Fluorescence labeling of an aminoacyl-tRNA at the 3′-end and its interaction with elongation factor Tu·GTP

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Received 19 September 1986

A new approach for the fluorescence labeling of an aminoacyl-tRNA at the 3′-end is applied to study its interaction with bacterial elongation factor Tu (EF-Tu) and GTP at equilibrium. The penultimate cytidine residue in yeast $tRNA^{Tyr}$-C-C-A was replaced by 2-thiocytidine (s^2C). The resulting $tRNA^{Tyr}$-C-s^2C-A was aminoacylated and then alkylated at the s^2C residue with *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (1,5-I-AEDANS). A >100% increase in the intensity of fluorescence emission of the modified Tyr-$tRNA^{Tyr}$-C-s^2C(AEDANS)-A was observed upon interaction with EF-Tu·GTP. A ternary complex dissociation constant of 1.27×10^{-8} M was calculated from this direct interaction. Using such fluorescent aminoacyl-tRNA, the affinity of any unmodified aminoacyl-tRNA can be determined by competition experiments. By this approach, we show here that the affinity of unmodified Tyr-$tRNA^{Tyr}$-C-C-A is identical to that of the modified Tyr-$tRNA^{Tyr}$. This indicates that the fluorescence labeling procedure applied does not alter the affinity of the aminoacyl-tRNA for EF-Tu·GTP. The introduction of 2-thiocytidine into nucleic acids and their labeling with spectroscopic reporter groups may provide a unique means of investigating various types of nucleic acid-protein interactions.

aminoacyl-tRNA *Elongation factor Tu* *2-thiocytidine* *Fluorescence titration*

1. INTRODUCTION

The bacterial elongation factor Tu (EF-Tu) forms a stable complex with aminoacyl-tRNA in the presence of GTP. This ternary complex binds to the ribosome [1] and participates in the decoding and proofreading steps of the elongation cycle of protein biosynthesis [2].

Primarily three different approaches have been used to estimate the affinity of EF-Tu·GTP for aminoacyl-tRNA: (i) the reduction by aminoacyl-tRNA of the amount of EF-Tu·GTP bound to nitrocellulose filters [3]; (ii) protection of aminoacyl-tRNA by EF-Tu·GTP from non-enzymatic deacylation [4]; (iii) protection of the 3′-end of aminoacyl-tRNA by EF-Tu·GTP from nuclease digestion [5]. By these methods, however, the concentration of the ternary complex is measured either indirectly or under non-equilibrium conditions.

Recently, Abrahamson et al. [6] investigated the stability of aminoacyl-tRNA·EF-Tu·GTP ternary complexes at equilibrium by monitoring a fluorescence change that accompanies the binding of EF-Tu·GTP to *Escherichia coli* Phe-$tRNA^{Phe}$ with a fluorescent dye covalently attached to 4-thiouridine (s^4U) at position 8. The fluorescence change of this tRNA upon ternary complex formation is most likely due to a conformational change near s^4U-8 base [7]. However, the occurrence of bases in tRNAs that can be labeled with fluorescent reporter groups or of fluorescing bases is very rare. Furthermore, the naturally occurring fluorescent Y-base [8] in the anticodon loop of yeast

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00145793/86/$3.50

$tRNA^{Phe}$ or a fluorescent dye introduced into the extra arm of *E. coli* $tRNA^{Phe}$ [9] do not show a change in fluorescence intensity upon binding to EF-Tu·GTP.

In this communication, we describe a general approach for the fluorescence labeling at the 3′-end of any aminoacyl-tRNA to study aminoacyl-tRNA·EF-Tu·GTP ternary complex formation at equilibrium.

2. MATERIALS AND METHODS

$tRNA^{Tyr}$-C-C-A from yeast (spec. act. 1485 pmol Tyr bound/A_{260} unit; 1 A_{260} unit of tRNA corresponds to 1500 pmol) was prepared as described [10].

Yeast ATP(CTP):tRNA nucleotidyltransferase (spec. act. 45 kU/mg) was kindly provided by Dr H. Sternbach (Max Planck Institut für Experimentelle Medizin, Göttingen). Yeast tyrosyl-tRNA synthetase (spec. act. 15 kU/mg) was purified as described [10], and *E. coli* EF-Tu·GDP (spec. act. 22 kU/mg) was prepared according to [11]. Pyruvate kinase was from Sigma (Taufkirchen, FRG) and inorganic pyrophosphatase from Boehringer (Mannheim, FRG). s^2CTP was synthesized as described previously [12]. [^{14}C]-Tyrosine (50 Ci/mol) was from Amersham (Braunschweig, FRG).

$tRNA^{Tyr}$-C was obtained from $tRNA^{Tyr}$-C-C-A by sodium periodate, lysine pH 9 and alkaline phosphatase treatments essentially as described [13]. $tRNA^{Tyr}$-C-s^2C-A was prepared by incubation of $tRNA^{Tyr}$-C in the presence of s^2CTP, ATP and ATP(CTP):tRNA nucleotidyltransferase [12].

2.1. *Aminoacylation of $tRNA^{Tyr}$-C-s^2C-A*

A solution (100 μl) containing 150 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 1 mM [^{14}C]tyrosine, 5 A_{260} units of $tRNA^{Tyr}$-C-s^2C-A, 200 mU of inorganic pyrophosphatase and 300 U of tyrosyl-tRNA synthetase was incubated at 37°C for 30 min. An aliquot was removed to determine the cold trichloroacetic acid-precipitable radioactivity and the remainder was phenol extracted. The aqueous phase was treated with ether and the tRNA was ethanol precipitated. The dried pellet was stored at −70°C. The yield of aminoacylation was 1485 pmol Tyr bound/A_{260} unit.

2.2. *Synthesis of 1,5-I-AEDANS*

1,5-I-AEDANS was synthesized by reaction of the *N*-hydroxysuccinimide ester of iodoacetic acid with *N*-(aminoethyl)-5-naphthylamine-1-sulfonic acid in analogy to [14].

2.3. *Alkylation of Tyr-$tRNA^{Tyr}$-C-s^2C-A with 1,5-I-AEDANS*

A solution (50 μl) containing 100 mM sodium acetate, pH 5.2, 100 mM NaCl, 10 mM $MgCl_2$, 5 A_{260} units of [^{14}C]Tyr-$tRNA^{Tyr}$-C-s^2C-A and 15 mM 1,5-I-AEDANS was incubated at 37°C for 4 h. The [^{14}C]Tyr-$tRNA^{Tyr}$-C-s^2C(AEDANS)-A was ethanol precipitated five times to remove unreacted 1,5-I-AEDANS. The dried pellet was stored at −70°C.

2.4. *Fluorescence measurements*

Fluorescence measurements were performed using an Aminco SPF 500 spectrofluorometer controlled by a Hewlett-Packard 9815 A microcomputer. The band pass was 5 nm for excitation and 10 nm for emission. The temperature was maintained at 5°C with a Haake type C circulating bath. All samples were excited at 335 nm and the emission spectra were run from 400 to 600 nm.

2.5. *Titration procedure*

E. coli EF-Tu·GTP was prepared by incubating EF-Tu·GDP (2.5 nmol) with 75 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM GTP, 5 mM phospho*enol*pyruvate and 15 U of pyruvate kinase in 100 μl at 37°C for 20 min. The complex was stored in ice and was stable for several hours under these conditions.

Titrations were performed in a 1 ml quartz cuvette at 5°C containing 50 mM sodium borate, pH 7, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM GTP and [^{14}C]Tyr-$tRNA^{Tyr}$-C-s^2C(AEDANS)-A. The emission was measured at 490 nm to determine the initial fluorescence intensity (F_0). Titrations were carried out by the sequential addition of the EF-Tu·GTP stock solution (in volumes ranging from 2 to 50 μl) to the cuvette. After each addition, the solution was gently mixed and the emission at 490 nm was measured after 2 min to ensure that equilibrium had been reached. Emission scans were run before adding EF-Tu·GTP and at the end of the measurements. No measurable deacylation of the tRNA was observed during the titra-

tion. In the competition experiments, the samples initially contained both [^{14}C]Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A and [^{14}C]Tyr-tRNA^{Tyr}-C-C-A. Titrations with EF-Tu·GTP were carried out as described above.

3. RESULTS AND DISCUSSION

It is now well established that the aminoacyl residue, its protonated α-amino group [1,4], the single stranded 3'-end, the continuous helix formed by the acceptor stem and the T stem provide the essential features in aminoacyl-tRNA required for its recognition by EF-Tu·GTP [15–17].

To study the interaction of aminoacyl-tRNAs with EF-Tu·GTP at equilibrium, we developed a new fluorescence titration assay. Toward this aim, 2-thiocytidine was incorporated into position 75 (the numbering of the nucleotides being according to [18]) of yeast tRNA^{Tyr}. 2-Thiocytidine is a modified nucleoside to which spectroscopic reporter groups can be attached under mild conditions [12,19]. The chemical reaction of 1,5-I-AEDANS with nucleic acids containing 2-thiocytidine is shown in fig.1. Although tRNA^{Tyr}-C-s^{2}C-A is fully aminoacylatable, tRNA^{Tyr}-C-s^{2}C(AEDANS)-A carrying a large aromatic residue on cytidine-75 is not a substrate for tyrosyl-tRNA synthetase. Therefore, to obtain Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A, the tRNA^{Tyr}-C-s^{2}C-A was first aminoacylated and then treated with 1,5-I-AEDANS. A similar procedure was used previously for the preparation of spin-labeled tRNA^{Phe} [20]. The fluorescence emission spectrum

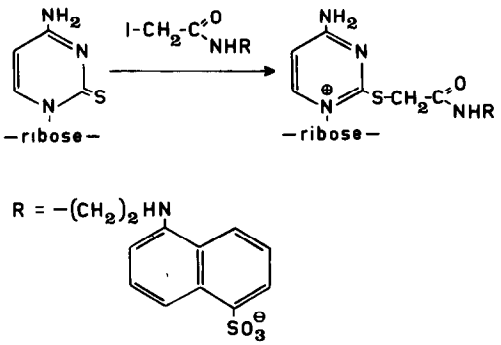

Fig.1. Fluorescence labeling of nucleic acids containing 2-thiocytidine by 1,5-I-AEDANS.

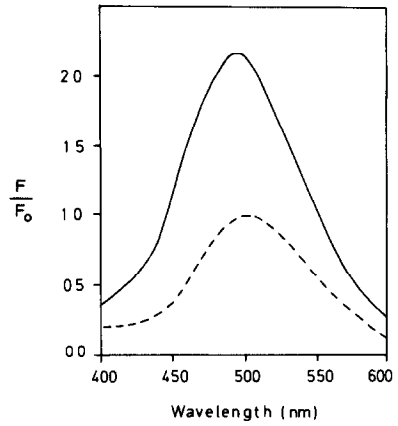

Fig.2. Fluorescence emission spectra (uncorrected) of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A (0.2 μM) before (– – –) and after (——) addition of excess (2 μM) EF-Tu·GTP. The excitation wavelength was 335 nm. The emission intensity of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A in the sample prior to EF-Tu·GTP addition is arbitrarily defined to be 1.

of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A is presented in fig.2. The fluorescence labeling of this tRNA occurred specifically at the s^{2}C residue of the 3'-end since no 1,5-AEDANS incorporation was observed into tRNA^{Tyr} with a normal 3'-C-C-A end (not shown).

Addition of EF-Tu·GTP to Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A resulted in a >100% enhancement of its fluorescence emission intensity (fig.2). This change in fluorescence is due to the binding of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A to EF-Tu·GTP in a ternary complex. No fluorescence change was observed when EF-Tu·GDP was added to Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A or when EF-Tu·GTP was added to uncharged tRNA^{Tyr}-C-s^{2}C(AEDANS)-A.

As shown in fig.3, the binding of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A to EF-Tu·GTP can be continuously monitored by the change in fluorescence emission intensity at 490 nm. In the presence of the non-fluorescent Tyr-tRNA^{Tyr}-C-C-A, the binding curve is expected to increase more slowly since it competes for the same binding site on the protein as the fluorescent tRNA. To generate the binding isotherms shown in fig.3, an equation relating the EF-Tu·GTP concentration (R) to the relative fluorescence intensity increase F/F_0, where F_0 is

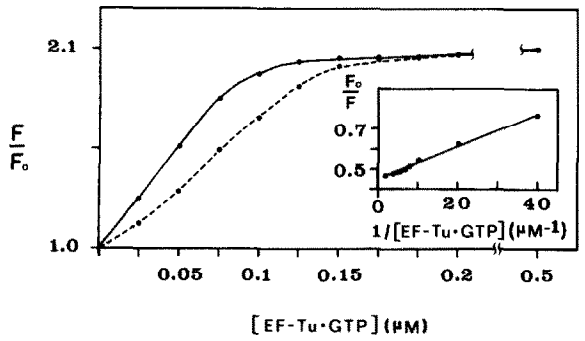

Fig.3. Titration of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A (0.05 μM) with EF-Tu·GTP in the absence (——) or presence (– – –) of Tyr-tRNA^{Tyr}-C-C-A (0.05 μM). The inset shows the double reciprocal plot of the binding data for Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A alone.

the initial and F the given relative fluorescence intensity, was derived:

$$R = K_w(F/F_0 - 1)/(F_\infty/F_0 - F/F_\infty) + W_0(F/F_0 - 1)/(F_\infty/F_0 - 1) + I_0/[1 + K_i/K_w(F_\infty/F_0 - F/F_0)/(F/F_0 - 1)] \quad (1)$$

In this equation K_w is the apparent dissociation constant of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A, F_∞/F_0 the maximal relative fluorescence change, W_0 the analytical concentration of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A, I_0 the analytical concentration of Tyr-tRNA^{Tyr}-C-C-A, and K_i the apparent dissociation constant of Tyr-tRNA^{Tyr}-C-C-A. In the absence of inhibitor (Tyr-tRNA^{Tyr}-C-C-A) the term $I_0/[1 + K_i/K_w(F_\infty/F_0 - F/F_0)/(F/F_0 - 1)]$ is equal to zero. By subjecting the data to a non-linear least-squares fitting analysis (Newton-Gauss method [21]) K_w was determined to be 12.7 nM. This value was then used as a constant for the calculation of K_i from data obtained in the competition experiment shown in fig.3. The K_i of Tyr-tRNA^{Tyr}-C-C-A was determined to be 11.8 nM.

The accuracy of the calculated dissociation constants depends on the accuracy of the given analytical values. The concentration of Tyr-tRNA^{Tyr}-C-C-A (I_0) was determined from the trichloroacetic acid-precipitable tyrosine radioactivity; it remained constant during the titration experiment since no measurable deacylation occurred. The concentration of Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A (W_0) was determined in two ways. First, W_0 could be measured by the trichloroacetic acid-precipitable tyrosine radioactivity under the assumption that the attachment of 1,5-AEDANS was complete [20]. Second, W_0 could also be calculated by a non-linear least-squares fitting analysis of the data according to eqn 1. Both methods led to the same value indicating that the assumption on the quantitative yield of the chemical modification of Tyr-tRNA^{Tyr}-C-s^{2}C-A was correct. Similarly, F_∞/F_0 was determined either from the experimental data as shown in the inset in fig.3 or by calculation using eqn 1. Again, both values were identical.

The very similar values of the dissociation constants for Tyr-tRNA^{Tyr}-C-s^{2}C(AEDANS)-A and Tyr-tRNA^{Tyr}-C-C-A, namely 12.7 and 11.8 nM respectively, indicate that the fluorescence labeling at the 3′-end of an aminoacyl-tRNA does not alter its affinity for EF-Tu·GTP. It can be assumed that the position 2 of the penultimate cytidine does not interact with EF-Tu·GTP. It was suggested that the -C-C-A end of tRNA can interact with 23 S ribosomal RNA via Watson-Crick-type pairing [22]. If this is the case, the 2-oxo functions of the C-74 and C-75 residues must be free during aminoacyl-tRNA·EF-Tu·GTP complex formation. Our present results, as well as the results of others [20], are in favor of this hypothesis.

The present study furthermore demonstrates that the introduction of 2-thiocytidine into any tRNA or other defined DNA or RNA molecules may provide a general means of labeling nucleic acids with spectroscopic receptor groups for the study of their interaction with proteins.

ACKNOWLEDGEMENTS

R.L.J. is grateful to the French Ministère de l'Industrie et de la Recherche for a fellowship, to Deutscher Akademischer Austauschdienst and the Ministère des Relations Extérieures for stipendiums under the French-German Cooperative programs (PROCOPE). This work was supported by the Deutsche Forschungsgemeinschaft (SFB 213-D5).

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