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A synthetic alanyl-initiator tRNA with initiator tRNA properties as determined by fluorescence measurements: comparison to a synthetic alanyl-elongator tRNA

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ABSTRACT

Two synthetic tRNAs have been generated that can be enzymatically aminoacylated with alanine and have AAA anticodons to recognize a poly(U) template. One of the tRNAs ($tRNA_e^{Ala/AAA}$) is nearly identical to *Escherichia coli* elongator $tRNA^{Ala}$. The other has a sequence similar to *Escherichia coli* initiator $tRNA^{Met}$ ($tRNA_i^{Ala/AAA}$). Although both tRNAs can be used in poly(U)-directed nonenzymatic initiation at 15 mM Mg^{2+}, only the elongator tRNA can serve for peptide elongation and polyalanine synthesis. Only the initiator tRNA can be bound to 30S ribosomal subunits or 70S ribosomes in the presence of initiation factor 2 (IF-2) and low Mg^{2+} suggesting that it can function in enzymatic peptide initiation. A derivative of coumarin was covalently attached to the α amino group of alanine of these two Ala-tRNA species. The fluorescence spectra, quantum yield and anisotropy for the two Ala-tRNA derivatives are different when they are bound to 70S ribosomes (nonenzymatically in the presence of 15 mM Mg^{2+}) indicating that the local environment of the probe is different. Also, the effect of erythromycin on their fluorescence is quite different, suggesting that the probes and presumably the alanine moiety to which they are covalently linked are in different positions on the ribosomes.

INTRODUCTION

Recombinant DNA techniques have been used to introduce specific mutations into naturally occurring tRNAs. The mutant tRNAs were expressed in *E.coli* and then isolated so that the roles of specific bases and sequences in aminoacylation and peptide synthesis could be examined (reviewed in 1,2). Recently, adequate quantities of these recombinant tRNAs have been produced by *in vitro* transcription (3) for use in *in vitro* studies. Insight into the role of modified bases in tRNA structure (4,5), aminoacylation (3) and the ability of tRNA to accurately participate in peptide synthesis (6) has resulted.

Most studies examining the interactions of tRNAs with ribosomes and the reactions that make up peptide elongation have used filter binding assays or applied chemical modification of the tRNA/rRNA followed by analysis of protected regions of the RNA (reviewed in 7 and 8). Although effective and sensitive, these methods require the disruption of ongoing biological processes. An alternative method we have previously employed in examining tRNA structure and function is fluorescence. This technique was used to demonstrate a change in the conformation of $tRNA^{Phe}$ upon deacylation during peptidyl transfer (9) and movement of $tRNA^{Phe}$ relative to ribosomal protein L1 upon peptide bond formation (10). Fluorescent signals can be readily followed throughout a series of reaction steps with little or no perturbation of the ongoing reaction sequences.

We have previously reported the synthesis of polycysteine and polyserine from a poly(U) template using novel $tRNA^{Cys/AAA}$ and $tRNA^{Ser/AAA}$, respectively (11). By attaching a fluorescent probe to the amino group of Phe-tRNA or the novel Ser-tRNA, we were able to monitor the ribosomal environment surrounding the amino terminus of the nascent peptides formed with these synthetic tRNAs (11).

In order to further examine the initial steps of nascent peptide formation and tRNA positioning on the ribosome, we have generated two novel tRNAs that are enzymatically aminoacylated with alanine and possess AAA anticodons (see Figure 1). The first tRNA ($tRNA_i^{Ala/AAA}$) is analogous to the *E.coli* initiator $tRNA_f^{Met}$ (12). Although the primary structure of this tRNA was modified to facilitate *in vitro* transcription and aminoacylation, the features believed to be necessary for initiator function were maintained. These features include a non-Watson–Crick base pair at the end of the acceptor stem (13,14), a pyrimidine-11-purine-24 base pair in the dihydrouridine stem (12), and a series of guanine and cytosine residues in the anticodon stem forming three consecutive G-C base pairs (13). The second tRNA ($tRNA_e^{Ala/AAA}$) was constructed to be analogous to the *E.coli* elongator $tRNA^{Ala/UGC}$ (12). Here we report on the properties of the novel initiator $tRNA_i^{Ala/AAA}$ and the elongator $tRNA_e^{Ala/AAA}$ with respect to *in vitro* translation.

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MATERIALS AND METHODS

Materials

Oligodeoxyribonucleotides were synthesized on an Applied Biosystems (Foster, CA) 381A DNA synthesizer. Plasmid pUC18 was from Bethesda Research Laboratories (Gaithersburg, MD), PCR reagents were from Perkin-Elmer Cetus (Norwalk, CT) and the Sequenase II kit used for double-stranded sequencing was from United States Biochemicals (Cleveland, OH). [α-^{35}S]dATP was from New England Nuclear (Boston, MA) and [^{14}C]phenylalanine and [^{14}C]alanine were from ICN Radiochemicals (Irvine, CA). 3-(4-maleimidophenyl)-7-diethylamino-4-methylcoumarin (CPM) was from Molecular Probes (Junction City, OR). Yeast tRNA^{Phe} was from Sigma Chemical Company (St. Louis, MO). Purified *Escherichia coli* initiation factor 2 (IF-2) was a generous gift from Dr. C.Gualerzi (Max-Planck-Institut fur Molekulare Genetik, Berlin). The preparation of *Escherichia coli* ribosomal subunits has been described before (15). T7 RNA polymerase was prepared as described by Davanloo *et al.* (16).

tRNA preparation and aminoacylation

Plasmids containing the synthetic elongator tRNA^{Ala} (pALA) or synthetic initiator tRNA^{Ala} (pFMET) were constructed by applying previously described PCR and recombinant DNA strategies (11). The procedure used for *in vitro* transcription of the tRNAs has been reported previously (11). The resulting synthetic tRNAs (shown in cloverleaf form in Figure 1) were enzymatically aminoacylated with [^{14}C]alanine (50 Ci/mol) using the aminoacyl-tRNA synthetases present in the 70% saturated ammonium sulfate precipitate of a wheat germ S-150 fraction (17) as described previously for other synthetic tRNAs (11). Aminoacylation of tRNA$_e^{Ala/AAA}$ routinely reached about 60% (pmol Ala incorporated/pmol tRNA) using the wheat germ synthetases or the tRNA-depleted *E.coli* S-150 fraction as the source of aminoacyl-tRNA synthetase. Aminoacylation of tRNA$_i^{Ala/AAA}$ reached about 20% with either synthetase preparation. In no case was either tRNA observed to be aminoacylated with any amino acid other than alanine (data not shown). Yeast tRNA^{Phe} was aminoacylated as described in Picking *et al.* (18).

Fluorescence labeling of Ala-tRNA$_e^{Ala/AAA}$ and Ala-tRNA$_i^{Ala/AAA}$

Labeling with CPM of a mercaptoacetic acid covalently linked to the amino acid group of each of the two synthetic [^{14}C]alanyl-tRNAs was performed as described previously for natural (18) and synthetic tRNAs (11). The CPM-Ala-tRNA was purified by C_1 reversed-phase high performance liquid chromatography essentially as described for CPM-Ser-tRNA$^{Ser/AAA}$ (11). The [^{14}C]alanine concentration in the collected fractions was determined by liquid scintillation spectrometry, tRNA concentration was determined by absorbance at 260 nm (1 A_{260} = 1.5 nmol) and the CPM concentration was measured by absorbance at 390 nm (ϵ^M = 30,000). Only CPM-Ala-tRNA with a CPM:Ala:tRNA ratio of 1:1:1 was used in these studies.

Poly(U)-dependent polypeptide synthesis

Polypeptide synthesis was facilitated by prebinding N-acetyl derivatives of Phe-tRNA, Ala-tRNA$_e^{Ala/AAA}$ and Ala-tRNA$_i^{Ala/AAA}$. N-acetylation of the aminoacyl-tRNAs was carried out with the N-hydroxysuccinimide ester of acetic acid

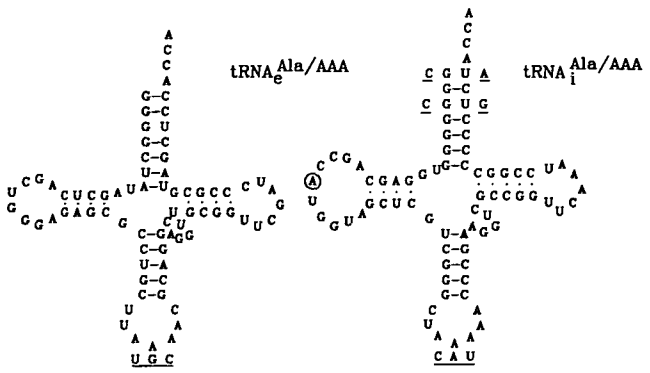

Figure 1. The nucleotide sequence of tRNA$_e^{Ala/AAA}$ and tRNA$_i^{Ala/AAA}$. The sequence of the synthetic elongator tRNA and initiator tRNA are given in cloverleaf form. Where sequence changes have been made to facilitate recognition of poly(U) and aminoacylation with alanine, the original bases are shown underlined next to the position they occupy in the natural tRNA. The circled A in tRNA$_i^{Ala/AAA}$ was inserted to destroy an internal BstNI restriction site which prevented formation of the full length DNA required for transcription with T7 polymerase. The addition of the A also created a new NcoI restriction site which was used to screen isolated plasmid inserts for the correct tRNA sequence.

according to the method of Rappoport and Lapidot (19). Nonenzymatic tRNA binding to ribosomes was performed in 50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, 5 mM 2-mercaptoethanol, 0.2 mg/ml poly(U), 0.5 μM ribosomes and 0.5 μM acylated tRNA. Polyalanine synthesis was initiated as described previously for polyphenylalanine (18) and polyserine (11) except that [^{14}C]alanine and either tRNA$_e^{Ala/AAA}$ or tRNA$_i^{Ala/AAA}$ were used as the amino acid and tRNA sources. tRNA$_e^{Ala/AAA}$ was used at a final concentration of 1 A_{260} units/ml while tRNA$_i^{Ala/AAA}$ was used at a concentration of about 3 A_{260} units/ml to reach nearly similar amounts of aminoacylated tRNA in all polyalanine assays. The incorporation of [^{14}C]alanine into polyalanine was determined as described previously (11).

Determination of nascent peptide chain length

For estimation of the average length of nascent polyalanine chains (given in Table II) it was necessary to experimentally determine the number of active ribosomes in the poly(U)-dependent polypeptide assay described above. To do this, 55 pmol of Ac[^{14}C]Phe-tRNA (100 Ci/mol), Ac[^{14}C]Ala-tRNA$_e^{Ala/AAA}$ or Ac[^{14}C]Ala-tRNA$_i^{Ala/AAA}$ (50 Ci/mol) was prebound to poly(U)-programmed ribosomes (50 pmol) by incubation at 37°C for 15 min prior to the addition of the other components required for protein synthesis. After prebinding the Ac[^{14}C]aminoacyl-tRNA, polypeptide synthesis was carried out using nonradioactive alanine. This provides a measure of those ribosomes on which the amino acid from prebound [^{14}C]-labeled Ac-aa-tRNA was subsequently incorporated into nascent polyalanine. When the total pmol of alanine incorporated into polyalanine (described above) is measured, the value can be divided by the number of active ribosomes to give an estimate of the average nascent polyalanine chain length.

Fluorescence measurements

A model 8000 photon-counting spectrofluorometer from SLM-Aminco Instruments, Inc. (Urbana, IL) was used for steady-state fluorescence measurements as described (20). Fluorescent tRNAs

Table I. Fluorescence Properties of CPM-Ala-tRNA$_e^{Ala/AAA}$ and CPM-Ala-tRNA$_i^{Ala/AAA}$

tRNA species	position	anisotropy	quantum yield	Em_{max}(nm)
CPM-Ala-tRNA$_e^{Ala/AAA}$	free	0.185	0.393	479
	bound (P site)	0.346	0.503	473
	bound (A site)[1]	0.312	0.472	475
CPM-Ala-tRNA$_i^{Ala/AAA}$	free	0.205	0.449	478
	bound (P site)	0.310	0.516	475
	bound (A site)[1]	0.208	0.466	478

[1] Deacylated tRNA^{Phe} was prebound to poly(U)-programmed ribosomes in a 1.1:1 ratio prior to the binding of the fluorescent tRNA species.

were bound to ribosomes as described above except that the concentration of these tRNAs was reduced to about 10% of the ribosome concentration to ensure maximum tRNA binding. Spectra were measured at 1-nm emission intervals at a scanning rate of 0.5 sec per wavelength increment with an excitation wavelength of 385 nm. Anisotropy and relative intensity measurements were carried out at an emission wavelength of 475 nm. Measurements were automatically corrected for the wavelength dependence of photomultiplier sensitivity. Fluorescence measurements were carried out at 20°C in a volume of 0.6 ml. All measurements were made with a sample absorbance of less than 0.1 at the excitation wavelength and fluorescence anisotropy measurements were made as previously described (18). The quantum yields of CPM-Ala-tRNA$_e^{Ala/AAA}$ and CPM-Ala-tRNA$_i^{Ala/AAA}$ were determined by comparison with quinine sulfate in 0.05 M H_2SO_4, which has a quantum yield of 0.508 at 25°C (21).

tRNA binding to 30S and 70S ribsomes by IF-2

CPM-Ala-tRNA$_e^{Ala/AAA}$ and CPM-Ala-tRNA$_i^{Ala/AAA}$ (25 pmol) were used to examine the effect IF-2 has on tRNA binding to 30S and 70S ribosomes. Fluorescence anisotropy and quantum yield were measured in the presence and absence of purified IF-2 (105 pmol) after the sequential addition of 30S subunits (195 pmol) and 50S subunits (195 pmol). Measurements were taken in 50 mM Tris-HCl (pH 7.5), 6 mM $Mg(OAc)_2$, 50 mM NH_4Cl, 1 mM GTP, 5 mM dithioerythritol and 0.2 mg/ml poly(U) in a final volume of 600 μl. When these measurements were finished, the Mg^{2+} concentration was raised to 15 mM to nonenzymatically facilitate tRNA binding and the anisotropy and quantum yield were measured again.

RESULTS

Fluorescence properties and binding to ribosomes of the synthetic tRNAs

Synthetic initiator tRNA^{Ala} (tRNA$_i^{Ala/AAA}$) and elongator tRNA^{Ala} (tRNA$_e^{Ala/AAA}$) were constructed; their sequences are shown in the cloverleaf form (Figure 1). To monitor binding of the synthetic tRNAs to the 70S ribosomes, each was fluorescently labeled with CPM. The fluorescence anisotropy and quantum yield were measured for each tRNA when free in solution, or bound to the A and P site of 70S ribosomes, respectively (Table I). Interestingly, these parameters differed for the two tRNAs under the conditions examined. The anisotropy of free CPM-Ala-tRNA$_e^{Ala/AAA}$ (0.185) was similar to that seen previously for the natural elongator tRNA CPM-Phe-tRNA^{Phe} (0.181) as well as the synthetic elongator tRNA CPM-Ser-tRNA$^{Ser/AAA}$ (0.175). Upon binding to 70S ribosomes the anisotropy and quantum yield

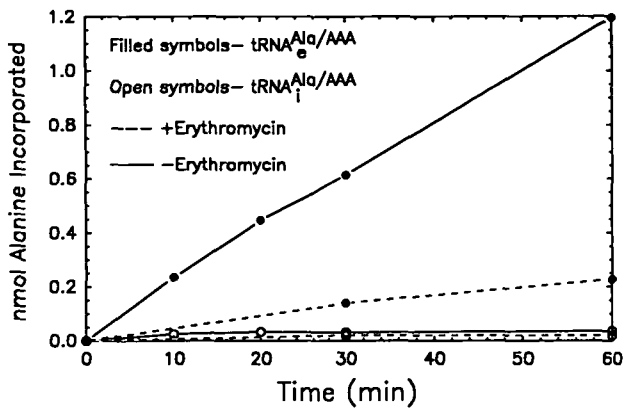

Figure 2. The poly(U)-directed synthesis of polyalanine with tRNA$_e^{Ala/AAA}$. AcPhe-tRNA (0.5 μM) was prebound to poly(U)-programmed ribosomes (0.5 μM) to facilitate the synthesis of polyalanine with either 0.3 A_{260} of tRNA$_i^{Ala/AAA}$ (open symbols) or 0.1 A_{260} tRNA$_e^{Ala/AAA}$ (closed symbols) as the elongator tRNA source in a final volume of 0.1 ml. [^{14}C]Alanine incorporation was measured after incubation at 37°C for the times indicated by deacylating all the aminoacylated tRNA with 0.5 M NaOH, then precipitating polyalanine with 5 ml ice cold 10% trichloroacetic acid and determining the radioactivity of the precipitate. Polyalanine synthesis in the presence of 4 μM erythromycin was also measured for comparative purposes (- - -).

of the CPM-Ala-tRNA$_e^{Ala/AAA}$ increased in the same way as previously studied tRNAs (18; Table I). Conversely, CPM-Ala-tRNA$_i^{Ala/AAA}$ had a higher anisotropy when free in solution (0.205) and a lower anisotropy when bound at the ribosomal P site (0.310) in comparison to free (0.185) and P site-bound (0.346) CPM-Ala-tRNA$_e^{Ala/AAA}$ (Table I). P site binding was confirmed by adding puromycin which resulted in a large decrease in the anisotropy of bound CPM-Ala-tRNA$_e^{Ala/AAA}$ (to 0.175) and CPM-Ala-tRNA$_i^{Ala/AAA}$ (to 0.183) as each CPM-Ala-puromycin product was formed and subsequently released from the ribosome (data not shown).

When deacylated tRNA^{Phe} was prebound to the 70S ribosomes to promote A site binding of CPM-Ala-tRNA$_i^{Ala/AAA}$, no change in the quantum yield, anisotropy or emission spectrum was observed indicating that no binding had occurred (Table I). This was confirmed by experiments demonstrating that, after pre-binding of deacylated tRNA^{Phe}, the CPM-Ala-tRNA$_i^{Ala/AAA}$ did not remain with the ribosome fraction when the reaction components were separated by gel filtration on Sephacryl S-300 (data not shown). Conversely, CPM-Ala-tRNA$_e^{Ala/AAA}$ appeared to bind at the ribosomal A site as judged by increased anisotropy (0.312) and quantum yield (0.472) (Table I). Typically less than 10% of this A site-bound CPM-Ala-tRNA$_e^{Ala/AAA}$ was reactive with puromycin (data not shown).

Table II. Polyalanine Synthesis after Prebinding Three Different N-blocked Aminoacyl-tRNAs

Initiating tRNA	pmol Ala incorporated	Nascent chains initiated pmol	Nascent chain length
AcPhe-tRNA	676	18	38
AcAla-tRNA$_e^{Ala/AAA}$	626	13	48
AcAla-tRNA$_i^{Ala/AAA}$	614	14	44

Ac[^{14}C]aminoacyl-tRNA was used in the presence of nonradiative alanine to determine the number of nascent chains initiated. [^{14}C]Alanine was used to measure pmol Ala incorporated into polyalanine. The average length of nascent polyalanine is given by pmol Ala/pmol nascent chains as described in Methods.

Table III. IF-2 Mediates the Binding of CPM-Ala-tRNA$_i^{Ala/AAA}$ to Ribosomes

tRNA	addition	anisotropy	quantum yield
CPM-Ala-tRNA$_i^{Ala/AAA}$	none	0.206	0.449
	+30S	0.204	0.450
	+50S	0.238	0.453
	+9mM Mg^{2+}	0.308	0.503
	+IF-2 & 30S	0.232	0.462
	+50S	0.300	0.516
	+9 mM Mg^{2+}	0.306	0.512
CPM-Ala-tRNA$_e$Ala/AAA	none	0.182	0.393
	+30S	0.187	0.389
	+50S	0.200	0.393
	+9 mM Mg^{2+}	0.340	0.483
	+IF-2 & 30S	0.188	0.393
	+50S	0.197	0.397
	+9 mM Mg^{2+}	0.343	0.488

IF-2 and/or 30S ribosomal subunits were incubated with the fluorescent tRNA for 15 min at 37°C with 6 mM Mg^{2+} present before measuring fluorescence. After each subsequent addition, the reaction mixture was incubated 10 min at 37°C and then 15 min at 20°C (in the cuvette used for fluorescence measurements) prior to reading the fluorescence. All reactions were carried out in a final volume of 600 μl as described in Methods. Following the addition of ribosomes, the Mg^{2+} concentration was increased 9 mM (to give a final concentration of 15 mM) to allow for the nonenzymatic binding of the fluorescent tRNAs. The anisotropy and quantum yield were then measured again for comparative purposes.

The data suggest that the synthetic initiator tRNA has retained the binding properties of natural tRNA$_f^{Met}$. These properties are paralleled by fluorescence characteristics that differ from those of the elongator Ala-tRNA.

Poly(U)-dependent translation

AcPhe-tRNA was prebound to poly(U)-programmed *E.coli* ribosomes (in the presence of 15 mM Mg^{2+}) to facilitate polypeptide synthesis as outlined under Methods. Then polyalanine synthesis was attempted with either tRNA$_e^{Ala/AAA}$ or tRNA$_i^{Ala/AAA}$. Only tRNA$_e^{Ala/AAA}$ supported poly(U)-dependent polyalanine synthesis (Figure 2). Synthetic elongator tRNA-dependent polyalanine synthesis was linear for more than 60 min at 37°C (Figure 2) and for longer than 2 h at 20°C (data not shown). Conversely, when tRNA$_i^{Ala/AAA}$ was used, polyalanine synthesis was only slightly above background levels (Figure 2). An interesting feature of polyalanine synthesis in this system is that it is sensitive to inhibition by erythromycin (Figure 2). This is in contrast to polyphenylalanine synthesis which is resistant to erythromycin, presumably because of the extremely atypical physical and chemical properties of this polypeptide (discussed in 15). The synthesis of other synthetic nad natural polypeptides is inhibited by erythromycin (22).

We then determined if AcAla-tRNA$_i^{Ala/AAA}$ or AcAla-tRNA$_e^{Ala/AAA}$ could be nonenzymatically prebound to ribosomes to facilitate polyalanine synthesis. Incorporation of alanine into polyalanine from Ala-tRNA$_e^{Ala/AAA}$ was similar whether AcAla-tRNA$_e^{Ala/AAA}$, AcAla-tRNA$_i^{Ala/AAA}$ or AcPhe-tRNA was prebound to poly(U)-programmed 70S ribosomes (Table II). The length of the nascent peptides produced was similar regardless of the initiating tRNA (Table II). The important feature of these data is that while tRNA$_i^{Ala/AAA}$ is not functional for peptide elongation, it is able to bind at the ribosomal P site to allow nonenzymatically-initiated peptide synthesis. It should be noted, also, that polyalanine synthesis was inhibited by erythromycin, regardless of the initiating acyl-tRNA (even AcPhe-tRNA; data not shown).

IF-2 mediates the binding of CPM-Ala-tRNA$_i^{Ala/AAA}$ to ribosomes

The ability of tRNA$_i^{Ala/AAA}$ to function in enzymatic peptide initiation was tested using purified *E.coli* initiation factor 2 (IF-2) to bind the tRNA to 30S and 70S ribosomes (in 6 mM Mg^{2+}). IF-2 facilitated binding of CPM-Ala-tRNA$_i^{Ala/AAA}$ to 30S subunits as judged by increased anisotropy and quantum yield (Table III). When 50S subunits were then added to the reaction mixture, over 80% of the CPM-Ala-tRNA$_i^{Ala/AAA}$ was bound to the 70S ribosomes in 6 mM Mg^{2+} as indicated by increased fluorescence anisotropy (Table III). Only a slight further increase in binding was observed when the Mg^{2+} concentration was

increased to 15 mM to mediate the nonenzymatic binding of the tRNA as discussed above (Table III). With poly(U), at 6 mM Mg^{2+}, CPM-Ala-$tRNA_i^{Ala/AAA}$ did not bind to 30S ribosomal subunits in the absence of IF-2 and only poorly to 70S ribosomes (Table III). Only after increasing the Mg^{2+} to 15 mM did the initiator tRNA bind to poly(U)-programmed ribosomes in the absence of IF-2 (Table III). These data indicate that IF-2 mediates the binding of CPM-Ala-$tRNA_i^{Ala/AAA}$ to 30S ribosomal subunits or to 70S ribosomes in 6 mM Mg^{2+}.

CPM-Ala-$tRNA_e^{Ala/AAA}$, in contrast, did not bind to 30S or 70S ribosomes in 6 mM Mg^{2+} in the presence or absence of IF-2 (Table III); however, in each case it did bind upon increasing the Mg^{2+} concentration to 15 mM. At the high Mg^{2+} concentration, the fluorescence anisotropy and quantum yield of CPM-Ala-$tRNA_e^{Ala/AAA}$ increased to the values previously observed for ribosome binding (see Table I).

The effect of ribosome binding and the subsequent binding of erythromycin or sparsomycin with puromycin on the fluorescence of the synthetic initiator and elongator tRNAs

Fluorescence from the coumarin moiety of CPM is very sensitive to hydrophobicity, charge, and other factors in the immediate vicinity of the probe. Entrance into a region of increased hydrophobicity typically results in a blue shift of the emission spectrum of CPM accompanied by an increase in fluorescence intensity, while a red shift and decreased intensity are usually indicative of entrance into a more polar environment. This environmental sensitivity was used to compare the two CPM-Ala-tRNAs under different conditions. The fluorescence emission spectra of CPM-Ala-$tRNA_e^{Ala/AAA}$ and CPM-Ala-$tRNA_i^{Ala/AAA}$ were measured when the tRNAs were free in solution, bound to poly(U)-programmed ribosomes, bound to ribosomes simultaneously with erythromycin and bound to ribosomes simultaneously with sparsomycin and puromycin (Figure 3A and B). Sparsomycin has been shown in this laboratory to bind to 70S ribosomes and inhibit puromycin reactivity with P site-bound N-acyl-Phe-tRNA without preventing the binding of puromycin (Odom and Hardesty, manuscript in preparation).

The intensity of the emission spectrum of CPM-Ala-$tRNA_e^{Ala/AAA}$ is increased and accompanied by a considerable blue shift (6 nm) upon binding to ribosomes (Figure 3A). The emission of CPM-Ala-$tRNA_i^{Ala/AAA}$ undergoes a similar although somewhat smaller increase in fluorescence intensity upon binding to ribosomes (Figure 3B), accompanied by a smaller blue shift (3 nm). When erythromycin was bound to ribosomes already containing CPM-Ala-$tRNA_e^{Ala/AAA}$, a 15% increase in the fluorescence intensity was observed, accompanied by a further 3 nm blue shift in the emission maximum (Figure 3A). Erythromycin resulted in only a 3% increase in the relative intensity of bound CPM-Ala-$tRNA_i^{Ala/AAA}$ accompanied by a 2 nm blue shift in the emission spectrum (Figure 3B). These observations suggest that the CPM-Ala moiety on the initiator tRNA is in a somewhat different position on the ribosome relative to the bound erythromycin. The fluorescence of each tRNA (after binding to poly(U)-programmed ribosomes) was also examined following the sequential addition of sparsomycin and puromycin. Sparsomycin binding had little effect on the emission spectrum of either fluorescent tRNA (data not shown). Sparsomycin, however, is an inhibitor of puromycin reactivity, and allows for examination of the effect of puromycin binding on the fluorescence of each tRNA (Figure 3A and B). Puromycin binding resulted in a 26% increase in the fluorescence intensity

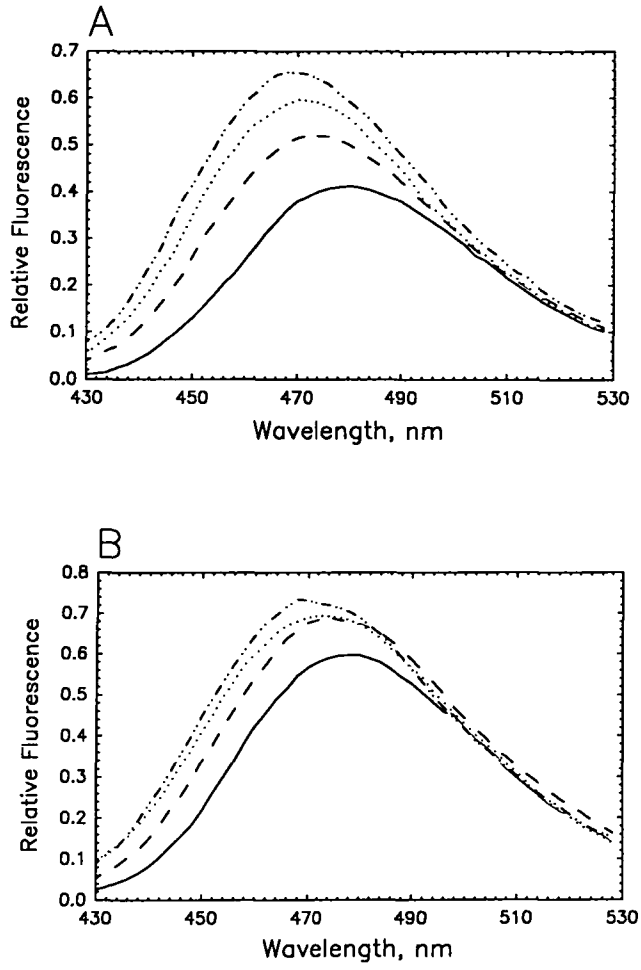

Figure 3. The emission spectra of free and ribosome-bound elongator and initiator CPM-Ala-$tRNA^{Ala/AAA}$. The emission spectrum of CPM-Ala-$tRNA_e^{Ala/AAA}$ (panel A) and CPM-Ala-$tRNA_i^{Ala/AAA}$ (panel B) (30 pmol each) was measured as described in Methods. Measurements were in a final volume of 600 μl for the free (——) and ribosome-bound (- - -) tRNAs. Ribosomes were present at 0.5 μM. After the ribosomes were added, the reaction mixtures were incubated for 15 min at 37°C. Fluorescence measurements were carried out at 20°C with an excitation wavelength of 385 nm. After the measurements were complete, erythromycin was added to a final concentration of 4 μM and the effect of erythromycin binding on the spectrum of each ribosome-bound tRNA was monitored (· · ·). In another experiment the emission spectrum of each tRNA was measured after ribosome binding. Sparsomycin was subsequently added (final concentration = 5 μM). The emission spectrum was taken. Then puromycin was added to give a concentration of 0.5 mM. The emission spectrum was again measured (- · - · -). After measuring the emission spectrum in the presence of puromycin, the fluorescence anisotropy of each sample was measured to assure that no drop in anisotropy had occurred which would indicate puromycin reactivity.

of bound CPM-Ala-$tRNA_e^{Ala/AAA}$ with a 4 nm blue shift in the emission maximum (Figure 3A). No change in anisotropy was observed, which confirms sparsomycin inhibited puromycin reactivity. Under identical conditions, puromycin binding resulted in only a 6% increase in the fluorescence of bound CPM-Ala-$tRNA_i^{Ala/AAA}$ with a 4 nm blue shift (Figure 3B).

The results with erythromycin and sparsomycin/puromycin support the conclusion that the CPM-Ala portion of this initiator tRNA is held in a different position on the ribosome than is the same portion of the elongator tRNA. An interesting feature of these results is that polyalanine synthesis initiated with either

AcAla-tRNA is inhibited by erythromycin and each N-acyl-tRNA is puromycin-reactive (in the absence of sparsomycin) when bound in the ribosomal P site. This suggests the CPM-Ala portions of the two synthetic tRNAs are held differently with respect to the binding sites of erythromycin and puromycin without any obvious effect on the function of either antibiotic.

DISCUSSION

We have generated analogues of *E.coli* initiator $tRNA^{Met}$ and $tRNA^{Ala}$ that can be enzymatically aminoacylated with alanine. Alanine was chosen because of the well characterized aminoacylation requirements of $tRNA^{Ala}$ (23), and both tRNAs were given AAA anticodons to promote recognition by poly(U). Although GAA is the naturally-occurring anticodon for UUU and UUC codons, we chose not to depend upon wobble for the recognition of poly(U) by the two synthetic alanyl tRNAs. The results presented above indicate $tRNA_i^{Ala/AAA}$ has initiator function, and only $tRNA_e^{Ala/AAA}$ functioned in peptide elongation.

The fluorescence properties of a CPM residue on the α-amino group of these two aminoacylated tRNAs reflect the local environment around the fluorophore when the tRNAs are either free in solution or bound to ribosomes. CPM-Ala-$tRNA_e^{Ala/AAA}$ binds to 70S ribosomes with many of the same properties we have previously reported for CPM-Phe-tRNA (18) and synthetic CPM-Ser-$tRNA^{Ser/AAA}$ (11). For CPM-Ala-$tRNA_i^{Ala/AAA}$, however, the fluorescence anisotropy data indicate that the fluorophore is held more rigidly than that of its elongator counterpart when each is free in solution. Conversely, the fluorophore on the initiator tRNA analogue appears to be able to move more freely than the same probe on the elongator tRNA when bound to 70S ribosomes. In addition, although each fluorescent tRNA binds to the ribosomal 'P site', each responds differently to the subsequent binding of erythromycin. Considered together these results suggest that the CPM probe and presumably the alanine to which it is linked are not in identical positions when the two tRNAs are in the ribosomal 'P site'. This difference in binding relative to the binding site of erythromycin does not prevent the antibiotic from inhibiting polyalanine synthesis started after prebinding N-blocked elongator Ala-tRNA, initiator Ala-tRNA, or Phe-tRNA.

We propose that the different fluorescence properties reported here may be general features of elongator and initiator tRNAs and that they are a direct result of the special sequence and structural features of these tRNAs. The results also suggest that neither methionine nor the AUG codon are uniquely required for peptide initiation with IF-2. Indeed, initiator tRNAs mutated to recognize UUC and GUC codons and aminoacylated with phenylalanine and valine, respectively, have been shown to initiate enzymatically active β-galactosidase *in vivo* when the mRNA contains the appropriate codon at the initiator codon position (24). In a similar study, initiation of active chloramphenicol acetyltransferase can occur from an amber (UA-G) codon, probably with glutamine, when an initiator tRNA altered to recognize the initiation amber codon is present (25).

ABBREVIATIONS

Poly(U), polyuridylic acid; Acaa-tRNA, aminoacyl-tRNA which has been acetylated on its α-amino group; $tRNA_e^{Ala/AAA}$, a synthetic elongator tRNA which has an AAA anticodon and is aminoacylated with alanine; $tRNA_i^{Ala/AAA}$, a synthetic initiator tRNA which has an AAA anticodon and is aminoacylated with alanine; $tRNA^{Ser/AAA}$, a synthetic elongator tRNA which has an AAA anticodon and is aminoacylated with serine; CPM-Ala-$tRNA_e^{Ala/AAA}$ and CPM-Ala-$tRNA_i^{Ala/AAA}$, elongator and initiator synthetic $tRNA^{Ala/AAA}$, respectively, which has been aminoacylated, mercaptoacetylated at its amino group and then reacted with CPM; CPM, 3-(4-maleimidophenyl)-7-diethyl-amino-4-methylcoumarin; IF-2, prokaryotic translation initiation factor 2; PCR, polymerase chain reaction.

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