The conformation and dynamics of nascent polypeptides emerging from the ribosome during biosynthesis are largely unexplored, to date. Yet, understanding these aspects of the protein folding question is very important, considering that most biomolecules need to be properly synthesized to function efficiently in the cell. The protein's fate in the cellular environment critically relies on the ability of the nascent chain to properly sample conformational space. This includes the need to avoid irreversible misfolded conformations before biosynthesis of the full-length protein is complete.

High-resolution structures of the large ribosomal subunit in the absence (1–3) and presence (4, 5) of the ribosome-associated chaperone trigger factor (TF) have recently become available. A close inspection of these structures suggests that both the ribosome exit tunnel and TF impose a number of geometrical constraints to conformational sampling during translation in Escherichia coli (2). These constraints have the potential to affect the folding and dynamics of nascent chains before biosynthesis has reached completion.

In addition to TF, a second major cotranslationally active chaperone, DnaK, is known to interact with elongating nascent chains in bacteria (6). A complex regulatory cycle involving the cochaperones DnaJ, GrpE, and nucleotide binding modulates DnaK's activity (7). The preferred binding motifs of DnaK and TF are not identical; however, both chaperones share an affinity for nonpolar and basic residues (8).

This work provides the first high-resolution spectroscopic characterization of the dynamics of ribosome-bound nascent polypeptides and proteins in Escherichia coli by dynamic fluorescence depolarization and assess the population of cotranslationally active chaperones trigger factor (TF) and DnaK. E. coli cell-free technology and fluorophore-linked E. coli Met-tRNA^Met enable selective site-specific labeling of nascent proteins at the N-terminal methionine. For the first time, direct spectroscopic evidence captures the generation of independent nascent chain motions for a single-domain protein emerging from the ribosome (apparent rotational correlation time ~5 ns), during the intermediate and late stages of polypeptide elongation. Such motions are detected only for a sequence encoding a globular protein and not for a natively unfolded control, suggesting that the independent nascent chain dynamics may be a signature of folding-competent sequences. In summary, we observe multicomponent, severely rotationally restricted, and strongly chain length/sequence-dependent nascent chain dynamics.
Figure 1. General features of ApoMb nascent chains. a) Schematic representation of apoMb ribosome-bound nascent polypeptides examined in this work. Chain lengths of N-terminal fragments are marked as subscripts. Colored segments (labeled A–H) denote α-helical regions of native full-length apoMb. Stars indicate the experimentally detected high- (red) and low- (pink) affinity DnaK binding sites (48). b) Pictorial illustration of the expected fraction of nascent polypeptides buried inside the ribosomal exit tunnel (length ~100 Å (1–3)) for limiting fully α-helical (1.5 Å residue$^{-1}$) and fully extended (3.5 Å residue$^{-1}$) conformations (see also Supporting Information). c) SDS-PAGE analysis of nascent apoMb polypeptides N-terminally tagged with BODIPY-FL-Met. Expression products for both wild-type (WT) and trigger factor (tig) deficient (Δtig) cell-free systems are displayed. Samples in this panel are shown after treatment with puromycin (Puro). d) Verification of the fully ribosome-bound status of the nascent chains by treatment of ribosome-associated peptidyl-tRNAs with Puro. In the presence of the ribosome, Puro catalyzes the conversion of peptidyl-tRNA (lane 1) to peptidyl-Puro (lane 2), giving rise to a lower molecular weight species. As an example, the effect of Puro on apoMb$^{153}$ RNCs is shown. Similar analysis was successfully performed on other nascent chains (Supplementary Figure 1).

Isotropy decay approach based on frequency-domain fluorometry known as dynamic fluorescence depolarization (10, 11) to discriminate the different types of motions occurring within RNCs. This methodology is uniquely tailored to discriminate complex motions, including the local motions of polypeptides bound to large macromolecules. Such dynamic properties are difficult to assess by other spectroscopies or functional assays.

The analysis is carried out for ribosome-bound nascent chains of apomyoglobin (apoMb) and the nativelyunfolded protein PIR, used as a control. The α-helical apoMb sequence has been selected because (i) the folding pathways (12, 13) and backbone motions (14) of the full-length protein have been extensively studied, and (ii) the production of native apoMb in E. coli cell-free systems has already been established in our laboratory by 2D NMR and ultracentrifugation (15). Although the in vitro chain elongation behavior of apoMb has already been studied on purified N-terminal fragments in buffered solution (16), it is important to extend the previous studies to include the effect of the translation machinery and cotranslationally active chaperones on polypeptide folding and dynamics.

RESULTS AND DISCUSSION

Experimental Design. Nascent polypeptides of increasing length derived from the apomyoglobin (apoMb) sequence (Figure 1, panel a) were analyzed in an E. coli cell-free system. The polypeptide length was chosen so that variable fractions of the chain are expected to be buried in the ribosomal exit channel (Figure 1, panel b). The natively unfolded PIR$^{90}$ control protein was examined as a reference because of its low nonpolar content (Supplementary Figure 3) and its intrinsic inability to adopt a compact structure in aqueous medium. Small angle X-ray scattering (SAXS) of pure full-length PIR shows that this sequence behaves as an unfolded self-avoiding Gaussian chain (17).

Initiation of protein biosynthesis is neither synchronized nor rigorously synchronizable across the ensemble of ribosomes in intact cells and cell-free systems. This limitation, which hampers both sensitivity and resolution, was overcome by generating ribosome-bound nascent chains of increasing length and monodisperse molecular mass. Creation of these homogeneous equilibrium populations of apoMb-RNCs is key to the success of our experiments (Figure 1, panel c).

Given that conformational sampling is typically much...
faster than translation (16), these equilibrium populations may be regarded as working models for transiently elongating polypeptides. Cell-free expression was carried out in the presence of BODIPY-FL-tagged Met-tRNAfMet, leading to the biosynthesis of N-terminal BODIPY-FL-labeled nascent chains. This strategy provides the advantage of ensuring that all nascent chains are labeled with only one fluorophore at a unique sequence site (the amino group of N-terminal methionine). Because of its fluorescence lifetime in the low nanosecond range (τF = 5.9 ns), BODIPY-FL is an ideal fluorophore here due to its small size and its ability to probe motions on a timescale close to the independent tumbling time of small globular proteins.

Biochemical Characterization. As a complement to the spectroscopy, the amounts of chaperones associated with resuspended RNCs were characterized. Immunoblotting (Figure 2, panel a) shows that the average amount of TF per active ribosome increases progressively with chain elongation, with a sharp rise near completion of biosynthesis. The absolute amounts of TF associated with apoMb153-RNCs after ultracentrifugation are comparable to the estimated amounts of translating ribosomes (data not shown). Given the known 1:1 stoichiometry for substrate—TF binding (18), the above results suggest that TF displays a higher affinity for nascent apoMb chains as they get longer and acquire a higher nonpolar content, in agreement with recent studies on other proteins (19, 20).

While the TF chaperone associates most tightly with RNCs bearing full-length apoMb, the biosynthesis of the natively unfolded control sequence PIR90 requires reduced amounts of TF (Figure 2, panel a). In addition to its low hydrophobicity, PIR90 has very few aromatic residues (Supplementary Figure 3). Both nonpolar content and aromatic residues of the substrate are important for TF affinity in solution (21, 22). Therefore, the observed low TF content in PIR90-RNCs is consistent with the prediction that this chaperone should not significantly interact with PIR90.

Immunoblotting also shows that the bacterial Hsp70 chaperone DnaK associates with apoMb-translating ribosomes more than with the PIR90 control sequence, in wild-type strains (Figure 2, panel b). However, in contrast with the behavior of TF, the amount of detectable ribosome-apoMb-associated DnaK is independent of substrate chain length within experimental error. The presence of residual DnaK even at short chain lengths is not entirely surprising given the known affinity of DnaK for E. coli ribosomes (23). Therefore some of the DnaK detected here may be due to association with the ribosome. DnaK concentrations increase for cell-free systems prepared from Δtig strains (lacking the gene for TF), in agreement with the overall complementary roles of DnaK and TF (8) and the likelihood that they

Figure 2. Normalized concentrations of TF and DnaK co-pelleting with RNCs. Normalized chaperone concentrations were obtained by dividing chaperone concentrations by ribosome-bound nascent chain levels (determined by fluorescence) and reporting the results relative to the values for the full-length apoMb153 wild-type (WT) sample. Reported values are the average of two to six separate experiments. Uncertainties are reported as standard errors. a) Normalized concentrations of TF associating with translating ribosomes carrying nascent polypeptides. Cell-free expression was performed in wild-type cell-free systems. b) Normalized concentrations of DnaK associating with translating ribosomes carrying nascent polypeptides. Cell-free expression was performed in both wild-type and Δtig cell-free systems.
Figure 3. Technical aspects of dynamic fluorescence depolarization data collection. a) Representative physical model (3-component) for multiple independent molecular motions and matching equations applicable to rotational correlation times ($\tau_i$) differing by at least 1 order of magnitude. The BODIPY-FL fluorophore is shown in green. $F_i$ is the fractional amplitude for the anisotropy decay corresponding to each of the motions. In the case of 2-component RNC dynamics, the intermediate timescale ($I$) term is lost. b) Representative experimental fluorescence anisotropy data for short (apoMb$_{16}$) and long (apoMb$_{153}$) ribosome-bound nascent chains and associated 2- and 3-component fits, respectively. c) Multiexponential curve fitting (2- and 3-component analysis) and residuals for ribosome-bound ApoMb$_{16}$. d) ApoMb$_{153}$ analyzed similarly to panel c and 2-component fit with variable $r_0$. e) Simulation illustrating the unique features of typical 2- and 3-component frequency-domain anisotropy decays. f) Expected phase changes upon varying the fraction of intermediate ($F_I$) and fast ($F_F$) timescale motions, to illustrate the progression from 2- ($F_I = 0$) to 3-component decays. The 2- and 3-component phase change plots of panel e coincide with simulations A and C. g) Parameters used for the simulations in panel f.
representative raw data collected by frequency domain fluorometry for short and long ribosome-bound nascent chains are shown (Figure 3). There is a dramatic difference between the dynamics of short and long nascent chains (Figure 3, panel b). The short chains experience a very fast motion, given that the sinusoidal maximum of the phase change falls at frequencies even higher than experimentally detectable. This fast motion has an apparent \( \tau \) similar to that of free BODIPY (0.12 \( \pm \) 0.04 ns, measured in the same medium) and must be rotationally restricted since the anisotropy does not completely decay over the experimental timescale.

The featureless modulation ratio profile further confirms the presence of local segmental dynamics and a very slow global tumbling (\( \geq 0.5 \) \( \mu \)s). Upon curve fitting (Figure 3, panel a, and Methods), the slowest anisotropy phase, which corresponds to the residual anisotropy at time infinity, is assigned to the global motions of the ribosome complex.

Remarkably, the long chains show one additional local motion, which is detectable as a characteristic spectral feature (i.e., a curved bump) of the phase-change data at intermediate modulation frequencies. This feature reveals the presence of a local motion with a 3–7 ns apparent rotational correlation time (\( \tau \))c. The above dynamic component is clearly detectable (Figure 3, panels b and d) and statistically well beyond experimental error (Table 1). Spectral simulations further confirm the correlation between the fraction of depolarization decay assigned to the nanosecond motion and its matching spectroscopic signature (Figure 3, panels e and f).

Data Analysis and Interpretation. A detailed analysis of the fluorescence depolarization data for all of the apoMb chain lengths (Figure 4 and Table 1) reveals that the number and type of observed motions are significantly dependent on the peptide elongation stage. Only two distinct classes of dynamics are identified for short nascent chains, whereas three types are detected for longer chains.

We assign the slow phase to the chain motions associated with the overall tumbling of the higher order complex, dominated by the size of the ribosome. Accordingly, the \( \tau \)c for this motion was set to 1 \( \mu \)s. While this motion cannot be resolved directly, as its timescale is much longer than the fluorophore lifetime, its presence is inferred from the fact that a nondecaying anisotropy...
TABLE 1. Dynamic fluorescence anisotropy parameters and average $\chi^2$ for the data in Figure 4$^a$

<table>
<thead>
<tr>
<th>Polypeptide chain</th>
<th>Slow</th>
<th>Intermediate</th>
<th>Fast</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c (ns)</td>
<td>Fraction$^b$</td>
<td>$\tau_c$ (ns)$^b$</td>
<td>Fraction$^b$</td>
</tr>
<tr>
<td>ApoMb$_{16}$</td>
<td>1000</td>
<td>0.72 ± 0.02</td>
<td>0.28 ± 0.02</td>
<td>1.90</td>
</tr>
<tr>
<td>ApoMb$_{35}$</td>
<td>1000</td>
<td>0.74 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>0.74</td>
</tr>
<tr>
<td>ApoMb$_{57}$</td>
<td>1000</td>
<td>0.73 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>0.67</td>
</tr>
<tr>
<td>ApoMb$_{89}$</td>
<td>1000</td>
<td>0.74 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>2.63</td>
</tr>
<tr>
<td>ApoMb$_{124}$</td>
<td>1000</td>
<td>0.70 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>3.39</td>
</tr>
<tr>
<td>ApoMb$_{357}$</td>
<td>1000</td>
<td>0.68 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>4.52</td>
</tr>
<tr>
<td>ApoMb$_{153}$</td>
<td>1000</td>
<td>0.65 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>7.01</td>
</tr>
<tr>
<td>PIR$_{90}$</td>
<td>1000</td>
<td>0.57 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>2.06</td>
</tr>
<tr>
<td>Ribosome-released ApoMb$_{153}$</td>
<td>na</td>
<td>0.67 ± 0.01</td>
<td>41 ± 2</td>
<td>0.74</td>
</tr>
</tbody>
</table>

$^a$Fit quality was separately assessed for each experimental sample. Three-component decays were chosen over 2-component decays only when a significant decrease in $\chi^2$ (by a factor $\geq 2.5$) was observed. The $\chi^2$ values corresponding to the best fits are shown in bold. $^b$The error reflects ± 1 standard error (for 2$^a$ ≤ 7 repeats, unless otherwise stated). In the case of a standard error smaller than the curve fitting error for any of the experimental repeats, the curve fitting error is reported. $^c$The reported error for the single 2-component sample of apoMb$_{57}$ is the curve fitting error.

component needs to be introduced for the fitting algorithm to converge. The fraction of anisotropy decay for this motion is treated as a floating parameter in the fits.

The phase corresponding to motions occurring on an intermediate timescale ($\tau_c = 3–7$ ns) provides evidence for the presence of an independent conformation of the ribosome-bound nascent protein. This phase is detected only for the longer nascent chains (apoMb$_{89–153}$), and its timescale corresponds to the approximate apparent rotational correlation time of a small structured domain. The apparent rotational correlation time for the intermediate timescale motion changes significantly upon release of apoMb$_{153}$ from the ribosome in the crowded medium, from 4.5 ± 0.7 to 41 ± 14 ns. As expected, the slow motion due to global RNC tumbling is no longer present for this species. Therefore it is clear that apoMb undergoes some structural and/or shape changes upon release from the ribosome. The fast timescale motion is detected for all samples, including ribosome-released apoMb$_{153}$.

The fact that the 3–7 ns intermediate timescale motion preserves a constant fraction and a similar $\tau_c$ for the 89- to 153-residue chains suggests that the general nature of this motion does not significantly vary as the protein emerges from the ribosome. While high resolution structural/dynamic details cannot be drawn by fluorescence depolarization alone, the above observations support the fact that this motion is a persistent feature of all RNCs bearing the first ~60 N-terminal residues of apoMb.

The apoMb$_{37}$-RNC has a peculiar experimental behavior. It displays 3-component dynamics in only 75% of the experiments, while the remaining 25% fit best to 2-state dynamics, lacking the nanosecond timescale motion (Figure 4). The poor data reproducibility at this chain length may reflect the fact that the independent nanosecond motions of nascent apoMb$_{27}$ (50–100% buried in the ribosomal tunnel, Figure 1, panel b) could arise from an unstable structure whose population is strongly affected by small sample-dependent changes in experimental conditions.

In summary, three distinct dynamic regimes are identified for nascent apoMb-RNCs, fast (sub-nanosecond), intermediate (3–7 ns), and slow (>1 µs). These motions are assigned to global tumbling of the ribosomal complex, nascent chain dynamics, and local fluorophore dynamics, respectively. The short nascent chains (16, 35 residues) display only the fast and slow motion while the longer chains (89–153 residues) also display the nanosecond motion.
The behavior of the nascent chains in the absence of molecular chaperones was analyzed by creating chaperone-free RNCs in a TF-deficient cell-free system (Δtig strain, −G conditions). We then treated the resuspended ribosomes bearing either apoMb or PIRnascent chains with GrpE and excess ATP (+G conditions, Figure 5, panel a). This procedure ensures release of any bound DnaK from the nascent chains, as confirmed by immunoblotting. The presence of additional chaperones (DnaJ or SecB) in the RNC has been excluded by immunoblotting (Supplementary Figure 2). As shown by the frequency-dependent phase changes (Figure 5, panel b, modulation frequency ~70 MHz), release of DnaK from full-length apoMb does not lead to a decrease in the fraction of the nanosecond motion. In fact, an increase in fraction (from 4% to 95%) is observed. This result is important because it shows that the 37 ns motion is not due to the binding of ribosome-bound nascent chains to DnaK but is a unique feature of the nascent chain. In other words, this inde-
Dependent motion is sampled by the nascent chains regardless of the presence of chaperones. Not surprisingly, no changes in nascent chain dynamics are detected for PIR90 under \( \text{H}11001 \) conditions.

**Models for RNC Dynamics.** On the basis of the above data, the following insights into the nature of the independent nascent chain motions are gained. We will focus the discussion on the nascent chains of sufficient length to emerge from the ribosomal tunnel, as they exhibit the most interesting behavior.

Representative cartoons (Figure 6) highlight the known presence of a dynamic exchange between chaperones and nascent chains and provide a framework for interpretation of the data on the RNCs generated in wild-type cell-free systems. Three important facts need to be considered, namely, (i) the existence of a reversible interaction between nascent chains and trigger factor or DnaK, (ii) the fact that TF is effectively “frozen on the ribosome” (i.e., ribosome-bound) on the timescale of TF binding to nascent chains (20, 26), and (iii) the microsecond to second on/off rates for nascent chain interaction with TF (20, 26) and microsecond to minute on/off rates for interaction with DnaK (27). The above observations support the idea that nascent chains emerging from the ribosome are transiently bound to TF and/or DnaK on a slow timescale (\( \mu \text{s} \sim s \)) relative to

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**Figure 5.** Dynamic fluorescence depolarization of RNCs generated in a trigger factor-depleted (\( \Delta \text{tig} \)) cell-free system, before and after release of DnaK. a) Illustration of DnaK release induced by the addition of 2 \( \mu \text{M} \) GrpE, 30 \( \mu \text{M} \) ATP, and 500 \( \mu \text{M} \) KCl (\( \text{+G} \) conditions). b) Western blot showing that the concentration of apoMb153-RNC-bound DnaK is significantly reduced under \( \text{+G} \) conditions. This panel also shows the phase shift of the frequency domain fluorescence anisotropy of apoMb153-RNC upon release of DnaK. The shift corresponds to an enhancement in the fraction of nanosecond motions. c) Western blot and fluorescence analysis (as in panel b) of PIR90. No shift from a 2-component fit is detected under \( \text{+G} \) conditions. Each experiment in panels b and c was repeated 3 times.

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**Figure 6.** Proposed models for ribosome-bound nascent polypeptides consistent with the observed RNC dynamics. Both TF (a) and DnaK (b) are involved in binding/release events. The nascent chains are likely to interact with both chaperones, in the wild-type cell-free system. Only species of type 2 and 4 contribute to the 3–7 ns motion.
the dynamic fluorescence depolarization timescale (ns). Therefore, although chaperone-bound nascent chain species are likely to exist, their motions remain spectroscopically unresolved here and merge into the fraction of nondecaying anisotropy of the entire RNC (Figure 4). We conclude that this study does not provide information about the dynamics of chaperone-bound species 1 and 3 (Figure 6). Conversely, BODIPY depolarization is sensitive to the independent chain motions of the chaperone-free species of type 2 or 4.

The possibility that approximately nanosecond chemical exchange processes between species of types 1 (or 3) and 2 (or 4) are responsible for the observed 3–7 ns timescale motions is ruled out on the grounds that this type of dynamics is also detected in the absence of any cotranslationally active chaperones (Figure 5). In addition, as discussed above, the literature reports much longer lifetimes (μs–s) for TF– and DnaK–nascent chain complexes.

In summary, we interpret the apoMb89–153 3–7 ns motions as the signature of an independently tumbling structural domain of nascent chains emerging from the ribosome. Shorter apoMb chains buried in the ribosomal tunnel and natively unfolded PIR90 lack this feature.

### Nascent Chain Structure and Dynamics

Which specific structure is compatible with such independent nanosecond dynamics? It is clear that this question cannot be answered on the basis of fluorescence depolarization data alone. On the other hand, it is possible to broadly define the class of structures consistent with the spectroscopic results.

The predicted size and shape compatible with the observed nanosecond dynamics of apoMb153 RNCs (Figure 7) prompt some broad speculations. The expected rotational correlation times corresponding to rigid extended chains, semicompact ellipsoids, and spheres are shown for different chain lengths. The effective viscosity (required to generate the plots and measured by us in the resuspended ribosome medium; see also Supporting Information) sensed by the nascent chain depends on both crowding of the medium and local structural confinement (28). Two representative limiting viscosities (η) are considered here: η of free BODIPY (panel a) and η of ribosome-released apoMb153 (panel b), measured in RNC solutions.

Both panels a and b exclude the presence of fully extended chains, given that the typical polypeptide persistence length is only 4–6 residues. Less elongated ellip-
soids and spheres are feasible for a wide range of molecular sizes. Panel a predicts longer chain lengths (90–130 residues) than could possibly emerge form the ribosome in apoMb$_{153}$. Panel b, conversely, estimates the possible presence of 10–15-residue-long spheres and semicompact ellipsoids.

Large nascent chains emerging from the ribosome may sense local crowding/confinement to a similar extent as ribosome-released apoMb$_{153}$, whereas smaller structures may sense a viscosity closer to that of the free fluorophore. While it is impossible to differentiate between the two scenarios on the basis of the currently available information, it follows from the above discussion that the observed tumbling unit should be at least partially compact and possibly correspond to a fairly small stretch of residues. In summary, we speculate that the independent nanosecond dynamics of full-length apoMb$_{153}$ may correspond to a semicompact ellipsoidal shape of chain length >8 residues.

Conclusions. We have presented direct spectroscopic evidence for the presence of independent nascent protein conformations during intermediate and late stages of biosynthesis. These species are absent in the case of a natively unfolded control protein inca-

METHODS

Bacterial Strains and Plasmids. The E. coli K12 strain A19 (35) was supplied by G. Kramer. The MCA100::Δtig::cat E. coli strain was provided by P. Genevaux and F. U. Hartl (36). Construction of the E. coli A19::Δtig strain was carried out by transferring the Δtig::cat allele from MC4100::Δtig::cat into the A19 strain by P1 transduction using the P1vir phage. Transductants were selected at 37 °C on Luria–Bertani agar containing chloramphenicol (15 μg mL$^{-1}$) and 10 mM sodium citrate. Colonies were further purified by restreaking on the same plates. The absence of TF was confirmed by Western blot.

The plasmid containing the sperm whale Mb gene was described previously (15). A pET-28b (Novagen) plasmid carrying the gene for Grb14 PIR domain was supplied by I. Broutin (15). The PIR stop codon (TGA) was mutated to GAT with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The PIR stop codon (TGA) was mutated to GAT with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The pET16 plasmid containing the wild-type tig gene was purchased from Takara Mirus Bio.

Generation of BODIPY-FL-Met-tRNA$_{Met}^{\text{35S}}$. BODIPY FL (Invitrogen) and tRNA$_{Met}^{\text{35S}}$ (Sigma-Aldrich) were used without further purification. Aminocacylation of tRNA$_{Met}^{\text{35S}}$ was performed as described previously (37) with overexpressed and purified E. coli methio-

The presence of ribosome-bound nascent chain structure has been detected only indirectly before (9, 29–32), and high resolution information has only been gained for the outer domains of multidomain proteins (33). Here, for the first time, we (i) present direct evidence for the conformational dynamics of nascent polypeptides encoding a single-domain protein both inside and outside the ribosomal exit tunnel, and (ii) demonstrate the distinctive dependence of nascent chain structural dynamics on the stage of chain elongation and amino acid sequence. While more investigations are needed to unveil further details at higher resolution, this work takes an initial step at exploring the structure/dynamics of nascent proteins.

In contrast with purified N-terminal fragments, which become structured only extremely close to chain completion or heavily aggregate (34), we show here that ribosome-bound apoMb explores independent conformations even when a large C-terminal fraction is missing.

Finally, this investigation introduces dynamic fluorescence depolarization as a tool to study cotranslational protein folding. The power of this technique stems from its simplicity. Only one site-specific label is needed, in contrast with other approaches (e.g., FRET), which typically require multiple probes. In addition, dynamic fluorescence depolarization enables the resolution of multiple concurrent motions in a single experiment.
Generation and Isolation of RNCs. Ribosome-bound nascent chains were generated by oligodeoxynucleotide-directed mRNA cleavage (Supporting Information). Ribosomal pellets (including ribosome-bound peptides and associated proteins) were isolated upon ultracentrifugation (Beckman TL-100 with TLA 100.1 equilibrium rotor) as follows. Cell-free expression mixtures (75 μL) were loaded onto a 150 μL sucrose cushion (1 M sucrose, 20 mM tris-HCl, 10 mM MgOAc₂, 500 mM NH₄Cl, 0.5 mM EDTA, and 1 mM α-dithiothreitol, pH adjusted to 6.9) (42) and ultracentrifuged at 160,000 × g for 60 min at 4 °C. The ribosominal pellet was resuspended in 15 μL (i.e., 1/5 of the original volume) of buffer (10 mM tris-HCl, 10 mM MgOAc₂, 60 mM NH₄Cl, 0.5 mM EDTA, 1 mM α-dithiothreitol, pH 6.9) by shaking for 60 min on ice in an orbital shaker. The ribosome-bound status of nascent chains was tested by treatment with puromycin (Figure 1, panel d) added post-translationally (final concentration 0.5 mM), followed by incubation for 15 min at 37 °C.

Generation of TF- and DnaK-Free Samples. To analyze nascent proteins in the absence of DnaK and TF, ΔDnaK RNCs were incubated with 2 μM GrpE, 30 μM ATP, and 500 μM KCl ( +G conditions). The presence of DnaK in resuspended RNCs was tested under −G and +G conditions by an additional ultracentrifugation/resuspension step.

Determination of DnaK and TF Concentrations. Cell-free proteins were separated by SDS-PAGE (8% tris-glycine polyacrylamide gels) (49) and transferred to a PVDF membrane, followed by probing with mouse monoclonal anti-DnaK (Stressgen) or rabbit anti-TF (Genevaux and Hartl) polyclonal antibodies. Goat antirabbit or goat antimaline alkaline phosphatase conjugates (Novagen) were used as secondary antibodies. Protein bands were detected by chemiluminescence, using CDP-Star substrate (Novagen) were used as secondary antibodies. Protein bands were detected by chemiluminescence, using CDP-Star substrate (Novagen) and CDP-Star substrate (Novagen) and CDP-Star substrate (Novagen).

Fluorescence Instrumentation and Analysis. All measurements were performed at RT. Anisotropy decay data were collected by the multifrequency phase and modulation technique with a Chronos spectrofluorimeter (ISS) equipped with calcite prism polarizers and a laser diode (λex = 473 nm, λem > 510 nm with a 51294 filter, Newport Corp.-U.K.). Lifetime measurements were performed on all samples with vertically polarized excitation and 547 nm polarized emission. All frequency-domain fluorescence data were analyzed using the GLOBALS software package (45). A frequency-independent standard deviation of 0.2° was used for phase and 0.004° for modulation was applied. Lifetime curve fitting required three discrete components, one kept fixed to 0.001 ns to account for minor contributions from light scattering (less than 2% of total intensity). The frequency-dependent G-factor was measured independently. Frequency-dependent polynomial corrections were applied to all phase and modulation data to take the G value into account. Depolarization data were fit to multieponential decay expressions (Figure 3, panel a) including either two or three discrete components (10). In the case of two components, only two terms, describing the fast (F) and slow (S) components, were included. The fractional amplitudes (F₀ and F₁) and apparent rotational correlation time for the fast motion (τ_r) were allowed to vary while the fundamental anisotropy and the slow correlation time were fixed (r(0) = 0.37, τ_r = 1 μs). In all cases, a three-component expression was also considered, with an added term including F₁ and τ_r. The three component analysis was retained only if a significant improvement in fit quality was achieved (Table 1). Spectral simulations were performed with the Vinci software (ISS).

Fluorescence-Detected Gel Electrophoresis. Puromycin-released chains were analyzed by SDS-PAGE with a three-layer tris-tricine gel (15, 46). Low pH gels (47) were employed to reveal puromycin treated peptide- tRNAs.

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Supporting Information Available: This material is free of charge via the Internet.

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