Making a Network of Hydrophobic Clusters

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PERSPECTIVES: PROTEIN FOLDING

Making a Network of Hydrophobic Clusters

Robert L. Baldwin

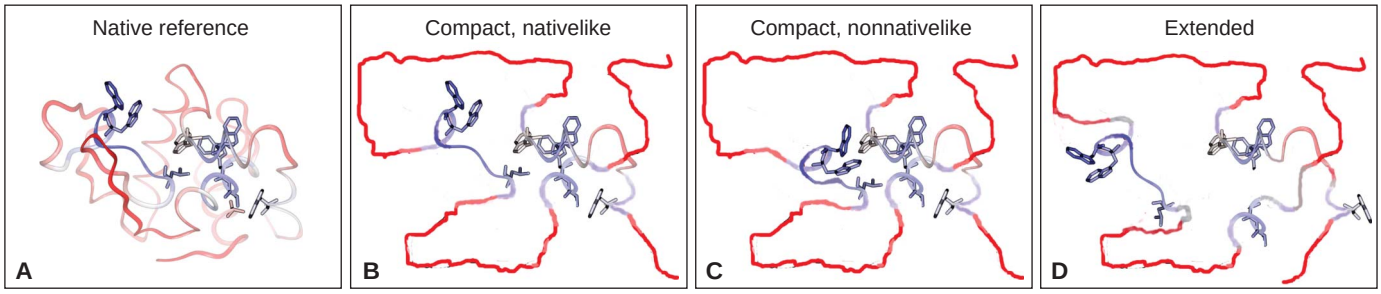
It has been hotly debated whether a hydrophobic collapse precedes or occurs concomitantly with formation of secondary structures at the beginning of protein folding (*1*). The role of secondary structures in guiding the folding pathway is readily understood because they provide the framework for the final native structure. It has also long been recognized (*2*) that burial of nonpolar (hydrophobic) side chains out of contact with water provides the major source of the free energy change that drives folding. But compelling models have been lacking for how this burial might produce a "hydrophobic collapse" that initiates folding.

On page 1719 of this issue, Klein-Seetharaman *et al.* (*8*) tackle a case of this kind. They show that in unfolded hen lysozyme, one particular residue, tryptophan 62 (Trp^{62}), stabilizes a network of hydrophobic clusters with cooperative, long-range interactions. At least some of these interactions must be nonnative, because Trp^{62} is freely exposed to solvent in the structure of the native protein. Earlier studies show that reflect nonnative interactions because they cannot be found in the structures of the native proteins. It remains unclear how these hydrophobic clusters affect the folding process.

Klein-Seetharaman *et al.* find six hydrophobic clusters for denatured hen lysozyme without S–S bonds, both in water and in 8 M urea (*8*). Four of the clusters contain at least one tryptophan residue. When the authors mutated Trp^{62} to glycine to examine the role of this residue in stabilizing its nonpolar cluster, they made a startling observation (*8*). Not only does the peak for the Trp^{62} nonpolar cluster in the R_2 plot disappear when Trp^{62} is replaced by glycine, almost all peaks for the whole system of clusters disappear. Evidently, the hydrophobic clusters form a network with cooperative, long-range interactions (see the figure), with Trp^{62} playing a central role in maintaining the network's stability.

How do these observations relate to the folding of hen lysozyme? Previous studies have provided insights into the role of Trp^{62} in lysozyme folding, and these older experiments may be interpreted in light of the new knowledge about hydrophobic clusters. Replacement of Trp^{62} by a tyrosine residue increases the folding rate of hen lysozyme with intact S–S bonds 13-fold (*11*) and causes only a 1° change in thermal stability. The increased folding rate of the Tyr^{62} mutant is good evidence for participation of Trp^{62} in a nonnative interaction that slows the folding process. This conclusion is consistent with the fluorescence properties of a folding intermediate formed in a fast (40 ms) phase of folding with intact disulfide bonds (*11*, *12*). It seems probable that the nonnative interaction made by Trp^{62} involves the network of hydrophobic clusters.

The folding of hen lysozyme in vivo includes formation of correct disulfide bonds. Once the correct S–S bonds have been formed, hen lysozyme can be unfolded and refolded without breaking the S–S bonds and then methylating the cysteine residues to prevent them from reacting further. Disulfide bonds stabilize the folded structures of proteins, and reducing the four disulfide bonds of hen lysozyme causes the protein to unfold.



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Hen lysozyme backbone conformation in different states (A to D). Four of the six hydrophobic clusters in hen lysozyme contain tryptophan residues: clusters 2 (Trp^{28}), 3 (Trp^{62} and Trp^{63}), 5 (Trp^{108} and Trp^{111}), and 6 (Trp^{123}). (C) shows the compaction of the unfolded molecule produced when the network of hydrophobic clusters is formed.

The most plausible model has been the "hydrophobic zipper" (*3*), in which clusters of nonpolar side chains stabilize secondary structures such as α helices or β hairpins. Examples are known in which such hydrophobic clusters guide the folding process (*4*, *5*). The clusters persist until folding is complete and can be visualized in the native structure of proteins. Hydrophobic patches have also been observed in native protein structures at sites where two α helices interact, and there is good evidence (*6*, *7*) that hydrophobic clusters of this kind can guide the folding process.

In these examples, hydrophobic clusters and secondary structures are formed concomitantly and produce nativelike structures. But hydrophobic clusters have also been found in denatured proteins, under conditions where secondary structures are unstable. Some of these clusters must replacing Trp^{62} by another amino acid residue strongly affects the folding process.

Hydrophobic clusters in denatured proteins can be detected by various nuclear magnetic resonance (NMR) probes, including the nuclear Overhauser effect (*9*) and chemical shifts of side chain protons (*10*). Clusters are formed not only in water but also in the presence of the denaturant urea (*8*–*10*). A particularly useful probe for examining which residues form a hydrophobic cluster in a denatured protein is the transverse relaxation rate, R_2, of a residue's amide group in the peptide backbone, measured by ^{15}N-^{1}H heteronuclear NMR. The value of R_2 expected for a structureless "random coil" can be read from the nearly horizontal plot of R_2 versus residue number. Residues forming a hydrophobic cluster have elevated values that are readily observed in this plot (*8*).

When the protein contains disulfide bonds, as does hen lysozyme, analysis of the transverse relaxation rates in the unfolded form is simplified by reducing the

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bonds. Correct disulfide bonds can be formed in a large fragment (residues 59 to 105) of hen lysozyme (129 residues). Trp^{62} is essential for the specific formation of correct S–S bonds in this fragment (*13*), suggesting that Trp^{62} exerts its effect through the network of hydrophobic clusters and that this network thus has a beneficial effect on the folding in vivo, despite the fact that Trp^{62} slows the lysozyme refolding reaction with preformed S–S bonds.

Could a network of hydrophobic clusters also explain the long-range order observed recently in urea-denatured staphylococcal nuclease (*14*)? It has been suggested (*15*) that steric clashes between side chains and backbone lead to the long-range structure. This model was used initially (*16*) to propose that folding begins locally at sites throughout an unfolded protein. The jury is still out on which model is correct. But the observations reported by Klein-Seetharaman *et al.* (*8*) show that accepted concepts about denatured proteins must be reexamined critically. More surprises are likely to emerge.

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PERSPECTIVES: STRUCTURE

Close Before Opening

Kathleen Postle

Wherever biological membranes are found, they are accompanied by proteins that direct the trafficking of ions, nutrients, and waste products across these cellular barriers. Some proteins form simple channels or pores that allow the concentration of substrates to be equalized across the membrane without the expenditure of energy. Other proteins are active transporters that use energy to pump substrates across membranes against a concentration gradient. Interesting variations on these simple themes have arisen as our knowledge has increased. Some channels can be gated (opened or closed) by, for example, voltage changes or binding of cyclic nucleotides. One of the most complex active transporters is now described on page 1715 of this issue by Ferguson *et al.* (*1*). They report the crystal structure of FecA, a protein found in the outer membrane of the Gram-negative bacterium *Escherichia coli*. FecA, which transports ferric iron from the environment, belongs to a class of active transporters that are gated by the inner membrane protein TonB. The crystal structure of FecA yields an unexpected finding that justifies solving crystal structures for multiple members of a protein family.

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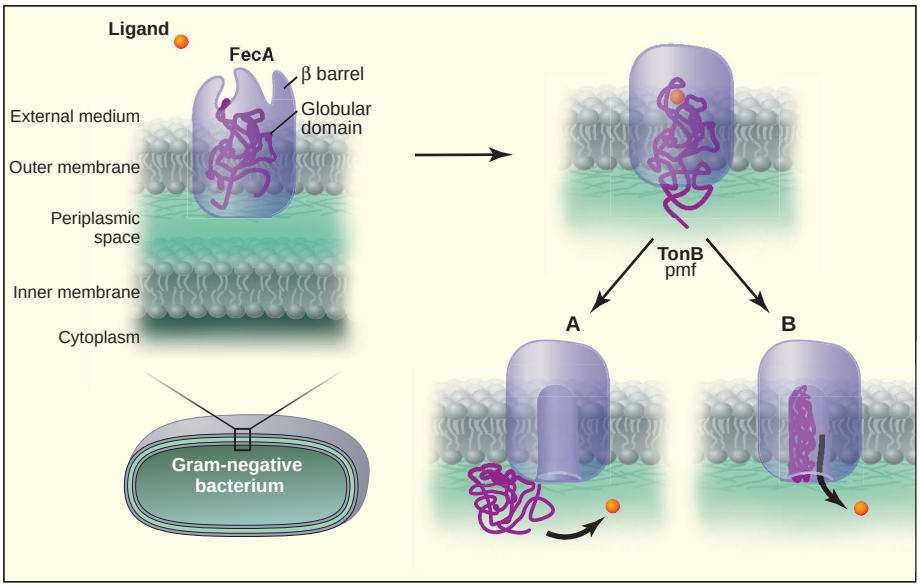
Most nutrients required by Gram-negative bacteria diffuse through outer membrane channel proteins (called porins) into the aqueous periplasmic space between the outer and inner bacterial membranes. They are then actively transported across the inner membrane into the bacterial cytoplasm (see the figure). Active transport is driven by either an ion electrochemical potential across the inner membrane or by hydrolysis of adenosine triphosphate (ATP). The acquisition of iron, an essential nutrient, presents bacteria with unique difficulties. Indeed, in vertebrate hosts, free iron levels are deliberately kept low to discourage survival of bacterial pathogens. In aerobic nonhost environments, iron is found as the virtually insoluble oxyhydroxide. Thus, being at the base of the food chain, bacteria must resort to extreme measures to obtain iron. They acquire iron by synthesizing and secreting chelator molecules called siderophores, which have an extremely high affinity for ferric iron. Iron-siderophore complexes are then bound by specific active transporter proteins in the outer membrane of Gram-negative bacteria with very high affinity (in the nanomolar range). These active iron transporters—including FhuA, FepA, and FecA and the vitamin B_{12} transporter BtuB—are closely related. They



Two gates for a TonB-gated transporter. (**Left**) The TonB-gated transporter FecA (lilac) of Gram-negative bacteria, when not bound to ligand (orange spot), is in a conformation that is ready to bind to ligand. (**Right**) Binding of ligand to FecA causes the newly discovered external gate to close (*1*). Meanwhile, large conformational changes (which are likely to be signals for TonB interaction) take place at the periplasmic face of the internal globular domain (TonB-responsive gate: purple) (*3, 4, 6–9*). Activated TonB opens this gate, allowing release of ligand into the periplasmic space. This can happen in two ways, both of which require TonB and the proton-motive force (pmf) of the inner membrane (*2, 10–13*). (**A**) The globular domain leaves the β barrel completely, perhaps through denaturation of its overall structure (*14*). (**B**) The globular domain remains inside the β barrel and becomes rearranged, thus providing a passage through which ligand can exit (*14, 15*).

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