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The Conformation of Nascent Polylysine and Polyphenylalanine Peptides on Ribosomes*

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Polypeptide synthesis using either phenylalanine or lysine was initiated on *Escherichia coli* ribosomes; then the position and conformation of the nascent peptide were monitored by fluorescence techniques. To this end, fluorophores had been attached to the amino terminus of each nascent peptide, and major differences were observed as chain extension occurred. Polyphenylalanine appeared to build up as a hydrophobic mass adjacent to the peptidyl transferase center while polylysine apparently was extended directly from the ribosome into the surrounding solution. An explanation for these differences may be provided by the physical and chemical properties of each polypeptide. These properties may be responsible for the route by which each peptide exits the peptidyl transferase center as demonstrated by the different sensitivity of each to inhibition by erythromycin.

Very little is known of the mechanisms involved in the establishment of the secondary and tertiary structures of proteins as they are synthesized on the ribosome. Correct folding of a peptide into the discrete, often metastable conformation of a native, soluble enzyme probably occurs in parallel with linear synthesis and is likely to be profoundly influenced by the physical nature of the peptide as it emerges from the ribosome as well as by the physical character of the adjacent regions of the ribosome through which it passes. Influences of external factors such as those involved in the cotranslation of the nascent peptide into or through cellular membranes present additional features to this general problem.

The nascent peptide is generated at the peptidyl transferase center of the large ribosomal subunit by the sequential addition of amino acids from aminoacyl-tRNA to the carboxyl terminus of the growing polypeptide. The tRNA binding sites are located between the ribosomal subunits, and the peptidyl transferase center is on the interfacing surface of the large subunit near its central protuberance. Growing evidence supports the notion that at least some nascent peptides synthesized *in vivo* emerge from the surface of the ribosome at a site called the exit domain located on the outer surface of the large ribosomal subunit at a point distal to the peptidyl transferase center (1). A segment of 30–35 amino acids on nascent peptides is protected from proteolytic degradation (2–4). This corresponds to a distance of about 50 Å for a polypeptide forming an α-helix. This peptide segment may span the distance between the peptidyl transferase center and the exit domain; however, there is no direct evidence to indicate how the nascent peptide transverses the intervening distance between these two points. A tunnel has been reported to span the large ribosomal subunit from the interfacing surface to a point on the outer surface (5). The outer portion of this tunnel appears to be in the region of the nascent peptide exit domain. It was suggested that the nascent peptide may follow this tunnel to the exit domain for some, if not all, newly synthesized proteins.

Erythromycin blocks the synthesis of most but not all polypeptides on *Escherichia coli* ribosomes by binding to a site that is very close to the amino acid of aminoacyl-tRNA in the peptidyl transferase center (6). It has been suggested that the antibiotic may bind to the 50 S subunit at the entrance to the tunnel mentioned above (7) thereby blocking extension of the newly formed nascent peptide. However, the fact that erythromycin does not inhibit the formation of all polypeptides clearly indicates that another or an alternate mechanism must exist. Ryabova and co-workers (8) have questioned the existence of a tunnel spanning the 50 S subunit through which the nascent peptide passes. They find that the nascent peptide is accessible to antibodies from a point very near the peptidyl transferase center. Here, we report the results from our own studies primarily using fluorescence techniques to characterize the extension of phenylalanyl and lysyl polypeptides as they are formed on *E. coli* ribosomes.

The sensitivities of the fluorescent probes used in these studies to environmental conditions in their immediate vicinity have been used to monitor changes in the relative positions of the amino termini of polyphenylalanine and polylysine at various points prior to and after synthesis of the first peptide bond. Results with erythromycin and a fluorescent derivative of erythromycin support the hypothesis that the antibiotic binds very near to the amino terminus of short nascent peptides at a site that is blocked by the peptide as it is extended. Polyphenylalanine can be formed in the presence of erythromycin, but polylysine synthesis is blocked by the antibiotic. The results suggest that the difference in sensitivity may be due to a difference in the physical properties or conformation of the nascent peptides. The data appear to indicate that neither polyphenylalanine nor polylysine nascent peptides are restrained in a tunnel but rather extend directly into the solution surrounding the ribosome from a region near the peptidyl transferase center.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—E. coli K12 strain A19 was provided to us by Drs. K. Nierhaus and H. G. Wittmann, Berlin. Erythromycy-

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lamine was a gift from Dr. Robert Hamill of Lilly. *E. coli* $tRNA^{Phe}$ and $tRNA^{Lys}$ were from Subriden RNA, Inc. (Rollingbay, WA). CPM[1] and CITC were from Molecular Probes, Inc. (Junction City, OR). IAEDANS, yeast $tRNA^{Phe}$, puromycin dichloride, poly(U), poly(A), ATP, GTP, *N*-hydroxysuccinimide, erythromycin, Sephadex, and Sepharose products were purchased from Sigma. DE23 cellulose was from Whatman. *N,N'*-Dicyclohexylcarbodiimide was from Schwarz/Mann. [^{14}C]Phenylalanine and [^{14}C]lysine were from ICN Biomedicals, Inc. (Irvine, CA). Phenol and dimethylformamide were from J. T. Baker Inc. and were redistilled before use. HPLC grade methanol was from Fisher. All other chemicals were of reagent grade.

Preparation of E. coli Ribosomal Subunits—The growth and maintenance of *E. coli* K12, strain A19, and the isolation of ribosomes and ribosomal subunits have been described previously (9).

Poly(U)-dependent and Poly(A)-dependent Peptide Synthesis—The poly(U)-directed synthesis of polyphenylalanine has been described previously and provides a convenient measure of ribosome activity (9). To accommodate the use of fluorescent aminoacyl-tRNA and erythromycin derivatives, some modifications of this published procedure were made. The postribosomal supernatant fraction (S-150), used in previous studies as the source of aminoacyl-tRNA synthetases and elongation factors, was chromatographed on DE23 cellulose to remove nucleic acid and other materials that interfere with measurements of fluorescence from some probes. A 1.2 × 30-cm column containing DE23 cellulose was equilibrated with 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NH_4Cl, 0.5 mM EDTA, 2 mM β-mercaptoethanol, and the sample was applied after dialysis in the same solution. After extensive washing, a fraction containing the synthetases and elongation factors was eluted with the same solution to which NH_4Cl was added to give a final concentration of 250 mM. To facilitate the nonenzymatic initiation of polyphenylalanine synthesis, AcPhe-tRNA or a fluorescent analogue of AcPhe-tRNA was preincubated with ribosomes. The fluorescent derivative was used at about 10–25% of the ribosome concentration to ensure maximum tRNA binding whereas AcPhe-tRNA was at about the same concentration as the ribosomes to allow the maximum proportion of ribosomes to initiate polypeptide synthesis. A typical reaction mixture contained 50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, 5 mM β-mercaptoethanol, 200 μg/ml poly(U), 7.5 A_{260} units/ml 30 S ribosomal subunits, 14 A_{260} units/ml 50 S ribosomal subunits, and the appropriate amount of AcPhe-tRNA or its analogue. This mixture was incubated at 35 °C for 10 min to allow for the tRNA species used to bind to the ribosomes. The reaction was then made to 2 mM ATP, 0.2 mM GTP, 2.5 mM dithioerythritol, 1.6 mg/ml creatine phosphate, and 7.5 A_{260} units/ml unfractionated *E. coli* tRNA. Creatine phosphokinase was then added to a final concentration of 30 μg/ml and S-150 was added to 900 μg/ml. Polymerization was initiated by adding [^{14}C]phenylalanine to give 45 μM. Polyphenylalanine synthesis was at 20 °C in the temperature-regulated sample compartment of the fluorometer unless otherwise indicated. The amount of [^{14}C]phenylalanine incorporated was determined by removing aliquots from the reactions for trichloroacetic acid precipitation. [^{14}C]Phe-tRNA was hydrolyzed by incubating the samples with 0.1 M NaOH prior to trichloroacetic acid precipitation.

Poly(A)-directed polylysine synthesis was carried out as described for polyphenylalanine synthesis except for the minor modifications noted below. Poly(A), used as an mRNA at 40 μg/ml or less, was heated to 70 °C and rapidly cooled before addition to the reaction mixture. The initiation of polylysine synthesis was carried out nonenzymatically using either Ac_2Lys-tRNA or its fluorescent derivative, αAc-ϵCPM-SAcLys-tRNA, as described above for AcPhe-tRNA. The rest of the reaction was set up exactly as with polyphenylalanine synthesis except that the tRNA hydrolysis step was omitted before trichloroacetic acid precipitation. For this reason, a control reaction was performed with each polylysine synthesis to use as a background so that radioactivity contributed by [^{14}C]Lys-tRNA could be subtracted.

Labeling of Yeast Phe-tRNA with CPM and IAEDANS—Labeling of the α-amino group of yeast Phe-tRNA will be described in more detail elsewhere.[2] Briefly, a mercaptoacetyl group was introduced at the α-amino group of Phe-tRNA by reaction of the aminoacyl-tRNA with the succinimidyl ester of dithiodiglycolic acid, the latter having been prepared by the dicyclohexylcarbodiimide method (10). Subsequently the disulfide bond was reduced to give a free sulfhydryl. CPM or IAEDANS was then reacted with the resulting sulfhydryl group for 30 min at 35 °C in 100 mM Hepes-KOH (pH 8.0) with a final probe concentration of 2 mM. Differences in the labeling procedures for CPM and IAEDANS based on the difference in solubility of each compound will be described in detail elsewhere as will the purification of the labeled tRNAs by reversed-phase HPLC.[2]

Labeling of E. coli Lys-tRNA with CPM—In order to introduce a sulfhydryl group specifically at the ϵ-amino group of *E. coli* Lys-tRNA, it was reacted with the succinimidyl ester of dithiodiglycolic acid essentially as described by Johnson *et al.* (11) for reaction of Lys-tRNA with succinimidyl acetate. The reaction mixture contained 0.1 M potassium phosphate (pH 7.0) and 10 A_{260} units/ml [^{14}C]Lys-tRNA. Succinimidyl dithiodiglycolate was added to 80 mM followed quickly by sufficient 8 M KOH to bring the final pH to 11.4. The reaction was allowed to proceed for 15 s at 0 °C after which the pH was lowered to 5 with glacial acetic acid. From this point, the labeling with CPM proceeded exactly as described for Phe-tRNA. ϵCPM-SAcLys-tRNA was purified by reversed-phase HPLC on a Beckman Ultrapore C_8 column. The column was equilibrated with 20 mM Tris HOAc (pH 5.5), 10 mM $Mg(OAc)_2$, and 400 mM NaCl. Elution was with a discontinuous gradient of methanol with the buffer and salt concentrations being held constant. The ϵCPM-SAcLys-tRNA eluted at 39% methanol. When desired, the α-amino group of ϵCPM-SAcLys-tRNA was acetylated by the method of Rappoport and Lapidot (12).

Preparation of Ac_2Lys-tRNA and AcPhe-tRNA—Yeast $tRNA^{Phe}$ was aminoacylated as described previously (13) except that a 0.5 M KCl salt wash of rabbit reticulocyte ribosomes was used as the aminoacyl-tRNA synthetase source (14).

E. coli $tRNA^{Lys}$ was charged with lysine in a similar reaction containing 50 mM Tris-HCl (pH 7.5), 10 mM $Mg(OAc)_2$, 2.5 mM dithioerythritol, 50 mM NH_4Cl, 30 μM [^{14}C]lysine, 3.6 mM ATP, 10 A_{260} units/ml *E. coli* $tRNA^{Lys}$, and about 200 μg/ml S-150 fraction that had been subjected to DE23 cellulose chromatography. The reaction mixture was incubated for 15 min at 35 °C. When the incubation was completed, 2 M NaOAc (pH 5.0) was added to give a final concentration of 0.2 M, and the mixture was extracted with phenol. The aqueous portion was then precipitated three times with 3 volumes of ethanol.

For use in the nonenzymatic initiation of polypeptide synthesis, the Phe-tRNA and Lys-tRNA were acetylated essentially according to Rappoport and Lapidot (12). The acetylation reaction was carried out in 0.1 M triethanolamine HCl (pH 7.8), 6 mM $Mg(OAc)_2$, 10 A_{260} units/ml Phe- or Lys-tRNA, and 10 mg/ml succinimidyl acetate that was added from a 120 mg/ml stock solution prepared in dimethylformamide. The reaction proceeded at 0 °C for 30 min at which time the mixture was made to 0.2 M NaOAc (pH 5.0) and precipitated with 3 volumes of ethanol. The ethanol precipitation was repeated twice.

AcPhe-tRNA and deacylated $tRNA^{Phe}$ were separated by C_{18} reversed-phase HPLC as described previously (15). Ac_2Lys-tRNA was separated from deacylated tRNA and Lys-tRNA that had not been acetylated completely by reversed-phase HPLC using a Beckman System Gold HPLC and a Beckman Ultrapore C_8 column (0.46 × 25 cm). The sample was loaded in 20 mM Tris-HOAc (pH 5.5), 10 mM $Mg(OAc)_2$, and 400 mM NaCl after the column had been equilibrated with the same buffer. A discontinuous gradient of methanol in the same solution was used for elution, Ac_2Lys-tRNA eluting at 15% methanol.

Nonenzymatic Formation of Diphenylalanine and Dilysine—Binding of AcPhe-tRNA, Ac_2Lys-tRNA, and the fluorescent analogues of each of these to the ribosomal P site was carried out by a modification of the procedure of Wurmbach and Nierhaus (16). As described above, initiation of polypeptide synthesis with the fluorescent derivatives of aminoacyl-tRNA was carried out at a concentration of the derivative

[1] The abbreviations used are: CPM, 3-(4-maleimidophenyl)-7-diethylamino-4-methylcoumarin; CITC, 3-(4-isothiocyanatophenyl)-7-diethylamino-4-methylcoumarin; IAEDANS, 5-[2-(2-iodoacetamido)ethylamino]-1-naphthalenesulfonic acid; αAc-ϵCPM-SAcLys-tRNA, Lys-tRNA that was mercaptoacetylated at its ϵ-amino group, reacted with CPM, and acetylated at its α-amino group; CPM-SAcPhe-tRNA, Phe-tRNA that was mercaptoacetylated at its α-amino group and then reacted with CPM; AEDANS-SAcPhe-tRNA, Phe-tRNA that was mercaptoacetylated at its α-amino group and then reacted with IAEDANS; CITC-erythromycin, erythromycylamine labeled at its amino group with CITC; HPLC, high performance liquid chromatography; Ac_2Lys-tRNA, Lys-tRNA that was acetylated at its α- and ϵ-amino groups; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] Odom, O. W., Picking, W. D., Tsalkova, T., and Hardesty, B. (1991) *Eur. J. Biochem.*, in press.

which was much lower than that of the ribosomes to ensure that a high proportion of the acyl-tRNA was bound. After binding the *N*-blocked aminoacyl-tRNA to the puromycin-reactive site, a second aminoacyl-tRNA was bound nonenzymatically in a similar fashion to the puromycin-nonreactive site (the A site according to the classical model). When the *N*-blocked aminoacyl-tRNA bound first was fluorescently labeled, the second aminoacyl-tRNA was added at a concentration approximately equal to the ribosome concentration.

Preparation of CITC-Erythromycin and the Binding of Labeled and Unlabeled Erythromycin—CITC-erythromycin was prepared from erythromycylamine as described in detail elsewhere.[2] The binding of labeled erythromycin reached rapid equilibrium at 35 °C and was carried out in the presence of *N*-blocked aminoacyl-tRNA with little or no interference. Previous results indicated that CITC-erythromycin bound to the ribosomes with a K_d of 8 nM[3] which is similar to the K_d of the unmodified antibiotic (17).

To measure the effect of polylysine synthesis on CITC-erythromycin fluorescence, 0.26 μM CITC-erythromycin was bound to 0.6 μM ribosomes in 50 mM Tris-HCl (pH 7.5), 15 mM $Mg(OAc)_2$, 100 mM NH_4Cl, and 5 mM β-mercaptoethanol for 5 min at 35 °C. Ac_2Lys-tRNA was then bound in the presence of poly(A) for subsequent initiation of polylysine synthesis as described above. Steps after the initial binding of CITC-erythromycin were performed directly in the cuvettes used for fluorescence measurements.

The ability of CITC-erythromycin to be exchanged for excess unlabeled erythromycin when the former had been prebound to ribosomes prior to initiation of polyphenylalanine synthesis was measured to ascertain the fate of the antibiotic after the synthesis of a peptide whose synthesis it is unable to inhibit. CITC-erythromycin (0.26 μM) was bound to 1 μM ribosomes in the cuvette to be used for fluorescence measurements exactly as described above. The poly(U)-directed synthesis of polyphenylalanine was then initiated. After 30 min at 35 °C, unlabeled erythromycin was added in a 20-fold excess over CITC-erythromycin and in a 5-fold excess over the ribosome concentration. The amount of CITC-erythromycin remaining bound over time was measured as a function of anisotropy and was calculated as described in the figure legends.

Isolation of Ribosomes with Bound tRNA and Nascent Peptides by Gel Filtration—In some cases, it was necessary to eliminate any contribution to fluorescence measurements of the portion of labeled *N*-blocked aminoacyl-tRNA that was not bound to the ribosomes. Reaction mixtures containing ribosomes with bound tRNA or nascent chains were isolated by gel filtration. A 4-ml column (0.7 × 10 cm) containing Sephacryl S-300 was equilibrated with 50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, and 5 mM β-mercaptoethanol. The binding of *N*-blocked aminoacyl-tRNA and the formation of nascent peptides were as described above except that the reaction volume was reduced from 0.5 to 0.2 ml. Fractions of 0.5 ml were collected, and their absorbance at 260 nm and associated radioactivity was measured. Fluorescence studies were then conducted on the ribosome peak fraction which eluted in the void volume well ahead of free tRNA and ribosome-unassociated proteins.

Sephadex G-15 Gel Filtration of Polylysine Nascent Peptides—To examine the relative sizes of the polylysine nascent peptide chains, polylysine synthesis was initiated with αAc-εCPM-SAcLys-tRNA in the presence or absence of excess unlabeled erythromycin. The ribosomes bearing bound tRNA nascent peptide chains were then isolated on Sephacryl S-300 for fluorescence measurements as already described. These ribosome fractions were then brought to a final concentration of 0.2 M NaOH to hydrolyze all acyl-tRNA present, neutralized with glacial acetic acid, and briefly centrifuged to remove any particulate material. To separate components of molecular mass greater than approximately 1,000 Da from smaller components, the samples were applied to a 16-ml Sephadex G-15 column (1 × 20 cm) equilibrated with 50 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, and 100 mM NH_4Cl. Fractions of 0.5 ml were collected and the presence of lysine monitored by determination of [^{14}C]lysine in a 25-μl aliquot of each fraction. Elution of free [^{14}C]lysine from the same column was also determined.

Fluorescence Measurements—A model 8000 photon-counting spectrofluorometer from SLM Instruments, Inc. (Urbana, IL) was used to carry out steady-state fluorescence measurements as described previously (18). Spectral data were acquired at 2-nm intervals with a scanning rate of 2 or 5 s/wavelength increment. The wavelength dependence of the sensitivity of the photomultiplier was automatically corrected, and all measurements were made at an absorbance of less than 0.1 at the excitation wavelength in a volume of 0.5 ml at 20 °C unless otherwise indicated. Steady-state fluorescence polarization and anisotropy measurements were made with SLM fluorometer as described previously (19).

When the fluorescence from CITC-erythromycin or the CPM derivatives was measured, excitation was at 385 nm. For the fluorescence polarization measurements used to determine anisotropy, the emission wavelength was 470 nm for the CITC and CPM derivatives. AEDANS fluorescence was measured with an excitation wavelength of 360 nm, and the emission for AEDANS fluorescence anisotropy and intensity was at 480 nm.

RESULTS

Initiation of Polyphenylalanine Synthesis with Fluorescent Acyl-tRNA—Fluorescence anisotropy, fluorescence intensity, and emission maximum were measured for AEDANS-SAcPhe-tRNA and CPM-SAcPhe-tRNA after initiation of polypeptide synthesis with these tRNA species. The fluorescence properties of CPM and AEDANS are greatly influenced by the environment immediately surrounding the probes. Thus, these types of measurements provide a way to monitor changes in the environment of the amino terminus of the nascent peptide as it is extended during synthesis on ribosomes. The effects of binding to ribosomes on anisotropy and fluorescence intensity of AEDANS- and CPM-SAcPhe-tRNA are shown in Fig. 1, *A* and *B*, respectively. Binding causes AEDANS anisotropy to increase from 0.03 to about 0.25 whereas CPM anisotropy increases from 0.17 to about 0.37. The higher numerical values for the fluorescence anisotropy of CPM apparently reflect its shorter fluorescence lifetime. These results indicate that the aminoacyl portion of aminoacyl-tRNA is held rather rigidly in the peptidyl transferase center. Upon binding to ribosomes, the fluorescence intensity of the AEDANS derivative of Phe-tRNA decreases about 30% with a shift in the emission maximum from about 502 to 490 nm (Fig. 1*C*) whereas the intensity of CPM fluorescence increases more than 30%, and the emission maximum shifts toward the blue from 481 to 471 nm (Fig. 1*D*). These are approximately the changes in fluorescence which would be observed if the probes were taken from water to 70% ethanol. Such changes in the fluorescence intensity and emission maximum for each fluorescent analogue indicate that the peptidyl transferase center is hydrophobic in nature as was suggested previously.[3]

Upon initiation of polyphenylalanine synthesis, the anisotropy of bound AEDANS-SAcPhe drops rapidly, indicating a decrease in the rigidity with which it is bound to the ribosome (Fig. 1*A*). This momentary drop is followed by a gradual increase in anisotropy, indicating that extension of the peptide restrains the movement of the amino-terminal probe. A similar although much smaller drop in anisotropy is observed when CPM-SAcPhe-tRNA is used instead of the AEDANS derivative (Fig. 1*B*). Fig. 1, *A* and *B*, also shows the corresponding changes in the intensity of fluorescence from the AEDANS and CPM derivatives during the course of polyphenylalanine synthesis. There is a small initial drop in the intensity of both CPM and AEDANS fluorescence. Experiments in which the rate of polymerization is slowed indicate that the drop in intensity occurs slightly ahead of the drop in anisotropy (results not shown). This initial drop is followed by a gradual increase in fluorescence intensity as the peptide is elongated further. Also, at early stages of polymerization the emission maximum of AEDANS (but not that of CPM) shifts to the red (results not shown). Considered together, these results for anisotropy, intensity, and emission maximum appear to indicate that the probe on the amino-terminal residue of polyphenylalanine passes quickly into a relatively

[3] Odom, O. W., Picking, W. D., and Hardesty, B. (1991) *Biochemistry*, in press.

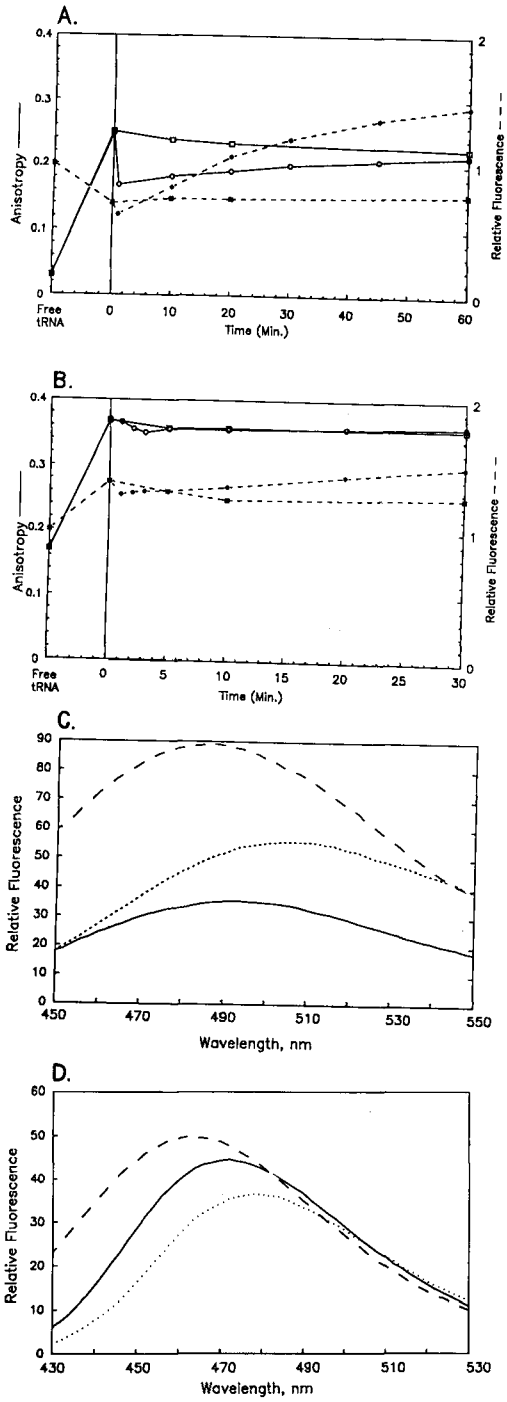

FIG. 1. **Changes in the fluorescence properties of AEDANS and CPM attached to the amino terminus of a growing polyphenylalanine nascent peptide.** In *panels A* and *B*, anisotropy (*open symbols*) and relative fluorescence intensity (*closed symbols*) are followed for AEDANS-SAcPhe-tRNA (*A*) and CPM-SAcPhe-tRNA (*B*) when free in solution (*free tRNA*), bound to ribosomes (time = 0 min), and throughout the synthesis of nascent polyphenylalanine (shown with *circles*). Control samples (shown with *squares*) were treated identically except that the S-150 fraction (added in experimental samples at 0 min) was omitted. In each case, nascent peptides approaching 100 phenylalanine residues in length were produced (data not shown). *Panels C* and *D* show the emission spectra of *A* and *B*, respectively, of free tRNA (.....), ribosome-bound tRNA (——), and after polyphenylalanine synthesis was completed (– – –). *Panel C*, AEDANS-SAcPhe-tRNA; *panel D*, CPM-SAcPhe-tRNA.

more polar environment in which it has higher flexibility as a very short peptide is formed. Subsequently, its movement becomes more restrained, and its environment becomes increasingly hydrophobic as the nascent peptide is extended.

Initial changes in these fluorescence properties may reflect movement of the amino terminus of the nascent peptide out of the peptidyl transferase center. Over time, the progressive increases in anisotropy and fluorescence intensity suggest that the probes become increasingly confined in an environment of increasing hydrophobicity. The blue shifts in emission maxima after extensive polymerization with AEDANS (Fig. 1*C*) and especially with CPM (Fig. 1*D*) also indicate an extremely hydrophobic environment. The length of the nascent polyphenylalanine chains in these studies has been estimated to approach 100 amino acids. This far exceeds the predicted length from the peptidyl transferase center to the peptide exit domain, suggesting that the observed fluorescence properties of the amino-terminal labels are not due to restriction within any particular region of the ribosome. Instead, these results may reflect accumulation of a mass of insoluble polyphenylalanine as this particular nascent peptide is extended. Polyphenylalanine is insoluble in aqueous solvents and is likely to have atypical secondary structure.

Synthesis of Diphenylalanine—To examine the events occurring immediately after initiation of polyphenylalanine synthesis, the AEDANS and CPM derivatives of Phe-tRNA were bound to ribosomes, and then Phe-tRNA was bound nonenzymatically to form the corresponding dipeptides. The fluorescence was monitored before and after the dipeptide was formed (Table I). Synthesis of AEDANS-SAcdiPhe-tRNA (bound in the A site according to the classical model) causes a slight decrease in anisotropy and a marked decrease in relative fluorescence intensity compared with that of AEDANS-SAcPhe-tRNA (in the classical P site). For comparison, data for each aminoacyl-tRNA analogue bound in the A site (by blocking the P site with deacylated $tRNA^{Phe}$) are also included. This comparison also indicates that dipeptide formation causes a large decrease in relative fluorescence of the AEDANS as well as a small blue shift in the emission maximum. These data indicate that the decrease in intensity seen during the synthesis of polyphenylalanine initiated with this aminoacyl-tRNA derivative (Fig. 1*A*) occurs during formation of the first peptide bond whereas most of the decrease in anisotropy occurs at a later stage. Also, the blue shift in the emission maximum indicates that the environment at the dipeptide stage is more hydrophobic than the original environment. As noted above, during polymerization there is first

TABLE I

The effect of dipeptide formation on the fluorescence of AEDANS-SAcPhe-tRNA and CPM-SAcPhe-tRNA

The tRNA analogues used in these experiments were bound to ribosomes that were not subsequently isolated over Sephacryl S-300. The anisotropies obtained here are equivalent to those following isolation.

Analogue	Site	Fluorescence anisotropy	Relative fluorescence intensity	Em_{max}[a]
				nm
AEDANS-Phe-	P	0.280	≡1.00	490
AEDANS-Phe-	A	0.273	0.92	490
AEDANS-diPhe-	A	0.271	0.57	489
CPM-Phe-	P	0.372	≡1.00	471
CPM-Phe-	A	0.370	0.97	474
CPM-diPhe-	A	0.370	0.96	471

[a] Em_{max} designates the wavelength (in nm) at which the maximum emission occurs for that sample.

a red shift in the emission maximum of the AEDANS probe followed by a blue shift upon extended polymerization. The dipeptide data indicate that the red shift occurs after formation of the first peptide bond.

Immediately after dipeptide formation, the product is unreactive with puromycin (thus by definition is in the classical A site). However, particularly the AEDANS-SAcdiPhe-tRNA derivative tends to become puromycin-reactive over long periods of time (data not shown). Bergemann and Nierhaus (20) have observed a similar phenomenon and attributed it to spontaneous translocation.

Formation of a dipeptide initiated with CPM-SAcPhe-tRNA causes only a slight decrease in intensity in comparison with the monomer in the P site and a slight blue shift in comparison with the monomer in the A site (Table I). CPM anisotropy is not greatly affected by dipeptide formation or polypeptide synthesis. The slight decrease in CPM relative fluorescence (Table I) may be related to the drop in intensity observed immediately after initiation of polyphenylalanine synthesis (Fig. 1*B*).

The Effects of Erythromycin and Puromycin on Fluorescent Analogues of the Mono- and Dipeptide Forms of Phe-tRNA—To compare further the initial stages of polyphenylalanine formation, the dipeptides discussed in relation to Table I were synthesized, and the effects of erythromycin and puromycin on each were examined. These data were then compared with the same parameters for the monoaminoacyl-tRNAs bound in the A site (Table II). CPM-SAcPhe-tRNA fluorescence increases 17% with a 6-nm bathochromic shift in the emission spectrum with erythromycin present (with or without puromycin added). Puromycin alone has a small, although significant, effect on fluorescence intensity and causes a 5-nm bathochromic shift in the emission spectrum. Formation of CPM-SAcdiPhe-tRNA has only a minor effect on fluorescence intensity but does result in a blue shift in the CPM emission spectrum. Erythromycin once again causes a 6-nm bathochromic shift in the CPM emission spectrum (in addition to the 3-nm shift seen upon dipeptide formation) accompanied by an even greater increase in fluorescence intensity. This effect again appears to be independent of any effect elicited by puromycin. Puromycin, when added alone to the bound CPM-SAcdiPhe-tRNA, affects the fluorescence intensity much as it affects the fluorescence intensity of bound CPM-SAcPhe-tRNA; however, the emission maximum of the dipeptidyl-tRNA analogue undergoes only a 2-nm bathochromic shift.

AEDANS-SAcPhe-tRNA is quenched by erythromycin with no change in its emission maximum whereas puromycin increases the AEDANS fluorescence 183% and causes a major bathochromic shift in the emission spectrum (Table II). Bound simultaneously, erythromycin and puromycin cause a major bathochromic shift in the emission spectrum and fluorescence intensity of the AEDANS to reach a value intermediate to those achieved with the individual antibiotics.

Formation of a dipeptide from AEDANS-SAcPhe-tRNA quenches the fluorescence from the probe and causes a small bathochromic shift in the emission maximum. Erythromycin further decreases the AEDANS intensity, and puromycin once again increases the fluorescence intensity and causes a bathochromic shift in the emission spectrum. The combination of erythromycin with puromycin, however, results in an increase in fluorescence intensity to a value greater than that seen with either individual antibiotic with a change in the emission maximum to an intermediate level. The reason for this result is unclear; it may reflect a steric interaction of erythromycin with the newly formed dipeptide, forcing the AEDANS group closer to the bound puromycin. Interestingly, erythromycin appears to retard, but not completely block, the spontaneous translocation (20) seen with fluorescent diPhe-tRNA analogues. This suggests that erythromycin is able to interact with the nascent diphenylalanyl-tRNA to affect its reactivity in the next round of peptide elongation.

The Effects of Polyphenylalanine Synthesis on Fluorescently Labeled Erythromycin—Erythromycin inhibits the synthesis of most polypeptides, including polylysine (21), on *E. coli* ribosomes. It does not inhibit polyphenylalanine synthesis (22) even though the antibiotic remains tightly bound to ribosomes on which polyphenylalanine chains are being extended, as judged from the fluorescence anisotropy of erythromycin labeled with a coumarin probe, CITC-erythromycin.[2] The fluorescence intensity of CITC-erythromycin bound to ribosomes increases as polyphenylalanine synthesis is initiated and continues to increase as the chain is extended. Eventually, values are reached similar to those for CITC-erythromycin in organic solvents, such as phenol.[2] This is accompanied by a bathochromic shift in the CITC emission spectrum. Considered with the data presented above, the results suggest that polyphenylalanine is synthesized as a hydrophobic mass that accumulates very close to the CITC-erythromycin binding site and the peptidyl transferase center. The extended nascent peptides of either natural proteins (23) or polyphenylalanine[3] prevent binding of erythromycin to the ribosomes presumably by blocking the erythromycin binding site. These observations prompt questions as to the relative position of the bound erythromycin and the nascent polyphenylalanine and polylysine chains. The conformation of these nascent peptides may be important for erythromycin inhibition. How is it possible for the nascent polyphenylalan-

TABLE II

Comparison of the effects of erythromycin and/or puromycin on the fluorescence of amino-terminal labeled Phe-tRNA or diPhe-tRNA in the puromycin-unreactive ribosomal site

I designates relative fluorescence intensity; *A* designates fluorescence anisotropy; and Em_{max} designates the wavelength (in nm) at which the maximum emission occurs for the sample.

Labeled tRNA bound	No addition			+Erythromycin			+Puromycin			+Erythromycin and puromycin		
	Em_{max}	*I*	*A*	Em_{max}	*I*	*A*	Em_{max}	*I*	*A*	Em_{max}	*I*	*A*
	nm			*nm*			*nm*			*nm*		
CPM-SAcPhe-	474	1.00	0.37	468	1.17	0.37	469	1.08	0.37	468	1.17	0.37
CPM-SAcdiPhe-	471	0.99	0.37	465	1.30	0.37	469[a]	1.13	0.35	465	1.32	0.37
AEDANS-SAcPhe-	490	1.00	0.28	490	0.38	0.28	472	2.83	0.28	482	1.42	0.28
AEDANS-SAcdiPhe-	489	0.57	0.28	489	0.33	0.28	482[a]	1.61	0.24	486	2.00	0.28

[a]These represent maximum values since some spontaneous translocation occurred during the time the spectra were being measured.

ine peptide to escape inhibition by erythromycin, and more importantly why is the extension of other peptides blocked?

A further indication of the relative position of the extended nascent polyphenylalanine chain with respect to bound CITC-erythromycin was obtained by allowing polyphenylalanine synthesis to proceed on ribosomes to which the antibiotic analogue had been already bound. We have found that ribosome-bound CITC-erythromycin will exchange with free unmodified erythromycin.[3] The effect of nascent polyphenylalanine on exchange of ribosome-bound CITC-erythromycin with unmodified erythromycin was determined with the results shown in Fig. 2. The time course for the exchange of excess unlabeled erythromycin for the bound CITC-erythromycin is plotted. The percentage of CITC-erythromycin bound to ribosomes was calculated from the difference in the fluorescence anisotropy of free and ribosome-bound CITC-erythromycin. The maximum change in anisotropy (from free CITC-erythromycin) upon ribosome binding (ΔA_{max}) and the change in anisotropy at any given time after unlabeled erythromycin addition (ΔA) were entered into the following equation:

$$\% \text{ (CITC-erythromycin) bound} = \frac{\Delta A}{2\Delta A_{max} - \Delta A} \times 100.$$

This takes into account the fact that bound CITC-erythromycin has twice the fluorescence intensity as does CITC-erythromycin free in solution and that the observed anisotropy is an intensity-weighted average.[3] The percentage of CITC-erythromycin bound to ribosomes containing nascent polyphenylalanine chains remains greater than 70% whereas that remaining bound to ribosomes without nascent peptides rapidly approaches 20% (Fig. 2). These results indicate that the nascent polyphenylalanine peptide prevents exchange of CITC-erythromycin. Probably this is due to shielding of the bound antibiotic by the nascent peptide.

Initiation of Polylysine Synthesis with αAc-εCPM-SAcLys-tRNA—Polylysine synthesis was initiated with αAc-εCPM-SAcLys-tRNA, and the fluorescence anisotropy, intensity, and emission maximum were monitored (Fig. 3) as was done previously for the fluorescent derivatives of AcPhe-tRNA.

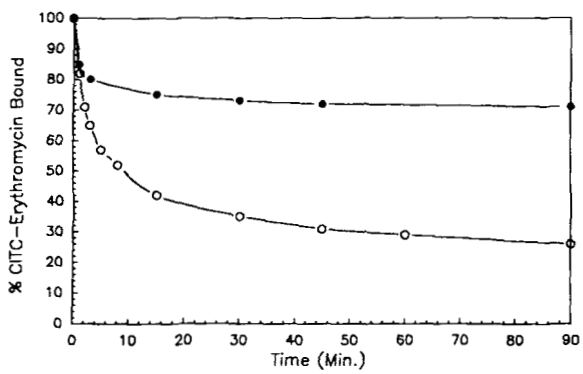

FIG. 2. **Exchange of ribosome-bound CITC-erythromycin before and after polyphenylalanine synthesis.** CITC-erythromycin (0.25 μM) and AcPhe-tRNA (1 μM) were bound to ribosomes (1 μM), and then other components required for polyphenylalanine synthesis were added except that the S-150 enzyme fraction was omitted from the control to prevent polymerization. The samples were incubated at 37 °C for 30 min. Unlabeled erythromycin (5 μM final concentration) was added to both sample and control, and changes in fluorescence anisotropy were monitored over time. The fraction of CITC-erythromycin remaining bound was determined by the equation: fraction bound = $\Delta A/2\Delta A_{max} - \Delta A$. *Open symbols*, erythromycin exchange on ribosomes without nascent peptides. *Closed symbols*, exchange on ribosomes containing nascent polyphenylalanine chains estimated to be approximately 30 amino acids long.

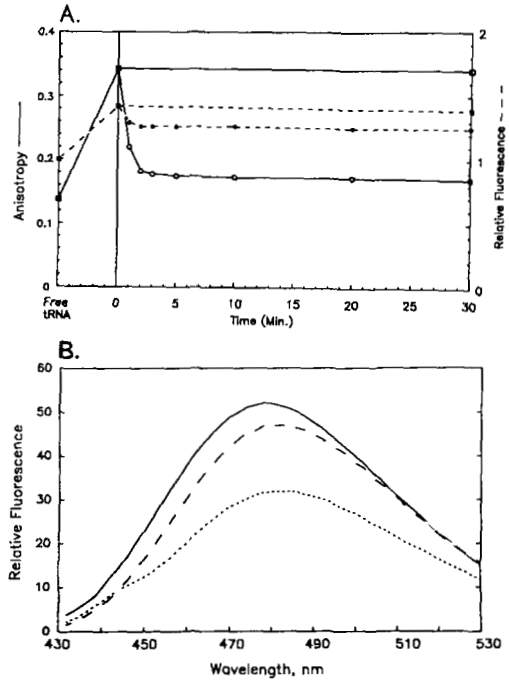

FIG. 3. **Changes in the fluorescence of αAc-εCPM-SAcLys-tRNA after initiation of polylysine synthesis.** In *panel A*, anisotropy (*open symbols*) and relative fluorescence (*closed symbols*) were monitored for control samples (*squares*) and for samples in which polylysine synthesis was induced (*circles*), as was shown in Fig. 1*B* for CPM-SAcPhe-tRNA. In *panel B*, the emission spectra for αAc-εCPM-SAcLys-tRNA free in solution (.....), bound to ribosomes (——), and after polylysine synthesis was completed (- - -) are shown. The nascent polylysine peptides were considerably shorter than the nascent polyphenylalanine peptides described in Fig. 1 (on the order of 10–15 lysine residues long); however, these polylysine peptides can be compared with the early events that occur during polyphenylalanine synthesis (about 10 min after polyphenylalanine initiation).

Upon binding to ribosomes, the anisotropy of the Lys-tRNA analogue increases from 0.14 to 0.34 with a 42% increase in relative fluorescence (Fig. 3*A*). These data are similar to those seen for CPM-SAcPhe-tRNA upon binding to ribosomes (Fig. 1*B*). αAc-εCPM-SAcLys-tRNA binding causes a bathochromic shift in the CPM emission maximum from 482 to 476 nm (Fig. 3*B*), which is also similar to the effect ribosome binding has upon CPM-SAcPhe-tRNA (Fig. 1*D*). At this point, the poly(A)-directed synthesis of polylysine could be initiated to follow the fate of the amino-terminal CPM as it was extended on a charged hydrophilic nascent peptide (Fig. 3*A*). As with polyphenylalanine synthesis, polylysine synthesis results in an initial drop in CPM anisotropy, but the decrease is much larger with polylysine. Unlike the result with polyphenylalanine synthesis, however, further extension of the nascent polylysine causes an even greater decrease in anisotropy rather than an increase (Fig. 3*A*). This apparent increase in the mobility of the amino terminus of nascent polylysine is accompanied by a decrease in intensity for CPM fluorescence and a hypsochromic shift in the emission maximum (Fig. 3*B*). The lengths of the polylysine chains in these experiments were only in the range of 10–15 amino acids but were well beyond the point at which the anisotropy of nascent polyphenylalanine had started to rise (*cf.* Fig. 1, *A* and *B*). In addition, at no point in the synthesis of polyphenylalanine initiated with CPM-SAcPhe-tRNA (Fig. 1*B*) did the anisotropy drop as low as it did during polylysine synthesis.

Despite forming a relatively short nascent peptide, the results indicate that the probe on the amino terminus of

nascent polylysine moves quickly into a more polar environment in which it has a high degree of mobility. From this point there appears to be very little change in the environment of the probe as the nascent peptide is extended. This is inconsistent with polylysine entering a constricted region of the ribosome which is 30–35 amino acids in length before exiting at a site distal to the peptidyl transferase center.

Synthesis of Dilysine and the Effects of Erythromycin and Puromycin on the Fluorescent Mono- and Dilysyl-tRNAs—As was done previously with the fluorescent analogues of AcPhe-tRNA (Table I), αAc-εCPM-SAcLys-tRNA was used to form dipeptidyl-tRNA nonenzymatically to examine the effects the formation of the first peptide bond has on CPM fluorescence (Table III). The changes in fluorescence properties associated with dilysine formation only remotely reflect the early stages that occur after the initiation of polylysine synthesis (shown in Fig. 3). Dilysine formation causes no change in anisotropy although it does cause a hypsochromic shift in the emission and a relatively small decrease in fluorescence intensity (Table III). Polylysine synthesis results in a rapid, substantial drop in anisotropy and decrease in fluorescence intensity (Fig. 3*A*) accompanied by a relatively large hypsochromic shift in the emission spectrum of CPM (Fig. 3*B*). Presumably, the relatively large changes observed with polylysine synthesis reflect extension of the nascent peptide beyond the dipeptide stage.

For further examination of the initial steps in polylysine synthesis, the mono- and dilysyl-tRNA analogues (each bound in the classical A site) were incubated with erythromycin and/or puromycin, and the effects of antibiotic binding on fluorescence were monitored. αAc-εCPM-SAcLys-tRNA behaves somewhat like CPM-SAcPhe-tRNA (Table II) upon erythromycin addition, puromycin addition, and the addition of both erythromycin and puromycin (Table III). Erythromycin (± puromycin) causes a 4-nm bathochromic shift in the CPM emission spectrum whereas puromycin alone only caused a 2-nm shift. Unlike the results with CPM-SAcPhe-tRNA, when αAc-εCPM-SAcLys-tRNA was used to form dilysine, a 3-nm hypsochromic shift was observed in the emission maximum. However, relative to the dipeptide emission spectrum, erythromycin (± puromycin) causes a 5-nm bathochromic shift whereas puromycin alone continues to cause a 2-nm bathochromic shift. These data indicate that erythromycin once again interacts more strongly with the dipeptidyl-tRNA than with monoaminoacyl-tRNA.

Initiation of Polylysine Synthesis in the Presence of CITC-Erythromycin—Polylysine synthesis is inhibited by erythromycin causing the accumulation of di- and tripeptides (21). Polylysine synthesis was initiated with diacetyl-Lys-tRNA in the presence of bound CITC-erythromycin to compare the effects on CITC fluorescence with those produced by the synthesis of polyphenylalanine. When CITC-erythromycin is bound to ribosomes, its fluorescence intensity increases substantially with a bathochromic shift in the CITC emission spectrum (Fig. 4). The binding of diacetyl-Lys-tRNA then results in a very slight decrease in intensity and hypsochromic shift in the emission maximum. When polylysine synthesis is initiated, the CITC emission spectrum shifts back toward the blue with a small increase in fluorescence intensity (Fig. 4). The result is similar to but less pronounced than that seen with polyphenylalanine synthesis. During the latter process, nascent peptides of 100 or more amino acids may be formed whereas only very short lysine peptides are formed in the presence of the antibiotic (see below) or its fluorescent derivative. The data appear to indicate that the nascent lysine peptide moves very close to the bound erythromycin before its extension is blocked. Polyphenylalanine synthesis, on the other hand, is somehow able to bypass erythromycin blockage.

The Effect of Erythromycin on the Fluorescence of αAc-εCPM-SAcLys-tRNA before and after Polymerization—The fluorescence properties of CPM-Lys-tRNA in various stages of polylysine synthesis in the presence and absence of erythromycin were examined (Table IV). Binding of αAc-εCPM-SAcLys-tRNA to ribosomes results in the characteristic in-

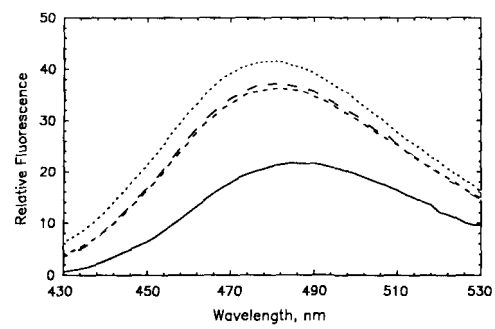

FIG. 4. **The effect of polylysine synthesis on fluorescence from CITC-erythromycin.** The emission spectra of CITC-erythromycin free in solution (*unbroken line*), bound to 70 S ribosomes (*long dashed line*), bound to 70 S ribosomes with Ac_2Lys-tRNA (*short dashed line*), and after initiation of polylysine synthesis (*dotted line*).

TABLE III

The effect of dilysine formation on the fluorescence of αAc-εCPM-SAcLys-tRNA and a comparison of the effects of erythromycin and/or puromycin on the mono- and dilysyl-tRNA analogues

	Site	Fluorescence anisotropy	Relative fluorescence intensity	Em_{max}[a]
				nm
CPM-Lys-	P	0.340	≡1.00	476
CPM-Lys-	A	0.338	1.10	475
CPM-diLys-	A	0.345	0.97	478
CPM-Lys- +erythromycin	A	0.340	1.13	471
CPM-Lys- +puromycin	A	0.340	1.09	473
CPM-Lys- +erythromycin +puromycin	A	0.340	1.14	471
CPM-diLys- +erythromycin	A	0.330	1.10	473
CPM-diLys- +puromycin	A	0.340	1.03	476
CPM-diLys +erythromycin +puromycin	A	0.350	1.08	473

[a] Em_{max} designates the wavelength (in nm) at which the maximum emission occurs for that sample.

TABLE IV

The effect of erythromycin on the fluorescence of αAc-εCPM-SAcLys-tRNA before and after polymerization

Position of αAc-εCPM-SAcLys[a]	tRNA position	Anisotropy	Intensity	Em_{max}
				nm
Free in solution		0.139	1.00	482
Bound to ribosome	P	0.340	1.42	476
Bound + erythromycin[b]	P	0.343	1.55	475
First in poly (Lys)		0.170	1.20	480
First in poly (Lys) + erythromycin[b]		0.221	1.50	477
First in poly (Lys)[c]		0.226	0.92	480
First in poly (Lys) + erythromycin[b,c]		0.266	1.40	478

[a] Used as the αAc-aminoacyl-tRNA.

[b] When used, erythromycin at 5.0 μM was bound simultaneously with the tRNA.

[c] After incubation for polylysine synthesis, the reaction mixtures were chromatographed on Sephacryl S-300 to seperate peptidyl tRNA bound to ribosomes from unbound εCPM-SAcLys-species.

crease in fluorescence intensity and anisotropy with a blue shift in the CPM emission spectrum. Erythromycin binding causes a further, although slight, blue shift in the emission maximum with little or no change in anisotropy. Initiation of polylysine synthesis with the CPM residue on the amino-terminal amino acid results in a large drop in anisotropy with a significant decrease in fluorescence intensity and a red shift in the emission spectrum (see also Fig. 3, *A* and *B*). However, the otherwise identical initiation of polylysine synthesis in the presence of erythromycin (concentration of erythromycin > ribosome ≫ αAc-εCPM-SAcLys-tRNA) results in a somewhat smaller decrease in anisotropy, essentially no decrease in fluorescence intensity, and only a small hypsochromic shift in the emission maximum (Table IV, top five entries). This erythromycin effect correlates with a 50% inhibition of [^{14}C]lysine incorporation (data not shown).

To examine more closely the effect of polylysine synthesis on amino-terminal fluorescence, ribosomes with attached nascent chains were isolated by gel filtration chromatography. In this way it is possible to measure fluorescence from only αAc-εCPM-SAcLys-tRNA incorporated into polylysine-tRNA which remains bound to the ribosomes (Table IV, bottom two entries). In comparison to free αAc-εCPM-SAcLys-tRNA, the bound aminoacyl-tRNA analogues show increased anisotropy, increased relative fluorescence intensity and a significant bathochromic shift in fluorescence. Initiation of polylysine synthesis in the presence of erythromycin results in a decrease in anisotropy and a slight red shift in the emission maximum, indicating that these are characteristics of the ribosome-bound short nascent peptide produced when this antibiotic is bound and are not simply due to release of a population of small peptides either free or as peptidyl-tRNA. The fluorescence intensity of these short peptides, however, does not change appreciably, suggesting that the attached fluorophore remains in the vicinity of the hydrophobic peptidyl transferase center. A further drop in anisotropy and a relatively large decrease in intensity accompanied by a further hypsochromic shift of CPM fluorescence from attached full-length polylysine chains also suggest that erythromycin causes the amino-terminal amino acid containing CPM to remain in the vicinity of the peptidyl transferase center (Table IV).

In addition to its effects on fluorescence, erythromycin also appears to be limiting the amount of [^{14}C]lysine in the polylysine that remains attached to the ribosomes (data not shown). The isolated ribosome fractions were treated with NaOH to release lysine peptides from tRNA, neutralized with glacial acetic acid, and clarified by centrifugation. The sample containing [^{14}C]lysine peptides was fractionated on Sephadex G-15 to separate products by size. The results with lysine peptides formed in the presence or absence of erythromycin are shown in Fig. 5. Also shown is the profile for free [^{14}C]lysine. The results indicate that the majority of the ribosome-bound lysine peptides synthesized in the absence of erythromycin are larger than 1,000 in molecular weight. This would correspond to at least 5 lysyl residues with a single CPM (M_r ~ 400) attached and fits well with an estimated minimal chain length of 10. Conversely, lysine peptides formed in the presence of erythromycin are appreciably smaller and are eluted only slightly before [^{14}C]lysine (Fig. 5). If only the amount of [^{14}C]lysine in the higher molecular weight radioactive peptides is considered, erythromycin appears to inhibit polylysine synthesis about 90%. Further determinations of the exact polylysine chain length in the presence of erythromycin have not given clear results, but the data do appear to agree with the results of Otaka and Kaji (21) that erythromycin causes an accumulation of di- and tripeptides.

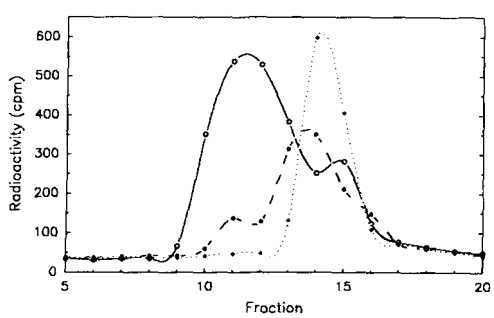

FIG. 5. **Chromatographic profile of the products of polylysine synthesis on Sephadex G-15.** Polylysine synthesis was carried out in the presence (*closed circles*) or absence (*open circles*) of excess erythromycin as described under "Experimental Procedures." Ribosome-bound peptidyl-tRNA was hydrolyzed with 0.2 M NaOH. Then the fraction was neutralized with glacial acetic acid prior to application to the column. The *closed diamonds* represent the position at which free [^{14}C]lysine elutes from the column. Fraction 10 is at the void volume of the column.

DISCUSSION

Peptides are elongated at a rate of up to 10 residues/s *in vivo*; however, the *in vitro* system used in these studies elongates at a considerably slower rate. This is advantageous in that elongation is slowed to a rate at which we can easily detect various changes in the amino terminus by fluorescence techniques. Despite the relatively slow rate of elongation *in vitro*, we were still forced to carry out these experiments at 20 °C since at 37 °C the changes occur too rapidly to measure accurately. After the incorporation of [^{14}C]phenylalanine and [^{14}C]lysine into their respective nascent peptides, we have determined that each is elongating at a nearly linear rate over the times reported here (data not shown). This rate is such that, for polyphenylalanine, nascent chains of greater than 100 amino acids are only produced after 60 min. The rate for polylysine synthesis is considerably less. Although the kinetics of elongation *in vivo* are quite different from the kinetics of this system, the results obtained here should nevertheless be valid as a tool for examining ribosome structure and nascent peptide conformation during elongation.

The decrease in fluorescence anisotropy which occurs as peptide synthesis is initiated with the peptidyl-tRNA analogues appears to reflect increased movement of the probe as it leaves the peptidyl transferase center. The apparent decrease in the constraints on the movement of the probe is associated with a change in fluorescence quantum yield and emission maximum, indicating that the probe enters a more hydrophilic environment. Some of the intensity changes occur very rapidly as the first peptide bond is formed, as indicated by experiments in which only a dipeptide is formed, but the anisotropy changes occur at a later stage of polymerization.

Fluorescence from nascent lysine peptides remains at levels established immediately after initiation as the peptides are extended. There is no indication that the probe at the amino terminus for the nascent peptide enters a tunnel or is constrained by the ribosome. The results appear to indicate that nascent polylysine peptides extend from the peptidyl transferase center directly into the aqueous environment surrounding the ribosome. At the very least, if polylysine does enter a channel or tunnel, the tunnel must be very large and hydrophilic in nature. If it does not enter a tunnel into which natural nascent peptides are normally channeled, this may be related to the fact that polylysine at neutral pH exists mainly as a random coil (24).

The situation for polyphenylalanine is quite different. After

an initial drop, fluorescence anisotropy increases at a decreasing rate throughout the incubation period, apparently due to extension of the nascent polyphenylalanine chain. This increase in anisotropy is associated with a change in quantum yield and a blue shift in emission maximum consistent with the probe entering an increasingly hydrophobic environment. We interpret these results as indicating that as the nascent peptide is extended, polyphenylalanine is accumulated, probably as an insoluble mass of undetermined structure, in the immediate vicinity of the peptidyl transferase center. This hypothesis is strengthened greatly by the results with CITC-erythromycin. This derivative binds to ribosomes with approximately the same affinity as unlabeled erythromycin, apparently at the same binding site on 50 S subunits. CITC-erythromycin bound to empty ribosomes is exchanged readily with unlabeled erythromycin. The exchange can be monitored effectively by changes in CITC-fluorescence anisotropy or fluorescence intensity. Both unlabeled erythromycin and CITC-erythromycin inhibit polylysine synthesis but have little or no effect on polyphenylalanine synthesis. The synthesis of polyphenylalanine on ribosomes to which CITC-erythromycin is bound causes an increase in the quantum yield of its fluorescence, indicating that the environment of the probe becomes increasingly hydrophobic as the nascent peptide is extended. The fluorescence anisotropy of the CITC-erythromycin remains high, demonstrating that the antibiotic is not displaced from the ribosome by the nascent peptide. Most important for the hypothesis that the nascent polyphenylalanine is accumulated as an insoluble mass near the peptidyl transferase center is that the exchange of CITC-erythromycin with unlabeled erythromycin decreases as nascent peptide is formed. We interpret this result, considered with the apparent increase in the hydrophobicity of the environment around the CITC-erythromycin, to indicate that the antibiotic is shielded directly by the nascent peptide.

The results considered above prompt questions about why polyphenylalanine synthesis is not inhibited by erythromycin and about the physical characteristics and function of the erythromycin binding site. We suggest that the lack of inhibition is related to the unusual chemical and physical characteristics of polyphenylalanine compared with those of most other polypeptides. It appears likely that the nascent polyphenylalanine peptide may collapse as it is formed into an insoluble mass lacking discrete secondary structure. In contrast, most natural peptides may take the form of an α-helix as they are extended on a ribosome. Lim and Spirin (25) have presented persuasive arguments that an α-helix inevitably would be formed by the corotational movement of the nascent peptide with the tRNA as a consequence of the codon-anticodon interaction as the tRNA and mRNA are moved through the ribosome.

The function of the erythromycin binding site is unclear. It is very close to the peptidyl transferase center, apparently within a distance occupied by a di- or tripeptide. It appears to be relatively hydrophobic in nature, as judged by changes in fluorescence from CITC-erythromycin which occur upon binding to ribosomes, and is occupied by the nascent peptide after it is extended beyond a critical length. It has been suggested (7) that erythromycin may block the tunnel spanning the large ribosomal subunit (5) through which the nascent peptide may be channeled to reach the exit domain on the outer surface of the subunit. This is a provocative hypothesis, but the data presented here provide very little indication as to whether or not it is correct. Erythromycin blocks the extension of nascent polylysine chains; however, it appears that these nascent peptides may extend directly into the aqueous environment from the peptidyl transferase center. Thus, inhibition of polylysine synthesis would not be anticipated if erythromycin blocks the entrance to the tunnel. Perhaps there is a channel or obligatory path immediately adjacent to the peptidyl transferase center through which most peptides must pass even before they enter the proposed tunnel. Erythromycin may block this path. Unfortunately, these suggestions and the data presented above provide little indication of how the nascent peptide is channeled from the peptidyl transferase center to the exit domain for proteins whose synthesis may involve this mechanism.

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