Fluorescence Characterization of the Environment Encountered by Nascent Polyalanine and Polyserine as They Exit *Escherichia coli* Ribosomes during Translation†

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ABSTRACT: The fate of the amino termini of nascent polyalanine, polyserine, and polylysine was monitored by fluorescence techniques as each was translated on *Escherichia coli* ribosomes. A coumarin probe was placed at the α-amino group of a synthetic elongator alanyl-tRNA or a synthetic initiator alanyl-tRNA or at the ϵ-amino group of natural lysyl-tRNA, and each was used to nonenzymatically initiate peptide synthesis. The fluorescent alanyl-tRNAs containing an AAA anticodon were used to initiate polyserine (with a synthetic $tRNA^{Ser}$) or polyalanine synthesis from a poly(uridylic acid) template. The fluorescent lysyl-tRNA was used to initiate polylysine synthesis from poly(adenylic acid). Changes in the fluorescence of the amino-terminal coumarin were examined to characterize the environment of the probe as the nascent peptides were extended. Protection from proteolysis and the binding of anti-coumarin antibodies or Fab fragments suggest that the amino terminus of each polypeptide is protected from interaction with proteins ($M_r > 28\,000$) until the peptides are extended to an average length of 40–50 residues; however, the fluorescence from the amino terminus of shorter nascent polyalanine and polyserine peptides was readily quenched by methyl viologen ($M_r = 257$), indicating ribosomes do not shield the nascent peptide from molecules of this size. The data appear to indicate that polyalanine, polyserine, and polylysine are extended from the peptidyl transferase into a protected region of the ribosome such as a groove or tunnel but that this region is readily accessible to small molecules.

Recently there has been much interest in the topology and physical character of the pathway followed by nascent peptides as they leave the peptidyl transferase center and exit the ribosome. The results from earlier studies indicated that a segment consisting of 30–40 amino acids of nascent peptides is protected from proteolytic degradation by ribosomes (Malkin & Rich, 1967; Blobel & Sabatini, 1970; Smith et al., 1978; Ryabova et al., 1988). These observations appear to be consistent with the movement of the nascent peptide through a protective path or tunnel within the ribosome. By three-dimensional image reconstruction from electron micrographs, Yonath and co-workers identified a cavity or tunnel through the 50S ribosomal subunit of *Bacillus stearothermophilus* (Yonath et al., 1987). The cavity extended from an area near the peptidyl transferase center on the interfacing surface to a region at or near the nascent peptide exit site (Bernabeu & Lake, 1982). Yonath and co-workers (Yonath et al., 1987) hypothesized that the tunnel may be the path followed by nascent peptides exiting the ribosome. A similar structure was observed in 80S ribosomes (Milligan & Unwin, 1986) and in *Escherichia coli* ribosomes (Frank et al., 1990). Frank and co-workers also proposed that the region of the hole distal to the peptidyl transferase center may be the nascent peptide exit site (Frank et al., 1990). Immunoelectron microscopy studies indicated that the nascent peptide was accessible to antibodies at two sites on the back side of the 50S subunit, but at no point between these areas and the peptidyl transferase center (Ryabova et al., 1988). This result was interpreted to suggest the existence of a channel along the surface of the 50S subunit from a point very near the peptidyl transferase center to the exit site. This alternative model was supported by Radermacher et al. (1988), who observed a channel leading from near the peptidyl transferase center toward an indentation on the back of the 50S subunit near the region where antibody binding to the exiting nascent peptide was seen. Both models suggest a specific pathway from the peptidyl transferase center to the nascent peptide exit site identified by Bernabeu and Lake (1982). A groove on the surface of the ribosome could easily accommodate a variety of polypeptide sequences as well as bulky side chains. Yonath et al. (1990) suggested that the tunnel through the 50S subunit also could easily provide these features. Which of these models is correct, if either, remains unclear. The characteristics of the path followed by the nascent peptide are the topic of this paper.

Previously we have examined changes in the environment of polyphenylalanine and polylysine as these nascent peptides were extended on ribosomes. Fluorescent signals emitted from coumarin probes attached to the amino terminus of each were followed (Picking et al., 1991a). The results did not indicate that either nascent polypeptide enters a region clearly identified as a tunnel. However, polyphenylalanine is a very atypical peptide with respect to its low solubility in most aqueous and organic solvents, its physical structure, and the fact that its synthesis is not inhibited by erythromycin. It appears to accumulate as an unstructured, hydrophobic mass which can cover erythromycin that is bound to the ribosome near the peptidyl transferase center (Odom et al., 1991; Picking et al., 1991a). It has been suggested that erythromycin acts to inhibit peptide elongation by sterically blocking the entrance to the tunnel described by Yonath's group (Arevalo et al., 1988).

Nascent polylysine was examined because its properties are in contrast to those of polyphenylalanine. Its synthesis is

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sensitive to erythromycin, and it is very soluble in aqueous solutions, but the fluorescence anisotropy from a coumarin probe linked to the amino-terminal residue of polylysine decreases rapidly as the nascent peptide is extended, perhaps suggesting immediate exit from the ribosome (Picking et al., 1991a). The combined results suggest the possibility that nascent peptides might exit the ribosome by different routes, either directly from the peptidyl transferase center into the solvent surrounding the ribosome or through a path or tunnel to the exit domain. Interpretation of our earlier results was compromised in that polylysyl-tRNA can be easily dissociated from ribosomes as peptide synthesis takes place (Spirin et al., 1988) and polylysine demonstrates unusual characteristics in solution in the presence or absence of poly(adenylic acid) [poly(A)][1] (Tsuboi et al., 1966). Also, only relatively short polylysine peptides (approximately 15 amino acids) can be efficiently formed in the poly(A)-directed system.

To alleviate the drawbacks of polyphenylalanine and polylysine synthesis, synthetic tRNAs that can be used for poly(U)-dependent synthesis of polycysteine and polyserine (Picking et al., 1991b) or polyalanine (Picking et al., 1991c) have been generated. In addition, a synthetic initiator tRNA species that contained an AAA anticodon and that can be enzymatically aminoacylated with alanine but is structurally and functionally similar to initiator $tRNA_f^{Met}$ has been formed. It is active with IF-2 and GTP in what appears to be normal enzymatic initiation of peptides with *N*-acylalanine from UUU codons (Picking et al., 1991c).

Here we present evidence that the amino termini of polyalanine and polyserine nascent peptides are apparently accessible to methyl viologen at all points following the start of poly(U)-dependent translation but that specific antibody molecules do not have ready access until the nascent polyalanine and polyserine have reached a length exceeding 50 residues. Proteolysis with proteinase K indicates that the amino terminus of polyalanine is protected from proteolysis until the nascent polypeptide exceeds 30 residues in length. From these data, we propose that polyalanine and polyserine synthesized with synthetic tRNAs enter a protected region on the ribosome and that this region has the characteristics of a groove or a tunnel which is easily accessible to small molecules and could thus accommodate a wide variety of amino acids with very bulky side chains.

Materials and Methods

Materials. [^{14}C]Serine and [^{14}C]alanine were from ICN Radiochemcials (Irvine, CA). [^{3}H]Alanine was from Amersham Corp. (Arlington Heights, IL). 3-(4-Maleimidophenyl)-7-diethylamino-4-methylcoumarin (CPM) was from Molecular Probes (Eugene, OR). Methyl viologen and β-galactosidase were from Sigma Chemical Co. (St. Louis, MO). Proteinase K was from E. Merck (Darmstadt, Germany). The preparation of *Escherichia coli* ribosomal subunits has been described in detail previously (Odom et al., 1980).

tRNA Preparation. Plasmids encoding the synthetic elongator $tRNA^{Ala}$ (pALA) or synthetic initiator $tRNA^{Ala}$ (pFMET) and synthetic elongator $tRNA^{Ser}$ (pSER) have been described previously (Picking et al., 1991b,c). Transcription of the linearized plasmids with T7 RNA polymerase has also been described previously (Picking et al., 1991b). The synthetic tRNAs were aminoacylated with [^{3}H]alanine using the aminoacyl-tRNA synthetases present in the 0–70% ammonium sulfate precipitate of a wheat germ S-150 fraction (Lax et al., 1986), as described previously (Picking et al., 1991b). CPM labeling of mercaptoacetic acid covalently linked to the amino group of the amino acid of each of the synthetic alanyl-tRNAs was performed as described previously (Odom et al., 1990). After separation from other components by C1-reversed-phase HPLC, only CPM-SAcAla-tRNAs with a CPM:amino acid:tRNA ratio of 1:1:1 were used in these studies.

Poly(U)-Dependent Polypeptide Synthesis. First, nonenzymatic binding of *N*-acylaminoacyl-tRNA to ribosomes was carried out in 50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, 2 mM 2-mercaptoethanol (solution A), and 0.2 mg/mL poly(U) with 300 pmol of ribosomes and 30–40 pmol of CPM-SAcAla-tRNA in a final volume of 300 μL. Polyalanine or polyserine synthesis was then performed as described previously (Picking et al., 1991b,c). Synthetic elongator tRNAs were used at a final concentration of 1 A_{260} unit/mL. Incorporation of [^{14}C]alanine (50 Ci/mol) into polyalanine and of [^{14}C]serine (50 Ci/mol) into polyserine was measured as described previously (Picking et al., 1991a). The nascent peptide chain length was calculated by determining the picomoles of amino acids incorporated and the number of active ribosomes for each type of nascent peptide examined [cf. Picking et al. (1991b,c)]. The number of active ribosomes was estimated by prebinding N-blocked radiolabeled alanyl-tRNA at a 1 to 1 molar ratio with ribosomes and then measuring the incorporation of radioactive amino acids after translation in the presence of unlabeled alanine or serine. The percentage of ribosomes active in polyalanine synthesis was about 25% and in polyserine synthesis was about 10%. In subsequent experiments, the picomoles of [^{14}C]amino acid incorporated into polyalanine or polyserine divided by the picomoles of active ribosomes used gave the average nascent chain length.

In all cases, the ribosomes bearing nascent peptides were separated from the rest of the translation components by gel filtration on Sephacryl S300 (16 mL) in solution A. The poly(A)-directed synthesis of polylysine (after prebinding εCPM-SAc-αAcLys-tRNA) was performed exactly as previously described (Picking et al., 1991a). The longest polylysine which can be synthesized in this way contains about 15 residues.

Nascent peptide chain lengths were also measured by polyacrylamide gel electrophoresis in the presence of SDS and urea (Anderson et al., 1983). Following the isolation of the nascent peptide-bearing ribosomes, an aliquot was removed to mesure the incorporation of [^{14}C]alanine so that the nascent peptide chain length could be calculated. The remainder of the fractions containing nascent polyalanine or polyserine was precipitated with 5% trichloroacetic acid. The precipitated components were centrifuged and washed twice with acetone. The precipitates were resuspended in 25% NH_4OH and incubated for 60 min at 37 °C to deacylate all the tRNA in the sample. The liquid was then removed by evaporation, and the samples were resuspended in electrophoresis sample buffer.

[1] Abbreviations: poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); $tRNA_f^{Met}$, natural *E. coli* initiator tRNA; CPM, 3-(4-maleimidophenyl)-7-diethylamino-4-methylcoumarin; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Fab, fragments of IgG molecules containing a single antibody binding site prepared by digestion of IgG with papain; CPM-SAcAla-tRNA, a synthetic $tRNA^{Ala}$ which contains an AAA anticodon sequence and which has been aminoacylated with alanine and then labeled on the alanine amino group via mercaptoacetic acid by CPM; initiator CPM-SAcAla-$tRNA_i$, a synthetic tRNA which contains an AAA anticodon and otherwise the known sequence of $tRNA_i^{Met}$ and which has been aminoacylated with alanine and labeled on the alanine amino group with mercaptoacetic acid and then CPM; εCPM-SAc-αAcLys-tRNA, $tRNA^{Lys}$ which has been aminoacylated with lysine, mercaptoacetylated and CPM-labeled at its ε-amino group, and acetylated at its α-amino group.

The polypeptides in the sample were separated on 30% polyacrylamide gels according to the procedure of Anderson et al. (1983). The gels were then dried and autoradiographed. Low molecular weight Rainbow markers (Amersham) were used to determine the size of the resultant polypeptides. The markers used included the insulin a chain (3450 Da), the insulin b chain (3400), aprotinin (6500), lysozyme (14 300), and trypsin inhibitor (21 500).

Steady-State Fluorescence Measurements. A Model 8000 photon-counting spectrofluorometer from SLM–Aminco Instruments, Inc. (Urbana, IL), was used for steady-state fluorescence measurements as described (Rychlik et al., 1983). Spectra were measured at 1-nm emission intervals at a scanning rate of 0.5 s per wavelength increment at an excitation wavelength of 385 nm. Fluorescence anisotropy and relative intensity measurements were performed 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 of solution A. All measurements were made with a sample absorbance of less than 0.1 at the excitation wavelength.

Preparation of Rabbit Anti-CPM IgG and Measurement of Their Binding to CPM-SAc-Alanine. CPM was attached to the sulfhydryl groups of β-galactosidase by incubating 2 mM CPM with 2 mg/mL β-galactosidase in 50% dimethylformamide. CPM-β-Galactosidase was then removed from the unreacted CPM by gel filtration on Sephadex G-100 equilibrated with 50 mM Hepes–KOH (pH 7.5). The CPM-labeled β-galactosidase was concentrated to 1 mg/mL using a Centricon-30 (Amicon).

Two female New Zealand White rabbits were injected subcutaneously with 100 μg of CPM-β-galactosidase mixed with an equal volume of Freund's complete adjuvant. Every 2 weeks, 30 mL of blood was drawn from the main artery of the ear, and the animals were boosted with CPM-β-galactosidase. IgG was obtained by passing the serum over protein G–agarose (Sigma) equilibrated with 0.1 M potassium phosphate (pH 7.2) and 1 mM PMSF. IgG was eluted with 0.1 M glycine hydrochloride (pH 3.0) and was then dialyzed against 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl. Fab fragments were generated from this IgG preparation by incubation with papain (Sigma) as has been described (Weir, 1986).

The binding of anti-CPM IgG or Fab fragments to CPM-SAc-alanine resulted in an increase in the intensity of the CPM fluorescence accompanied by a large blue shift in the emission maximum (from 470 to about 450 nm). The fraction of CPM associated with the nascent peptide bound by IgG was calculated directly from the emission spectra by using the following equations: observed F at 450 nm = $f_f(I_f$ at 450 nm) + $f_b(I_b$ at 450 nm); observed F at 470 nm = $f_f(I_f$ at 470 nm) + $f_b(I_b$ at 470 nm). F is the observed fluorescence intensity; f_f and f_b are the fractions of unbound and bound CPM, respectively. I_f is the intensity of the unbound CPM, and I_b is the intensity of the bound CPM. Either of the above equations can be used to calculate f_b, because by substituting $1 - f_b$ for f_f and rearranging, f_b = (observed $F - I_f$)/($I_b - I_f$). For these calculations, the emission spectrum of CPM was measured; then the antibodies were added, and the spectrum was taken again. When the CPM-containing moiety (as CPM-SAc-Ala-tRNA or at the N-terminus of a nascent peptide) was attached to ribosomes, the emission spectrum was measured before and after adding immune IgG; then the fluorescence emission spectrum was measured again at the end of the experiments after adding puromycin to a final concentration of 1 mM to release all the associated nascent peptides.

Quenching of CPM Fluorescence with Methyl Viologen. To examine the accessibility of the amino terminus of polyalanine and polyserine to small molecules in the solvent surrounding the ribosome, methyl viologen (M_r = 257) was used as a specific quencher of coumarin fluorescence (Jones et al., 1984). The fluorescence of various samples containing CPM-SAcAla (as elongator CPM-SAcAla-tRNA, as initiator CPM-SAcAla-tRNA$_i$, or as N-terminal CPM-SAcAla in polyalanine or polyserine) was quenched with increasing amounts (0–20 mM) of methyl viologen. The degree of fluorescence quenching was then plotted as F_0/F versus the methyl viologen concentration according to the Stern–Volmer equation $F_0/F = 1 + K_Q[Q]$, where F_0 is the starting fluorescence intensity, F is the fluorescence intensity in the presence of quencher, and [Q] is the methyl viologen quencher concentration (Stern & Volmer, 1919). The Stern–Volmer quenching constants (K_Q) are defined by the slopes of the resulting plots. K_Q can be used as a relative measure of the accessibility of a given fluorophore to the quenching agent used. All fluorescence quenching experiments were performed at an excitation wavelength of 385 nm. The emission wavelength for quenching was 475 nm in all cases except when CPM-containing moieties were bound by anti-CPM antibodies in which case the emission was measured at 460 nm. The degree of quenching observed was not altered by the emission wavelength used for measurement, so a wavelength near the emission maximum of CPM bound by anti-CPM antibodies was used when these antibodies were present.

For comparison, ribosomes bearing nascent peptides with CPM-SAcAla at the amino terminus were isolated by gel filtration and divided into two equal fractions. Each fraction was made to 0.6 mL with solution A. Methyl viologen quenching of CPM fluorescence was measured directly on one fraction, while the other fraction was first incubated with 1 mM puromycin for 60 min at 20 °C to release nascent peptides from the ribosomes. In this way, it was possible to compare the ability of methyl viologen to quench the N-terminal CPM fluorescence of a ribosome-associated nascent peptide with its ability to quench the N-terminal fluorescence of the identical polypeptide after release from the ribosome. This was done to compare the degree of quenching of the amino termini of identical peptides which are either ribosome-associated or puromycin-released.

Proteinase K Digestion of the Nascent Polypeptides. Proteinase K was suspended at 1 mg/mL in water. Ribosomes bearing CPM-SAcAla-tRNA or CPM-SAcAla at the amino terminus of polyalanine or polyserine were prepared and isolated by gel filtration on Sephacryl S300. The fluorescence anisotropy of CPM was determined in solution A (time = 0 min). Proteinase K was then added to a final concentration of 5 μg/mL, and the change in fluorescence anisotropy was monitored over time at 20 °C. The ability of the proteinase K to hydrolyze the amino terminus of the nascent polypeptide was assessed by the initial rate (as ΔA/min) of the decrease in anisotropy. A decrease in fluorescence anisotropy would be expected as the amino-terminal CPM-SAcAla residue is proteolytically removed from nascent polyalanine and polyserine. The rate of the initial decrease in anisotropy would be expected to increase proportionally with increased accessibility to proteinase K.

Results

Synthesis of Polyalanine and Polyserine with CPM-SAc-Alanine at the Amino Terminus. In vitro translation of

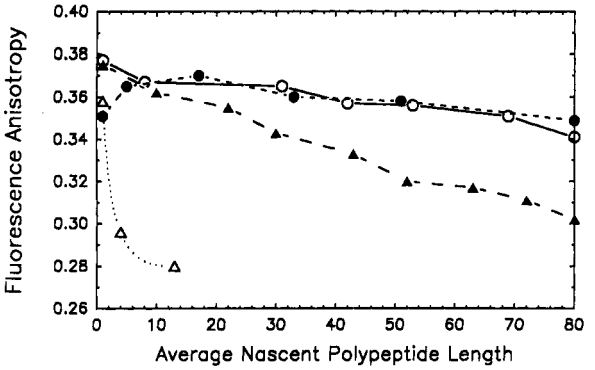

FIGURE 1: Change in fluorescence anisotropy of the amino terminus of polyalanine and polyserine as a function of average nascent peptide chain length. CPM-SAcAla-tRNA (40 pmol) or CPM-SAcAla-tRNA$_i$ (40 pmol) was prebound to ribosomes (250 pmol) as described under Materials and Methods. Synthesis of polyalanine (behind the elongator or initiator CPM-SAcAla-tRNA) or polyserine (behind the elongator CPM-SAcAla-tRNA) was then initiated and allowed to proceed at 25 °C for various lengths of time. The nascent peptide-bearing ribosomes were isolated by chromatography on Sephacryl S300, and the nascent peptide chain length was determined as described under Materials and Methods. The fluorescence anisotropy of ribosome-associated polyserine initiated with elongator CPM-SAcAla-tRNA (closed triangles, long dashed line), polyalanine initiated with elongator CPM-SAcAla-tRNA (open circles, solid line), polyalanine initiated with initiator CPM-SAcAla-tRNA (closed circles, short dashed line), and polylysine initiated with ϵCPM-SAc-αAcLys-tRNA (open triangles, dotted line) was determined and is shown as a function of the calculated average chain length.

polyalanine and polyserine from a poly(U) template was carried out for different lengths of time with CPM-SAc-alanine as the amino-terminal residue. The fluorescence anisotropy of the fluorophore at the amino terminus of each peptide was monitored as a function of the average length of the ribosome-associated peptides.

As shown in Figure 1, the fluorescence anisotropy of elongator CPM-SAcAla-tRNA bound to ribosomes is very high (0.375). This value is lower for the synthetic initiator CPM-SAcAla-tRNA$_i$ (0.353), apparently indicating that the two tRNA species are not bound to the ribosomes in exactly the same way. The anisotropy of the CPM-SAcAla-tRNA prior to ribosome binding was 0.185, 0.200 for initiator CPM-SAcAla-tRNA$_i$. Polyalanine or polyserine synthesis was carried out, and the nascent peptide-bearing ribosomes were isolated by gel filtration; then the anisotropy was measured. A chain length dependent decrease in fluorescence anisotropy was observed for the amino terminus of each nascent peptide except that of polyalanine which was started after prebinding initiator CPM-SAcAla-tRNA$_i$. In the latter case, the anisotropy initially increased until it reached a value similar to polyalanine initiated with elongator CPM-SAcAla-tRNA, before beginning to decrease slightly (Figure 1). The anisotropy of fluorescence from CPM-SAcAla at the amino terminus of polyserine decreased at a quicker rate, perhaps suggesting that polyserine is less structured during elongation than is polyalanine. The fluorescence anisotropy of ϵ-CPM-SAc-αAcLys at the amino terminus of polylysine decreased rapidly as elongation proceeded with poly(A) (Figure 1). This could reflect the immediate exit of this polypeptide from the ribosome or the propensity of polylysine to form a random coil. An additional effect of the CPM moiety being at the lysine ϵ-amino group must also be considered. In all cases, when puromycin was added to the solution containing ribosome-bearing nascent peptides, the anisotropy dropped to less than 0.250 (about 0.17 for puromycin-released CPM-SAcAla; 0.246 for Ala$_n$ peptides where n approached 100 residues). Greater than 80% of the ribosome-bound CPM-SAcAla-tRNA was reactive with puromycin on the basis of release of radioactive CPM-SAcAla (data not shown).

Polyacrylamide gel electrophoresis with autoradiography was used to confirm the length of nascent peptides. Autoradiographs indicated that continued translation of polyalanine resulted in a time-dependent increase in the apparent molecular weight of the Ala$_n$ peptides. Polyalanine peptides calculated to be 34 residues in length (from incorporated [^{14}C]alanine) migrated on SDS–polyacryalmide gels as polypeptides of about 2100–2600 Da, corresponding to about 29–36 alanine residues (data not shown). This is in reasonable agreement with the polyalanine chain length estimated by [^{14}C]alanine incorporation. Similar results were obtained for determinations of polyserine chain length by urea–SDS–polyacrylamide gel electrophoresis and by [^{14}C]serine incorporation, although the bands containing polyserine were more diffuse than those with polyalanine.

The results presented in Figure 1 show that the environment surrounding the fluorescent amino terminus of these peptides changes as they are extended. The decrease in anisotropy indicates that as these peptides grow in length, their amino termini become more mobile, in turn suggesting they enter a less confined environment. In no case, however, does the anisotropy reach that of the fluorescent polypeptides that have been released from ribosomes with puromycin (Figure 1), although the fluorescence anisotropy of the amino terminus of polylysine rapidly drops to less than 0.3. The fact that the fluorescence anisotropy for polylysine decreases faster than that for polyserine, which decreases faster than that for polyalanine, may reflect a decreasing tendency for each of these polypeptides to form a random coil. Polyalanine is the only one usually found in a highly structured α-helix in aqueous solutions (Chou & Fasman, 1974; Padmanabhan & Baldwin, 1991).

Accessibility of the CPM Residue Attached to the Amino Terminus of Nascent Polyalanine and Polyserine to Antibodies Generated Against CPM. Antibodies directed against CPM were prepared and used to measure the accessibility of the amino termini of nascent polyalanine, polyserine, or polylysine on ribosomes. When the immune IgG fraction was added to CPM-SAcAla-tRNA, a large increase in fluorescence intensity (to a quantum yield approaching 1.00) and a blue shift in its emission spectrum (Δ of 25 nm) were observed (Figure 2A). No change in fluorescence was observed when CPM-SAcAla-tRNA was incubated with the preimmune IgG fraction (Figure 2A). The unusually large blue shift (to less than 455 nm) in the emission maximum and the large increase in fluorescence intensity of the CPM fluorescence were the largest ever observed for the probe in this laboratory and provided convenient measures for antibody binding.

When the anti-CPM IgGs were titrated with CPM-SAcAla-tRNA, which had been prebound to ribosomes, no change in fluorescence was observed (Figure 2B). If puromycin was then added to the reaction mixture, causing release of CPM-SAcAla-puromycin, a blue shift in the emission maximum was observed which was nearly identical to that observed for unbound CPM-SAcAla-tRNA (cf. Figure 2A). Identical results were observed if the synthetic initiator tRNA species, CPM-SAcAla-tRNA$_i$, was substituted for the elongator tRNA (data not shown). As a control, CPM-SAcAla-tRNA was prebound to 70S ribosomes, antibodies were added, and then the reaction mixture was chromatographed on Sephacryl S300. All of the fluorescent material remained with the ribosome fraction and had an emission maximum of 471 nm, indicating that no

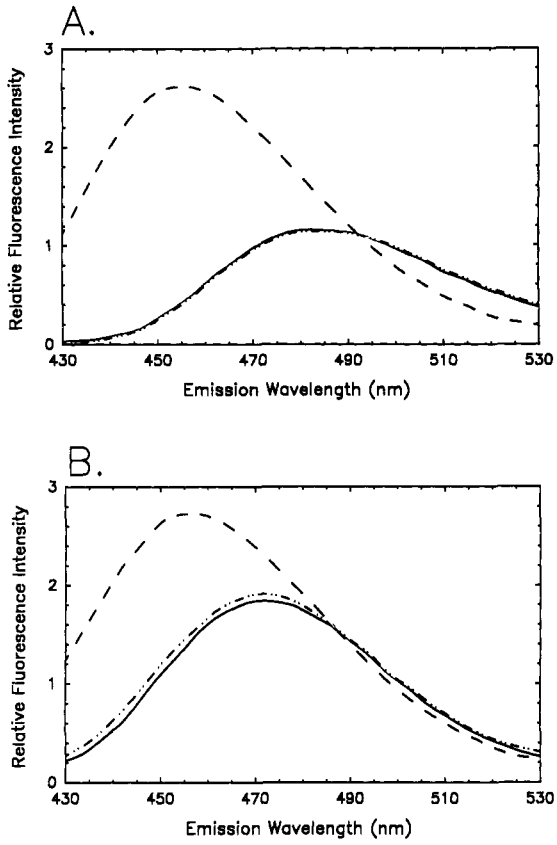

FIGURE 2: Changes in the emission spectrum of CPM-SAcAla-tRNA by IgG isolated from rabbit antiserum prepared against CPM. (A) the emission spectrum of elongator CPM-SAcAla-tRNA (30 pmol) is shown as a solid line, the emission spectrum after the addition of 300 pmol of preimmune IgG fraction is shown by the dot–dot–dash line, and the emission spectrum after the addition of 300 pmol of anti-CPM antibodies (IgG fraction) is shown by the dashed line. (B) The emission spectrum of ribosome-bound elongator CPM-SAcAla-tRNA is shown as the solid line. The dot–dot–dash line is the emission spectrum after the addition of the anti-CPM antibodies (IgG). The dashed line is after releasing CPM-SAcAla-puromycin in the presence of the anti-CPM antibodies by reaction with 1 mM puromycin for 45 min. All emission spectra were measured with an excitation wavelength of 385 nm at 20 °C.

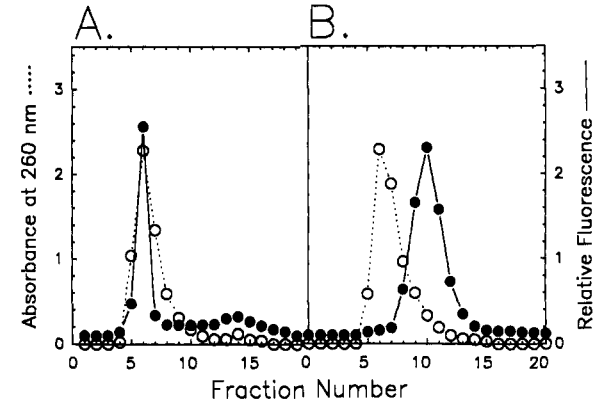

FIGURE 3: CPM-SAcAla-tRNA binds to either ribosome or anti-CPM IgG, but not to both. In (A), CPM-SAcAla-tRNA (40 pmol) was prebound to ribosomes (300 pmol) plus poly(U), and then anti-CPM antibodies (300 pmol) were added in a total final volume of 300 μL. The ribosomes were then isolated by gel filtration on Sephacryl S300. The ribosomes eluted at the void volume as shown by the elution of material absorbing at 260 nm (dotted line). All the fluorescent material also eluted at this point (solid line) and had an emission maximum at 471 nm. In (B), CPM-SAcAla-tRNA was first incubated with the anti-CPM antibodies; then ribosomes and poly(U) were added. The ribosome fraction was again eluted from Sephacryl S300 at the void volume (dotted line). In this case, however, the fluorescent material eluted after the void volume (solid line) and had an emission maximum at 453 nm. Fluorescence was measured with an excitation wavelength of 385 nm.

antibody binding had occurred (Figure 3A). Conversely, if CPM-SAcAla-tRNA was prebound to the antibodies, then ribosomes were added, and the mixture was fractionated on Sephacryl S300, no fluorescence was seen in the ribosome fraction, but fluorescence was observed in the protein fraction which trailed the ribosome fraction (Figure 3B). The emission maximum of this peak was at 453 nm, indicating antibody·CPM interaction and that the antibodies prevented binding of the tRNA to the ribosomes. Once CPM-SAcAla-tRNA was bound to ribosomes and the ribosomes were isolated by gel filtration, no time-dependent release of the bound fluorescence tRNA was observed as judged by adding anti-CPM IgG and looking for changes in intensity and the emission maximum.

Various lengths of polyalanine and polyserine were synthesized after prebinding either initiator or elongator CPM-SAcAla-tRNA followed by isolation of the nascent peptide-bearing ribosomes by chromatography on Sephacryl S300. The effect of the antibodies on the emission spectrum of the ribosome-associated fluorescence was determined. Puromycin was then added to release the nascent peptides from the ribosomes, and then the emission spectrum was remeasured. The change in the fluorescence intensity at 450 and 470 nm before adding puromycin was then used to calculate the fraction of CPM bound by the antibodies as described under Materials and Methods (Figure 4). The maximum spectral blue shift was obtained by reacting the ribosome-associated nascent peptides with puromycin in the presence of the anti-CPM antibodies and was used in each case to represent complete antibody·CPM binding. Previous work had indicated that greater than 80% of the fluorescence was released from the ribosomes after reaction with puromycin (data not shown). Binding of CPM-SAcAla-tRNA to ribosomes in itself caused an increase in the relative fluorescence intensity (of about 30–40%) and a blue shift in the emission maximum (from 480 to 470 nm). A slight further decrease in the emission maximum to about 467 nm occurred as the nascent peptides were extended with alanine. The data indicate that the amino terminus of very short polyalanine and polyserine (<10 residues) is entirely shielded from antibody binding until after reaction with puromycin. Only after the average nascent polypeptide lengths exceeded 20 residues was a significant amount of the amino-terminal CPM bound by antibodies prior to the addition of puromycin. When the polyalanine and polyserine chains were extended to about 60 residues, the fraction of the CPM bound by antibodies reached half of the maximum binding achieved upon reaction with puromycin (Figure 4). About 70% was reached at twice that length. That the amino termini of the polypeptides appear to never become entirely accessible to the antibodies may be due to a small population of ribosomes that bind CPM-SAcAla-tRNA but are not involved in peptide synthesis. These data suggest that the amino termini of polyalanine and polyserine become accessible to the anti-CPM IgG only after they grow to an average length of about 60 residues. Similar results were seen when peptides were initiated with initiator CPM-SAcAla-$tRNA_i$ (data not presented).

When the anti-CPM IgGs were incubated with ribosomes bearing nascent polylysine that averaged either 4 or 13 residues in length, no binding was observed until after incubation with puromycin (data not shown). This result is consistent with the data for polyalanine and polyserine and occurs even though the anisotropy of the N-terminus of polylysine rapidly de-

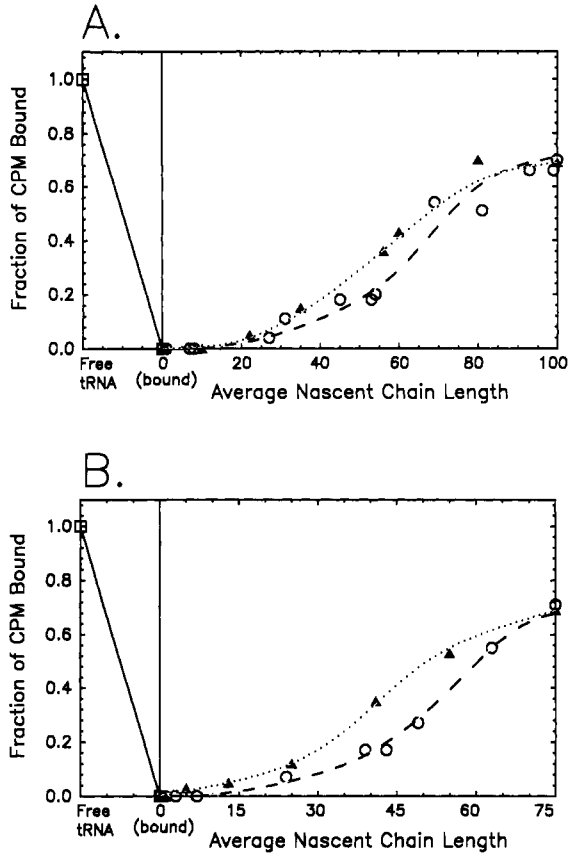

FIGURE 4: Binding of anti-CPM IgG and Fab fragments to CPM at the amino terminus of polyalanine and polyserine. (A) Ribosomes bearing nascent polyalanine (open circles) or polyserine (closed triangles) were prepared after prebinding CPM-SAcAla-tRNA (open squares). The ribosomes were then isolated by gel filtration on Sephacryl S300 and the average nascent chain length was calculated as described under Materials and Methods. Rabbit IgGs (300 pmol) prepared against CPM were then added, and the resultant change in the emission spectrum of CPM-SAcAla was measured after 5 min at 20 °C. Puromycin was then added to a final concentration of 1 mM and allowed to react for at least 45 min, and the emission spectrum was measured again. Shown on the y axis is the amount of the ribosome-associated amino-terminal CPM bound as a fraction of the total possible amount bound after the release of the nascent peptides with puromycin. All spectra were measured at an excitation wavelength of 385 nm at 20 °C. (B) The accessibility of CPM at the amino terminus of polyalanine to Fab fragments (300 pmol) was determined exactly as the accessibility to IgG was measured (shown in panel A). The Fab fragments were prepared from the IgG preparation used in part A. The spectra were measured at an excitation wavelength of 385 nm at 20 °C.

creased upon peptide synthesis (see Figure 1).

To extend these results, the immune IgG was treated with papain to generate Fab fragments (M_r = 50000). These fragments had the same effect on the fluorescence properties of free and ribosome-bound CPM-Ala-tRNA as did the intact IgG (data not presented). They were then used in experiments similar to those described above. As with the anti-CPM IgG, little or no CPM binding was observed until the average nascent poly(Ala) chain length exceeded 20 residues. Subsequent release by reaction with puromycin resulted in substantial Fab binding (Figure 4B). Also, the fraction of CPM bound by Fab did not become half-maximal until the nascent polypeptides averaged about 50 residues in length (Figure 4B). These data suggest that M_r > 50000 proteins have limited access to the amino terminus of nascent polyalanine and polyserine until the polypeptides exceed 50 residues in length. Apparently they also have no access to the amino terminus of polylysine 13 residues long despite the very low anisotropy

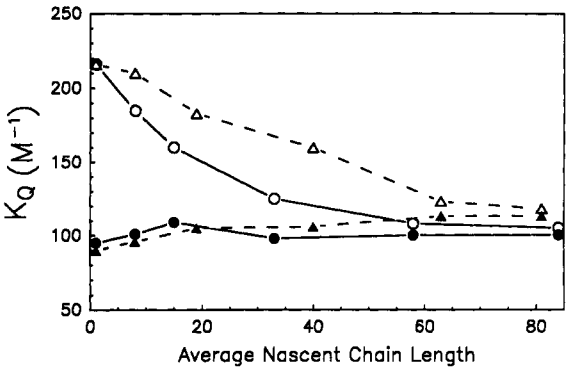

FIGURE 5: Methyl viologen fluorescence quenching of the N-terminal CPM-SAc-alanine of polyalanine and polyserine. CPM-SAcAla-tRNA$_e$ was bound to ribosomes, and different lengths of polyalanine or polyserine were synthesized (Materials and Methods). The ribosomes were then isolated by gel filtration and divided into two fractions. Methyl viologen quenching was performed directly on one set and was performed on the second set after the nascent peptides were released by puromycin. In each case, the calculated K_Q was then plotted versus average peptide length. The solid lines represent polyalanine (○, ●), and the dashed lines represent polyserine (△, ▲). Open symbols are the ribosome-associated peptides, and the closed symbols are those peptides that were released by reaction with puromycin prior to the quenching experiments. On the basis of anisotropy measurements, no significant release of the ribosome-associated peptides occurred during the course of the quenching experiments.

of the polylysine amino terminus.

Ability of Methyl Viologen To Quench the Fluorescence of the Amino-Terminal CPM of Polyalanine and Polyserine. Fluorescence quenching of the amino-terminal CPM by methyl viologen (M_r = 257) was examined to test protection of the probe from small molecules in aqueous medium. The results for CPM-SAc-alanine at the amino terminus of various lengths of nascent polyalanine and polyserine are presented in Figure 5. Initially, the ability of methyl viologen to quench fluorescence from the synthetic elongator and initiator CPM-SAcAla-tRNAs was examined. The tRNAs were tested free in solution and when each was first incubated with anti-CPM antibodies. The quenching constant was relatively high for the free CPM-Ala-tRNA (K_Q = 90 M^{-1}) but was reduced to about 20 M^{-1} when bound to IgG. The K_Q for the corresponding serine derivative, CPM-SAcSer-tRNA, was the same (90 M^{-1}). The K_Q for free synthetic initiator CPM-SAc-Ala-tRNA was 73 M^{-1}, which is somewhat lower than that of the elongator CPM-SAcAla-tRNA.

Quenching constants for methyl viologen with the CPM-SAcAla-tRNAs were also examined after each was bound to poly(U)-programmed ribosomes and after each was released from the ribosomes by reaction with puromycin. Interestingly, the ability of methyl viologen to quench the fluorescence of both CPM-SAcAla-tRNAs increased greatly when each was bound to 70S ribosomes (K_Q = 216 M^{-1}). The basis of this phenomenon is not known but could reflect either an electrostatic interaction between negative charges within the peptidyl transferase center of the ribosome and cationic methyl viologen (resulting in an elevated local concentration of the latter) or an increase in the fluorescence lifetime (τ) of the CPM following tRNA binding to the ribosomes. Increased fluorescence intensity of each CPM-SAcAla-tRNA is observed upon ribosome binding (in the range of 30–40%), and τ is usually proportional to fluorescence intensity; however, an increase in τ should not be greater than the relative increase in quantum yield, yielding a minimum bimolecular quenching rate constant (k_Q) for the bound material which is still greater than that for the free material. This and the fact that ribosome binding eliminates quenching by iodide (data not shown) would

suggest that increased τ and a negatively charged ribosomal environment may both be contributing to the increase in K_Q observed upon ribosome binding. A quantitative assessment of the ribosomal environment around the CPM-SAcAla-tRNA, however, will require fluorescence lifetime data. In any event, the results indicate that CPM-SAcAla-tRNA within the peptidyl transferase center is readily accessible to collision with methyl viologen. The release of CPM-SAcAla-puromycin from these ribosomes results in a decrease in the K_Q to 75 M^{-1} for each tRNA species. This is similar to the K_Q for initiator CPM-Ala-tRNA and to that of CPM which has been reacted with dithioerythritol (65 M^{-1}), which is assumed to be fully accessible.

To further examine methyl viologen quenching of CPM at the amino terminus of polyalanine and polyserine, several lengths of each nascent peptide were synthesized, and the ribosomes bearing them were isolated by gel filtration as described above. The ribosome fractions were then divided, and methyl viologen quenching was determined directly and after reaction with 1 mM puromycin to release the nascent peptides (Figure 5).

As ribosome-associated polyalanine and polyserine increased in average length, the K_Q of each rapidly decreased, approaching values similar to that of free elongator CPM-Ala-tRNA (Figure 5). In each case, the K_Q of the puromycin-released polypeptide was similar to that of free elongator CPM-SAcAla-tRNA, even at very short polypeptide lengths (Figure 5). That the K_Q for methyl viologen of the ribosome-associated polypeptides never becomes less than that of the identical set of polypeptides free in solution suggests that the environment encountered by CPM at the amino terminus of polyalanine and polyserine remains aqueous throughout translation and does not protect the polypeptides from collision with methyl viologen. No release of peptidyl-tRNA from the ribosomes was observed for the lengths of times required for the quenching experiments (as judged by IgG binding over time).

Release of the Amino-Terminal CPM-SAcAla Residue from Nascent Polyalanine by Proteinase K. Hydrolysis by proteinase K (molecular weight $\simeq$ 27 000) was also used to examine the accessibility of the amino terminus of the nascent polypeptides. The rate of proteolysis of nascent peptides of different lengths was monitored by following the decrease in fluorescence anisotropy of the amino-terminal CPM-SAcAla as it was released from the ribosome. Limiting amounts of protease were used to minimize degradation of ribosomal proteins and loss of the ribosomal integrity, and only the initial rate of proteolysis (as the change in anisotropy per minute) was used as an index of accessibility. These experiments were performed to give a comparison (by fluorescence) to previous proteolysis experiments involving natural nascent proteins (Malkin & Rich, 1967; Blobel & Sabatini, 1970; Smith et al., 1978; Ryabova et al., 1988).

There was very little release of CPM-SAcAla from short nascent polyalanine averaging 1–25 residues in length (<2 ΔA/min); however, the rate of hydrolysis increased rapidly when peptides reached an average length of 40 residues (to 4 ΔA/min). Increasing the average nascent chain length to 50 residues resulted in a large increase in the rate at which the amino-terminal CPM-SAcAla was released (7 ΔA/min). Further polyalanine synthesis (to yield an average nascent peptide length of 70 or more residues) resulted in a maximum accessibility by proteinase K (12 ΔA/min).

These data indicate that although proteinase K has access to the nascent polyalanine sooner than IgG or Fab fragments, it still does not readily interact with the polypeptide until it has reached a length of about 40 or more amino acids. When the same procedure was used to examine the exit of polyserine from 70S ribosomes, the results were similar, although this polypeptide appeared to become accessible somewhat sooner (at about 25–30 residues). It was difficult to assess the accessibility by proteinase K to polylysine because only short polylysine chains could be synthesized; however, at 13 residues in length, it also appears to be protected (data not shown). The data indicate that the fluorescent nascent peptides studied here behave similarly to natural peptides examined previously by proteolytic techniques.

Discussion

The experiments described in this paper were designed to characterize, by fluorescence techniques, the environment encountered followed by synthetic nascent polypeptides as they are extended on *E. coli* ribosomes. Polyphenylalanine and polylysine turned out to be poor models for such an examination (Picking et al., 1991a). These obstacles have been overcome by the use of synthetic alanyl-tRNAs (Picking et al., 1991c) and seryl-tRNA (Picking et al., 1991b) which contain AAA anticodons and therefore can be used for polypeptide synthesis in a poly(U)-dependent translation system. The choice of these amino acids is based partially on the fact that neither requires a specific anticodon sequence for aminoacylation [reviewed by Normanly and Abelson (1989)] and that each has physical properties compatible with solubility and/or the formation of an α-helix. Polyserine is soluble in aqueous solution but tends to form a random coil under these conditions (Chou & Fasman, 1974), while polyalanine, although less soluble in aqueous solution, forms a stable α-helix (Chou & Fasman, 1974). It was proposed by Spirin and Lim (1986) that an α-helix is inevitably formed between adjacent amino acids during transpeptidation. When the core of an α-helix is formed within an existing polypeptide, the energy required for the generation of additional helical conformation is reduced (Creighton, 1983). For this reason, it could be expected that both polyalanine and polyserine could contain a significant region of α-helical structure. With this in mind, we have attempted to characterize the environment of the route taken by these synthetic polypeptides as they are translated by attaching a probe to an alanyl residue incorporated at their amino termini.

In following the fluorescence anisotropy of the amino terminus of polyalanine and polyserine, it becomes clear that extension of these peptides results in a continual increase in the mobility of each amino terminus during elongation. That the amino terminus of polyserine undergoes a more rapid decrease in anisotropy may be due to its propensity to form a random coil, at least relative to polyalanine (Chou & Fasman, 1974). The rapid decrease in the anisotropy of short nascent polylysine may be due in part to its tendency to form a random coil and in part to the location of the CPM moiety on the ϵ-amino group of lysine. Rotation around the carbon–carbon bonds between the ϵ-amino group and the α-carbon could result in an aberrantly low fluorescence anisotropy.

One explanation for the decrease in anisotropy of the nascent peptides examined here is that they are extended directly into the solution surrounding the ribosome. Quenching experiments were performed to examine the accessibility of the amino termini of polyserine and polyalanine to small molecules in the surrounding solution. The results suggest that at no point is the amino terminus of these polypeptides clearly protected from collision with methyl viologen molecules (M_r = 257). On

the other hand, when anti-CPM antibodies were used to examine the accessibility of the amino termini of the nascent peptides for very large molecules, the results clearly demonstrated that polyalanine and polyserine are not accessible to the IgG molecules until they approach a length of 60 or more residues. Similar results were obtained with Fab fragments prepared from these IgGs.

To further decrease the size of the probe used in these experiments, and for comparison to the result of other groups applying nonfluorescence methods, proteinase K was used to see at what point the amino terminus of the nascent polypeptides could be cleaved. The amino termini of very short nascent peptides were protected from proteolysis for a relatively long time, but the amino termini of peptides exceeding an average length of 30–35 residues were readily released by the protease, as judged by a rapid decrese in the fluorescence anisotropy of the amino-terminal CPM probe. This is comparable to the length of peptide observed by others to be protected when natural nascent peptides are exposed to limited proteolysis (Malkin & Rich, 1967; Blobel & Sabatini, 1970; Smith et al., 1978; Ryabova et al., 1988).

The data are consistent with the notion that the polypeptides enter a channel at the surface of the ribosome or even a tunnel if that tunnel is rather large and contains an aqueous environment. Indeed, Yonath and co-workers have characterized the tunnel (observed to pass through the 50S subunit) as being capable of accommodating any sequence of polypeptide, as well as being large enough to accommodate nearly any size amino acid side chain (Yonath et al., 1990), which would suggest that small solute molecules should be able to interact with polypeptides within it.

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Registry No. Polyalanine, 25191-17-7; polyserine, 25821-52-7; polylysine, 25104-18-1; poly(L-alanine), SRU, 25213-34-7; poly(L-serine), SRU, 25821-94-7; poly(L-lysine), SRU, 38000-06-5; peptidyltransferase, 9059-29-4.

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