On the Phe-tRNA induced binding of fluorescent oligonucleotides to the ribosomal decoding site

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ABSTRACT

Fluorescent oligonucleotides were prepared by dansylation of 5'-amino uridylates of varying chainlength. Except for the trinucleoside diphosphate, they stimulated the binding of Phe-tRNA to 70S E. coli ribosomes as efficiently as underivatised oligouridylic acids of comparable chainlength. The ternary ribosomal complex [70S x Phe-tRNA x dansyl-n 5'U(pU)₄] was separated from excess oligonucleotide and its fluorescence spectra were measured. The quantum yield of the dansylated pentauridylate was enhanced 2.5 fold when bound to the ribosomal decoding site, but no shift of the emission spectrum was observed. The ribosomal complex is considered useful for topographic investigations by singlet energy transfer, using the functionally defined decoding site as reference point.

INTRODUCTION

Due to its sensitivity and versatility fluorescence techniques have been successfully used to investigate the structure of ribosomes (1 - 11). Random labelling with fluorescent probes yields heterogeneous populations of ribosomes, which permit the determination of overall properties (1 - 5). By use of isolated ribosomal proteins, which were labelled at random, the kinetics of 30S reconstitution (9) was studied and protein neighbours were determined (10, 11). The latter results were obtained by singlet energy transfer experiments, assuming the corresponding proteins to be globular in the ribosomal RNA-protein complex, which does not hold for all proteins (12). Nevertheless, the results are in good agreement with those obtained by other techniques.

In contrast to randomly labelled ribosomes, the introduction of fluorescent probes at distinct, functionally defined sites has the advantage of making the interpretation of the results less ambiguous. This goal has been achieved by Cantor et
al. (13) with the aid of fluorescent antibiotics, which permitted the determination of the distance between the erythromycin binding site and the proteins L7, L12 (14). Wintermeyer et al. (15) were able to form ribosomal complexes with a macromolecular ligand, tRNA^Phe and Phe-tRNA^Phe from yeast, which was specifically derivatised with ethidium bromide in the dihydrouridine loop or the anticodon loop. Despite of these successes, alternative ways for the specific labelling of ribosomes are warranted, because thereby the possibilities for both topographic and functional studies are enlarged.

Encouraged by the finding, that derivatised oligonucleotides retain their ability to function as messenger analogues (16, 17), I considered the specific introduction of a fluorescent label into the ribosomal decoding site as feasible. The present paper demonstrates, that this is possible and that the use of dansylated oligonucleotides has two advantages: Due to the separation of products, quantitatively labelled messenger analogues of high purity can be synthesised, and their binding to ribosomes is made specific by the interaction with the cognate aminoacyl-tRNA.

MATERIALS AND METHODS

Preparation of dansyl-\(5'\) U(pU)_x:

Introduction of the dansyl group was always the last step, starting from the corresponding 5'-amino uridylates, which were prepared by standard procedures (18 - 21). Usually 0.5 \( \mu \)mol of the potassium salt of \(5'\) U(pU)_x was dissolved in 2 ml 50 mM sodium bicarbonate buffer (pH 9.2), which contained 50\% (v/v) acetone and 50 \( \mu \)mol of 1-dimethylamino-5-naphtalenesulfonyl chloride (Aldrich Europe). After stirring for 9 hours at room temperature in the dark, the clear solution was extracted with ethyl acetate and dansyl acid was precipitated with acetic acid. The supernatant was diluted with 20 ml 0.1 M triethyl ammonium bicarbonate (pH 7.4) and chromatographed on a DEAE cellulose column (1.5 cm x 6 cm), which was developed with a linear triethyl ammonium bicarbonate gradient (250 ml 0.0 M to 250 ml 0.4 M). The reaction products chromatographed well ahead of the starting oligonucleotides and were separated from fluorescent impurities. Homogeneity and composition of dansyl-\(5'\) U(pU)_x was
Nucleic Acids Research

determined by paper electrophoresis, pH 2.5 and pH 7.5, paper chromatography in n-butanol:acetic acid:water 5:2:3, and by nucleoside analysis (22). The products used for fluorescence measurements were free of fluorescent impurities.

Ribosomes and Phe-tRNA, Formation of Complexes:

70S ribosomes tight couples were prepared from mid log phase E. coli MRE 600 (E. Merck, Darmstadt) according to Noll (23). Crude tRNA from E. coli (Boehringer, Mannheim) was charged with [\(^3\)H]phenylalanine (Amersham Buchler), specific activity \(10^3\) Ci/mol, according to Traub (24), the degree of charging was 2%. The stimulation of Phe-tRNA binding to 70S ribosomes was determined in absence of supernatant factors as a function of oligonucleotide concentration. The incubation mixture contained in 0.1 ml 39 pmol 70S ribosomes, 44 pmol [\(^3\)H]Phe-tRNA, and varying amounts of oligonucleotides. The salt concentration was identical to that in buffer A: 50 mM Tris-HCl (pH 7.4), 20 mM MgCl\(_2\), 150 mM NH\(_4\)Cl. The incubation time was 10 minutes at 37°, followed by chilling in ice and millipore filtration according to Nirenberg and Leder (25). Blank values were determined in absence of oligonucleotide and amounted to 20% of the optimal value found at high messenger concentration. They were subtracted in each case. Formation of the ternary ribosomal complex was done by incubation of 1.4 nmol 70S ribosomes with 1.3 nmol [\(^3\)H]Phe-tRNA and 75 nmol dansyl \(\text{n}^5\text{U(pU)}_4\) in 0.15 ml buffer A (fig. 2a) under the conditions described above. In the control experiment Phe-tRNA was omitted (fig. 2b). Gel filtration was performed by use of a P-60 (Bio-Rad, München) column (1 cm x 30 cm) at 4° with buffer A as eluant. The arrow in fig. 2a indicated the fraction which was used for the kinetic and spectroscopic experiments. It contained 0.46 \(\mu\)M 70S ribosomes and 0.09 \(\mu\)M [\(^3\)H]Phe-tRNA bound to ribosomes immediately after its collection. This was 30 minutes after the termination of the incubation at 37°. The kinetics of the ternary ribosomal complex dissociation (fig. 3) was analysed by plotting \(\ln Y\) as function of time, where \(Y = (F_t - F_\infty)/(F_0 - F_\infty)\). F is the relative intensity of emission at 520 nm or CPM found after millipore filtration, while subscripts refer to time. For \(F_\infty\) the value after EDTA addition was used.
Recording of spectra:
Absorption spectra of dansyl-n U(pU) \_x in buffer A were recorded at room temperature by use of a Cary 118C spectrophotometer. Excitation and emission spectra were obtained under the same conditions using a Perkin Elmer MPF-3 fluorescence spectrophotometer operating in the ratio mode. Slit settings were at 10 nm and the emitted light was filtered through a F 39 filter. Fluorescence spectra of the ribosomal complex were obtained by use of a Schoeffel RRS 1000 fluorescence spectrophotometer. Slit settings were at 6 nm for excitation and 15 nm for emission. The cuvette holder was thermostated at $0^\circ$ and flushed with dry air. Emitted light was filtered through a GG 435 filter, because otherwise the solutions containing 0.5 $\mu$M 70S ribosomes showed very high fluorescence background with maxima at 558, 590 and 635 nm when excited at 340 nm. With the filter inserted, the background was small, but was corrected for by use of an equally concentrated solution of 70S ribosomes. The optical density at 340 nm, the excitation wavelength, was below 0.05 in case of the ribosomal complex and much lower in case of free oligonucleotides. No correction for inner filter effects was made due to the short pathlength (5 mm) of the rectangular cuvettes (Hellma GMBH, Müllheim/Baden). If not otherwise mentioned, the excitation wavelength was always 340 nm and the emission wavelength 520 nm.

RESULTS

Coding properties of dansyl-\(n U(pU)_x\):
Stimulation of Phe-tRNA binding to 70S E. coli ribosomes in absence of factor EF-Tu was determined as function of dansyl-\(n U(pU)_x\) concentration. As shown in figure 1, no stimulation of Phe-tRNA binding is observed, when $x$ was equal to two. For $x$ greater than two, the curves are indistinguishable from that obtained for $U(pU)_3$, which was used as a reference in each binding assay. The same results were found when the stimulation of Phe-tRNA to 30S ribosomal subunits was investigated at $0^\circ$ (data not shown).

Due to the high excess of oligonucleotide needed to induce Phe-tRNA binding, the isolation of the ribosomal complex is nec-
Fig. 1  Stimulation of the Phe-tRNA binding to 70S ribosomes by
(*) U(pU)_3, (•) dansyl-n U(pU)\_2, (○) dansyl-n U(pU)\_3,
and (▲) dansyl-n U(pU)\_4

necessary in order to investigate its spectral properties. Figure
2a shows, that this can be achieved by gel filtration over a
P-60 column. Omission of Phe-tRNA in the incubation mixture
leads to the loss of dansyl-n U(pU)\_4 in the fractions con-
taining the ribosomes (fig. 2b). The same finding was made when
tritium labelled hexauridylate was used as messenger analogue
in the presence and absence of Phe-tRNA (data not shown).

In order to determine, whether the dissociation of [3H]Phe-
tRNA and dansyl-n U(pU)\_4 were correlated, the time dependence
of the complex stability was followed by radioactivity and flu-
orescence intensity. For this purpose, an aliquot of the incu-
bation mixture was diluted 1:300 with buffer A, and kept under
the same conditions as the incubation mixture during gel fil-
tration and recording of the spectra. At appropriate times, the
complex concentration was determined in this solution by milli-
pore filtration (25) and by measurement of the relative emis-
sion intensity at 520 nm in the fraction 15 (see figure 2a). A
semilogarithmic plot, which was normalised to the value at 30
minutes, yields the same straight line for the dissociation of
Phe-tRNA and dansyl-n U(pU)\_4 from the ribosomal complex (fig.
3). The value chosen for infinite time was that obtained after
Fig. 2  Elution profile during gel filtration of 70S ribosomes and dansyl-\(5^{'}, U(pU)_4\) in presence (fig.2a) and absence (fig.2b) of Phe-tRNA. \(A_{260}\) (---) is absorbance and \(F_{520}\) (——) is intensity of emission (in arbitrary units) at the respective wavelength.

addition of EDTA in equal concentration to \(\text{Mg}^{2+}\). This treatment was observed to dissociate Phe-tRNA from ribosomes within less than three minutes at 0°. As shown in figure 3, the dissocia-

Fig. 3  Time course of the ribosomal complex dissociation. (X) refers to the intensity of emission at 520 nm and (o) refers to radioactivity of bound \(^3\text{H}\)Phe-tRNA
tion of the ternary ribosomal complex is slow at 0°C, with a half life time of about 210 minutes, and follows first order kinetics with respect to both Phe-tRNA and dansyl-\(5'U(pU)\) dissociation.

**Spectral properties of dansyl-\(5'U(pU)\)** and the ternary ribosomal complex:

Excitation and emission spectra of dansyl-\(5'U(pU)\) at room temperature are shown in figure 4 as a function of the solvent. In aqueous solution, buffer A, the excitation spectrum exhibits a broad maximum around 335 nm, which does not exactly match the absorption spectrum, which has a shoulder at 330 nm. The extinction coefficient in 0.1 M sodium phosphate buffer (pH 7) at room temperature was determined to \(\varepsilon_{330} = 6 \times 10^3\) \(\text{cm}^{-1}\) by use of tritium labelled oligonucleotide. This value is somewhat higher than that found for comparable dansyl derivatives (26). Upon transfer of dansyl-\(5'U(pU)\) from aqueous to methanolic solvent, the relative fluorescence intensity increases considerably (fig. 4) and the emission spectrum is shifted to shorter wavelengths (fig. 6). No change of the emission spectrum was observed for dansyl-\(5'U(pU)\) at 0°C in buffer A upon variation of the \(Mg^{2+}\) concentration between 0 and

![Fig. 4](image-url) 

**Fig. 4** Corrected excitation spectra and uncorrected emission spectra of 1.2 \(\mu M\) dansyl-\(5'U(pU)\) in buffer A (a) and methanol (b) at room temperature. For details see materials and methods.
20 mM, or upon addition of EDTA (data not shown). Furthermore, the fluorescence intensity at 440, 480 and 520 nm of the oligonucleotide in buffer A decreases linearly with increasing temperature between 0° and 32° by about 1% per centigrade (data not shown).

Uncorrected emission spectra of the isolated complex are given in figure 5. The fluorescence intensity decreases slowly with time at all wavelengths (see fig. 3) without shifting the emission spectrum. In contrast to free dansyl-\(^{5'}\)U(pU)\(_4\), addition of EDTA in equal amounts to Mg\(^{2+}\), leads to an instantaneous drop of the emission intensity and then remains constant with time. In order to determine whether the dissociation of dansyl-\(^{5'}\)U(pU)\(_4\) from the ribosome by EDTA shifts the emission spectrum, the ratio of relative intensities at each wavelength before (fig. 5a) and after (fig. 5d) dissociation was plotted as a function of wavelength (fig. 6). This procedure is preferred to the comparison of corrected emission spectra, because it is more sensitive to small changes and completely independent from instrumental parameters. Figure 6 shows, that upon dissociation of the ternary ribosomal complex by EDTA, the relative

![Fig. 5](image)

**Fig. 5** Uncorrected emission spectra of the isolated ribosomal complex at 0° after (a) 30 minutes, (b) 80 minutes, (c) 200 minutes, and (d) after addition of EDTA. The lower curve (e) is the background due to ribosomes, for which all spectra are corrected.
Fig. 6 Ratio of relative emission intensities of (●) a 1.2 μM solution of dansyl-n5'U(pU)4 in methanol/buffer A at room temperature and of (▲) (ribosomal complex)/(EDTA dissociated complex) at 0°.

Fig. 7 Corrected excitation spectra of (a) the ribosomal complex (as in fig.5a) and (b) the EDTA dissociated complex (as in fig.5d) at 0° in buffer A. The spectra are corrected for the background (c) due to ribosomes fluorescence intensity drops by a factor of 2.5, which is virtually independent from the wavelength.

The corrected excitation spectra of the ternary ribosomal complex before and after dissociation by EDTA are given in figure 7. As was found for the emission spectrum, the decrease in fluorescence intensity upon dissociation is independent from the excitation wavelength. This indicates, that no shift of the absorption spectrum of the dansyl moiety takes place when the oligonucleotide is bound to the ribosomal decoding site.
DISCUSSION

The assumption, that the isolated ribosomal complex contains the fluorescent oligonucleotide bound to the ribosomal decoding site, rests on the following findings: (a) dansyl-n\(^{5'}\)U\((pU)\_x\) fully substitutes U\((pU)\_3\) as a messenger analogue in the EF-T\(^u\) independent binding of Phe-tRNA to 70S and 30S ribosomes, if the chainlength x is greater than two. The observed stimulation of Phe-tRNA binding cannot be explained by contaminating underivatised oligonucleotides, which were removed. If they were present in undetectable amounts and responsible for Phe-tRNA binding, the binding curves had to be shifted to much higher oligonucleotide concentrations, which is not the case. (b) If the cognate macromolecular ligand Phe-tRNA is omitted in the incubation mixture, no binding of dansyl-n\(^{5'}\)U\((pU)\_4\) to ribosomes is observed. This indicates, that unspecific binding is absent or much weaker than binding to the decoding site in presence of Phe-tRNA. (c) The time dependence of Phe-tRNA and dansyl-n\(^{5'}\)U\((pU)\_4\) dissociation from the complex is identical, which is in perfect agreement with the assumption, that dissociation is an all or none process for both aminoacyl-tRNA and messenger analogue. (d) The fast dissociation of both Phe-tRNA and dansyl-n\(^{5'}\)U\((pU)\_4\) from the complex by EDTA addition also confirms the interrelationship between the binding of the two ligands. Although none of this evidence by itself can be taken as proof, together it strongly indicates, that the ribosomal complex, formed according to the given procedure, contains the fluorescent oligonucleotide bound to the decoding site.

The lack of codon activity found for dansyl-n\(^{5'}\)U\((pU)\_2\) permits some assumptions to be made about the mode of dansyl-n\(^{5'}\)U\((pU)\_x\) binding to the decoding site. While little difference exists between U\((pU)\_2\) and U\((pU)\_3\) with respect to the stimulation of Phe-tRNA binding, the observed difference in case of the dansylated uridylates suggests, that here the 5' terminal uridine is unavailable for base pairing with the anticodon loop. In the higher homologues, the dansyl residue therefore should be separated from this loop by at least one uridine base. Although halfsaturation points and plateau values determined for oligonucleotide induced binding of Phe-tRNA do not yield molecular parameters, the identity of the binding curves for
U(pU)₃ and dansyl-n⁵'U(pU)ₓ (with x greater than two) suggests, that these parameters are identical as well.

The solvent dependence of the quantum yield and the fluorescence emission spectrum of 1-dimethylamino-5-naphtalenesulfonyl derivatives has been reported (26, 27) and were accounted for by a two state model (28). For dansyl-tryptophane Chen (27) reported a sixteenfold increase of fluorescence emission intensity at 510 nm and a pronounced blueshift of the emission spectrum, when the solvent was changed from water to methanol. Qualitatively the same finding is made for free dansyl-n⁵'U(pU)₄ (fig. 6), although the differences are not as pronounced as for dansyl-tryptophane. On dissociation of dansyl-n⁵'U(pU)₄ from the ribosomal decoding site, the quantum yield is much less affected and no blueshift of the emission spectrum is observed. From this it can be concluded, that the polarity in the close vicinity of the decoding site is higher than that of methanol and very close to that of water.

The formation of the ternary ribosomal complex [70S x dansyl-n⁵'U(pU)₄ x Phe-tRNA] offers an alternative route to introduce a fluorescent label specifically into the close vicinity of the ribosomal decoding site. Despite such drawbacks as lack of stability and the necessity to remove excess oligonucleotide, the complex is considered useful for the investigation of elementary steps of protein biosynthesis, such as translocation, and for topographic studies by singlet energy transfer. Furthermore, the small, but significant increase of quantum yield upon binding to the decoding site, makes investigation of the codon-ribosome interaction by fast kinetic techniques feasible.

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