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CHARACTERIZATION OF THE FLUORESCENT BIMANE DERIVATIVE OF E. COLI INITIATOR TRANSFER RNA ($tRNA_f^{Met}$)#

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SUMMARY: The invariant modified base 4-thiouridine of the E. Coli initiator tRNA was chemically modified using a sulfhydryl specific fluorogenic probe, monobromobimane. The modified $tRNA_f^{Met}$ is virtually indistinguishable biochemically from the native form in the aminoacylation and formylation reactions, and in its binding behavior to the ribosomal P site. Fluorescence quenching by I^- increases 40% when the modified tRNA is charged with formylmethionine, even at this relatively well-shielded position in the tRNA elbow. Most important, the fluorescence polarization increases by a factor of 2, to almost the irrotational value, when fMet-$tRNA_f^{Met}$ binds to the ribosomal P site, providing a useful tool for studying fMet-$tRNA_f^{Met}$-ribosome interaction equilibria and kinetics.

Initiation is believed to be the rate limiting step in protein biosynthesis in E. Coli, and requires the formation of a complex between fMet-$tRNA_f^{Met}$, mRNA and ribosomes in a precise manner (1). Serious questions regarding the sequence and even the actors in this process remain (1,2). Recent filter binding studies have raised as many questions as they have answered (3-6). While many fluorescent derivatives of tRNA have been used to explore diverse aspects of ribosome structure and function, specific studies of the kinetics, steady states, and equilibria of initiation have, by and large, used mainly yeast AcPhe-$tRNA^{Phe}$, a good, but after all triply-exotic analogue (7-10); although fMet-$tRNA_f^{Met}$ labeled at $^4SU_8$ with AEDANS (11), and quite recently, with ethenoadenosine at A_{73} (12), have been suggested as possible candidates for kinetic studies.

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Abbreviations: Monobromobimane, 3,6,7 trimethyl-4 bromomethyl-1,5-diazabicyclo [3.3.0]octa-3,6-diene-2,8-dione; $tRNA_f^{Met}$-mB, B.S.A.-mB, and ^{4}Srd-mB represent bimane derivatives of initiator tRNA, bovine serum albumin, and 4-thiouridine, respectively.

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Bimanes, recently synthesized and introduced into biochemical use (13), bind to sulfhydryl groups with high specificity; because the ring system is quite small, neutral and closely but flexibly linked to the S atom, binding produces little strain either in the bimane or in the binding site, and tends not to interfere with later interactions involving other macromolecules. We report here the biochemical and spectroscopic characterization of $tRNA_f^{Met}$-mB, alkylated at the thio group of $^4SU_8$ with monobromobimane, which make it a useful close analogue of native initiator $tRNA_f^{Met}$.

MATERIALS AND METHODS

Ribosomes were prepared according to previously published methods (14). Crude Synthetase-Formylase extract and initiation factors were prepared by slight modification of the standard methods (15,16). Monobromobimane (Thiolyte) was purchased from Calbiochem-Behring Corp., and $tRNA_f^{Met}$ of E. Coli MRE 600 from Boehringer. All other chemicals were of reagent grade.

Preparation Of The Bimane Derivative Of $tRNA_f^{Met}$: To render the fairly inaccessible $^4SU_8$ residue reactive, derivatization was carried out in 90% DMSO (17,18). Typically 1.0 ml of the reaction mixture contained 1 mM phosphate (pH 7.4 at 22°C), 0.9 ml DMSO, 10 A_{260} $tRNA_f^{Met}$ and 0.5 mM monobromobimane (introduced as 50 mM solution in acetonitrile). These were allowed to react in the dark for ~ 5 h at room temperature, then dialysed against double distilled deionized water overnight to remove all reagents. The tRNA was then precipitated twice from alcohol under standard conditions (18) and stored.

Bioassay: Aminoacylation and subsequent formylation of the aminoacylated tRNA was done by standard methods (15). The degree of the aminoacylation was determined by TCA precipitation, and the extent of formylation of the aminoacylated tRNA determined by the Tris-Cu^{++} differential hydrolysis method (19).

Binding of the charged $tRNA_f^{Met}$, native or bimane modified, to the ribosome was studied using the Nitrocellulose filter binding assay (20). P site binding was tested using puromycin reactivity.

Fluorescence Measurements: All measurements were made in a Perkin Elmer MPF-44A fluorescence spectrophotometer equipped with a corrected spectra unit and the polarization accessory.

RESULTS AND DISCUSSION

Biochemical Properties: Bimane modified $tRNA_f^{Met}$ was almost indistinguishable from the native form: enzymatic methionylation exceeded 80% of the native values; both charged forms were equally formylated (≥ 85%). Binding to poly(A,U,G)-programmed ribosomes proceeded to the same rate and extent, and occurred exclusively at the ribosomal P site (defined as puromycin reactivity).

Spectroscopic Properties: The reaction of free ^4Srd with excess monobromobimane followed pseudo-first order kinetics to completion (Fig. 1). From the 260/ 380 nm absorbance ratio of $tRNA_f^{Met}$-mB it could be concluded that the derivatization exceeded 90%, with no evidence that binding occurred

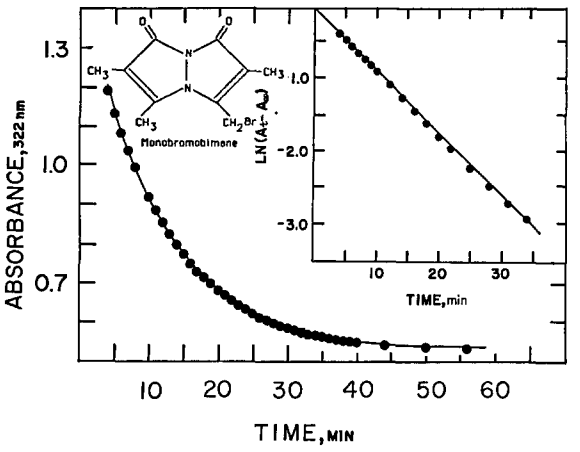

Fig.1. Kinetics of the reaction of 0.1 mM 4-thiouridine with 1 mM monobromobimane at 22°C in 60 mM phosphate buffer (pH 7.4). Inset shows a log plot of the absorbance and corresponds to a rate constant of 85.1 $M^{-1}min^{-1}$.

at sites other than the $^4SU_8$ position. Note that the excitation peaks at 267 and 380 nm (Fig. 2) are common to all bimane thioether derivatives including those of simple aliphatic mercaptans, and presumably represent optical transitions of the bimane itself, but that the 305 nm band is unique to both the free ^{4}Srd and the $tRNA_f^{Met}$ derivative. Excitation of the bimane derivative of $tRNA_f^{Met}$ at 305 nm, unlike that at 380 nm, results in completely

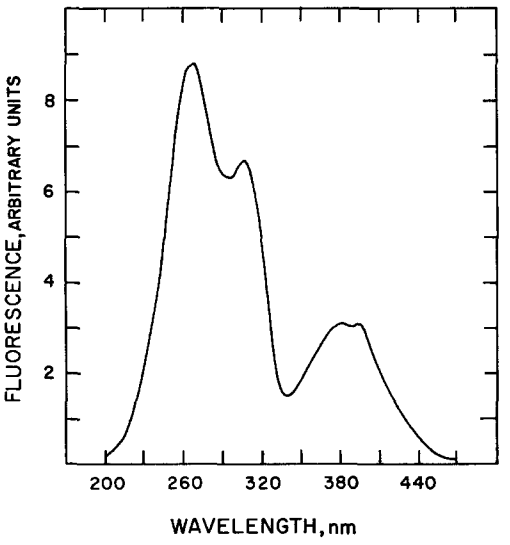

Fig. 2. Corrected fluorescence excitation spectrum of $tRNA_f^{Met}$-mB (0.8 A_{260}/ml) in a buffer containing 2 mM KAc (pH 5.5) and 1 mM $MgCl_2$. The emission wavelength was 480 nm.

Table 1

Sample	Excitation(nm)	P(480 nm)
^{4}Srd-mB	380	0.018
$tRNA_f^{Met}$-mB	380	0.171 ± 0.005
[^{3}H]fMet-$tRNA_f^{Met}$-mB	380	0.172 ± 0.005
[^{3}H]fMet-$tRNA_f^{Met}$-mB bound to the ribosomal P site	380	0.32 ± 0.01
B.S.A.- mB*	380	0.26
$tRNA_f^{Met}$-mB	305	0.00
[^{3}H]fMet-$tRNA_f^{Met}$-mB	305	0.00
[^{3}H]fMet-$tRNA_f^{Met}$-mB bound to the ribosomal P site	305	0.00
$tRNA_f^{Met}$-mB	267	0.05 ± 0.01
B.S.A.-mB*	267	0.04

* Since in this case the emission maximum is at 460 nm, the polarization was measured at this wavelength.

depolarized emission (see Table 1 and below); this must result from efficient energy transfer from the alkyl-thiouridine moiety to the bimane ring system.

Perrin analysis of the viscosity dependence of $tRNA_f^{Met}$-mB polarization yielded a linear plot of (1/P -1/3) vs T/η (T=293°K, 1.59 cp ≤ η ≤ 15.43 cp with sucrose) and a limiting polarization P_o of 0.46. Such a value of P_o indicates that the absorption and emission dipoles are nearly colinear in this compound, and so presumably in all syn-bimanes. The linearity of the plot is important because it indicates that the lifetime is long and that the fluorophore is rather firmly oriented in the tRNA. In fact, the polarization of the bimane attached to the species listed in Table 1 is consistent with their rigid-rotation correlation times (21,22). Changes in polarization thus directly report on tRNA dynamics in solution. In particular, the polarization of fMet-$tRNA_f^{Met}$-mB doubles on binding to the P site of the 70S ribosome: the bimane is still irrotationally bound in the complex. The two-fold increase is much larger than that observed for the proflavin derivative of yeast $tRNA^{Phe}$ (8); the net decrease in fluorescence intensity of the horizontal component is

about as large as the quantum yield increase of the ethanoadenosine derivative (12) on binding. This observed change in polarization is large enough to use in kinetic studies.

Iodide quenching of fluorescence is a measure of the accessibility of a fluorophore to I^- and therefore to the solvent. The Stern-Volmer quenching constant of bimane decreases 7 fold, from 69.4 M^{-1} to 10.1 M^{-1}, between ^{4}Srd and $tRNA_f^{Met}$ derivatives (Fig. 3), a result entirely consistent with the known shielded position of $^4SU_8$ in $tRNA_f^{Met}$ (23). Quenching is slightly greater in the charged fMet-$tRNA_f^{Met}$-mB (14.0 M^{-1}); since there is no change in polarization, this must involve a local change that does not increase the flexibility of the bound bimane. This result is consistent with the spin label studies on the $tRNA_f^{Met}$ (24). There is, however, no significant difference in the I^- quenchability of the fluorescence between the free and the ribosomal P site bound fMet-$tRNA_f^{Met}$-mB at low I^- concentrations (the difference at higher concentration may represent an artifact due to the complication arising from

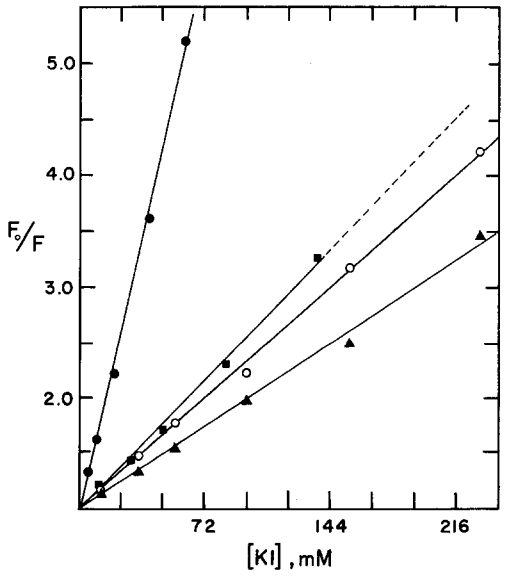

Fig. 3. Iodide quenching of the bimane fluorescence: The temperature was maintained at 25°C, the excitation and emission wavelengths were 380 nm and 480 nm, respectively. ●, 40 µM ^{4}Srd-mB in 60 mM phosphate buffer (pH 7.4). ▲, 0.10 A_{260}/ml $tRNA_f^{Met}$ in buffer containing 50 mM NH_4Cl/ 50 mM HEPES (pH 7.5)/ 5 mM $MgCl_2$. ○, 0.16 A_{260}/ml [^{3}H]fMet-$tRNA_f^{Met}$-mB in the same buffer as above. ■, Same solution as above, but also containing 200 µM GTP, 1.7 A_{260} poly(A,U,G), and 8 A_{260} of ribosomes in a total volume of 800 µl. Under these conditions, [^{3}H]fMet-$tRNA_f^{Met}$-mB was bound completely to the ribosomal P site.

the ribosomal dissociation under these conditions). This suggests that there is no appreciable change in the vicinity of the fMet-$tRNA_f^{Met}$ upon binding to the ribosomal P site. Consistent with this result is the fact that neither the fluorescence quantum yield nor the emission maximum of the modified tRNA, which are environment sensitive, change upon binding to the ribosomal P site. Similar I^- quenching results were obtained in studies with proflavin labeled in the dihydrouridine loop of the yeast NAcPhe-$tRNA^{Phe}$ (8).

It should be noted that after our study had been completed, the use of a somewhat different cationic bimane for double-label energy-transfer distance studies in $tRNA^{Phe}$ was reported. The degree of labeling was 35% and biochemical activity was not reported (25).

In conclusion, we report the characterization of the bimane derivative of the initiator tRNA of E. Coli which is biochemically indistinguishable from the native tRNA. This analogue provides a means for studying the $tRNA_f^{Met}$ conformational dynamics. Its emission polarization properties make it a suitable candidate for studying the tRNA-ribosome interactions which, we hope, will answer some fundamental questions regarding the initiation of protein synthesis.

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