Review

Pre-Molten, Wet, and Dry Molten Globules *En Route* to the Functional State of Proteins

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Review

Pre-Molten, Wet, and Dry Molten Globules *en route* to the Functional State of Proteins

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Abstract: Transition between the unfolded and native states of the ordered globular proteins is accompanied by accumulation of several intermediates, such as pre-molten globule, wet molten globule, and dry molten globule. Structurally equivalent conformations can serve as native functional states of intrinsically disordered proteins. This overview captures the characteristics and importance of these molten globules in both structured and intrinsically disordered proteins. It also discusses examples of engineered molten globules. The formation of these intermediates under the conditions of macromolecular crowding and their interactions with nanomaterials are also reviewed.

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1. Introduction

In the classical picture of enzymology, native structure of a protein is intimately correlated to its function [1], and the functional 3D structure of proteins is determined solely by their amino acid sequences [2–5]. A deviation from the native structure accompanied by the loss of biological activity was defined as protein denaturation. Hence, study of the process of the unfolding of a protein molecule (under various denaturing conditions) was as much responsible for gaining crucial knowledge on the structure-function relationships in proteins as investigating protein refolding. In the three somewhat related phenomena, protein folding (i.e., spontaneous formation of 3D structure by the nascent polypeptides in the cell), protein unfolding, and protein refolding from the unfolded state in the test tube, the transition between native structure and unfolded/denatured structure(s) was the common thread [6]. The transition between the native and denatured states of small globular proteins was initially considered as a two-state process (two-state model of protein unfolding) [7–9]. Over the years, two generic folding intermediates were identified: the molten globule (MG) [10–21] and the pre-molten globule (PMG) [22–26]. Curiously, the existence of such folding intermediates was predicted in 1973 by Oleg B. Ptitsyn (1929–1999) based on the theoretical considerations of the potential mechanisms by which hierarchical structure of a native globular protein can be rapidly formed despite the astronomically large number of possibilities by which a polypeptide chain can be packed in a compact globule [27]. More recently, the concepts of "wet" and "dry" molten globules have emerged, where dry molten globular (DMG) intermediates represent an expanded form of the native protein with a dry core [28–38]. This is an interesting observation, as early studies indicated that the molten globule represents a highly hydrated state, with water inside the molten globule interior possessing characteristics of a highly associated liquid [21]. However, already in 1989, theoretical analysis of the denatured states of globular proteins suggested that since the compactness

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of a denatured protein may vary within a wide range, several denatured forms can be distinguished, such as coil, swollen globule, the "wet" molten globule (the compact state with pores occupied by solvent), and the "dry" molten globule where solvent does not penetrate inside the protein [39]. This overview captures the evolving nature of our understanding of the protein folding process. It also covers the interfaces of protein folding with macromolecular crowding and intrinsic disorder in proteins [40,41].

2. Molten Globule as an (un)Folding Intermediate

Historically, protein denaturation and unfolding studies are based on the well-accepted and rather obvious (at least new) mantra stating "Structure does exit since it can be broken". These studies played crucial roles in establishing or protein science in general and in understanding the basis of the correlation between protein amino acid sequence and function in particular. Already in 1931, Hsien Wu (1893-1959) proposed the first theory of protein denaturation: the active structure is known to exist because it is destroyed by denaturation [42,43]. His paper published in the *Chinese Journal of Physiology* contained the first statement that protein function depends on prior structure [42,43]. However, even earlier, in 1925, Mortimer Louis Anson (1901-1968) and Alfred Ezra Mirsky (1900-1974) showed that intact hemoglobin can exist as such near the neutral point only, whereas dilute acid or alkali changed it to the denatured form, which could fold back to its native state upon restoration of native conditions, indicating that protein denaturation and unfolding are reversible processes [44]. In 1936, the first Western review on protein denaturation that represents the first modern theory of native and denatured proteins was published, where Alfred Mirsky and Linus Pauling (1901-1994) stated that the loss of certain highly specific properties constitutes the most significant change that occurs in denaturation of a native protein [45]. By 1944, it became clear that native proteins have unique structures, that the denaturation processes are manifold in nature and magnitude, and that the addition of high concentrations of strong denaturants, such as guanidine hydrochloride (GdmHCl) or urea, to a protein causes a complete (or almost complete) disruption of all conformational interactions and, as consequence, to the transformation of a protein molecule into the highly disordered state of a random coil [46]. Furthermore, the authors of this seminal review stated: "The term denaturation has been used rather loosely and indiscriminately to denote ill-defined changes in the properties of proteins, caused by a variety of chemical, physical, and biological agents. The observation that many unrelated processes may cause similar changes in a protein early led to the belief that any single change, such as the formation of a coagulum, suffices to characterize a "denatured" protein, and that all denaturing agents are alike in their action. Although proteins are now known to respond differently to various kinds of denaturation, the supposition of the singleness of the denaturation process has persisted" [46]. They also defined denaturation as "any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties" [46]. It is obvious that a clear distinction should be made between the denaturation and unfolding terms. Here, as defined above, denaturation is a process leading to the elimination of protein functionality resulting from the disruption of functional 3D structure. This can be triggered by a wide range of conditions, with the resulting denatured forms possessing a wide spectrum of properties depending on the conditions at which they were achieved. On the contrary, protein unfolding is defined as a process leading to the complete elimination of all the conformational forces stabilizing protein native structure and therefore resulting in the formation of a coil-like conformation.

Retrospectively, finding partially folded species of globular proteins under variety of denaturing conditions should not be too surprising. This is because the unique 3-D structure of a protein molecule is stabilized by specific non-covalent interactions, such as hydrogen bonds, hydrophobic interactions, electrostatic interactions and salt bridges, and van der Waals interactions. Since these conformational forces have different physical nature, it is quite possible that they would react differently to the changes in the environment, where under specific conditions, some forces would decline and dissipate, whereas others would stay unchanged or even strengthen. In these cases the protein molecule is obviously losing its biological activity; i.e., it is becoming denatured, but since

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not all the conformational forces are "shutdown", denaturation is not necessarily accompanied by the complete unfolding of a protein, giving rise to the appearance of new conformations with properties intermediate between those of native and completely unfolded states. Therefore, various degrees of denaturation/unfolding must exist, depending on the extent to which the structure of the protein has been modified under given conditions. Clearly, the fact that the extent of denaturation can be different is incompatible with the "all-or-none" hypothesis that a given protein can exist in only one of two states, the completely native or the completely denatured/unfolded [46].

These important considerations were rooted in the experimental evidence accumulated in the thirties and forties, when the incomplete unfolding and existence of some intermediate stages of denaturation have been recognized in several instances [47–51]. Furthermore, as it followed from the later studies, some denatured forms produced at milder denaturing conditions (e.g., heat- or pH-denatured proteins) can undergo additional structural alterations in the presence of strong denaturants, such as urea or GdmHCl [52]. Therefore, since the final denatured conformations of proteins are strongly dependent on the denaturing agent, not all denatured states are structurally similar, and under certain conditions the protein molecules are not completely unfolded. These very logical conclusions were formulated in a classical review by Charles Tanford (1921-2009) [9], which was one of the first papers providing in-depth analysis of the possibility that during the unfolding of globular proteins, accumulation of some equilibrium intermediate states might be expected. Unfortunately, since the results that were available at that time were too scanty, no serious generalization could be made. Furthermore, the vast majority of then reported studies suggested that accumulation of an intermediate during protein unfolding was regarded as an exception to the rule, whereas a conformational transition described by a two-state model represented the "normal" response of a protein to changes in its environment. Although proteins were shown to respond differently to various kinds of denaturation, the supposition of the singleness of the denaturation process has persisted [46].

For the first time, an intermediate accumulating during the unfolding process was identified as early as in 1973 by Tanford's group while looking at the chemical unfolding of bovine carbonic anhydrase B (BCAB) by GdmHCl [53]. It is notable that the intermediate identified by far- and near-UV circular dichroism (CD) spectroscopy was described as having the secondary structure of the native state but losing of the tertiary structure [53]. A year later, Kin-Ping Wong and Larry M. Hamlin used circular dichroism, difference spectrophotometry, enzymatic activity, and viscosity to study acid denaturation of this proteins and showed that the acid denatured BCAB is enzymatically inactive and does not have unique 3D structure as judged by near-UV CD, but does not exist in the random-coiled state as indicated by viscosity and far-UV CD [54]. Around the same time, Pititsyn's group [27] initiated their work, which eventually led to further early insights into folding intermediate. It was suggested that formation of native like secondary structure precedes the protein acquiring its tertiary structure. The results of the analysis of acid- and temperature-induced denaturation from this group were found to support this notion [10,11,13]. It was Ohgushi and Wada who in 1983 coined the term "molten globule" to describe such folding intermediates [12].

The most defining characteristics of a "classic" MG are outlined below [14–16,25,55–65]. A protein molecule in the MG state is characterized by the presence of significant secondary structure (which is often classified as native-like secondary structure) with no or very little tertiary structure (tight packing of side chains of amino acid residues is absent). Furthermore, 2D-NMR coupled with hydrogen-deuterium exchange showed that the protein molecule in the MG state is characterized not only by the native-like secondary structure content, but also by the native-like folding pattern [66–75]. Small-angle X-ray scattering (SAXS) analysis revealed that the molten globular proteins possess globular structure typical of native globular proteins [76–81]. In agreement with the preservation of globular structure, the protein molecule in this state is characterized by high compactness degree, as its expansion typically leads to a general increase of 10-20% in radius of gyration or hydrodynamic radius (over the native state), which corresponds to the volume increase of ~50% [57,82]. A considerable increase in the accessibility of a protein molecule to proteases was noted as a specific property of the MG state [83–89]. There is also an increase in the solvent exposure of the hydrophobic

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core, which is now less compact than the core of a native globular protein. This is reflected in the characteristic capability of MG to specifically bind hydrophobic fluorescent probe 1-anilino-naphthalene-8-sulfonate (ANS) or 1,1'-Bis(4-anilino-5-naphthalenesulfonic acid) (bis-ANS) [90–92]. MGs can show substantially levels of structure in some cases [55]. Lynne Regan reported that one part of a protein can retain the native structure whereas another part forms a MG [93]. That is not unexpected as proteins in general are characterized by noticeable structural heterogeneity, and conformational stability/flexibility can vary across the protein regions [94–96]. Presence of intrinsically disordered protein regions (IDPRs) in numerous proteins serve as extreme examples of this phenomenon [83,94–96].

While earlier data on the denaturation/unfolding and refolding of small proteins were compatible with two state model comprising of N→D and D→N transitions, the fact that many proteins were shown to form MG during their unfolding indicated that the reality is more complex, and one should consider protein unfolding as a sequential process N↔MG↔U. This clearly raised a question on the physical and thermodynamic nature of the corresponding N↔MG and MG↔U transitions. The answer to this question was retrieved first from the results of the multiparametric experimental analysis of equilibrium GdmHCl-induced unfolding of BCAB and S. aureus β-lactamase at 4oC, which clearly showed the molten globule is separated from the more unfolded states by the "all-or-none" transition (this was evidenced by the bimodal distribution function of the molecular dimensions within the transition from the molten globule to the unfolded state) [97]. Later, similar bimodal distribution in the HPLC gel-filtration profiles was observed within the unfolding pathways of the NAD+-dependent DNA ligase from the thermophile *Thermus scotoductus* [22,23]. In line with these observations, an analysis of then available data on the equilibrium urea- and GdmHCl-induced N→U, N→MG, and MG→U transitions of globular proteins revealed that the cooperativity of all these unfolding processes increases linearly with the increase of the molecular weight of the protein up to 25-30 kDa, indicating that the solvent-induced transitions from the native to the unfolded state, from the native to the molten globule state and from the molten globule to the unfolded state are characterized by the "all-or-none" nature therefore suggesting that he molten globule represents a third thermodynamic state of a protein molecule [98,99]. The validity of this model was later supported by Vijay S. Pande and Daniel S. Rokhsar, who in 1998, used analyzed the equilibrium properties of proteins by Monte Carlo simulations and showed that in addition to a rigid native state and a nontrivial unfolded state, a generic phase diagram contained a thermodynamically distinct MG state, further supporting the idea that MG represents a third phase state of proteins [100].

3. Potential Functionality of Folding Intermediates

Even before the acknowledgement of the prevalence and biological importance of intrinsically disordered proteins with their amusing structural heterogeneity, it was recognized that folding intermediates, including MGs, might have biological relevance. One of the first notes about this scenario was a hypothesis that MG state may be involved in the translocation of proteins across membranes [101]. This idea was successfully supported by experiments, and there is now enough evidence that translocation of proteins and their insertion into membranes involve MG state [102–106]. Model systems with α-lactalbumin showed the binding of MG to lipid bilayers [107]. In general, globular proteins can be transformed into the MG states on interaction with the membrane surface [108]. Such N→MG transitions in the vicinity of a membrane can be induced by the action of the so-called "membrane field", which is a combination of the local decrease in the effective dielectric constant of water near the organic surface with the effect of negative charges located on the membrane surface [109–112]. Release and loading of the large, tightly packed hydrophobic ligands from and to the globular proteins might be facilitated by the partial unfolding of the carrier (N→MG transition) resulting from the concerted action of the moderate local decrease of pH and the moderate local decrease of dielectric constant in proximity to the target membranes [113]. Furthermore, many proteins responsible for the transport of large hydrophobic ligands might have a MG properties in their preloaded apo-forms [114–116]. It was also shown that many carbohydrate- and amino acid-binding periplasmic protein in *E. coli* form molten globule, which bind to their respective ligands

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[117]. Chaperones interact with MGs and prevent their aggregation [118]. Earlier, Martin *et al*. discussed how a chaperone-mediated folding has a MG as an intermediate [119]. It was also pointed out that compact, MG-like intermediates are localized within a central cavity of the chaperonin GroEL [120–122]. Facilitated folding of actins and tubulins occurs via a nucleotide-dependent interaction between the cytoplasmic chaperonin and distinctive folding intermediates [123]. The presence of MG during nascent peptide folding has been inferred [124].

Importantly, although aforementioned functionalities have been attributed to the MG-like conformations, the major emphasis of all these and similar studies was still focused on the assumption that these functional MGs are folding intermediates kinetically trapped by the chaperones just after the protein biosynthesis but before proteins become completely folded [25,101,125], or appear as a result of point mutations preventing polypeptides from complete folding [25,126], or originating from the denaturing effects of the membrane field [101–112] or ligand binding or release [114–116]. However, the presence of MGs in the cells become an established fact. [127]. All these observations provided strong support to the validity and importance of the concept of MG as a folding intermediate of globular proteins *in vivo*.

4. How One Can Find Molten Globules, and Where They Can Be Found?

MGs of globular proteins are generally obtained by their mild denaturation that can be induced by acid, alkali, low to medium concentrations of chemical denaturants, such as urea and GdmHCl, chaotropic salts, moderately high temperature, and, for some proteins, even by low temperature [128–147]. Later studies revealed that in some proteins, MG can be also induced by various organic solvents [148–151]. However, it was also shown that fluorinated alcohols can preferentially stabilize α-helices leading to the formation of non-native helical structure in some all-β-sheet proteins. For example, such highly helical state was induced by 2,2,2-trifluoroethanol (TFE) in several all β-sheet proteins, such as cardiotoxin analogue II (CTX II), from the Taiwan cobra (*Naja naja atra*) [152], procerain, a cysteine protease from Calotropis procera [153], β-lactoglobulin [154–157] and mellitin [154,157] to name a few. All β-sheet to mostly α-helical structure in β-lactoglobulin and mellitin was also induced by hexafluoroisopropanol (HFIP), as well as by non-fluorinated alcohols, isopropanol, ethanol, and methanol [154,157]. Curiously, it was pointed out that an alcohol-induced α-helical state of β-lactoglobulin structurally resembles a transiently populated folding intermediate with high levels of non-native α-helical structure, which is formed within a few milliseconds during the refolding of this protein [158], suggesting that an intermediate with the non-native α-helical structure can accumulate during the refolding process of β-lactoglobulin, emphasizing that hierarchical model cannot correctly describe folding of some β-structural proteins including β-lactoglobulin [156,158].

The secondary and tertiary structures are evaluated generally by far- and near-UV CD respectively. Secondary structure can also be evaluated with Fourier-transform infrared spectroscopy (FTIR) or optical rotatory dispersion (ORD), whereas viscosity measurements, gel-filtration chromatography, dynamic light scattering (DLS), SAXS, and electron microscopy are used to track expansion of the molecular volume [63,64]. The decrease in the compactness accompanied by the increased solvent accessibility of the hydrophobic core is normally estimated by looking at the binding of the fluorescent dye ANS to a protein molecule [90–92]. However, it was also pointed out that since ANS and bis-ANS have strong affinity to partially folded MG state, they can shift the equilibrium from favoring the native state (N) to favoring the MG state [91]. As a result, the apparent destabilization of the native state is observed, as it was shown for the nucleotide-binding chaperone DnaK [91]. On the other hand binding of ADP or ATP to the native state of this protein resulted in a shift of the equilibrium from the MG toward the N state [91]. Furthermore, as early as 1995, Anthony L. Fink (1943-2008) had cautioned that "It is important to note that the presence of ANS tends to increase the propensity of molten globules and compact denatured states to aggregate, and that aggregation increases the ANS fluorescence emission" [64].

Some other techniques like hydrogen-deuterium exchange, NMR, X-ray, isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), and computational methods have also been increasingly applied in later years [73]. In general, all the techniques/methods applicable to

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looking at protein structure and stability can give valuable information about partially folded intermediates like MGs [159]. For example, various fluorescence techniques, such as analysis of the intrinsic and extrinsic fluorescence (both steady-state and time-resolved), fluorescence anisotropy, Förster resonance energy transfer (FRET), dynamic and static fluorescence quenching, as well as proteolytic susceptibility are also used quite often [160].

In additional to classical examples of α-lactabumin, BCAB, and β-lactamase, equilibrium and kinetic MGs have been described for a number of proteins and their mutants [161–163]. One interesting comparison is between the MGs formed by α-amylases from a thermophile and a mesophile [164]. This analysis revealed that the MG of the thermophile was more stable, which is not surprising. The polyols were less effective in refolding of the MG of the mesophilic enzyme [164].

Another interesting class of proteins are from halophiles. These generally require > 0.5 M KCl to be functional. In several cases, these proteins just like those from thermophiles are fairly stable towards unfolding. The mechanism of halo-adaption was investigated by Gloss *et al*. [165] by looking at the kinetics of folding of urea denatured dihydrofolate reductases (DHFR) from *E. coli* and a halophile. In both cases, after a burst intermediate, formation of two intermediates was detected. The data was consistent with salt ions destabilizing the unfolded states in both cases. The authors concluded that halo-adaption involves affecting the solvent via hydrophobic effect, Hofmeister effect, preferential hydration, and crowding. This is in line with the X-ray crystallography structural data, which showed extensive solvation but little salt binding in case of many halophilic proteins [165].

Yet another example of complexity in halo-adoption by halophile proteins is the role of protein hydration [166]. Given their higher surface charge density, it is widely believed that these are highly hydrated even in their native forms. This excessive hydration was expected to be responsible for the exceptional stability of corresponding proteins under saline conditions. The results obtained with an engineered protein with high number of acidic residues on its surface suggested that not only the surface hydration of a halophilic protein is not much larger than that of a mesophilic counterpart, even its hydration dynamics during unfolding was not very different [166].

Study of the proteasome from the extremely halophilic archaeon *Haloarcula marismortui* revealed that while other enzymes unfolded under sub-saline condition, the proteasome was more resistant [167]. The biological significance of this is in understanding how proteasome degrades the damaged proteins under sub-saline conditions as the stress situation for the organisms [167].

Uversky had compared the stabilities of proteins from mesophiles with those from halophiles, thermophiles and barophiles while advancing a hypothesis about the role of protein dielectricity in affecting the solvent properties in the context of protein-protein interactions [168]. The article mentions the earlier work with β-lactoglobulin, in which it was reported that the molten globule formation by the protein in alcohol-cosolvent mixtures was directly dependent on the decrease in the dielectric constant of the water as a result of mixing the simple alcohols [111]. Interesting enough, in an independent observation, Gupta *et al*. around the same time observed that for a number of proteins, the enzyme stability in aqueous-organic cosolvent mixtures was dictated by the polarity index of the organic solvent [169]. Solvents with polarity index of 5.8 and above were good cosolvents which did not destabilize the protein when added even up to 50% (v/v) to the aqueous buffer [169]. Both dielectric constant and polarity index are a measure of the solvent polarity.

Another interesting observation has been reported about MG formed by chymotrypsinogen [170]. A single cysteine reacts with glutathione at a very rapid rate. Such hyperactive cysteine residues are also present in serum albumin, lysozyme, and ribonuclease [170]. However cysteine present in two proteins of a thermophile (in which glutathione is absent) did not display this hyper-reactivity. The authors infer that this unusually high reactivity of cysteine residues is relevant to the oxidative refolding of proteins in the organisms, which have oxidized glutathione-reduced glutathione system [170].

Furthermore, many IDPs exist as MGs under physiological conditions, and hence many important biological functions of such proteins, including cell signaling and other regulatory activities depend upon these molten globular states [56,69,171–186].

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5. Baroenzymology, Cryoenzymology and Molten Globules

While the effects of the temperature on protein conformation are widely known, the influence of pressure on protein structure and function has also attracted considerable attention and referred to as baroenzymology [187]. The effect of pressure on protein refolding has been especially intriguing and has been discussed in a recent book [188]. Masahiro Watanbe *et al*. [189] used ultraviolet spectroscopy to compare the effect of pressure on native and molten globules of canine milk lysozyme with the corresponding behavior of the homologous protein bovine α-lactalbumin (BLA). Notably, MG state of the lysozyme was found have a more compact hydrophobic core; unlike the "swollen hydrophobic core of the MG state of BLA" [189]. This is an interesting result in the context of concepts of DMG and WMG, which now are commonly accepted kinds of molten globules.

High hydrostatic pressure at 600 MPa was shown to induce MG formation in another model protein, β-lactoglobulin, where this pressure-induced MG remained stable for at least 3 months [190]. High pressure induced a native dimer to a molten globule monomer transition in Arc repressor [191] and lactate dehydrogenase (LDH) [192], as well as promoted disassembly of the cowpea mosaic virus (CPMV) capsid into the molten globular monomers [193,194]. For many other proteins, such as trypsin [195], carboxypeptidase Y [196], butyrylcholinesterase (BuChE) [197], staphylococcal nuclease (SNase) [198], horse liver alcohol dehydrogenase (HLADH) [199], human Q26 and murine Q6 ataxin-3 [200], human serum albumin (HSA) [201], human acetylcholinesterase (hAChE) [202], and X-prolyl dipeptidyl aminopeptidase from *Streptococcus thermophilus* [203]. Taken together, all these data clearly indicate that high hydrostatic pressure has the unique property of stabilizing partially folded states or MG states of a protein [204].

Again, while heat denaturation is quite well acknowledged, cold denaturation of proteins is not so extensively mentioned in enzymology. Yet, this phenomenon has been known for a long time [205–208]. For example, cold denatured states were described for myoglobin [206], a mutant of phage T4 lysozyme [209], α-lactalbumin [210,211], equine β-lactoglobulin [212], ubiquitin [213], and cytochrome c [214]. To prevent freezing of the aqueous buffered solution at subzero temperatures and to assist destabilization, organic solvents are also generally present in order to study cold denaturation. Kumar *et al*. has discussed at length the cold denaturation of horse ferricytochrome c at extreme pH [215]. During acidic denaturation in the presence of anions, the partially folded state of the protein is referred to as an A state. Similarly, the partially folded state obtained under alkaline conditions in the presence of cations as counterions is referred to as B state. Although the A state and corresponding structural transitions have been studied in several cases, Kumar *et al*. have mentioned that the analysis of the B state has attracted much less attention [215].

6. Molten Globules and Intrinsic Disorder in Proteins

Coming back to the hypothesis on the potential role of protein of protein dielectricity in affecting the solvent properties mentioned earlier [168], in the context of functional relevance of partially unfolded protein intermediates, it was proposed that a protein lowers the dielecric constant of the local medium around its interface with the aqueous solvent/water rich medium. This facilitates the behavior of proteins acting as "unfoldases". Many proteins, in order to be functional have to be unfolded (referred to as conditionally disordered protein) [216,217]. In many cases, this conditional unfolding is initiated by the interacting protein, which acts as an unfoldase by lowering the local dielectric around it; this leads to the binding between the two as a part of a biologically relevant process. Example includes unfolding of BCL-xL while interacting with the intrinsically disordered PUMA, which in turn folds upon binding as entropic compensation [168]. Unfoldases also include ATP-dependent proteases (such as in proteomes) and molecular chaperones. Early examples in which this unfoldase behavior was observed were pore-forming domains of some toxins and carrier proteins of large nonpolar ligands. The aggregation including where it leads to amyloid formations (and is responsible for many diseases) may also be initiated by protein lowering the dielectric around it. Few other examples relevant to this are available [217–220]. Therefore, this hypothesis provided a common thread running through diverse phenomena [168]. Interesting enough, later work has

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confirmed that functionally relevant unfolded structures of many bacterial toxins are molten globules [221–223].

7. Engineered Molten Globules

Recently, one of us has described a number of examples of engineered proteins which form molten globules [83]. These examples give good idea of what kind of amino acid sequences favor formation of molten globules. Some of those examples are briefly recalled below.

Dihydrofolate reductase (DHFR, E.C.1.5.1.3) binds to the structural analogs of the substrate dihydrofolate. These inhibitors such as methotrexate and trimethoprim are well-known antifolate drugs. Mutants (Thr35Asp; Thr35Asp/Asn37Ser/Arg57His) of DHFR formed existed as MGs, which were catalytically active even though the mutations were made in the active site of the enzyme [224]. Binding of trimethoprim and NADPH to the MGs converted these mutants to a stable conformation close to the one obtained with the native enzyme [224]. Even more extensive mutations were carried out by circular permutation via linking the N- and C- termini of DHFR by a tripeptide and creating the new termini elsewhere [225,226]. One such circularly permutated variant existed as a MG, but addition of the ligands (substrates or antifolates) induced the MG→N transition [226].

An engineered Chorismate mutase from *Methanococcus jannascii* (MjCM) had the same k_{cat} as the native enzyme [227–231], though K_m value with substrate chorismate was increased 3-fold [227]. The mutant possessed all the properties of a MG [228], and the induced-fit binding of the substrate took place on the same time scale as the native enzyme [229]. The native enzyme is a dimer whereas this mutant was a monomer. For this MG, the conformations in equilibrium were higher in number as compared to the native enzyme. The stopped flow kinetic studies showed that on and off rates of a mutant form were higher than those of the native enzyme [230]. All these data fits well to the model, where the less structured monomeric conformation of MjCM represents a catalytic MG.

The same group also designed a variant of MjCM with an amino acid sequence consisting of just 9 amino acids [232]. This variant with considerably reduced diversity in amino acid composition (the native enzyme sequence is made up from 19 different amino acids) was also a MG but was more structured (with higher helical content) than the mutant described above. This engineered protein is a good system to evaluate the value of diversity in amino acid composition in native enzymes [232].

Another interesting system studied under this approach has been glutathione transferase A1-I (GSTA1-1), a promiscuous enzyme critically important in detoxification [233,234]. Its remarkable catalytic promiscuity results from its molten globular structure. It exists as a rather broad conformational ensemble, wherein the conformations are freely interconvertable [235]. Curiously, GSTA4-4, another glutathione transferase of this α-class is highly specific [236–238]. The question which this raised is how GSTA1-1 detoxifies structurally diverse substrates. An extensive study of many variants of both GSTA1-1 and GSTA4-4 enzymes suggested that the active site is structurally act as a molten globule with rest having a well-defined structure [235].

5-Aminolevulinate synthase [ALAS] is the first enzyme of heme biosynthesis. A pyridoxal phosphate dependent enzyme, it catalyses the condensation reaction between gly and succinyl CoA. An engineered murine ALAS is described which forms a catalytically active MG but with considerably reduced k_{cat} [239].

A transient folding intermediate during refolding of unfolded acylphosphatase from the archeon *Sulfolobus solfataricus* (Sso AcP) had an active site, which had only little structure but had enzyme activity [240]. Further refolding of this intermediate led to an ensemble of conformations with properties of an MG [241]. About 10% of molecules of this MG folded more slowly (than rest) to the native states due to requirement of cis-trans isomerisation of Leu49-Pro50 peptide bond. This is another example of incompletely folded yet catalytically active protein conformations [240,241].

Staphycoccal nuclease (SNase) was modified to form a deletion mutant, from which 9 amino acid residues from both ends were removed. The resulting Δ131Δ mutant was mostly unfolded and yet retained the activity of the native enzyme. The variant folded to the native state when high concentrations of the substrate was present [242,243]. NMR analysis revealed that the mutant (in the

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unfolded form) had retained most of the secondary structure elements of the wild-type protein, though these had far more flexibility [242–245].

A later study examined a double mutant F34W/W140F of this nuclease. This was found to exist as a MG, which could be folded to the native state [246]. The K_m values of this MG for DNA, calcium ion and pdTp were quite similar to that of the native enzyme. Its V_{max} was also mostly unaltered. This indicated that the MG acquired the folded/native conformation in the presence of ligands/substrate [246].

Yet another interesting engineered MGs were obtained by "de-evolution" of the dephospho-CoA kinase (DPCK) from *Aquifex aeolicus* by substituting its aromatic amino acids (including histidines) to Leu residues or to non-aromatic amino acids based on the best preservation of thermodynamic stability [247]. The 4 variants created had about 10 % of the protein sequence altered. Two variants (DPCK-LH and DPCK-M), unlike the wild-type enzyme, displayed ATPase activity in the absence of dCoA. Moreover, the DPCK-LH also showed phosphotransferase activity in the presence of dCoA. The variants had secondary structure similar to that of the wild enzyme, but contained less tertiary structure, indicating that these were MGs [247].

Another example of the catalytic engineered MGs is given by "artificial, rationally designed catalytic polypeptides" termed oxaldies [248]. These 14 amino acid residue-long polypeptides were capable of the spontaneous formation of the amphiphilic α-helix and self-oligomerization to a four-helix bundle and higher order aggregates, and they had a reactive amine anchored to them [248]. Notably, these MGs were able to catalyze oxaloacetate decarboylation rather efficiently [248].

These examples show that MGs probably are extreme case of flexible proteins with adequate potential for catalysis left undisturbed at least partially. The terms inactivation and denaturation refer to unfolding process viewed from the different windows of biological activity and structure. Catalytic MGs underline this notion.

8. Macromolecular Crowding and Molten Globules

It is realized that the intracellular environments are vastly different from the dilute solutions of enzymes in aqueous buffers, which are mostly used in enzymology. The intracellular volume is crowded with diverse macromolecules [249,250], where the concentrations of proteins, polysaccharides, and nucleic acids (in free or the conjugated forms) are estimated to be in a range of 80-400 mg/ml [251–254], and where diverse macromolecules and small molecules together occupy about 40% of the cytosolic volume [255]. Large number of studies have tried to simulate these environments by adding macromolecules (such as "inert" polymers, e.g., Poly(ethylene glycol) PEG, Dextran, Ficoll, and Poly(sodium 4-styrene sulfonate) (PSS) of different molecular mass and some "inert" proteins, such as bovine pancreatic trypsin inhibitor (BPTI), ribonuclease A, lysozyme, β-lactoglobulin, hemoglobin, and bovine serum albumin (BSA)) and high concentrations of low molecular weight substances [40,250,256]. The following examples discuss the implications of the crowding on formation of MGs.

Molten globule of apo-myoglobin was obtained by using high salt concentrations from the pH-denatured protein [257]. Being utilized as a crowding agent, dextran stabilized this MG against unfolding by both heat and cold. CD was used to look at the transitions and the results agreed with the excluded volume theory [257]. Similarly, the acid-unfolded form of cytochrome *c* at pH 2.0 was shown to undergo folding transition to the MG state after addition of high concentrations of dextran to protein solution [258]. On the other hand, a recent study showed that even under physiological condition of pH 7.0 and 25 °C, cytochrome c adopted MG in the presence of high PEG-400 concentrations [259]. This crowding-induced N→MG transition was attributed to the soft interactions between PEG and protein, indicating that macromolecular crowding effects are more complex than the excluded-volume [259].

More recently, it has been shown that Ficoll-70 interacts with the heme group of myoglobin, which converts the protein into a MG at physiological pH [260]. An FTIR study of the C-terminal domain of histone H1 in the presence of crowders PEG and Ficoll showed that this IDP becomes folded and gain noticeable levels of regular secondary structure [261]. However, fluorescence studies

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revealed that this is actually a MG. Perhaps, similarly, formation of this MG under the crowded conditions of the cell "may increase the rate of the transition towards the DNA bound state and facilitate H1 diffusion inside cell nuclei" [261]. Alkali pH-unfolded ferricytochrome c and lysozyme at pH 12.9 (±0.1) were shown to adopt MG conformations in the presence of various crowding agents (Dextran-40, Dextran-70, and Ficoll-70) [262].

The MGs of α-lactalbumin (LA) are easily obtained by subjecting its calcium ions depleted form (apo-form) to different denaturing conditions [263]. MG of the apo-form has been shown to play a role in apoptosis of tumor cells [264]. It was reported that the presence of PEG-2000 as a crowding agent also could lead to the formation of MG by human apo-LA [265]. Curiously, the other two polymeric crowding agents tried, Ficoll-70 and dextran-70, did not have this unfolding effect [265]. ITC analysis revealed that while these two crowders did not with the human apo-LA, PEG had a weak non-specific interaction with the protein [265]. Presumably, this weak interaction was sufficient to overcome the stabilizing effect of the crowder due to the excluded volume [265].

Atomistic MD simulations of three proteins with different structural organization (an intrinsically disordered 47-residue activator for thyroid hormone and retinoid receptor (ACTR), a molten globular 51-residue nuclear coactivator-binding domain of CREB (NCBD), and an ordered 191-residue interferon regulatory transcription factor (IRF-3)) were performed to assess the effect of small-sized synthetic (PEG500) and protein crowders (at concentrations of 175-300 g/L) on the structure, dynamics and interactions of these query proteins [266]. The results showed that the degree of disorder in a protein plays a critical role in its response to crowding. The excluded volume effects pushed the conformation towards compact structure, quinary (weak transient) interactions favored extended conformation [266]. Interestingly enough, while crowding slowed down protein flexibility and restricted the conformational landscape thereby resulting in a bias toward the "bioactive conformations", it simultaneously diminished the biologically relevant interactions. Another important finding was that PEG 500 as the synthetic crowder had different consequences than protein-induced crowding [266].

9. Interactions of Nanomaterials with Molten Globules

The foundations of our understanding of protein adsorption onto non-porous materials were laid by Wilem Norde [267]. Perceptively, he stated that proteins can be either hard or soft in the context of their structural changes upon adsorption. The latter kind undergoes significant structural changes upon adsorption. With the advent of nanoscience, the interactions of proteins with diverse nanomaterials has been extensively studied [268]. Proteins bound to the nanoparticles are referred to as their corona. The importance of corona in drug design/delivery has been discussed at a number of places [269,270]. In 2013, Saptarshi *et al*. have mentioned that the human carbonic anhydrase when bound to silica nanoparticles forms an MG [269], whereas detaching of nanoparticles resulted in the formation of three catalytically active intermediates with "native like structures" [269].

In a large number of cases, conformational changes/unfolding of proteins upon binding to the nanoparticles have been documented [269]. Carbonic anhydrase is one of the most extensively studied proteins in the context of molten globules. Billsten *et al*. looked at the adsorption of one of its mutant and two truncated forms, in which 4 and 16 amino acids had been deleted from the N-terminal, on silica nanoparticles [271]. While the whole-length mutant did not show any structural change, the truncated forms were present as MGs on the nanoprticles. These MGs were very similar to the MG formed when the whole mutant enzyme was unfolded by GdmHCl [271].

Furthermore, in some instances, interactions with nanoparticles were shown to induce fibrillation [272]. It has been observed that HbAO, the major component of human blood hemoglobin, formed an MG upon interaction with copper nanoparticles, which led to the protein aggregation [273]. This did not happen with HbA2, a protein isoform associated with β-thalassemia. The authors suggested that this behavior could form a basis for screening for thalassemia [273]. An excellent review discussing how the nanoparticle properties along with the protein nature influences the outcome of their interaction in terms of conformational changes leading to MGs and/or fibrils is available [274].

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10. Dry and Wet Molten Globules

An excellent paper published by Denisov *et al*. in 1999 focused on the hydration of non-native states of proteins Hydration of denatured and molten globule proteins. Early SAXS, DLS, and heat capacity data suggested that MGs have "substantial internal hydration" [275]. Disruption of tertiary structure during the MG formation is expected to lead to the increased hydration of protein interior, as water molecules are believed to become competing H-bond partners. ^{1}H nuclear Overhauser effect (NOE) spectroscopy and ^{17}O magnetic relaxation dispersion (MRD) data for several few structurally unrelated proteins (α-lactalbumin, lysozyme, ribonuclease A, apomyoglobin, and carbonic anhydrase) provided a finer picture. It was found that the MG states and native structures of these proteins have comparable levels of internal hydration, suggesting that the MG forms of these proteins are more structured and less solvent exposed than commonly believed [275].

Dry molten globules (DMGs) are molten globules characterized by the low hydration and efficient shielding of hydrophobic core from the solvent [34]. Thus, these precede WMGs during unfolding and are structurally closer to the native state in general, though these have all other characteristics commonly ascribed to MGs. DMGs do have expanded volumes, and their formation is accompanied by the conformational unlocking of the side chains (and related gain of the conformational entropy) though liquid-like van der Waals interactions are still present. The U→DMG step is a major free energy barrier in the entire U→N transition due to the large enthalpic contribution, though DMG is stabilized by the compensation via increase in the conformational entropy. While WMGs can be detected by fluorescence and near-UV CD, DMGs (with almost no water invading to change microenvironments of Trp and other side chains), these techniques can miss the DMG formation. Their detection can be done by NMR and FRET techniques [34]. However, analysis of the refolding kinetics could track DMG→N transition in the case of RNAse A by the ratio test, which measures kinetics by both tracking secondary structure by far-UV CD and tertiary structure by near-UV CD [34]. In cases of both dihydrofolate reductase (DHFR) and monellin (a sweet protein that has two noncovalently associated polypeptide chains: an 44-residue-long A chain, and a B chain with 50 residues), it was shown that ANS dye was bound to WMG but not to DMG [34].

Although the early reports on the existence of DMGs started appearing in 1995 [38,276], it is only in late 2000 that these intermediate states were accepted as real intermediates, which are distinct from the WMG (which is considered as a classic MG now) on folding/unfolding pathway [35,36,277]. The extended simulation of unfolding of lysozyme be urea at 37°C caught the formation of DMG though a similar study at higher temperature had failed to do so, indicating that higher temperature destabilized DMG [277]. Urea (unlike water) interacted with the peptide backbones [277].

Looking at the unfolding of barstar and its mutants with several techniques has revealed some finer details about formations of DMG and WMG [28]. In this study, near-UV CD indicated that urea-induced unfolding started with the loss of tertiary contacts to form an intermediate N*. FRET showed that this early intermediate expanded to form I. Fluorescence spectral measurements showed that both of these intermediates, N* and I were DMGs. Dynamic quenching of the single buried Trp in core suggested the later formation of WMG. Slowest step in the unfolding process of barstar was unfolding of WMG and had a solvated transition state [28].

The villin headpiece subdomain HP35 has been studied by triplet-triplet energy transfer and locked (native state) and an unlocked state (DMG) were identified [32]. DMG was characterized by a solvent free core but showed increased flexibility and "local unfolding" behavior. High pressure triplet-triplet energy transfer measurements revealed that while increasing pressure (which favors the N→DMG transition) was not accompanied by any expansion, the reverse transition showed a volume change. This indicated the existence of two DMG forms, where one is as compact as a native structure and another with an expanded volume [32].

In the case of multidomain proteins, it is possible that not all domains are in DMG states [30,278]. A recent review focusses on DMGs and their roles in diseases [29].

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11. Pre-Molten Globule States

The term pre-molten globule was coined in 1991 by Mei-Fen Jeng and S. Walter Englander to refer to a folding intermediate, which was observed during the unfolding of cytochrome c and which was less structured than even MGs [279]. Here, cytochrome c molecules at low pH and sodium chloride concentration of < 0.05 M were found to expand beyond the MG state as seen by the viscosity and fluorescence. While the helical content are nearly all retained, "tertiary structural hydrogen bonds are largely broken (hydrogen exchange rates), some normally buried parts of protein are exposed to water (fluorescence) and many of the native side chain contacts must be lost" [279].

In the same year, Chaffotte *et al*. were looking at a C-terminal peptide F2 of the β-2 subunit of *E. coli* tryptophan synthase using a number of biophysical techniques [280]. They concluded that "neither the secondary nor the tertiary structure of isolated F2 resembled those of native F2. In this respect, isolated F2 is not a molten globule" [280]. Few years later, the same laboratory found that transient intermediates formed within 2-4 msec during refolding of several proteins gave different estimates of the secondary structure contents with different techniques [281]. Again, looking more closely in the case of F2 fragment, they found that the isolated F2 folded into a "condensed, but not compact" conformation [281]. This conformation was in rapid equilibrium with the conformations with the native and non-native secondary structures and it was described as a pre-molten globule (PMG) [281].

In 1994, Uversky and Ptitsyn [ref] reported that β-lactamase at low temperature and in the presence of GuHCl formed two partially folded intermediates, the classic MG and a new equilibrium state of protein molecules, which they originally called "partly folded" state [26]. However, in 1996, the same group described four-state GdmHCl-induced unfolding of BCAB at low temperature [24] and concluded that in both proteins, unfolding is described as a four-state processes, N→MG→PMG→U. Furthermore, in these studies, PMG was found to have an expanded volume of "no more than two-fold" and had solvent-exposed clusters of nonpolar amino acids. It was found that significant levels of secondary structure content is retained at this stage of unfolding. Similar results were earlier reported with beta-lactamase as well [24,26]. Therefore, cryoenzymology could be used to detect formation of PMG.

When the salt sodium sulphate was gradually added to the unfolded Barstar (present under highly alkaline conditions), first PMG of the protein was observed [37]. At higher concentration, PMG got converted to MG, which was a dry MG as no water was present in the core. This dry MG had about 65% secondary structure and 40% tertiary contacts (as compared to the native form). It was also shown that this MG was a productive intermediate on the folding pathway [37]. The solvation dynamics around the active site of glutaminyl-tRNA synthetase was found to increase during the transition from MG→PMG [282]. Both partially folded intermediates had similar level of secondary structure, though PMG was more flexible. Furthermore, DLS revealed that in both cases, protein aggregates were present [282].

A series of further papers have confirmed this four-state picture comprising of pre-molten globule (PMG) and MG as intermediates during folding or unfolding of several proteins. Georlette *et al*. showed that the equilibrium GdmCl-induced unfolding of the NAD+-dependent DNA ligase from the thermophile *Thermus scotoductus* follows the four state N→MG→PMG→U model, where, similar to BCAB and β-lactamase, the MG→PMG transition was characterized by the presence of a bimodal distribution of the molecular dimensions in HPLC gel-filtration profiles, indicating that this process represents an "all-or-none" transition [22,23].

Khan *et al*. carried out experiments on unfolding and refolding of a mutant Leu94Gly of cytochrome c, which was earlier recognized as a MG [283,284]. Denaturation with LiCl led them to identify a PMG of the mutant, which was less stable than the mutant by about 5.4 KCal/mole and more stable than the unfolded protein by merely about 1.1 KCal/mole [283,284].

It has been shown that myoglobin in the presence of PEG of intermediate sizes formed PMG [285]. This was probably the first report of formation of a PMG under physiological conditions but in the presence of a crowding agent. Thus, it is likely that both MGs and PMGs are formed under the overcrowded conditions [285].

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In another example, cytochrome c under acidic condition was found to form MG and PMG when glucose and dextran-70 were used as crowders [286]. The authors believe that the polymeric crowder stabilized the protein [286].

12. Conclusion and Future Perspectives

Quite often, our insights about protein folding process have come by looking at protein unfolding! It started with protein denaturation, the data about that segues well with studies on protein stability. In general, protein stability under one stress condition is often accompanied by the stability under other denaturing conditions. The protein unfolding starts with denaturation of the polypeptide chain followed by numerous physicochemical changes [287].

With numerous biological phenomena, we have learnt that a two-step model is often the result of our inability to "see" multiple steps in the process. As our tools become more powerful and we develop better computational tools, we become able to see multiple intermediates. Curiously, this is also so in the enzyme kinetics. We had steady state kinetics. As fast kinetic methods became available, we had pre-steady state kinetics and more detailed picture of "on" and "off" processes [288]. So, it is not surprising that two-state models of protein unfolding have increasingly been replaced by four-state model, where during the unfolding process, protein undergoes sequential transitions from the native state to the molten globule, then to the pre-molten globule before eventually reaching the unfolded state. Thermal denaturation does not produce totally unfolded conformation; one needs some kind of another stress like a chemical denaturants to complete unfolding. Similar is also applicable to most proteins at extremely acidic and basic conditions. This means that we have more than one kind of denatured state. The fast accumulating data on the wet and dry MGs further indicate that journey is not over yet and we still are on the road. Recently, trapping the co-populated protein conformers during acid-induced unfolding of cytochrome c, myoglobin, and lysozyme indicated the existence of finer details of unfolding process [289]. Of especial interest is the "conformational shuttling", in which the population of an unfolded form of cytochrome c at low pH first increases and then decreases with time [289].

Since deep mutational scanning represent a fast and convenient way of providing knowledge on the residue-specific contribution to protein interaction involving IDPs, it is likely that such approaches will be able to further shed light on the ways by which molten globules play a biological role beyond being just a folding intermediate [290].

The classical way has viewed the unfolding/folding processes purely from the blinkered view of structure. With increasing understanding about the role of intrinsic disorder, this is slowly opening up another dimension [41]. However, we still do not have a clear and comprehensive answer to a question on what all roles these various MGs and PMGs may play under the *in vivo* conditions. After all, crowded intracellular conditions seem to affect protein conformation. Another unexplored aspect is related to specificity of these different unfolded (or differently partially folded) forms. Are they promiscuous (more or differently so in comparison with the ordered globular forms) [291]? Do they play a role in protein evolution? Are they involved in moonlighting [292]? Do they play a role in immune responses [293]? What are the various ways we can use them in applied biocatalysis or drug delivery designs [294–300]? We already know that partially unfolded states of some proteins are precursors to aggregation, and many diseases originate from aggregation via intrinsic disorder [301–310]. Let us not forget that unfolding increases disorder. Also, the roles of flexible conformations in protein assemblies and protein-protein interactions is likely to be more important than explored so far [311].

This review, by describing a thread running through different phenomena/approaches, such as cryoenzymology, baroenzymology, macromolecular crowding, intrinsic disorder and interactions of partially unfolded proteins with nanomaterials hopefully stimulate further research into the various facets of molten globules.

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