Volume 8 Number 4 1980 Nucleic Acids Research

# Characterisation of a new, fully active fluorescent derivative of E.coli tRNA $^{Phe}$

Jackie A.Plumbridge*, Hans G.Bäumert**, Måns Ehrenberg† and Rudolf Rigler†

Department of Molecular Biology, Wallenberg Laboratory, Uppsala University, Box 562, S-751 22 Uppsala, and †Department of Medical Biophysics, Karolinska Institutet, Box 60 400, S-104 01 Stockholm 60, Sweden

Received 15 January 1980

ABSTRACT

E.coli tRNA$^{Phe}$ has been labelled with fluorescein isothiocyanate taking advantage of the reactivity of this compound for primary aliphatic amino groups as exist in this tRNA as the modified base X(3-(3-amino-3-carboxypropyl)uracil). The extent of labelling was calculated as 1.6 nmole/$A_{260}$ unit suggesting one dye molecule per tRNA. The FITC-tRNA showed full activity in aminoacylation and polypeptide synthesis. The absorption and fluorescence of the label respond markedly on addition of $Mg^{++}$ to the tRNA. The label appears to be a sensitive probe of tRNA$^{Phe}$ tertiary structure.

## INTRODUCTION

Fluorescent labels provide sensitive probes for investigating the structure and function of macromolecules (1,2). Certain natural tRNA species contain odd bases which are intrinsically fluorescent (3) but apart from the base Y, present in eukaryotic tRNA$^{Phe}$'s (4), they have very low quantum yields. Several fluorescent derivatives of eukaryote (5-7) and prokaryote (8-11) tRNAs with varying biological activity have been described.

Transfer RNA molecules are uniquely suited for chemical modification because of their high content of unusual bases. These are often more reactive than the parent bases and so allow modification to be achieved under mild conditions. One such base in E.coli tRNA$^{Phe}$ is that designated X(3-(3-amino-3-carboxypropyl)-uracil) (12,13) which is found in the extraloop. The primary aliphatic amino group of X is unique amongst the modified nucleosides in E.coli tRNA$^{Phe}$ and has been used for attachment of a dansyl-derivative (10) and of fluorescamine (11). In search of a fluorescent label with superior spectroscopic properties we have chosen fluorescein-isothiocyanate (FITC) which was expected to

react with primary aliphatic amino groups to give a substituted thiourea (14).

## MATERIALS

E.coli $tRNA^{Phe}$ was purchased from Boehringer, Mannheim and was found to give and aminoacid incorporation of 900-1050 pmoles [$^3$H]Phe/$A_{260}$ unit. FITC Isomer I and phenoxyacetic acid N-hydroxyssuccinimidester were from Sigma, as were HEPES, PIPES, TRIS (Σ grade), ATP, GTP, PEP, and Pyruvate kinase.

[$^3$H]phenylalanine (TRK 266, 16Ci/Nmole) was from Amershams.

Phenyalalanine-tRNA ligase was either prepared from an S100 fraction by subsequent chromatography on DEAE Sephadex (Pharmacia), hydroxylapatite (Biorad), and G150 (Pharmacia) or was a gift from Dr. U. Lagerkvist (Göteborg).

EF-Tu·GDP was prepared by the method of Arai et al. (15) with slight modifications. The product was homogeneous as judged by polyacrylamide gel electrophoresis and bound 1 nmole of [$^3$H]GDP per 43.000 ngm protein.

Buffer A is 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA.

Buffer B is 100 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 20 mM KCl, 1 mM DTE.

Buffer C is 50 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 80 mM $NH_4Cl$, 80 mM KCl, 1 mM DTE.

Buffer D is 10 mM PIPES, 10 mM $Mg(OAc)_2$, 100 mM $NH_4Cl$, 150 mM KCl, 1 mM DTE.

## METHODS

### Labelling of $tRNA^{Phe}$ with FITC

25 $A_{260}$ units $tRNA^{Phe}$ were dialyzed overnight versus 50 mM HEPES, 1 M NaCl, pH 8.0, and then incubated in the dark for 8 hours at 30°C with 25 fold excess FITC. Unreacted dye was removed by extracting four times with phenol (distilled and saturated with buffer A) and then six times with ether. The product was extensively dialyzed versus buffer A.

After labelling the reacted $tRNA^{Phe}$ was applied to a BD cellulose (Serva) column, 3x0.5 cm, equilibrated with 50 mM NaOAc, pH 5.0, 0.3 M NaCl. The column was washed with the equi-

librating buffer, eluted with a linear gradient of 0.3 to 2.0 M NaCl in 50 mM NaOAc, pH 5.0 and then finally washed with 20% ethanol, 2 M NaCl, 50 mM NaOAc, pH 5.0. The tRNA was concentrated by ethanol precipitation in the presence of 2% NaOAc and subsequently dialysed versus buffer A.

Measurement of aminoacylation

The reaction mixture, 100 µl, contained 20 µM [$^3$H]phenylalanine (500 mCi/mmole), 2 mM ATP, 10 nM Phe-tRNA ligase and approximately 0.2 µM tRNA in buffer B. Samples were incubated for 15 or 30 minutes at 37°C. Reaction was stopped by addition of 2 ml ice cold 5% TCA and 50 µg carrier tRNA added. Samples were filtered through Whatman GF/C filters, washed with 15 ml cold 5% TCA and 4 ml 70% ethanol. After drying radioactivity was determined in a Beckman scintillation counter using a toluene base scintillator. $K_m$ and $V_{max}$ measurements were made under similar conditions except that the tRNA concentration was varied between 0.1 and 1.0 µM and the enzyme concentration was 0.1 nM. 20 µl aliquots were removed after 2, 4, 6 and 8 minutes.

Measurement of polyphenylalanine synthesis

Poly-U dependent polyphenylalanine synthesis was measured in a $tRNA^{Phe}$ dependent assay system with washed ribosomal subunits (16) and an SF II fraction (17) to supply aminoacid-tRNA ligases and protein elongation factors.

The reaction mixture (200 µl) contained 40 µg poly-U (which had been dialysed versus buffer C) 20 µM [$^3$H]phenylalanine, 1.6 µm washed 30S and 50S ribosomal subunit, 8 µl SF II fraction (containing 4 mg/ml protein) and approximately 0.2 µM $tRNA^{Phe}$ in buffer C. After preincubating for 5 minutes at 37°C the reaction was initiated with 10 µl of buffer C containing 20 mM ATP and 0.5 mM GTP. Aliquots were removed and quenched in 5% TCA. After heating to 90°C for 20 minutes samples were filtered and counted as described for aminoacylation.

Complex formation between Phe-FITC-$tRNA^{Phe}$ and EF-Tu

15 $A_{260}$ units of FITC-$tRNA^{Phe}$, were immediately after the labelling procedure dialysed versus buffer D and then charged with [$^3$H]phenylalanine, under the same conditions as above but in a total volume of 5 ml. The tRNA was two times phenol extracted and

two times ethanol precipitated and then dialysed versus 8 mM NaOAc, pH 5.0.

Phospho-enol-pyruvate (PEP) and pyruvate kinase were used to convert EF-Tu·GDP to EF-Tu·GTP (18,19). The reaction mix contained in a volume of 300 µl 1 mg EF-Tu·GDP, 20 µg pyruvate kinase, 5 mM PEP and 1 mM GTP in buffer D. After incubating 15 minutes at 30°C the reaction mix was cooled in ice. Five times concentrated buffer D was added to the charged FITC-$tRNA^{Phe}$ to make normal strength buffer D in the tRNA sample (total about 150 µl) and then immediately mixed with the EF-Tu·GTP. After 5 minutes in ice the whole mix was applied to an Ultrogel ACA 54 (LKB) column, 100x1.2 cm, equilibrated with buffer D at 5°C. The flow rate was 10 ml/hr and 1 ml fractions were collected.

Absorption and fluorescence spectra

Absorption spectra were measured in a Cary 118C spectrophotometer. Fluorescence spectra were measured in a laboratory built instrument consisting of a stabilized high pressure Xenon arc lamp as excitation source, Zeiss M4QIII prism monochromators for separating excitation and emission wavelengths and an SSR model 1140 quantum photometer as detection unit. Cuvettes with 0.44 cm pathlength were used for both absorption and fluorescence measurements, and all measurements were made in buffer A at 20°C.

Correction factors for wavelength dependent transmission of the monochromator and sensitivity of the photomultiplier were determined. For each wavelength the energy distribution of the exciting light source was measured with an HP 8334A Radiant Flux detector and an HP 8330A Radiant Flux meter and compared with the response of the monochromator-photomultiplier combination. The corrected fluorescence spectra were transposed to relative quantum spectra by normalizing the absorption of the sample at the excitation wavelength (20).

Titrations with $Mg^{++}$

The titrations were performed by adding aliquots of $Mg(OAc)_2$ with a Hamilton syringe to the cuvette. The concentration of FITC-$tRNA^{Phe}$ was kept constant by having FITC-$tRNA^{Phe}$ present in the $Mg^{++}$ solution at the same concentration as in the cuvette. The fluorescence was excited at 480 nm with a band width of 12 nm and was measured at 530 nm with a band width of 18 nm.

Polarisation measurements

For measurements of the polarized emission a polarizer and analyzer were placed before and after the sample compartment in the excitation and fluorescence light paths. They consisted of Polarex KSW 78 polarizing filters of Käsemann, Oberaudorf, W. Germany.

The polarisation was determined by measuring the light components vibrating in parallel with ($I_{||}$) and perpendicular to ($I_{\perp}$) the direction of the exciting light beam. To correct for polarisation effects in the optical system the polariser was turned at right angles to both components of the emitted light. In this position both components of the emitted light attain, in theory, the same magnitude (21). From their ratio a correction factor for the superimposed polarisation was determined (22). The degree of polarisation was expressed by the anisotropy of fluorescence, which can be calculated from the relationship $r=(I_{||}-I_{\perp})/(I_{||}+2I_{\perp})$. r is related to the rotational tumbling time, $\tau_r$, of the fluorescing species by

$$r = \frac{r_o}{1+\tau/\tau_R} \qquad (I)$$

where $\tau$ is the lifetime of fluorescence and $r_o$ is the limiting anisotropy when $\tau_R >> \tau$ (23). Thus r increases as $\tau_R$ increases (corresponding to reduced motion) up to the value of $r_o$. Experimentally $r_o$ was estimated by extrapolarition of measurements of r made at increasing solvent viscosity (24,25).

Measurement of fluorescence lifetime and rotational relaxation time

The lifetimes of fluorescence ($\tau$) and rotational relaxation time ($\tau_R$) were measured in a single photon spectrometer using pulsed excitation in a laboratory built instrument. To compensate for systematic drifts the fluorescence events detected by single photon counting techniques were related to the shape and intensity of the exciting light pulse by an optical switching system. A detailed description of the instrument and of the data evaluation procedure is given in (26).

## RESULTS

### Preparation of FITC-tRNA$^{Phe}$

The tRNA, after labelling with FITC, reproducibly gave three peaks on BD cellulose chromatography (Fig. 1). Peaks I and II emerged during the salt gradient while peak III was eluted with the 2 M NaCl 20% ethanol wash. Peaks II and III showed fluorescence whereas peak I showed none or only very slight fluorescence which is probably due to contamination from peak II.

The size of peak III in the absorption curve in Fig.1 is distorted because the wash of ethanol, through the flow-through cell of the detection unit causes changes in the refractive index and magnifies the $A_{254}$ reading. After ethanol precipitation the ratios of tRNA in the three peaks were 60:35:5 with some variation between different batches of tRNA. The aminoacylation capacity of the tRNA of these peaks was 900, 950 and 300 pmoles/$A_{260}$ unit when untreated tRNA$^{Phe}$ gave a value of 1000 pmoles/$A_{260}$ unit.

The tRNA from peak III was apparently labelled to the same extent as that from peak II, as judged by its absorption at 490 nm, the peak of the fluorescence absorption. As it was only a minor component it has not been further investigated and the

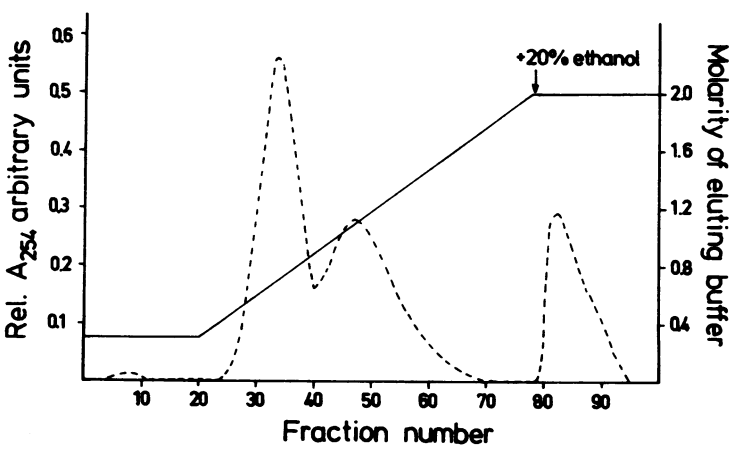


Figure 1. Elution of FITC-tRNA$^{Phe}$ from BD cellulose. See text for details. --- absorbance at 254 nm as measured on Uvicord photometer (LKB).

reason for its reduced aminoacid charging is unknown. The lack of labelling of tRNA from peak I with FITC is not due to incomplete reaction because attempts to relable the tRNA peak I under identical conditions produced tRNA with only 5% of the fluorescence of the original labelled tRNA. We would suggest that $tRNA^{Phe}$ is not homogeneous, with either only a fraction having the U-47 in the extra loop modified to X, or X having a masked (acetylated) amino group. Non stoichiometric labelling at the X-base has been reported previosly (10,27). All subsequent experiments (except where stated) were carried out with the fraction designated peak II.

Position of the label

To demonstrate that FITC-$tRNA^{Phe}$ was uniquely labelled at X-47 in the small extra loop we masked this base with phenoxyacetic acid as described by Friedman et al (13). They have shown that the X-base in E.coli tRNA is the only nucleoside which reacts with the N-hydroxysuccinimide ester of phenoxyacetic acid. The attempt to subsequently label this tRNA with FITC failed. The tRNA showed an $A_{490}/A_{260}$ ration of 0,0009 as measured in buffer A. This corresponds to 1,4% FITC labeling as compared with fully labelled $tRNA^{Phe}$ ($A_{490}/A_{260}$ = 0.066) or to 3.5% when compared with unfractionated $tRNA^{Phe}$ of which only 40 % can be labelled with FITC (see above). Further prove is provided by the fact that tRNAs not containing the X-base produce a comparable background of FITC labeling (see below).

Biochemical activity

Three tests of the FITC-$tRNA^{Phe}$ activity have been applied and show that it is a fully competent analogue of $tRNA^{Phe}$.

*(i) Aminoacylation*

The data in Table I show that $K_m$ and $V_{max}$ values for $tRNA^{Phe}$ are not appreciably affected by the FITC label.

*(ii) Polyphenylalanine synthesis*

FITC-$tRNA^{Phe}$ is also fully competent in its reaction with the ribosome, as measured by its ability to substitute for $tRNA^{Phe}$ in poly-U-programmed polyphenylalanine synthesis. The

TABLE I

Values for FITC-tRNA and $tRNA^{Phe}$

| $K_m$ and $V_{max}$ | | | |
|---|---|---|---|
| | units | $tRNA^{Phe}$ | FITC $tRNA^{Phe}$ |
| $K_m$ | µM | 0.16±0.02 | 0.11±0.04 |
| $V_{max}$ | pmole/min/ml | 13.7±0.6 | 9.2±1.1 |

Conditions are given in Methods. The values are calculated from a Hofstee analysis (28) of the initial rate data, using a standard linear regression program. Molarity of tRNA is calculated from 1 $A_{260}$ unit = 1000 pmoles.

rate of reaction was linearly dependent on tRNA concentration up to (at least) 0.5 µm (data not shown). Under conditions when tRNA is limiting (0.16 µm), FITC-$tRNA^{Phe}$ and $tRNA^{Phe}$ are equally proficient for polyphenylalanine synthesis. (Figure 2).

*(iii) Interaction with EF-Tu*

FITC-$tRNA^{Phe}$ was also shown to interact directly with EF-Tu·GTP (Fig. 3). EF-Tu alone elutes from the column with a peak at fraction 53 while Phe-$tRNA^{Phe}$ appears centered at fraction 70. After incubating with PEP and pyruvate kinase in the presence of

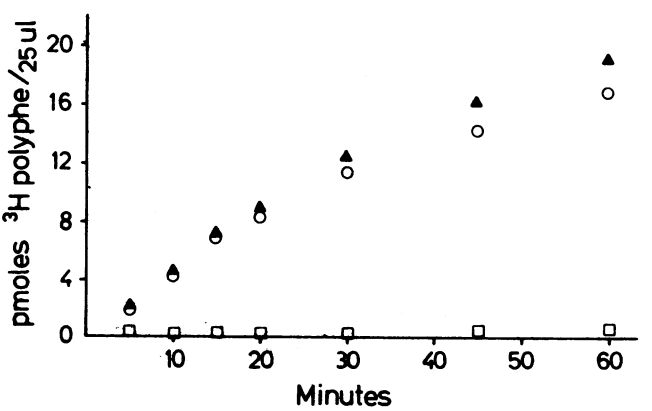


Figure 2. Rate of polyphenylalanine synthesis with limiting concentrations of $tRNA^{Phe}$ and FITC-$tRNA^{Phe}$. Polyphenylalanine synthesis was measured as described in Methods. ○ = 0.16 µM $tRNA^{Phe}$, ▲ = 0.16 µM FITC-$tRNA^{Phe}$, □ = no added tRNA.

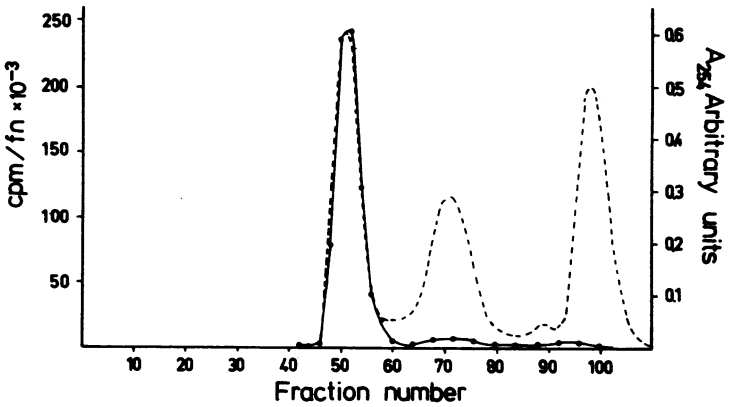


Figure 3. Complex formation between Phe-FITC-tRNA$^{Phe}$ and EF-Tu. See text for details. - - - adsorbance at 254 nm as measured by Uvicord photometer, -●- cpm [$^{3}$H]phenylalanine.

GTP followed by addition of Phe-FITC-tRNA extensive ternary complex formation was detected by the appearance of 90% of the [$^{3}$H]phenylalanine and 60% of the absorption at 254 nm at (or slightly ahead) of the position of EF-Tu.

The FITC-tRNA$^{Phe}$ used had not been purified on a BD cellulose column and so contained both FITC-tRNA$^{Phe}$ and unlabelled tRNA$^{Phe}$. It was charged with 800 pmoles/$A_{260}$ unit. Both the EF-Tu complex and the tRNA not involved in ternary complex formation showed FITC fluorescence. tRNA recovered from the complex by phenol extraction as well as the non bound tRNA were concentrated by ethanol precipitation. The tRNA derived from the EF-Tu complex had an $A_{490}/A_{260}$ ratio of 0.027 a measure of the amount of FITC bound to tRNA and was chargeable to 1250 pmoles/$A_{260}$ unit. The tRNA not involved in complex formation (from the free tRNA peak) had the same $A_{490}/A_{260}$ ratio and a charging capacity of 150 pmoles/$A_{260}$ unit. Thus EF-Tu had selected both FITC labelled and unlabelled tRNA$^{Phe}$ discriminating only on the basis of aminoacylation. The corresponding ratio for the FITC-tRNA$^{Phe}$ from peak II of the BD cellulose column, where all the tRNA molecules are labelled, is 0.066.

Spectral results

Absorption and fluorescence spectra

The absorption spectra of free FITC, FITC-tRNA$^{Phe}$ in the presence and absence of 20 mM $Mg^{++}$ are shown in the left of Fig. 4. $Mg^{++}$ produces an 60% increase in the absorption maximum at 490 nm of FITC-tRNA$^{Phe}$. The stochiometry of FITC reacted with tRNA was calculated from the extinction coefficient given for conjugated FITC (29) and the absorption of FITC-tRNA in the absence of $Mg^{++}$ (0.066 $A_{490}/A_{260}$).A value of 1.55 nmoles FITC/$A_{260}$ unit tRNA$^{Phe}$ was found.The same value (1.6 nmoles FITC/$A_{260}$unit tRNA$^{Phe}$)was obtained from the extinction coefficient of free FITC measured in buffer A (6.88 $\pm$ 0.4x10$^{4}$M$^{-1}$cm$^{-1}$)and the absorption of FITC-tRNA in the presence of $Mg^{++}$ (0.11 $A_{490}/A_{260}$).

Relative quantum spectra of the fluorescence emission are given in the right of Fig. 3. The shape is the same for FITC and

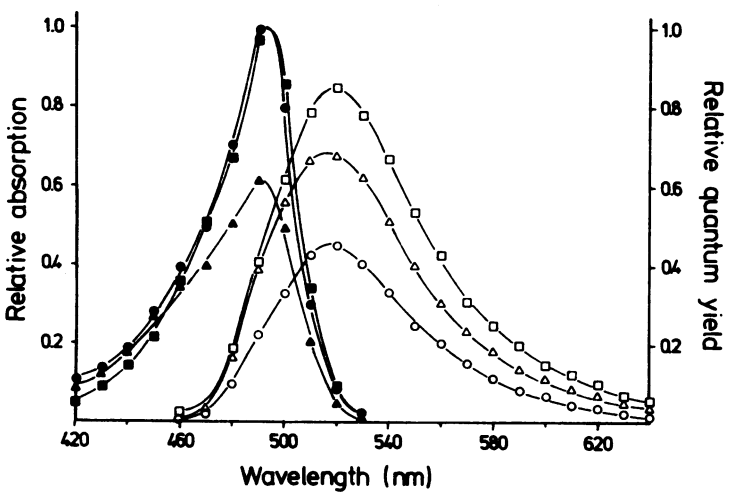


Figure 4. Absorption and quantum fluorescence spectra of FITC and of FITC-tRNA$^{Phe}$ in the presence and absence of $Mg^{++}$. The absorption of free FITC and FITC-tRNA$^{Phe}$ with 20 mM $Mg^{++}$ were both arbitrarily normalized to 1.0 and the absorption of FITC-tRNA$^{Phe}$ in the absence of $Mg^{++}$ expressed relative to that in the presence of $Mg^{++}$. Quantum fluorescence spectra were computed as described in the text. The quantum yields of FITC-tRNA$^{Phe}$ in the presence and absence of $Mg^{++}$ were expressed relative to the literature value for free FITC (20).

■ Absorption and □ fluorescence of free FITC.
▲ Absorption and ○ fluorescence of FITC-tRNA$^{Phe}$.
● Absorption and △ fluorescence of FITC-tRNA$^{Phe}$ with 20 mM $Mg^{++}$.

FITC-tRNA$^{Phe}$ in the presence and absence of $Mg^{++}$, but there are large differences in the quantum yields of the three species. The quantum yield (calculated as the area under the fluorescence curve) is highest for free FITC and is reduced to nearly one half when bound to tRNA$^{Phe}$. Addition of $Mg^{++}$ to FITC-tRNA$^{Phe}$ enhances the quantum yield as well as the absorption (Table II).

Since $Mg^{++}$ is most important for folding and tertiary structure of tRNA (30) it was of interest to investigate the influence of $Mg^{++}$ on the FITC-tRNA derivative. Both titration curves showing the effect of $Mg^{++}$ on fluorescence and absorption (Fig. 5) indicate the presence of at least two binding processes for $Mg^{++}$. A strong binding process saturating at about 0.5 mM and a weaker binding process saturating at about 30 mM.

FITC treated tRNAs lacking the X-base exhibit a fluorescence (measured at 520 nm) which amounts to 12% (E.coli tRNA$^{fMet}$) and 13% (yeast tRNA$^{Phe}$) of the fluorescence found for FITC labelled E.coli tRNA$^{Phe}$. This background fluorescence moreover is insen-

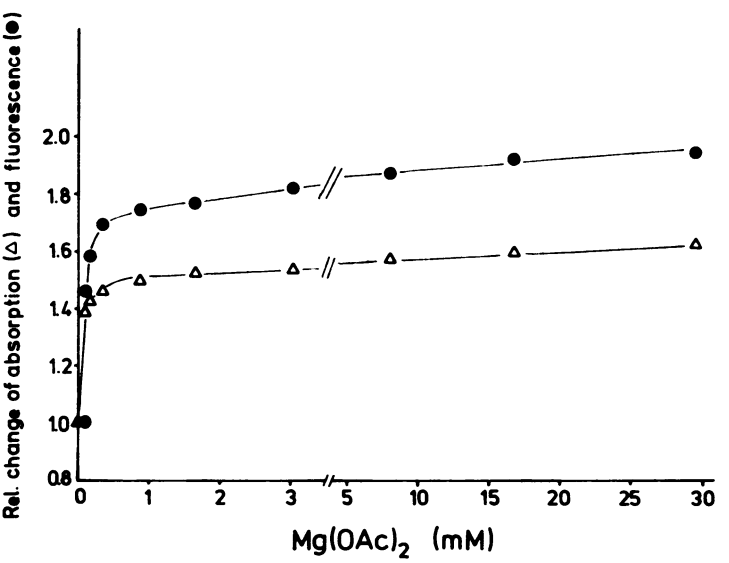


Figure 5. Effect of $Mg^{++}$ on the fluorescence and absorption of FITC-tRNA$^{Phe}$.
-●- Relative fluorescence intensity at 530 nm when exciting at 480 nm.
-△- Absorption at 492 nm.

sitive to $Mg^{++}$ in contrast to the FITC-fluorescence of E.coli $tRNA^{Phe}$. (Fig. 4 and Fig. 5).

Effect of $Mg^{++}$ on quantum yield

From the above results it is evident that the increase in fluorescence of FITC-$tRNA^{Phe}$ with $Mg^{++}$ is partly due to an increase in absorption and partly to a change in quantum yield. Evaluation of the lifetime of fluorescence ($\tau$) of FITC-$tRNA^{Phe}$ shows only small differences in the presence or absence of $Mg^{++}$ (Table II) which cannot account for the large change in the quantum yield. Closer analysis of the lifetime as a function of $Mg^{++}$ concentration shows a more complex behaviour (Fig. 6). There is a significant decrease in the lifetime at $Mg^{++}$ concentrations when the strong binding process is observed. The lifetime subsequently increases again at higher $Mg^{++}$ concentration. Further analysis indicates that more than one lifetime is involved and that the complex function seen in Fig. 6 probably is a weighted mean of at least two lifetimes.

The change in quantum yield must be due to a change in the natural lifetime, $\tau_e$, (i.e. the radiative lifetime in the absence of deactivation processes). Using the definition of quantum yield $Q=\tau/\tau_e$ and the data in Table II we calculate that $\tau_e$ for FITC-$tRNA^{Phe}$ is reduced from 7.5 nsec to 5.3 nsec when saturated with $Mg^{++}$. The decrease in natural lifetime can be understood from the increased absorption coefficient enhancing

TABLE II

Flurorescence properties of FITC-$tRNA^{Phe}$

| Unit | Q | $\tau$ nsec | $\tau_e$ nsec | r | $\tau_R$ nsec |
|---|---|---|---|---|---|
| Free FITC | 0.85 | N.D. | - | 0.03 | N.D. |
| FITC-$tRNA^{Phe}$ | 0.452 | 3.515±0.01 | 7.8 | 0.114 | 0.5±0.2 |
| FITC-$tRNA^{Phe}$ + 20 mM $Mg^{++}$ | 0.677 | 3.537±0.007 | 5.2 | 0.126 | 2.8±0.8 |

Quantum yields were related to free FITC as described in the text. The value for FITC is taken from (20). $\tau$ and $\tau_R$ were determined by pulsed fluorescence measurements.
Error limits are the standard errors for the determination.
$\tau_e$ was calculated from Q and $\tau$. N.D. = not determined.

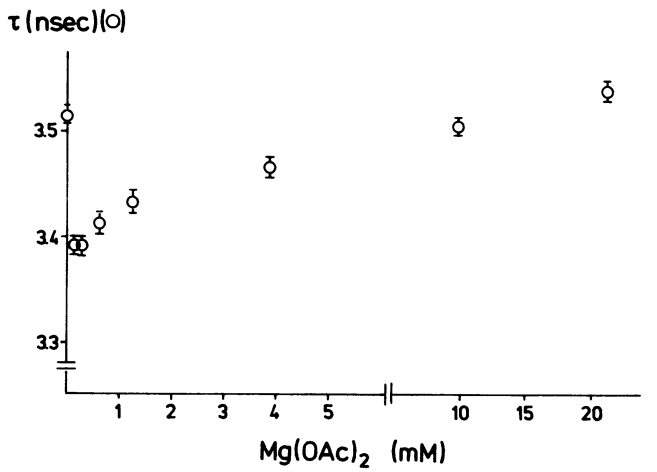


Figure 6. Effect of $Mg^{++}$ on the lifetime of fluorescence. Lifetimes were measured by pulsed fluorescence decay data. Error bars indicate the standard error in the determinations.

the emission rate, $k_e=1/\tau_2$ (21).

Rotational motion

Knowledge of the lifetime for molecular probes is of particular interest since it defines the time window in which rotational motion can be observed. Measurements of the anisotropy for FITC and FITC-tRNA with and without $Mg^{++}$ are given in Table II. For FITC-$tRNA^{Phe}$ a value of $r_o=0.3$ was extrapolated for infinitely high solvent viscosity. This agrees with the literature value of $r_o=0.35$ for fluorescein (31). Using equation (I) and the values of r in Table II, $\tau_R$ is estimated as 2.6 nsec for FITC-$tRNA^{Phe}$ in the presence of $Mg^{++}$. Direct determination of $\tau_R$ from the pulsed fluorescence measurements gives a similar value (2.8 nsec, Table II). It also suggests that the rotational motion of FITC is faster in the absence of $Mg^{++}$. A tumbling time of 2.8 nsec is very much faster than the 30 nsec expected for a molecule of the size of tRNA (32,33). Thus the label must have a considerable degree of freedom of motion even when attached to tRNA. The label cannot be intercalated with neighbouring base pairs.

DISCUSSION

Chemical modification of tRNA can affect its biological function. Full activity was found for the FITC derivative of E.coli $tRNA^{Phe}$ described in this paper. Using three different activity assays, aminoacylation, polyphenylalanine synthesis and complex formation with EF-Tu, no difference between parental and derivatized tRNA was detected. Moreover, after complex formation with EF-Tu the FITC-tRNA exhibits better aminoacylation properties than the parental $tRNA^{Phe}$. This observation provides a method of isolating fully functional tRNA from a partially degraded preparation. Unfortunately the time involved for gel filtration at pH 7.5 is such that a significant fraction of the charged tRNA ( 20%) in the complex is deacylated on emerging from the column.

The failure to label tRNA with FITC after blocking the X-base and of tRNAs without X-base as well as the observed stoichiometry of about 1 mole FITC/mole E.coli $tRNA^{Phe}$ shows that the X-base must be the labelled nucleoside.

A similar FITC derivative of E.coli $tRNA^{Tyr}$ has been described (9). This tRNA is also fully competent in aminoacylation and interaction with EF-Tu but, because the modification is adjacent to the anticodon, functions involving codon-anticodon interaction are inhibited.

A dansyl derivative of the X-base of E.coli $tRNA^{Phe}$ has been prepared and was found to be unchargeable (10). Like the fluorescamine derivatized E.coli $tRNA^{Phe}$ (11), the FITC labelled E.coli $tRNA^{Phe}$ exhibits unchanged biological activity. The reason for chosing FITC is its excellent fluorescent properties (high quantum yield and extinction coefficient) as well as the large overlap of the emission spectrum with the absorption spectrum of rhodamine isothiocyanate (RITC) thus providing an ideal couple for energy transfer measurements. An RITC derivative of E.coli $tRNA^{Phe}$ can be prepared as well (H.Bäumert and R.Rigler, to be published).

The response to $Mg^{++}$ clearly indicates the existence of weak and strong interactions occurring in the same concentration ranges as found for other tRNAs (34-36).

The fluorescence changes are very similar to those reported

for the Y-base of yeast $tRNA^{Phe}$ (37,38). Three strong binding sites for $Mg^{++}$ have been found by crystallographic analysis of yeast $tRNA^{Phe}$ (30) of which one is linking the dihydrouridine and TΨCG loops. It is tempting to postulate that it is this $Mg^{++}$ binding which causes the marked change in absorption of FITC-$tRNA^{Phe}$. For the free FITC hardly any change in absorption (about 5%) is observed on shifting the pH from 7.5 to 9.5. The same increase in pH produced an increase in FITC-$tRNA^{Phe}$ absorption in the absence of $Mg^{++}$ of about 60%, a value comparable to that observed when $Mg^{++}$ was added at pH 7.5. Although we cannot provide a direct explanation for this hyperchromic effect we conclude that both changes seen with pH and $Mg^{++}$ on FITC-tRNA reflect changes in this very important region of tertiary interactions which are monitored by the label.

The complex behaviour of the fluorescence lifetime (Fig.6) suggests the existence of different conformations of FITC-tRNA. Temperature relaxation studies (not shown) indicate the presence of two isomerisation steps with transitions in the time range of about 25 and 600 msec at 20°C. This is in agreement with previous observations on ethidium labelled yeast $tRNA^{Phe}$ (33, 36) pointing to the existence of three conformers of tRNA in solution.

The short rotational relaxation time of the fluorescence probe means it is sensitive to local changes which can affect its relatively free motion. Changes of this type can be conveniently monitored by following the anisotropy. Indeed preliminary data indicate that the label does become more rigid on binding to ribosomes.

ACKNOWLEDGEMENTS

We thank Prof. C.G.Kurland for valuable discussions and constant support. J.A.Plumbridge was the recipient of an EMBO Long Term Fellowship and H.G.Bäumert the recipient of a fellowship from Deutsche Forschungsgemeinschaft. Research was supported by funds from the Swedish Natural Sciences Research Council and the Swedish Cancer Society.

Present address: *Institut de Biologie Physico-Chemique,
13, rue Pierre et Marie Curie, F-75005 Paris, France.
**Institut für Biochemie, Johann Wolfgang Goethe Universität,
D-6 Frankfurt/M.-Niederrad 1, W.Germany.
Reprint requests should be sent to R.R.

REFERENCES

1. Biochemical Fluorescence (1977) Chen,R.F. and Edelhoch,H. Eds., Vol. 2, Marcel Dekker, New York.
2. Rigler,R. (1977) Trends in Biochemical Sciences 2, 252-254.
3. Favre,A. and Yaniv,M. (1971) FEBS Letters 17, 236-240.
4. Beardsley,K., Tao,T. and Cantor,C.R. (1970) Biochemistry 9, 3524-3532.
5. Wintermeyer,W. and Zachau,H.G. (1971) FEBS Letters 18, 214-218.
6. Wintermeyer,W. and Zachau,H.G. (1974) In Methods in Enzymology, Colowick,S.P. and Kaplan,N.O. Eds., Vol. 29, pp. 667-673,
7. Pachmann,U., Cronvall,E., Rigler,R., Hirsch,R., Wintermeyer, W. and Zachau, H.G. (1973) Eur.J.Biochem. 39, 265-273.
8. Yang,H.-C. and Söll,D. (1974) Biochemistry 13, 3615-3621.
9. Pingoud,A., Kownatzki,R. and Maass,G. (1977) Nucleic Acids Research 4, 327-338.
10. Schiller,P.W. and Schechter,A.N. (1977) Nucleic Acids Research 4, 2161-2167.
11. Sprinzl,M. and Faulhammer,H.G. (1978) Nucleic Acids Research 5, 4837-4851.
12. Ohashi,Z., Maeda,M., McCloskey,T.A. and Nishimura,S. (1974) Biochemistry 13, 2620-2625.
13. Friedman,S., Li,H.-T., Nakanishi,K. and Van Lear,G. (1974) Biochemistry 13, 2932-2937.
14. Fluorescent protein tracing (1976) Nairn,R.C. Ed., Chruchill Livingstone, Edinburgh-New York.
15. Arai,K.-I., Kawakita,M. and Kaziro,Y. (1972) J.Biol.Chem. 247, 7029-7037.
16. Hardy,S.J.S., Kurland,C.G., Voynow,P. and Mora,G. (1969) Biochemistry 8, 2897-2905.

17. Wood,W.B. and Berg,P. (1962) Proc.Nat.Acad.Sci. USA 48, 94-104.
18. Arai,K., Kawakita,M. and Kaziro,Y. (1974) J.Biochem. 76, 283-292.
19. Brot,N., Redfield,B. and Weissbach,H. (1971) Biochem.Biophys. Res.Comm. 41, 1388-1395.
20. Parker,C.A. and Rees,W.T. (1960) The Analyst 85, 587.
21. Förster,T. (1971) Fluoreszenz organischer Verbindungen. Vandenhoeck and Ruprecht, Göttingen.
22. Azumi,I. and McGlynn,S.P. (1962) J.Chem.Phys. 37, 2413-2420.
23. Rigler,R. and Ehrenberg,M. (1973) Quart.Rev.Biophys. 6, 139-199.
24. Perrin,P. (1936) J.Phys. Radium, Paris 7, 1-11.
25. Weber,G. (1952) Biochem.J. 51, 145-167.
26. Rigler,R. and Ehrenberg,M. (1976) Quart.Rev.Biophys. 9, 1-19.
27. Sprinzl,M., Personal communication.
28. Hofstee,B.H.J. (1952) Science 116, 329-331.
29. Tengerdy,R.P. and Chang,C.-A. (1966) Anal.Biochem. 16, 377-383.
30. Jack,A., Ladner,J.E., Rhodes,D., Brown,R.S. and Klug,A. (1977) J.Mol.Biol. 111, 315-328.
31. Luminescence of organic substances, Schmillen,A. and Legler,R., Eds., Landolt-Börnstein, Vol. 3, p. 276, Springer-Verlag, Berlin/Heidelberg 1967.
32. Tao,T., Nelson,J.H. and Cantor,C. (1970) Biochemistry 9, 3514-3524.
33. Rigler,R., Ehrenberg,M. and Wintermeyer,W. (1977) In Molecular Biology, Biochemistry and Biophysics, Pecht,I. and Rigler,R. Eds., Vol. 24, pp. 219-244, Springer-Verlag, Heidelberg.
34. Römer,R. and Hach,R. (1975) Eur.J.Biochem. 55 271-284.
35. Stein,A. and Crothers,D. (1976) Biochemistry 15, 157-160.
36. Ehrenberg,M., Rigler,R. and Wintermeyer,W. (1979) Biochemistry 18, 4587-4599.
37. Eisinger,J., Feuer,B. and Yamane,T. (1970) Proc.Nat.Acad. Sci. USA 65, 638-644.
38. Robinson,B. and Zimmerman,T.P. (1971) J.Biol.Chem. 246, 110-117.