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Packing in molten globules and native states

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Close packing of hydrophobic residues in the protein interior is an important determinant of protein stability. Cavities introduced by large to small substitutions are known to destabilize proteins. Conversely, native states of proteins and protein fragments can be stabilized by filling in existing cavities. Molten globules (MGs) were initially used to describe a state of protein which has well-defined secondary structure but little or no tertiary packing. Subsequent studies have shown that MGs do have some degree of native-like topology and specific packing. Wet molten globules (WMGs) with hydrated cores and considerably decreased packing relative to the native state have been studied extensively. Recently there has been renewed interest in identification and characterization of dry molten globules (DMGs). These are slightly expanded forms of the native state which show increased conformational flexibility, native-like main-chain hydrogen bonding and dry interiors. The generality of occurrence of DMGs during protein unfolding and the extent and nature of packing in DMGs remain to be elucidated. Packing interactions in native proteins and MGs can be probed through mutations. Next generation sequencing technologies make it possible to determine relative populations of mutants in a large pool. When this is coupled to phenotypic screens or cell-surface display, it becomes possible to rapidly examine large panels of single-site or multi-site mutants. From such studies, residue specific contributions to protein stability and function can be estimated in a highly parallelized fashion. This complements conventional biophysical methods for characterization of packing in native states and molten globules.

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Introduction

A compact state of a protein with well-formed secondary structure but a fluctuating tertiary structure was first reported in 1981 [1] while the term molten globule (MG) was coined in 1983 [2] to describe such structures. However, over the years, an overwhelming amount of evidence has shown that many MGs have native-like packing interactions in at least some regions of the molecule. Studying kinetic MG like intermediates that occur during protein folding can provide valuable information about complex and poorly understood folding and unfolding pathways. MG like structures have been shown to be involved in chaperone binding [3,4], protein function [5•] and in various genetic diseases [6,7]. This review summarizes current views, recent studies and approaches to study the role of packing in molten globules (MGs) and native states of proteins.

Molten globules

The molten globule state of α-lactalbumin

The structure of α-lactalbumin (α-LA) consists of two subdomains. The alpha-helical domain consists of helices A, B, C, D and a small 3_{10} helix, while an antiparallel beta sheet and a 3_{10} helix comprise the beta-sheet domain. The MG state of α-LA has been characterized under a variety of conditions, including low pH, removal of bound Ca^{2+} ions at neutral pH, reduction of disulfide bonds and intermediate denaturant concentrations [8]. Multiple studies have revealed a bipartite structure of the α-LA MG, where the α-helical domain adopts a near native-like conformation, while the beta-sheet remains largely unstructured [9]. Systematic mutagenesis of buried hydrophobic residues [10] and alanine scanning mutagenesis [11] of a simplified model of a neutral pH α-LA MG showed that the alpha-helical domain was structured with packing of specific hydrophobic residues. Heteronuclear NOE measurements at low temperatures [12] demonstrate that the MG of α-LA has more native-like structure than suggested by earlier studies.

The solvent accessible surface area of the low pH MG and the native state of α-LA were recently probed [13] using the photochemically reactive radiolabelled probe diazirine (DZN). This probe has approximately the same volume as water and has been shown to chemically modify accessible peptide bonds. Besides being an accessibility probe, DZN is mildly hydrophobic, thus increased labelling is observed for residues that are parts of hydrophobic clusters. In the MG, only the α-domain showed increased labelling relative to the denatured state, indicating the presence of a hydrophobic cluster. In the native state neither domain showed significant labelling. Using a photochemically induced dynamic nuclear polarization NMR pulse labelling technique (photo-CIDNP) in the MG state of full-length α-LA, it was shown [14] that two tryptophan residues are buried in both the native state

and the MG states, indicating the presence of native-like structure. However, four aromatic amino acid residues which are all buried in the native state were found to be substantially exposed to solvent in the MG, indicating loss of structure in certain areas of the molecule. More recently, it was shown that tryptophan residues in Ca^{2+} depleted MG and native α-LA exhibited high red edge excitation shift (REES) in their fluorescence emission maxima [15], indicating a significantly restricted environment and slow relaxation of water-dipoles around the chromophores. The denatured state however showed much smaller REES.

Though studies on bovine α-LA have shown that the equilibrium MG formed at pH 2 and the kinetic MG formed during refolding at neutral pH are similar [16], this observation has been challenged by recent work on human α-LA [17]. Wild-type recombinant α-LA protein with four intact disulfides forms a MG at pH 7 due to addition of a methionine at the N-terminus. NMR spectroscopy as a function of urea concentration revealed that both α and β domains of the MG were destabilized at pH 7 compared to pH 2, possibly due to electrostatic effects exerted by the large number of acidic amino acid residues in the protein. Particularly, the C-helix and the Ca^{2+} binding loop are well structured in the pH 2 MG, but not in the pH 7 MG, explaining why simpler models of MGs studied so far at neutral pH failed to detect any appreciable structure in these regions.

Molten globule state of apomyoglobin

Holo-myoglobin forms a compact structure made up of eight α-helices (A–H). In the heme-free form or apomyoglobin, while the majority of the protein retains its native structure, certain regions including the helix F and the C-terminus of helix H are destabilized and undergo conformational fluctuations [18]. Unlike the MG of α-LA, unfolding and refolding of the equilibrium MG of apomyoglobin are highly cooperative [19]. Hydrogen exchange probed by 2-D proton-NMR studies show that helices A, G and H are formed in this MG, while the rest of the protein is less structured [20]. Mutations that disrupt packing interactions in the AGH region in native apo-myoglobin also destabilize the equilibrium molten-globule intermediate [21,22]. This suggests that the AGH helices are packed in a native-like 3-D orientation in the MG. Apomyoglobin refolds at pH 6 through an obligatory on-pathway burst-phase kinetic intermediate [23] which was found to have properties very similar to the previously characterized equilibrium MG. Rapid-mixing quench-flow hydrogen exchange monitored by NMR spectroscopy showed that the AGH core of the MG folds within the dead time of mixing (~400 μs) and parts of B, D and E helices fold within milliseconds [24–26]. This has also been recently supported by NMR relaxation dispersion experiments [27•]. Mutagenesis experiments coupled to H/D exchange have shown that while B/C helices and B/G helices in the kinetic intermediate pack through native-like contacts [25], B/E and H/G helices appeared to involve non-native contacts [28]. Mutations that increase helical propensity at the N-terminus of the B-helix have been shown to confer increased protection to H/D exchange for residues in the B and E helices [29]. Non-native hydrophobic contacts along with lack of substantial structure in the N terminus of helix B are believed to be kinetic traps that must be overcome to fold to the native state. In addition to characterizing the MG, NMR relaxation dispersion experiments [27•] demonstrated the existence of a transiently populated intermediate between the native and the MG state, which showed chemical shifts very similar to the native state, except for residues in the F-helix. The F-helix forms part of the heme-binding pocket and is known to be conformationally flexible in the apo form of the protein.

Overhauser dynamic nuclear polarization NMR was used to measure the dynamics of water molecules around engineered cysteine residues that were labelled with nitroxide spin labels [30•]. Residues in the H-helix participate in the formation of the ABGH core of the MG. The experiments revealed while the H-helix residues in the core of the native state are in a dry environment, the nonpolar core of the MG was 'wet', with hydration dynamics which were only marginally slower than bulk water. Recent single molecule pulling experiments using optical trap force spectroscopy showed that unlike the native state of apomyoglobin which is brittle and hence does not undergo much deformation upon application of force, the MG state of apomyoglobin is highly deformable [31•].

Overall, the AGH core of the kinetic MG of apomyoglobin appears structured but hydrated with the B, D and E helices docking onto it later in the folding process.

Other molten globules

Flavodoxins are single-domain proteins with an α–β parallel topology (32% helix and 18% sheet). During folding to the native state a kinetic off-pathway intermediate is populated for apoflavodoxin, which has properties of a MG [32]. Under equilibrium conditions, a similar MG intermediate is formed in the concentration range of 1–3 M guanidine hydrochloride. However, substitution of a buried and highly conserved phenylalanine residue with tyrosine (F44Y) gives rise to a highly helical MG under native, low salt conditions [33]. Both native and non-native helices are present in the MG [34]. The hydrodynamic radius of the apoflavodoxin MG is only 11% greater than its native counterpart, as shown by fluorescence correlation spectroscopy experiments [34]. Hydrogen exchange studies have shown residues in the helical region from 110 to 125 have the highest protection factors against exchange, though the majority of the rest of the molecule also shows better protection than random

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