Molten globules move into action

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One of the important goals of protein engineering and design is to create proteins with novel binding specificities. In addition to the intrinsic interest in understanding how the affinity and specificity of protein–ligand interactions is modulated, one can imagine a variety of practical applications for such a technology.

The most relevant natural proteins are antibodies, in which an incredible range of binding specificities is displayed on the same basic scaffold. The specificity and affinity of antibodies for different target molecules is amazing, and yet to be matched by human design.

In addition to the role for which antibodies naturally evolved, protection of the host organism from a wide range of invading pathogens, they are used in a variety of biotechnological applications such as affinity purification, *in situ* localization, immunoprecipitation, immunoblotting, and many others.

Yet, despite the awesome properties of antibodies, they are not without problems. Monoclonal antibodies can be difficult and time-consuming to produce, animals must be killed, and the antibody protein is of high molecular weight and quite delicate in its storage and handling requirements.

It would therefore be very useful if one could create a different protein scaffold, with none of the intrinsic problems of the antibody molecule, yet which could exhibit the positive binding properties of antibodies.

How can this be done? One needs a means by which to select or screen for proteins that display the binding properties of interest. Although there are several emerging strategies for such *in vitro* selections, the most widely used to date has been "phage display."

The essential component of any selection strategy is that the genotype must be tied to phenotype. That is, when a protein, which displays a particular binding specificity, is selected for, there must be some way to know what changes in the protein have occurred and to obtain a "clone" of that protein.

With monoclonal antibody production, the desired activity is screened for in monoclonal cell lines, which naturally contain the DNA encoding the antibody of interest, and the desired "clone" can be propagated. In phage display the protein of interest is "displayed" on the surface of the phage, as a fusion to one of the phage's own coat proteins. The phage particle contains the DNA encoding the fusion protein and is thus tagged.

Single-chain antibodies, Fv domains, and other engineered small fragments of antibodies have been displayed in this fashion on the surface of phage. The companion papers by Wahlberg *et al.* and Högbom *et al.* in a recent issue of PNAS (1, 2) take the strategy a step further. They chose a small, robust, well characterized protein and used phage display to evolve this molecule to have specific protein-binding activity.

These papers describe the properties of a variant of the Z domain of staphylococcal protein A that was selected to bind to its parent, wild-type Z domain of protein A. This particular target was chosen for a convenient "proof of principle" experiment.

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The surprising and unique result of this study is the solution behavior of the selected Z domain variant. The selected Z domain variant, which binds wild-type Z domain, does not display the properties of a native protein: it has many of the distinguishing features of a "molten globule" (3).

What is a molten globule? This question could stimulate hours of discussion, but here are the basics. The molten globule state was first described for certain proteins and could be induced by a variety of conditions, including low pH or removal of a cofactor (apo-myoglobin, for example). This nonnative state was recognized by the physical properties it displays. A molten globule exhibits some or all of the following: a high level of secondary structure (significant short wavelength CD signal), no defined tertiary structure (no long wavelength CD signal), poor dispersion of its NMR spectrum, rapid backbone amide exchange with solvent, a noncooperative thermal denaturation transition, low stability, a tendency to aggregate, and a high affinity for hydrophobic dyes (most typically ANS, which displays a large increase in fluorescence on binding to this state, but typically has no affinity for the unfolded or native state of the same protein).

The Z domain of staphylococcal protein A is a 58-aa, well characterized, well behaved three-helix bundle protein. It is homologous to one of the "B domains" of protein A, which is known to bind the Fc portion of IgG. On the basis of this homology and the binding mode of the B domain, 13 surface amino acids on helices 1 and 2 of the Z domain were chosen for randomization.

With this rationale for residue selection, a library of variants of the Z domain, with the potential to display novel binding specificities, was created and displayed on the surface of phage. These proteins are optimistically named "affibodies."

After several rounds of selection, the affibody "Z_{SPA-1}" was isolated and characterized, both alone and as a complex with the wild-type Z domain. Interestingly, the affibody Z_{SPA-1} alone displayed several features reminiscent of a molten globule. It has low solubility, poor amide dispersion, a low Tm, and noncooperative thermal denaturation transition. In addition, it has high secondary structure, as evidenced by CD, and binds the hydrophobic dye ANS with significant fluorescence enhancement.

Despite these apparently less-than-ideal features, Z_{SPA-1} does bind wild-type Z domain. Moreover, the binding is coupled to the adoption of a uniquely defined tertiary structure and loss of the molten globule characteristics. Both solution NMR and x-ray crystal structures of the Z domain–Z_{SPA-1} complex are presented. Interestingly, in the complex, Z_{SPA-1} adopts a fold that is closely similar to that of wild-type Z domain (for residues 8–56, the rms deviation for backbone atoms between wild-type Z domain and Z_{SPA-1} is 0.9 Å).

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What is the nature of the protein–protein interface? In a series of protrusions into a hydrophobic groove on the surface of the antigen, 9 of the 13 mutated residues participate at the binding interface. The surface area occluded from solvent on complex formation is quite substantial, a total of ≈1,665 Å^2, and 64% hydrophobic. This finding compares remarkably well with the surface area buried in typical antibody–antigen complexes, 1,600–1,700 Å^2.

How tightly does the affibody bind antigen and how does this compare with typical antibody–antigen affinities? The Z_{SPA-1}–Z domain complex has a dissociation constant of ≈1 μM, whereas antibody–antigen complexes are typically much tighter, 1 nM or less. What is the cause of this dramatic difference in affinity for two complexes with similar areas of contact? The authors suggest the importance of buried water molecules at typical antibody–antigen interfaces, which are not evident in the Z_{SPA-1}–Z domain complex. There is, moreover, clearly a substantial energetic cost associated with constraining the molten-globule-like Z_{SPA-1} molecule into the specific unique conformation of the complex.

These results lead to many interesting questions. For a small protein, is such a molten globule form an inevitable consequence of mutating a large fraction of surface residues? Does the molten globule character of the protein aid in binding by enhancing flexibility and the potential for "induced fit"? Are there other variants of the Z domain that resulted from this selection that do not exhibit molten-globule-like properties? If so, do they bind more or less tightly to antigen? Do proteins with molten-globule-like properties result from selections that use the same Z domain but present a different molecular binding target? The answers to these and other questions will provide an important extension of the exciting studies presented here.

1. Wahlberg, E., Lendel, C., Helgstrand, M., Allard, P., Dincbas-Renqvist, V., Hedqvist, A., Berglund, H., Nygren, P.-Å. & Härd, T. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 3185–3190.
2. Högbom, M., Eklund, M., Nygren, P.-Å. & Nordlund, P. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 3191–3196.
3. Ptitsyn, O. (1995) *Trends Biochem. Sci.* **20,** 376–379.