

manipulate antigen presentation on class I MHC molecules for immune therapy. □

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focus on **MEMBRANE TRAFFIC**

Protein folding *in vivo*: the importance of ribosomes

Thomas O. Baldwin

The folding of proteins into their native conformation is more favourable kinetically if it occurs at the same time as protein synthesis, but there are few examples of such co-translational folding. The folding of the Semliki Forest virus capsid protein is one example.

One of the most intriguing problems of modern molecular biology is that of co-translational protein folding, and, more generally, the question of how proteins fold within the living cell. In a major recent advance, Nicola *et al.*¹ (page 341 of this issue) have shown that a specific protein can fold co-translationally within living cells, both eukaryotic and prokaryotic, and express its biological activity while still a nascent polypeptide. Although this study does not prove that all proteins fold co-translationally *in vivo*, it does show clearly that co-translational folding may occur in viable cells, and, more fundamentally, that the underlying laws of thermodynamics and chemical kinetics that govern protein folding are the same for both eukaryotes and prokaryotes.

All proteins have in common the biological function of folding, and all proteins must fold to express their biological activity. The molecular details of the processes by which proteins fold to achieve their native, biologically active structures have intrigued scientists for several decades and this area remains among the most exciting and challenging fields of investigation today. Most of our understanding of this fundamental biological process comes from studies of refolding of denatured proteins *in vitro* or folding during biosynthesis in a cell-free translation system. The main objective of these studies has been to gain a detailed understanding of the processes by which proteins fold *in vivo* to arrive at the ensemble of conformations that define the native, biologically active state.

The subject of co-translational folding has been reviewed recently^{2–4}. Much of the debate surrounding the nature of this process probably stems from a failure to define terms precisely. I believe that the best definition of co-translational folding is operational. Co-translational folding has occurred if, following extrusion from the ribosome, the native structure is achieved more quickly than if the full-length, unfolded polypeptide were diluted from chemical denaturant into the same folding milieu as that in which protein biosynthesis occurred (Fig. 1). If the native structure is formed more quickly as a consequence of biosynthesis, then it must be concluded that, by some mechanism, some folding of the polypeptide, whether to the final native structure or not, occurred during the process of biosynthesis. The formation of some secondary structure in the growing polypeptide chain would help to stabilize the nascent chain and so constrain the population of final conformations assumed by the polypeptide.

Nicola *et al.*¹ provide solid evidence for co-translational folding *in vivo*. These authors use the Semliki Forest virus capsid protein to study folding events within living mammalian and bacterial cells. The capsid protein is produced as the amino-terminal portion of a polyprotein, and possesses chymotrypsin-like protease activity which allows it to cleave itself from the remainder of the polyprotein. The protease acts *in cis* only, and as the catalytic residues involved in the protease activity are dispersed in the linear sequence, it is obvious that the pro-

tein must fold before the self-processing reaction can take place.

Using straightforward pulse-chase labelling methods, Nicola *et al.*¹ find that no full-length polyprotein can be detected in the cells used and that capsid-protein production is completed long before the rest of the polyprotein is released from the ribosomes. When the protease activity of the capsid protein is inhibited by the alkylating reagent *N*-ethylmaleimide, polypeptides that are longer than the full-length capsid protein are detected. Analysis of the size of the longest capsid-protein-containing nascent chains shows that cleavage must occur when the ribosome has proceeded roughly 43 residues beyond the carboxy terminus of the capsid protein, consistent with protection of 30–40 residues of the C terminus of nascent chains by the ribosome large subunit. These results indicate that the process of folding of the capsid domain into the active conformation must be fast on the timescale of protein synthesis, and that cleavage must occur almost immediately after extrusion of the cleavage site from the ribosome.

Another point of interest in Nicola *et al.*'s results is that Hsp70, a molecular chaperone that can enhance the correct folding of certain proteins, does not appear to be involved in the folding of the capsid protein. Co-immunoprecipitation with anti-Hsp70 antibody shows that Hsp70 does associate with many other nascent chains, but not with the capsid protein. This may be an example of an experiment for which a negative result is more informative than a positive one. In the field of protein folding, observation of co-immunoprecipitation with a chaperone is often interpreted as evidence of an absolute requirement for the interaction for folding to occur.

The elegant simplicity of Nicola *et al.*'s experiments underscores the difficulty of studying biophysical processes within the context of living cells. Their study is fundamentally a comparison of the rate of polypeptide elongation with that of formation of the biological activity that cleaves the capsid protein from the polyprotein. It is clear that the biologically active structure forms significantly more rapidly than the full-length polyprotein is produced. The

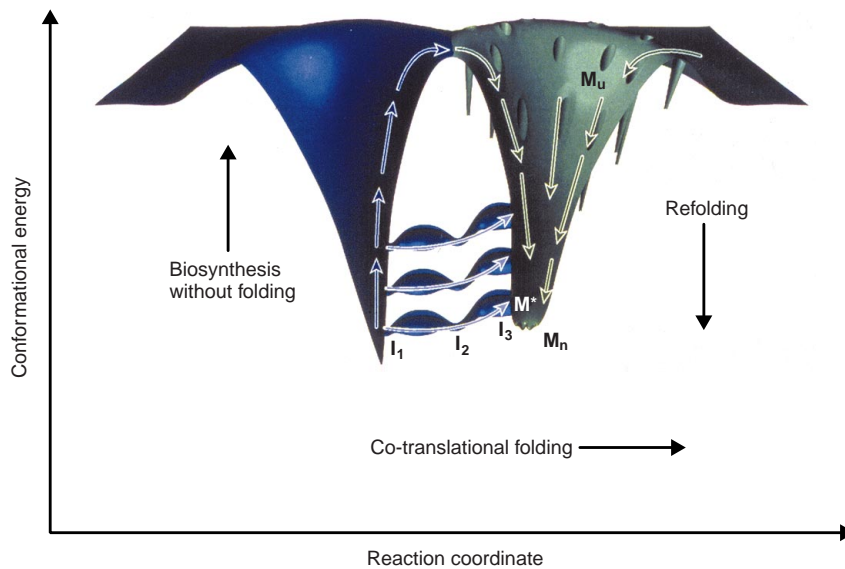


Figure 1 Cross-section of basic thermodynamic funnels adapted to include the processes of protein biosynthesis and folding. The y-axis shows the system's conformational energy; the circumference and surface of the funnels depict the conformational space available to the polypeptide; the x-axis shows the process of co-translational folding. The left funnel shows the hypothetical situation of protein biosynthesis without concomitant folding. The right funnel shows protein refolding following transfer from denaturing conditions to conditions that favour maintenance of the native structure. During refolding, the unfolded polypeptide (M_u ; M denotes a monomeric polypeptide unit) must avoid kinetic traps to arrive at the subset of structures (M_n) that define the protein's native state. During biosynthesis, as the nascent polypeptide is extruded without folding from the ribosome, the increased solvent-exposed surface and hydrophobicity of the constituent amino acids result in an increase in the system's thermodynamic instability. When the polypeptide reaches full length and is released from the ribosome, its folding reaction would be the same as if it had been diluted from denaturing conditions. Alternatively, the nascent chain might assume some structure as it becomes long enough to have interacting amino-acid side chains (I , intermediate). These interactions would stabilize the emerging polypeptide and constrain it to a smaller number of conformations, shown as local minima in tunnels joining the funnels. When the full-length polypeptide is released (intermediate M^*) it is already partially folded and can fold to the final native ensemble of conformers more quickly than if it had been released in a fully unfolded state. (Adapted, with permission, from ref. 2.)

interpretation that the protease activity is a property of the nascent polypeptide appears to be solid.

There are few other firm examples of co-translational protein folding *in vivo*⁵⁻⁸. Co-translational folding within the lumen of the endoplasmic reticulum has been demonstrated convincingly through detection of native disulphide bonds⁷. But apart from such examples, there have been few tools available to detect co-translational folding. Most studies have relied on the use of cell-free translation systems and examination of nascent polypeptides following translational arrest, using a variety of low-resolution methods. For example, the ability of nascent polypeptides to form native epitopes has been taken as evidence of co-translational folding². Other studies have relied on comparison of the rate of biosynthetic folding with the rate of refolding of

unfolded but covalently intact protein⁶⁻⁹. In studies of the folding of the β -subunit of bacterial luciferase, which must assemble with the α -subunit to become active, the $\alpha\beta$ structure was achieved more quickly when the β -subunit was produced on ribosomes in the presence of folded α -subunit than when the unfolded β -subunit was allowed to refold in the translation mix containing free α -subunit^{6,9}. These studies show that the structure of the polypeptide when released from the ribosome is such that it can assume the native structure much more speedily than it can refold when diluted out of chemical denaturant, so the ribosome and/or the vectorial aspect of protein biosynthesis may be critical in the process of protein folding *in vivo*^{6,9}.

Co-translational folding seems to me to be a thermodynamic imperative (Fig. 1). The alternative is to suppose that the polypeptide

must exit the ribosome into an aqueous medium without assuming any prioritized array of structures. This would obviously be a thermodynamically unfavourable situation, and, with each amino-acid residue added, the system would become less stable. If the full-length polypeptide were released in a fully unfolded state from the ribosome, it would refold with the same kinetics as if it were diluted from a chemical denaturant into the cellular milieu, which does not appear to be the case^{2,4-6,8,9}.

Recent studies *in vitro* have shown that the processes of protein folding are intrinsically fast; small unfolded proteins can, under optimal conditions, achieve the native structure on the microsecond timescale¹⁰. In comparison, the process of protein biosynthesis is exceedingly slow, even in the optimized environment of the living cell. Refolding of larger proteins is also often slow and inefficient, compromised by numerous off-pathway kinetic traps, including aggregation. It is generally thought that the many molecular chaperones and folding catalysts in living cells act to assist proteins in finding their native structure by avoiding off-pathway processes that lead to aggregation. A fundamental problem with this hypothesis is that the stoichiometry of the proposed participants is wrong. In *Escherichia coli*, for example, the amount of the chaperone GroEL in cells is adequate to assist the folding of only a small fraction of all nascent chains^{11,12}.

How then do nascent chains fold? Perhaps many proteins are capable of co-translational folding without the assistance of molecular chaperones, both in eukaryotes and in prokaryotes, as suggested by Nicola *et al.*'s results¹. And perhaps the vectorial aspect of protein biosynthesis itself plays a central part. These are questions for which there are as yet no clear answers, although recent advances hold much promise^{1,13}. □

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