Fluorophores at the N Terminator of Nascent Chloramphenicol Acetyltransferase Peptides Affect Translation and Movement through the Ribosome*

(Received for publication, September 10, 1999)

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Structurally different fluorescent probes were covalently attached to methionyl-tRNA\textsubscript{f} and tested for their incorporation into nascent peptides and full-length protein using an \textit{Escherichia coli} cell-free coupled transcription/translation system. Bovine rhodanese and bacterial chloramphenicol acetyltransferase (CAT) were synthesized using derivatives of cascade yellow, eosin, pyrene, or coumarin attached to [\textsuperscript{35}S]Met-tRNA\textsubscript{f}. All of the probes tested were incorporated into polypeptides, although less efficiently when compared with formyl-methionine. Eosin, the largest of the fluorophores used with estimated dimensions of 20 × 11 Å, caused the largest reduction in product formed. The rate of initiation was reduced with the fluorophore-Met-tRNA\textsubscript{f} compared with fMet-tRNA\textsubscript{f} with pyrene having the least and eosin the biggest effect. Analysis of the nascent polypeptides showed that the modifications at the N terminus affected the rate at which nascent CAT peptides were elongated causing accumulation of peptides of about 4 kDa, possibly by steric hindrance inside the tunnel within the 50 S ribosomal subunit. Fluorescence measurements indicate that the probe at the N terminus of nascent pyrene-CAT peptides is in a relatively hydrophilic environment. This finding is in agreement with recent data showing cross-linking of the N terminus of nascent peptides to nucleotides of the 23 S ribosomal RNA.

Initiation of protein synthesis by a specific initiator tRNA\textsuperscript{Met} at an AUG codon is a universally conserved step in gene expression for both eukaryotes and prokaryotes. In prokaryotes, methionine linked to the initiator tRNA\textsuperscript{Met} is formylated to provide a more efficient start. However, peptide initiation can be carried out with other N-acyl derivatives of Met-tRNA\textsubscript{f}, many of which can be synthesized chemically from Met-tRNA\textsubscript{f}.

We have synthesized coumarin maleimide-S-acetyl-Met-tRNA\textsubscript{f} to incorporate a fluorescent probe at the N terminus of polypeptides during their synthesis in a cell-free coupled transcription/translation system derived from \textit{Escherichia coli} (1–4). A considerable amount of information on the role of the molecular chaperones DinJ and DnaK in folding of the nascent protein and on cotranslational folding of nascent peptides was obtained through this method. In addition, we showed recently (5) that incorporation of coumarin at the N terminus of bacterial chloramphenicol acetyltransferase (CAT) resulted in increased ribosomal pausing at the already existing pause sites provided translation was slowed. Translational pause sites and their potential causes have been extensively discussed in this publication.

The studies cited above have prompted the question of what are the limits in terms of size and chemical character of the modification on the N terminus of nascent peptides under optimal translation conditions. Will the ribosomes initiate with bulky (pyrene, eosin) or a charged group (cascade yellow) covalently attached to the a-amino group of methionine on the initiator-tRNA as efficiently as with fMet-tRNA\textsubscript{f}? Initiation of protein synthesis is usually the rate-controlling step and a major point for regulation of translation (6). Do the fluorophores at the N terminus affect the amount and the rate at which polypeptides are formed after they are initiated? Are they incorporated into native full-length protein? The last two questions imply that the bulky modification at the N terminus may hinder the required folding of the nascent peptide to first pass through the tunnel inside the large ribosomal subunit (7), then to acquire the three-dimensional structure of the native protein.

Here we report the effect on translation of three additional N-terminal fluorophores (pyrene, cascade yellow, eosin), which vary in size and structure compared with coumarin. Synthesis of bovine rhodanese (RHO, a thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) or bacterial CAT (EC 2.3.1.28) was initiated in the presence of fluorophore-Met-tRNA\textsubscript{f}. Formation of nascent peptides and full-length product was analyzed and compared with polypeptides formed with N-formyl-methionine at the N terminus. The results indicate that RHO and CAT could be produced when pyrene-Met or cascade yellow-Met were at the N terminus of the protein; however, using eosin-Met-tRNA\textsubscript{f}, the translational machinery worked less efficiently. With all fluorophore-Met-tRNA\textsubscript{f} species, peptide initiation was an impaired step. With CAT, but not with RHO, pausing during translation was increased, resulting in accumulation of low molecular weight nascent peptides. The results are discussed under the aspect of folding of the nascent polypeptides on the ribosomes.

EXPERIMENTAL PROCEDURES

Materials

3-(4-Maleimidophenyl)-7-(diethylamino-4-methyl) coumarin, N-(1-pyrene)-maleimide, eosin-5-maleimide, and cascade yellow succinimidyl ester were from Molecular Probes, Inc. (Eugene, OR). The structures of coumarin-maleimide, N-(1-pyrene)-maleimide, and eosin-5-maleimide are given in Ref. 25. The structure of the succinimidyl ester of cascade yellow is given in Ref. 26.

\textsuperscript{35}S Methionine was purchased from NEN Life Science Products. Puromycin-CPG and 5’-dimethoxymethyl-N-acetylcysteine, 2’-(O-TBDMS)-[(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite were purchased from Glen Research (Sterling, VA). Cytidyl acid-puromycin (C-puro) was synthesized from these components by Dr. John Lee (Department

This paper is available on line at http://www.jbc.org

* This work was supported by National Institutes of Health Grant GM 53152 and Welch Foundation Grant F 1348. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\[ \text{Received for publication, September 10, 1999} \]
of Biochemistry, University of Texas Health Science Center, San Antonio, TX). Low molecular weight protein standards for SDS-PAGE were from Promega (Madison, WI). The original rhodanese plasmid was a kind gift from Dr. Paul Horowitz (University of Texas Health Science Center, San Antonio, TX), and the plasmid containing CAT coding sequence was provided by Dr. A. S. Spirin (Institute of Protein Research, Pushchino, Russia). Both the coding sequences of rhodanese and CAT were inserted under the T7 promoter in a PGEM vector.

Methods

Synthesis of Fluorophore-Met-tRNA Species—Coumarin-Sac-Met-tRNA, prepared as described previously (1). Both eosin-succinimidyl-propionate (DTSSP) was used instead of succinimide monester of dithiodiglyceric acid. DTSSP is water-soluble and will cross-link two tRNA molecules, even though it is present at a high molar excess of the latter. The product including any DTSSP-([35S]Met-tRNA) was that formed was reduced by dithiothreitol. The resulting thiopropionate derivative of [35S]Met-tRNA was then reacted with maleimides of eosin or pyrene.

Cascade yellow-[35S]Met-tRNA was synthesized by reacting the [35S]Met-tRNAf with maleimides of eosin or pyrene.

In vitro Translation Assay—The in vitro translation assay was used (9) with some modifications in the salt concentration of the reaction mixture, which included 30 mM of Tris-HCl, pH 7.5, 10 mM magnesium acetate, 400 mM NaCl (solvent A), and 60% methanol in solvent A (solvent B). Coumarin-Met-tRNA and eosin-Met-tRNA were eluted from the C3 column with 50% of solvent B, whereas pyrene-Met-tRNA eluted with 80% of solvent B. Cascade yellow-Met-tRNA was eluted with 20% of solvent B.

The Cell-free System—Preparation of the plasmides, isolation of T7 RNA polymer, and preparation of the E. coli cell-free extract (S30) were carried out as described (8). The in vitro coupled transcription/translation assay was used (9) with some modifications in the salt concentration of the reaction mixture, which included 30 mM of (NH₄)₂SO₄. However, unfraccionated tRNA and formic acid were omitted. Bovine RHO and CAT were synthesized using f[35S]Met-tRNA or fluorescence-[35S]Met-tRNA (generally at 10,000 Ci/mmol) as the radioactive precursors and the E. coli S30 fraction with non-linearized plasmids and T7 RNA polymerase plus rifampicin. In the absence of added plasmid were 0.08 ± 0.01 pmol. This value was subtracted. Similarly, enzymatic activity determined in samples lacking plasmid was subtracted from the values given for enzyme activity. The data presented are the average values from three separate experiments.

RESULTS

Incorporation of Coumarin, Cascade Yellow, Eosin, and Pyrene Derivatives of Methionine at the N Terminus of Polypeptides—Coupled transcription/translation in the in vitro E. coli system provides a method by which a modified methionine can be incorporated from derivatized Met-tRNA into a nascent peptide. This approach has been used here to study whether fluorescent probes differing in size and charge can be incorporated at the N terminus of bovine RHO (33 kDa, Ref. 14) or CAT (25.6 kDa, Ref. 15) nascent peptides.

First, we analyzed whether the fluorophore-Met modification at the N termini of RHO or CAT affected the extent to which these polypeptides were recovered in comparison to the initiation with fMet-tRNA. Production of full-length protein released from the ribosomes was monitored by their enzymatic activity (cf. Ref. 9). The data are presented in Table I. For these experiments, polypeptide synthesis was carried out in the presence of f[35S]Met-tRNA or fluorophore-[35S]Met-tRNA. The radioactively labeled methionine can be incorporated only at the N terminus of the proteins; the incubation was continued for 30 min to achieve maximal extension of the polypeptides initiated with the labeled initiator tRNA. Previous unpublished data showed that coumarin-Met covalently attached to a peptide cannot serve as a substrate for deformylase and methionine aminopeptidase. In addition, earlier unpublished observations

#TABLE I

| Initiator tRNA | RHO | CAT |
|---------------|-----|-----|
| tRNA | pmol synthesized | units x 10⁻⁷ | pmol synthesized | units x 10⁻⁶ |
| fMet-tRNA | 2.3 ± 0.6 | 53 ± 8 | 2.1 ± 0.4 | 30 ± 4 |
| Cascade yellow-Met-tRNA | 1.2 ± 0.3 | 21 ± 5 | 1.1 ± 0.1 | 9 ± 2 |
| Pyrene-Met-tRNA | 1.4 ± 0.4 | 6 ± 2 | 1.4 ± 0.3 | 2 ± 1 |
| CPM-Met-tRNA | 1.2 ± 0.2 | 31 ± 7 | 1.2 ± 0.2 | 7 ± 4 |
| Eosin-Met-tRNA | 1.0 ± 0.3 | 19 ± 6 | 0.7 ± 0.3 | 5 ± 3 |

S. Seliger, O. W. Odom, G. Kramer, and B. Hardesty, unpublished data.
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The data in Table I indicate a reduction in incorporation of radioactively labeled N-terminal methionine of 40–60% for both RHO and CAT when peptide synthesis was initiated with fluorophore-Met-tRNAf compared with fMet-tRNAf. In parallel, the measured enzymatic activities were reduced; yet, the fact that enzymatic activity was associated with the fluorophore-Met-RHO or -CAT polypeptides that were produced indicates that apparently full-length protein was formed and folded into its native structure. Note, however, that CAT activity was reduced more strongly than would have been expected from incorporation of radioactivity from Met when synthesis was initiated with fluorophore-Met-tRNAf.

Earlier observation by Kudlicki et al. (1) indicated that full-length rhodanese whose synthesis was initiated with coumarin-Met-tRNAf contained coumarin at its N terminus. We tested whether pyrene and cascade yellow can be detected at the N termini of RHO polypeptides released from the ribosomes. Both pyrene and eosin derivatives of Met-tRNAf were synthesized by the same reactions and offer to be equally stable. Cascade yellow, on the other hand, is a zwitterion, has a more flexible structure, and is linked to the α-amino group of methionine by reaction of its succinimidyl ester.

After coupled transcription/translation in the presence of pyrene- or cascade yellow-Met-tRNAf, the ribosomal and the supernatant fractions were separated. Released pyrene- or cascade yellow-labeled RHO was purified by gel filtration chromatography (1). Their fluorescence spectra are shown in Fig. 1 in comparison to the respective fluorophore-Met-tRNAf. The cascade yellow-RHO spectrum (panel A) is shifted to the blue with a maximum of the emission spectrum of 518 nm compared with the spectrum of the fluorophore-Met-tRNAf whose emission maximum is at 542 nm. Pyrene-labeled RHO (panel B) with an emission maximum of 398 nm showed a large increase in quantum yield compared with pyrene-Met-tRNAf, indicating that this environmentally sensitive probe is in a more hydrophobic surrounding at the N terminus of rhodanese. The data presented in Fig. 1 demonstrate that pyrene and cascade yellow are indeed present on the protein, which was initiated with the respective fluorophore-Met-tRNAf.

The Pattern of Nascent Peptide Formation—The pattern of nascent polypeptides of the two test proteins initiated with fluorophore-Met-tRNAf was reduced compared with binding of fMet-tRNAf. For this reason, we analyzed the pattern of nascent polypeptides with the results given in the following section.

The Pattern of RHO and CAT Pause-site Peptides—The pattern of synthesized polypeptides of the two test proteins initiated
with fMet-tRNA\textsubscript{f} or each of the four forms of fluorophore-Met-tRNA\textsubscript{f} was revealed after SDS-PAGE, followed by phosphorimaging. These "pause-site peptides" are in the size range of 5–33 kDa, the mass of full-length RHO. These different bands represented points at which translation is temporarily slowed or stopped. The cause of this pause in translation has not been established. One of our objectives was to determine if modification of the N terminus of the test proteins by attaching different fluorophores affects the pattern of these pause-site peptides. This does not seem to be the case, at least on the qualitative level.

The results given in Fig. 3 for RHO confirm the reduction in overall synthesis of polypeptides presented in Table I for fluorophore-Met-RHO. Synthesis of eosin-RHO was the lowest. The same is true for CAT (Fig. 3, lane 10). In addition, with CAT, there is a remarkable reduction in full-length product formed when synthesis was initiated with all fluorophore-Met-tRNA\textsubscript{f}. Full-length CAT is reduced on the expense of small polypeptides of 6.5 kDa and below, with the most prominent CAT peptides having a mass of 3.5–4 kDa (Fig. 3, lanes 7–10). Pyrene-Met at the N terminus of CAT exerts the strongest effect.
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Fig. 5. Kinetics of RHO synthesis initiated with either fMet-tRNA$_f$ or fluorophore-Met-tRNA$_f$. The experiment was carried out as described in the legend to Fig. 4, except that RHO was synthesized. Panel A, synthesis with N-formyl-[35S]Met-tRNA$_f$; panel B, synthesis with cascade yellow-[35S]Met-tRNA$_f$; panel C, synthesis with pyrene-[35S]Met-tRNA$_f$; panel D, synthesis with eosin-[35S]Met-tRNA$_f$.

Fig. 6. Reaction of nascent peptides with C-puro. RHO (lanes 1 and 2) or CAT (lanes 3 and 4) synthesis was initiated with unlabeled fMet-tRNA$_f$ (lanes 1 and 3) or unlabeled pyrene-Met-tRNA$_f$ (lanes 2 and 4). Synthesis was carried out with 5 μl of S30 in the reaction mixture during an incubation at 37 °C for 30 min. Immediately after this incubation, [35S]C-puro was added to the reaction mixtures and the incubation was continued as described under "Experimental Procedures." The reaction products were analyzed by SDS-PAGE and visualized by phosphorimaging.

of the emission spectrum, and in anisotropy will indicate interaction with other molecules or changes in the environment.

CAT peptides were synthesized with pyrene-Met at their N terminus under conditions in which low molecular weight nascent peptides accumulate on the ribosomes, and then the ribosomal fraction was isolated (see "Experimental Procedures") and subjected to fluorescence measurements. Fig. 7 (spectrum 1) shows the fluorescence emission spectrum of the resuspended ribosomes bearing nascent pyrene-CAT peptides. This fraction was then treated with C-puro (see "Experimental Procedures") and analyzed again in the fluorometer. The spectrum after puromycin reaction is shown by line 2 in Fig. 7. A large increase in fluorescence intensity was observed. This increase in quantum yield signals a move of the probe to a much more hydrophobic environment. Calculated fluorescence parameters for the spectra shown in Fig. 7 as well as anisotropy calculations are compiled in Table II. Included in this table are the corresponding values for pyrene-Met-tRNA$_f$, free in buffered solution.

After the measurements indicated in Fig. 7 (spectrum 2) were completed, the sample was centrifuged to analyze whether the pyrene-CAT-C-puro peptides were separated from the ribosomes. Both fractions, the resuspended ribosomes and the resulting supernatant, were subjected to fluorescence measurements with the results shown in Fig. 7 (spectra 3 and 4, respectively). The results indicate that the pyrene-CAT peptides were released into the soluble fraction. The quantitative fluorescence data for spectra 3 and 4 are included in Table II.

We interpret these results to indicate a change in the environment of the pyrene moiety at the N terminus of the nascent CAT peptides from a less hydrophobic (relatively hydrophilic) to a very hydrophobic surrounding. We emphasize the less hydrophobic/relatively hydrophilic aspect, because the relative fluorescence quantum yield of the nascent pyrene-Met-CAT peptides on the ribosomes is appreciably higher than the quantum yield of free pyrene-Met-tRNA$_f$ (Table II). Further, we interpret the results to indicate a relatively hydrophobic environment of the probe when the pyrene-CAT peptides are bound to the ribosomes probably inside the tunnel of the 50 S ribosomal subunit as discussed below. When released, the peptides may collapse with the pyrene buried inside the peptide in a very hydrophobic pocket.

**DISCUSSION**

The fluorescent probes used in the present study differ in their molecular size, shape, charge, and hydrophobicity. Computer-aided calculations of the dimensions of the probes indicated that the fluorophores used varied from 15 to 20 Å in length and about 5 Å in diameter with the exception of eosin,

**TABLE II**

| Fraction analyzed | Fluorescence characteristics |
|-------------------|-----------------------------|
|                   | $E_{max}$ | $A$  | Rel Q  |
|                   | nm       |      |        |
| 1. Ribosome fraction with bound pyrene-CAT peptides | 400 | 0.34 | 0.25 |
| 2. Puromycin-treated ribosome fraction | 404 | 0.22 | 3.10 |
| 3. Ribosomal fraction after second Airfuge centrifugation | 400 | 0.36 | 0.50 |
| 4. Supernatant fraction (contains the released peptides) | 404 | 0.20 | 4.50 |
| 5. Free pyrene-[35S]Met-tRNA | 380 | 0.16 | 0.10 |
which has a diameter of about 11 Å with 4 bromine atoms bonded to the xanthene ring system. All fluorophore-Met-tRNA$_f$ molecules tested cause a reduction in both CAT and RHO polypeptides formed, which in the case of RHO can be traced to less efficient initiation. Eosin-Met-tRNA$_f$ has the most pronounced effect.

In addition, the results presented above indicate that translational pausing at some of the early pause sites is prominent when probes are incorporated at the N terminus of CAT. Pyrene caused a greater increase in pausing than coumarin in producing CAT nascent peptides of about 30–35 amino acids in length. A stronger effect than with coumarin is also observed with cascade yellow, which is zwitterionic in nature. The pyrene derivative appears to be the most hydrophobic (based on the HPLC elution profile of the fluorophore-Met-tRNA) of the fluorophores tested, although it is in a similar size range to cascade yellow and coumarin. The results indicate that neither hydrophobicity nor the ionic character of the probe is the unique cause of pausing at specific sites.

Low molecular weight pause-site peptides of CAT were observed previously when protein synthesis was initiated with coumarin-Met-tRNA$_f$ and elongation was slowed by reducing the amount of the E. coli extract used in the assay (5). It was suggested that the primary amino acid sequence at the N terminus might be the determining element in the accumulation of pause-site peptides. CAT with a very hydrophilic N terminus was assumed to be strongly affected by the hydrophobic derivative of N-terminal Met during the required folding of the nascent peptide to allow for its path through the tunnel of the large ribosomal subunit (5). We wish to modify this hypothesis, because similar experiments as reported above on the elongation of RHO and CAT were carried out with another protein, E. coli release factor RF-1. Its N-terminal amino acid sequence (17) gives a hydrophobicity plot (18) very similar to CAT; however, there was no accumulation of low molecular weight nascent peptides, when RF-1 synthesis was initiated with pyrene- or cascade yellow-Met-tRNA$_f$ (data not presented).

What causes CAT peptides to accumulate when pyrene, cascade yellow, or coumarin forms the N terminus? It should be pointed out that an N-terminal probe on nascent CAT peptides became accessible to specific antibodies only when a mass of 8.5 kDa was reached. The corresponding values for RHO and for MS2 coat protein were 6.0 and 45.5 kDa, respectively (4). The nascent CAT peptides inside the ribosomal tunnel may form a more compact structure, which is affected by the bulky N-terminal extension in contrast to the secondary structures acquired by the other probes tested. The crystal structure of monomeric CAT (19) is very compact with no formation of domains as is the case with RHO (20). The crystal structure of RF-1 is not known yet. Secondary structure prediction according to Garnier and Robson (21) indicates an α-helical structure for the first 40 amino acids of RF-1. The N terminus of RHO is quite flexible; then an α-helix is formed by amino acids 11–22. The crystal structure of CAT indicates an N-terminal β-sheet (amino acids 1–12), followed by a short α-helix before another β-sheet is formed. We do not know whether these differences in the N-terminal secondary structures are the cause for the effects seen when pyrene- or cascade yellow-Met are at the N terminus of the nascent peptides. We assume this might be the case.

An important conclusion from the results presented above is that the N-terminal pyrene on the nascent CAT peptides is in a relatively hydrophobic environment. The most prominent CAT peptides are in the range of 4 kDa: about half the mass before the N terminus becomes accessible to antibodies. The recent model of the 70 S ribosome developed from image reconstruction of electron micrographs (22) confirms a structured tunnel through the 50 S subunit. The images at a resolution of 15 Å position the CCA end of P-site bound tRNA at the mouth of the tunnel, which is assumed to be the conduit for the nascent polypeptides. We estimated the length of the tunnel to be about 70 Å and its width between 16 and 19 Å, based on these cryoelectron microscopy data together with recently published crystallographic data on the 50 S subunit (23). Combining these numbers with the secondary structure for the N-terminal 40 amino acids of the CAT still leaves the question open where the N-terminal pyrene is localized within the tunnel. Recent data (24) obtained with an N-terminal photo-activatable probe on different nascent polypeptides indicated cross-linking to specific nucleotides of the 23 S RNA. When the synthesized peptides had a length of about 30 amino acids, cross-linking to several nucleotides of the ribosomal RNA was observed. For all three polypeptides of this length (ompA peptide, tetracycline resistant gene-peptide, and T4 gene 60 peptide), cross-linking to nucleotides 2062 and nucleotide 2609 was found consistently; additional cross-links were determined with the second peptide. These results are quite remarkable; they show that different peptides of the same length gave different cross-links. Furthermore, nucleotide 2609 is in domain V, the peptidyl-transferase center. This finding indicates that nascent peptides are not traversing the ribosomal tunnel in a linear arrangement. Most importantly, the results suggest a hydrophilic environment that the nascent peptide may encounter inside the ribosomal tunnel. Our fluorescence results with nascent pyrene-CAT peptides support this notion.

Acknowledgments—We appreciate the initial gift of cytidylic acid-puromycin from Dr. Rachel Green (Department of Molecular Biology and Genetics, Johns Hopkins University, Baltimore, MD). We thank Suleyman Baheeci (Department of Chemistry and Biochemistry, University of Texas, Austin, TX) for calculating the dimensions of the probes by Molecular Mechanics software. In addition, we thank Delbert Brod for excellent technical assistance and Barbara Jann for help with the typescript.

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