-methylantraniloyl derivative could be charged to an extent of 5% as compared to native tRNA^{Phe}. The fluorescence emission spectra of the derivatives are indicative of a slightly hydrophobic environment for both fluorophores. The results suggest that the integrity of the polar amino acid group of the X-base is required for the maintenance of the biologically active conformation.

INTRODUCTION

Transfer RNA derivatives containing a fluorescent probe covalently attached to a specific site are useful for both conformational studies of tRNA and investigations of its interactions with various proteins in the course of protein biosynthesis²⁻⁷. Fluorescent labels have been covalently attached to specific sites in tRNA by various chemical methods²⁻⁷. The resulting derivatives could be aminoacylated to an extent ranging from 0 to 100% as compared to that of the corresponding native tRNA. Recently tRNAs from *Escherichia coli* containing the X-base in position 48 have been shown to react significantly with the N-hydroxysuccinimide ester of phenoxyacetic acid, whereby their chromatographic behavior on BD-cellulose was altered^{8,9}. The subsequent determination of the X-base structure as 3-(3-Amino-3-carboxypropyl) uridine^{10,11} offered the explanation for the reactivity with N-hydroxysuccinimide esters as well as possibilities for specific chemical modification through reaction with the unique amino acid moiety.

In the present study purified tRNA^{Phe} from *E. coli* was reacted with N-hydroxysuccinimide esters of Dansylglycine¹² and N-methylantranilic acid.

Evidence is presented that the covalent attachment of the fluorescent probes occurs specifically at the X-base. The biological and spectroscopic properties of the fluorescent tRNA derivatives are described.

MATERIALS AND METHODS

Purified tRNA^{Phe} and tRNA^{Tyr} from *E. coli*·MRE 600 were products from Boehringer-Mannheim with acceptor activities of 1100 pmoles/A₂₆₀ unit and 1000 pmoles/A₂₆₀ unit, respectively. Ribonuclease T₁ and T₂ were obtained from Sigma. Dansylglycine and N-methylantranilic acid were commercial products and their N-hydroxysuccinimide esters were prepared in the usual manner¹³.

For the preparation of Dns-gly-X-tRNA^{Phe} 12 mg (4×10^{-4} mM) of tRNA^{Phe} were dissolved in 3 ml of triethanolamine-HCl (10 mM, pH 4.7) containing 10 mM magnesium chloride. After cooling with ice a solution of 16.2 mg (4×10^{-2} mM) N-hydroxysuccinimide ester of Dansylglycine in 1.5 ml of dry acetonitrile was added and the reaction mixture immediately brought to pH 8.0 with 1 M sodium hydroxide. After one and two hours of reaction two additions of 8.1 mg solid N-hydroxysuccinimide ester of Dansylglycine each were made and reacted for another six hours. The pH was then lowered to 5.0 with 1 M acetic acid and the tRNA precipitated several times with cold ethanol. The product was dissolved in 2 ml of 10 mM sodium acetate (pH 4.5), containing 0.3 M NaCl, for application on a column (1.0 x 10 cm) of BD-cellulose (Boehringer-Mannheim). After washing with the same buffer the column was eluted with a linear 200 ml gradient of 0.7–2.0 M sodium chloride containing 10 mM sodium acetate (pH 4.5) at a flow rate of 0.5 ml/min. At the end of the gradient the column was washed with final buffer (60 ml) and subsequently further eluted with a second 200 ml gradient of 2 M sodium chloride, 0–30% ethanol, containing 10 mM sodium acetate (pH 4.5). The tRNA derivative was concentrated, precipitated and dried in a vacuum dessicator. The reactions of tRNA^{Phe} with the N-hydroxysuccinimide ester of N-methylantranilic acid and of tRNA^{Tyr} with the N-hydroxysuccinimide ester of Dansylglycine and the chromatography of the reaction products were carried out similarly.

Digestions with Ribonuclease T₁ and T₂ were carried out according to published procedures¹⁴. Two-dimensional thin-layer chromatography was performed on Eastman cellulose chromatogram sheets (no. 13255) and the solvent systems used were: (A) isobutyric acid–0.5 M ammonium hydroxide, 1:1; (B) tert-butanol/ammoniumformate (pH 3.8), 1:1.

The assay for amino acid acceptor activity contained per milliliter: 100 μ moles of potassium cacodylate (pH 7.5), 10 μ moles of magnesium chloride, 2

μ moles of ATP, 10 nmoles of phenylalanine, 1.7 nmoles of [3 H]phenylalanine (New England Nuclear), 0.5 μ moles of dithiothreitol, 20 μ grams of bovine serum albumin and crude aminoacyl-tRNA synthetase prepared as described in the literature¹⁵. The incubations were performed with 200 microliters of incubation mixture and aliquots of 60 microliters were removed at three different times and mixed with 1 milliliter of cold trifluoroacetic acid (10%). The acid-insoluble radioactivity was determined by the filter paper method.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatography of the reaction product of tRNA^{Phe} with the N-hydroxysuccinimide ester of Dansylglycine on a BD-cellulose column.

Figure 1. Purification of Dns-gly-X-tRNA^{Phe} on BD-cellulose. The elution conditions are described in "Materials and Methods." The intensity of fluorescence emission was monitored at 530 nm and the excitation wavelength was 335 nm.

Unreacted tRNA^{Phe} (first peak) was eluted with the salt gradient while the elution of the fluorescent tRNA^{Phe}-derivative (second peak) required the use of an ethanol gradient (0-30%) as expected because of the presence of the hydrophobic Dansyl group. No fluorescent material was eluted by the salt gradient and evidently a complete separation between unreacted tRNA^{Phe} and Dns-gly-X-tRNA^{Phe} was achieved. The reaction yield was only 66% despite a 200-fold excess of acylating reagent being used; similarly low yields have been observed in other specific reactions with tRNA^{16,17}. Since the unreacted tRNA^{Phe} had the same acceptor activity as the native starting material (1100 pmoles/A₂₆₀ unit) it can be concluded that the reaction conditions used for acylation did not cause any other irreversible changes in the tRNA molecule.

The specificity of the attachment of the Dansylglycyl residue to the amino acid group of the X-base was assessed in several ways. By difference absorption spectroscopy an extinction coefficient at 340 nm of $4480 \text{ M}^{-1} \text{ cm}^{-1}$ was determined which demonstrates the presence of exactly one Dansyl group per tRNA molecule. A digest of the fluorescent tRNA-derivative with Ribonuclease T_1 was subjected to thin-layer chromatography (systems A and B), whereby one single, fluorescent spot was observed. The spot was eluted and a fluorescence emission spectrum of the eluted material showed the typical Dansyl emission at 540 nm. The eluted material was then further digested to nucleotides with Ribonuclease T_2 and also subjected to two-dimensional thin-layer chromatography which again resulted in a single fluorescent spot. The specific reaction of the X-base with N-hydroxysuccinimide esters is based on the uniqueness of its amino group which is strongly basic (pK 8-9) in contrast to the amino groups and heterocyclic nitrogen atoms of the tRNA bases. Thus it has been shown that the four major nucleotides as well as synthetic polyribonucleotides do not react with N-hydroxysuccinimide esters^{16,18} and neither do the odd bases pseudouridine and 4-thiouridine under reaction conditions similar to ours¹⁶. We also reacted tRNA^{Tyr}_{E. coli} with the N-hydroxysuccinimide ester of Dansylglycine under the same conditions used in the acylation of tRNA^{Phe}. Since tRNA^{Tyr}_{E. coli} does not contain the X-base, no reaction was expected and indeed none was observed. On the basis of these observations we conclude that the acylation occurred exclusively at the amino acid group of the X-base. The reaction of tRNA^{Phe} with the N-hydroxysuccinimide ester of N-methylantranilic acid under identical conditions occurred with the same specificity but with a lower yield (24%). The low yield is probably due to steric hindrance to nucleophilic attack on the ester group caused by the N-methyl group in ortho-position. The specificity of the reaction of N-hydroxysuccinimide esters with the amino acid group of the X-base has recently been confirmed by a spin-labeling experiment¹⁹.

The Dns-gly-X-tRNA^{Phe} is completely devoid of acceptor activity, while the Anth-X-tRNA^{Phe} can be charged to an extent of 5% as compared to native tRNA^{Phe}. In the latter case even prolonged incubation does not result in an increase in the number of charged tRNA molecules once a plateau has been reached after about 15 minutes. One possible explanation for this observation is a change in the balance between acylating and deacylating activity in favor of the latter one. This might involve a conformational change brought about by the presence of the hydrophobic fluorescent probe. Friedman reported⁸ that modification of crude tRNA_{E. coli} with the N-hydroxysuccinimide ester of phenoxyacetic acid did in general not result in a loss of amino acid acceptor

activity. In confirmation of these results, Nauheimer and Hedgcoth²⁰ reported no change in acceptor capacity after phenoxyacetylation of tRNA^{Phe}_{E. coli}. This finding seems to suggest that the positive charge of the 3-amino-3-carboxyl group is not essential for the formation of a productive complex between tRNA and enzyme. However, our present results indicate that modification of the X-base by attachment of hydrophobic labels to its amino acid group leads to a more or less pronounced reduction in acceptor capacity depending on the size and hydrophobicity of the label. The total loss of acceptor activity observed with Dns-gly-X-tRNA^{Phe} can be explained on the basis of a hydrophobic interaction of the bulky Dansylglycyl moiety with neighboring groups. Such an interaction could produce either a conformational change of the tRNA or a disturbance of the interface topography in the enzyme-tRNA complex.

As revealed by the Dixon-plot in Fig. 2, Dns-gly-X-tRNA^{Phe} is a strong competitive inhibitor of phenylalanine-tRNA synthetase with a K_i of 8×10^{-7} M which is close to the K_M value for native tRNA^{Phe} ($K_M = 5 \times 10^{-7}$ M)²¹. Obviously, the modification of the X-base has a minimal effect on the binding to the enzyme.

Figure 2. Dixon plot of phenylalanine-tRNA synthetase inhibition by Dns-gly-X-tRNA^{Phe}. The tRNA^{Phe} concentrations used were 1.25 μmolar (□), 2.51 μmolar (○) and 4.81 μmolar (●). For the determination of initial velocities aliquots were removed from the assay mixture after 30", 1' and 2'.

The fluorescence emission spectrum of Dns-gly-X-tRNA^{Phe} (Fig. 3) shows a maximum at 530 nm and thus a hypsochromic shift of 10 nm relative to the emission maximum of Dansylglycine in aqueous solution. This shift is indicative of a slightly hydrophobic environment of the Dansyl group which may be caused by hydrophobic interaction (intercalation) with adjacent bases. Such an interaction could be the reason for a slight conformational change leading to the loss of acceptor activity. A similar small shift in the emission spectrum was observed with Anth-X-tRNA^{Phe} ($\lambda_{\text{max}} = 418 \text{ nm}$).

Figure 3. Uncorrected fluorescence emission spectra of Dns-gly-X-tRNA^{Phe} (—) and Anth-X-tRNA^{Phe} (---). The excitation wavelength was 335 nm; measurements were made on a Perkin Elmer MPF-2A fluorospectrophotometer.

It will be of interest to assess the influence of the X-base modification on the biological activity of tRNA^{Phe} in other steps of protein biosynthesis.

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