Point of VIEW

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Folding on the Assembly Line

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ABSTRACT Deciphering the mechanism of folding of newly synthesized proteins in the cell is a major challenge because of the large size and multiplicity of molecular components involved and the asynchrony of biosynthesis. Fluorescently labeled ribosome-bound nascent chains of a defined length were prepared and subjected to dynamic fluorescence depolarization spectroscopy measurements. Nanosecond anisotropy decay correlation times of proteins' nascent chains at different stages of polypeptide elongation were determined for the first time. Striking dependence of the chain dynamics on the stages of elongation was observed and revealed chain length dependence of folding on the ribosome.

Researchers studying the mechanism of protein folding in the test tube are often asked, "How relevant are your results to real life, to the folding of a newly synthesized polypeptide in the cell?" The ability of a protein to fold to its functional state, whether after synthesis in the intracellular environment or in the test tube, is indeed an amazing feature at the heart of the phenomenon of life. This amazement and the implicated possibility to predict a protein's structure and function from its sequence information are the driving force for generations of researchers to study the reversible denaturation/renaturation of proteins *in vitro*. Beyond doubt, the study of the "real problem", the mechanism of folding *in vivo*, also bears exciting promise for medicine and biotechnology.

Monitoring the folding and dynamics of the nascent chain *in vivo* is at least an order of magnitude more complex than monitoring the folding of a single protein in the test tube for three reasons: (i) the size and complexity of the cell system, which includes many macromolecular components, with nascent protein chains constituting $<0.1\%$ of the total protein in the sample; (ii) the binding dynamics of multiple chaperoning components; and (iii) the asynchrony of biosynthesis—how can we observe only those nascent chains of a protein, which are at a certain stage of peptide elongation? On page 555 in this issue, Ellis *et al*. (*1*) took a further step toward overcoming these issues by combining resources from chemistry, biology, and physics. They add a powerful experimental approach to the so-far very limited arsenal of biophysical methods that were used to characterize the structural transitions of a nascent polypeptide chain in the heterogeneous environment of the ribosome. They generated ribosome-bound nascent chains (RNCs) of apomyoglobin by cell-free biosynthesis and introduced at the same time and by the same process a synthetic fluorescent probe, BODIPY, into the N-terminus of the protein; this unique and elegant labeling method was first reported by Johnson and McMillan (*2*). They separated the RNCs by ultracentrifugation, resuspended the pellets, and thus were able to spectroscopically investigate the nascent chain of apomyoglobin at different stages of peptide elongation. The first to apply dynamic fluorescence depolarization spectroscopy to cotranslational folding, Ellis *et al*. resolved three independent dynamic processes by rigorous analysis of the obtained multiexponential fluorescence anisotropy decays of the BODIPY probe. Global analysis of multifrequency phase shifts and modulation of the fluorescence of the probe yielded three depolarization relaxation rates and their relative contributions. The longest correlation time was assigned to the global tumbling of the ribosomal complex (1 μs), and the shortest to the local rotational diffusion of the probe (subnanosecond). The medium component (3−7 ns) indicated that local fluctuations of the labeled chain sections were slowed down by structural constraints, for instance, by the formation of folded structures in the labeled polypeptide. This component appeared only for longer nascent chains of apomyoglobin and did not appear when the experiment was repeated with PIR, an intrinsically

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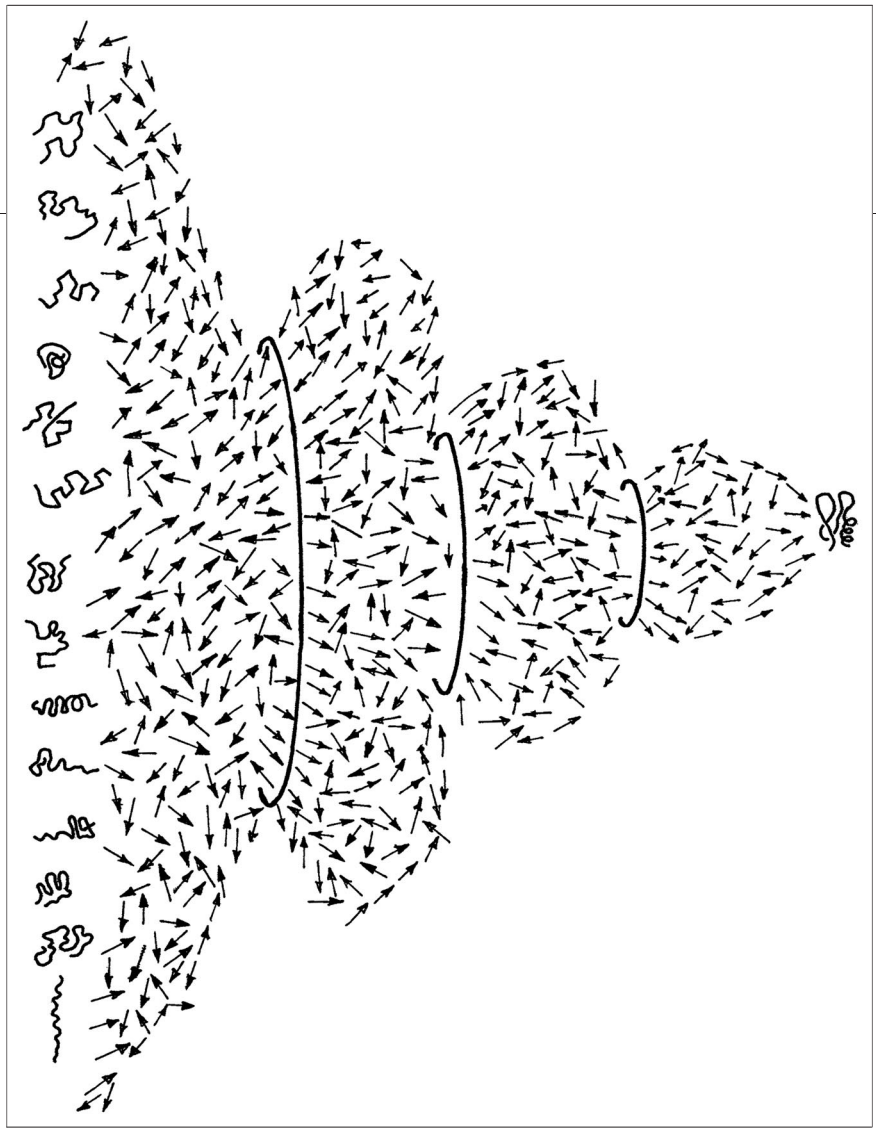

Figure 1. Schematic description of a 2D section of the multidimensional conformational space available to a globular protein. The left column represents the ensemble of fully unfolded protein molecules. Elementary, random conformational fluctuations, represented by the small arrows, move the chain around in the conformational space. Encounters of interacting chain sections result in weakly stable intramolecular interactions, imposing constraints on the available conformational space. These constraints are represented by the closed circles. A series of consecutive restrictions reduce the conformational space to the space available for the ensemble of native conformations (right corner). The result is a set of biased motions that form a folding pathway. Many different pathways can be followed in this structural funnel; some of them are those facilitated by additional constraints imposed by the ribosome and by the factors and chaperone proteins associated with it. The folding on the ribosome is a subset of the folding pathways available in solution.

unfolded protein, which lends support to the conclusion that it is indeed caused by folding. Ellis *et al*. thus were able to demonstrate a nascent chain that becomes conformationally restricted (folded) at later stages of elongation while still in the exit tunnel of the ribosome.

The elementary steps of the protein folding transition are stochastic. The random fluctuations of the chain are biased by constraints imposed by native-like inter-residue interactions that are on average more stable than non-native interactions. Gradual formation of such local and nonlocal interactions increases the number of constraints, and as a result the conformational space is reduced to the native subspace (Figure 1). Additional structural constraints restrict the alternative pathways available to a nascent polypeptide during and right after its biosynthesis. These are imposed first by the walls of the exit tunnel of the ribosome, then by the interactions with the surface residues of the supramolecular structure of the ribosome and its associated chaperone proteins, and eventually by the excluded volume effect of the crowded cellular environment. The pathways of folding *in vivo* are thus a subsection of the myriad of alternative pathways that a molecule can follow in solution. The stochastic nature of their folding transition and the low free-energy difference between their native and non-native states allow globular proteins to significantly populate alternative folds. The constraints imposed by the ribosomal complex evolved to reduce the probability of populating such non-native folds involved in numerous degenerative diseases (*3*).

How can we visualize and characterize the folding transition of the nascent polypeptide? An ideal experiment would yield a time-dependent series of images of the changing ensemble of nascent polypeptides, step by step, from the initiation of its synthesis to its final release. In the absence of such an experiment, multiple biophysical methods should be combined to compose such a motion picture.

In a series of heroic efforts initiated by Yonath *et al*. (*4, 5*), X-ray crystallography and cryoelectron microscopy were combined to reveal the structure of the ribosome in a variety of functional states. Detailed insights into the protein translation machinery have been obtained since then, yet the nascent protein chain has never been observed with any certainty, likely because of its inherent flexibility.

The ~100 Å long narrow tunnel between the peptidyl-transfer center and the surface of the ribosome explained the protease-protection experiment conducted 40 years ago (*6, 7*). Yet, the giant supramolecular structure composed of 52 protein molecules and very large RNA chains is a dynamic machine that acts as the most important assembly line in the cell. Full understanding

of its action and malfunction requires a combination of advanced molecular biological and biophysical methods, for which the crystal structure is the starting point.

Pioneering and very demanding FRET studies by Johnson and co-workers (*8*) gave solid evidence for cotranslational folding, even inside the narrow tunnel where the folding options appear to be very restricted. Johnson et al. (*9*) further found that a nascent secretory protein was fully extended inside the tunnel but that a nonpolar section of a nascent membrane protein folded into an α-helix during its passage through the tunnel. He concluded that the ribosome is actively involved in determining the folding of the nascent polypeptide in the tunnel on the basis of its sequence.

Recent advances in NMR spectroscopy of high-molecular-weight protein structures give some hope for future success in monitoring the conformations of bound nascent polypeptides on the ribosome. Using a combination of selective isotope labeling and ultrafast NMR spectroscopy, Dobson and coworkers (*10*) reported that NMR spectra of significant quality could be obtained from a nascent polypeptide chain attached to a ribosome. The work reported in this issue by Ellis *et al.* adds to the composite picture the important dimension of the nanosecond dynamics of the ribosome-bound nascent polypeptide.

The common theme of all current experimental approaches to the problem is the combination of site-directed labeling, based on molecular biological methods, with advanced spectroscopic methods. Advances in the methods applied so far, as well as new innovative biophysical methods that can be anticipated, will provide sections of a motion picture showing the fate of a nascent polypeptide all the way through the molecular assembly line of the ribosome. All methods are essential, but this goal will be achieved when they are applied synergistically. Time-resolved FRET methods that yield distributions of intramolecular distances in partially folded polypeptides in equilibrium and in kinetics experiments are available. Methods for single-molecule detection of intramolecular FRET efficiency are well established (*11*), and these two major FRET-based methods will hopefully soon be applied in studies of the nascent chain. These, as well as fast determination of high-resolution anisotropy relaxation rates of nascent polypeptides in real time, which might be practical with current modern devices, will probably be combined in the foreseeable future to yield sections of the future movie picture.

If folding on the ribosome is indeed the "real game", should we continue our efforts studying the simpler case, folding *in vitro*? In my view, the answer is positive. First, reversible unfolding and refolding is a natural process that occurs, for instance, in the cytoplasm during and after translocation. Second, the native structure is at any time in equilibrium with the unfolded structure as determined by the environmental conditions and by the sequence regardless of the pathway of folding. Finally, the pure-science physical–chemical question of the relation between sequence information and structure is of interest and even of biotechnological importance regardless of the "natural history" proteins in the cell. Given this, it is important to emphasize again that understanding the details of the mechanism of folding in the cell is of high importance not only for pure cell biological interest. This mechanism includes the capacity of avoiding the stabilization of alternative folds, commonly known as misfolding. This is a major source of interest and significance for all efforts invested in understanding the mechanism of folding of nascent polypeptides in the complex and crowded cellular environment.

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