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Molten globules, entropy-driven conformational change and protein folding

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The exquisite side chain close-packing in the protein core and at binding interfaces has prompted a conviction that packing selectivity is the primary mechanism for molecular recognition in folding and/or binding reactions. Contrary to this view, molten globule proteins can adopt native topology and bind targets tightly and specifically in the absence of side chain close-packing. The *molten globule* is a highly dynamic form with native-like secondary structure and a loose protein core that admits solvent. The related (but still controversial) *dry molten globule* is an expanded form of the native protein with largely intact topology but a tighter protein core that excludes solvent. Neither form retains side chain close-packing, and therefore both structure and function must result from other factors, assuming that the reality of the dry molten globule is accepted. This simplifying realization calls for a re-evaluation of established models.

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Introduction

The two-state approximation in protein folding [1] dates back almost half a century [2] and has conditioned the field's thinking for the duration. Specifically, a wealth of experimental evidence is consistent with the view that the folding of sufficiently small proteins is highly cooperative, akin to a first order phase transition [1]. As folding proceeds, the equilibrium population partitions into two predominant species, U(nfolded) and N(ative), with only a negligible contribution from intermediate species. Accordingly, the folding reaction can be written as U ⇌ N, with an equilibrium constant K_{eq} = [N]/[U] that can be obtained under any given set of conditions by measuring experimentally-accessible probes of the native state, such as Trp fluorescence or far-UV circular dichroism.

The two-state model has led to the implicit assumption that conspicuous features in N are prerequisite to its formation, none more so than side chain close-packing. Packing efficiency is assessed by the packing density, a dimensionless measure of a protein's summed atom volumes normalized by its molecular volume. Typically, proteins are well packed with few internal voids of atomic dimension and with mean packing densities around ⟨0.75⟩, similar to the packing densities of small organic solids [3]. The supposition that side chain close-packing and native conformation are linked events is the basis for knowledge-based potentials, Gō models, and reaction coordinates based on counting native contacts.

Consequently, the surprising discovery of the molten globule (MG) conformation, which is not close-packed, led to much discussion about its role – if any – in the mechanism of protein folding [4,5]. A related consideration regarding function is whether or not MGs can participate in specific binding reactions.

Initially, the term *molten globule* referred to the 'wet' molten globule, a highly dynamic state with native-like secondary structure and a protein core that admits solvent and lacks close-packing [4]. Later, there has been a growing realization that the 'dry' molten globule [6] is another distinct state along a graduated MG spectrum. The dry molten globule (DMG) is an expanded form of the native protein in which close-packing has been released at the start of unfolding, but native-like conformation persists and water does not penetrate the core [7•].

A paradigm-shifting insight from both types of molten globules, MGs and DMGs, is the enormously simplifying realization that both native topology and biological function can emerge in states that precede side chain close-packing and in the absence of ligand/substrate binding. Remarkably, formation of native topology and side chain close-packing are separable, unlinked events in the DMG model for folding/unfolding [7•], contrary to well-entrenched expectations arising from a strict two-state model.

This review focuses on specific binding by MGs (Part I) and entropy-driven conformational change (Parts II and III). Part I discusses two important systems in detail, highlighting the surprising finding that MGs can bind their substrates selectively and even tightly.

Parts II and III explore the pervasive influence of side chain conformational entropy. Many studies assume, if

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only tacitly, that conformational entropy does not play a guiding role in protein folding because it has already been dissipated when the native state emerges. According to this view, favorable interactions between and among side chains select the native state from other conceivable conformers while the population entropy diminishes haphazardly [8]. The studies in part II challenge this assumption. When a DMG is formed at the start of unfolding, the process is driven by the increase in conformational entropy. Similarly, when two proteins form a complex, conformational entropy is not a passive bystander but instead plays an active role in driving complex formation. Recent evidence from NMR shows that side chain conformational entropy in solution is complex, again reinforcing the realization that the thermodynamics of close-packing cannot be inferred from inspection of protein X-ray structures.

Some thermodynamic preliminaries

The energetics of close-packing in DMGs can be modeled by data from the crystal-melting equilibria of alkane hydrocarbons [7•,9,10]. Key thermodynamic features that govern the release of close-packing are: (1) a favorable change in conformational entropy that drives the process, (2) a compensating unfavorable change in enthalpy, and (3) an overall change in free energy that is much smaller than the change in $-T\Delta S$. A central question is: how cooperative is the release of close-packing and what controls its cooperativity? Thermodynamic and kinetic data are available for villin headpiece [11], and the thermodynamic results are consistent with the crystal-melting model. In this case at least, the release of close-packing is highly cooperative. Fortunately, the villin headpiece DMG is stable, and therefore it can be analyzed experimentally.

The defining difference between a dry molten globule (DMG) and a conventional molten globule (MG) is that water has been squeezed from the core of a DMG. Initially, the DMG was proposed as the transition state ($I^{\ddagger}$, which cannot be observed directly) of a two-state ($N = I^{\ddagger} = U$) folding reaction [6]. However, later experiments indicated that the DMG is a stable unfolding intermediate that can be studied experimentally [7•]. Operationally, the DMG can be unambiguously distinguished from a wet MG by the fact that its peptide backbone NH protons are well protected against exchange [7•]. Another standard test for distinguishing a DMG from an MG is that the fluorescent indicator dye ANS (8-anilino-1-naphthalene sulfonate) can penetrate and bind within the hydrophobic core of an MG but not a DMG. ANS-binding corroborated other results using circular dichroism and fluorescence resonance energy transfer, which suggest that a DMG is formed when single-chain monellin starts to unfold [12]. A related study based on molecular dynamics simulations found that urea, but not water, can penetrate the protein core of a presumed DMG that forms at the initial stage of unfolding of hen lysozyme [13].

Part I: Specific binding by molten globules

Provocative early studies of secondary structure in MGs by NMR-hydrogen exchange suggested specific binding between helices. In detail, helices A, G and H of myoglobin (Mb), which have little tendency to form in isolation, are present in the pH 4 molten globule of apoMb, as reported by backbone NH protons that are protected against exchange [14]. How could A and GH, situated at opposite ends of the polypeptide chain, stabilize each other without specific binding? A related result was reported in an early folding kinetics study of horseheart ferri-cyt *c* [15]. There, the N-terminal and C-terminal helices develop rapidly with similar kinetics, apparently forming a complex between these distant chain segments before the central helix forms. A later mutational study of yeast iso-1-cyt *c* confirmed that native-like interactions between the N-terminal and C-terminal helices are present when the acid MG is formed [16].

These studies imply that specific binding interactions can occur when MGs are formed. In retrospect, this might have been anticipated. Specific binding is also observed between complementing protein fragments such as the disordered S-peptide and its cognate S-protein, which together form ribonuclease S [17]. The basic question is: can specific binding occur in the absence of close packing?

The physical–chemical mechanism of specific binding is a relevant question in all of these systems. It is often supposed that tight, specific binding is not possible in the absence of close-packing, but clearly this is not the case for the S-peptide:S-protein system [17]. Still, it is surprising to find that an MG apparently can function as an enzyme, where tight, specific binding is needed but typically is tied to close-packing, as discussed next (Figure 1).

An active enzyme that appears to be an MG

In 2004, Hilvert and co-workers obtained a 109-residue designed enzyme that fortuitously has the properties of an MG [18••]. The authors' intention was simply to convert a dimeric enzyme (chorismate synthase, CM, from *methanococcus*) to a monomeric form by inserting a hinge-loop peptide within the dimer interface. Their resultant monomeric enzyme, mCM, had high specific enzymatic activity, like that of CM, but unlike the parental dimer, other properties of mCM were characteristic of an MG: rapid H/D exchange (HX) measured by mass spectrometry, thermal unfolding that shows little or no cooperativity, poor NMR signal dispersion, and the ability to bind the MG-indicator dye ANS.

The authors conducted a thorough investigation of both binding and activity for the MG enzyme, made possible

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