The Protein Unfolded State: One, No One and One Hundred Thousand

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ABSTRACT: Many in vitro studies, in which proteins have been unfolded by the action of a variety of physical or chemical agents, have led to the definition of a folded versus an unfolded state and to the question of what is the nature of the unfolded state. The unstructured nature of this state could suggest that “the” unfolded state is a unique entity which holds true for all kinds of unfolding processes. This assumption has to be questioned because the unfolding processes under different stress conditions are dictated by entirely different mechanisms. As a consequence, it can be easily understood that the final state, generically referred to as “the unfolded state”, can be completely different for each of the unfolding processes. The present review examines recent data on the characteristics of the unfolded states emerging from experiments under different conditions, focusing specific attention to the level of compaction of the unfolded species.

INTRODUCTION: SAME AND YET DIFFERENT

Understanding how globular proteins manage to retain their structures under adverse conditions is a major theme of modern Biology as it directly relates to protein function and to the rules that dictate protein folding. For this reason, a plethora of in vitro studies have become available over the span of almost a half century, in which proteins have been unfolded in vitro by the action of a variety of physical or chemical agents. These studies have led to the definition of a folded versus an unfolded state and to the question of what the unfolded state is like.

Referring to the three-dimensional structure of an unfolded species is almost oxymoronic, since unfolded species are, by definition, assumed to be species without any structure. At room temperature and ambient pressure, globular proteins generally adopt a determined conformation that is almost the same for all molecules. At temperatures higher or lower than room temperature, there is significant conformational freedom. In the ensemble of unfolded (or denatured) molecules, the peptide chains adopt different conformations in different molecules, virtually a different conformation in each of the enormous number of molecules.

While this is undoubtedly true, it is not uncommon to find in the literature statements that refer to “the” unfolded state as if it were a unique entity which holds true for all kinds of unfolding processes. This is likely wrong in most cases: the unfolding processes under physical or chemical stress conditions are dictated by entirely different mechanisms. For instance, it is well established that protein unfolding can occur as a consequence of both temperature increase (heat denaturation) and temperature decrease (cold denaturation).¹ The two processes are however inherently different, as demonstrated experimentally.² Heat-induced unfolding is entropically driven, while cold denaturation is enthalpically driven. In the case of pressure unfolding, the unfolded state is also often regarded, at least implicitly, as a unique entity similar or identical to species deriving from other unfolding processes.³ Instead, pressure unfolding originates by physical compression and the resulting state has no reason for sharing features with other unfolded states. The unfolded species induced by chemical denaturants can be expected to be yet different, because most denaturants are known to interact directly with the unfolded species. As a consequence, it can be easily understood that the final state, which is generically known as the unfolded state, can be completely different for each of these unfolding processes. For this reason, we chose the title of this review after the famous novel by the Italian writer Luigi Pirandello. It is also important to add that many people seem to think that a “truly” unfolded state must be characterized by the total absence of any residual structure following the rules of polymers. Such an assumption does not correspond to what happens in proteins: also in their unfolded states often proteins retain some local secondary structure tendency (even though in the absence of a stable three-dimensional fold). Accordingly, there have been numerous NMR studies on unfolded species suggesting that significant secondary structure elements, or at least extensive clusters of hydrophobic structure, may persist in unfolded proteins even at high concentrations of chemical denaturants.⁴,⁵

The present review wishes to analyze these differences because much may be learned from investigating the nature of unfolded species originating from the different events. We will focus on physical unfolding and on how pressure modulates the unfolded states obtained by cold and heat denaturation. This

Published: November 30, 2022
choice was dictated by the consideration that, as compared to chemical and thermal denaturation, pressure denaturation studies have probably received less interest, likely because not all laboratories are easily equipped with suitable facilities. We will examine recent data on the characteristics of the unfolded states obtained under different conditions, focusing specific attention to the level of compaction of the unfolded species. Finally, we will discuss how molecular crowding might affect physical unfolding as this effect may be of direct relevance also for the cellular environment.

**PRESSURE UNFOLDING**

A crucial aspect of protein unfolding is the change of volume from the folded species to the unfolded one. A plethora of theoretical models as well as direct experimental evidence on the change of volume upon unfolding are present in the literature. The way to define the volume of an unfolded protein might be divided into two large families: in one, the molar volume corresponds to the volume of the system that is not occupied by the solvent. This is the relevant volume for pressure-dependent investigations. In the second family, the volume occupied by an unfolded protein is derived from its dimensions: this is what is relevant for single-molecule Förster resonance energy transfer (smFRET) or small angle scattering (SAS) investigations. The second family will be discussed at length in the following subchapter, dealing with thermal unfolding. A relevant approach to the first family is represented by volumetric measurements. Papers dealing with volume changes based on volumetric measurements date back to the 1950s. The main outcome of these studies is that volume changes upon denaturation are of the order of 100 mL/mol and can represent an increase or a decrease of volume. This conclusion apparently indicates that there may be proteins that do not unfold under increasing pressure, because thermodynamics favors the species with lower volume. However, the situation is more complicated, partly because when measuring global volume changes, we are observing cooperative effects from several complex causes. The observed changes also represent the difference between two large quantities, and it is difficult to measure accurately the relative volumes of the two species. It is thus in order to examine the effects of pressure in more detail.

The first studies of pressure induced denaturation were performed by Hawley on chymotrypsinogen and by Zipp and Kauzmann on metmyoglobin. These authors observed that the spectral changes induced by pressure were apparently similar to those induced by other agents. Zipp and Kauzmann, for instance, stated that “the conformational change that occurs on raising the pressure at fixed pH and temperature is similar to the conformational change when the temperature is raised or when the solution is acidified at a fixed pressure”. It may however be argued that the authors do not specify whether the changes lead to more or less expanded states. This is almost certainly because these early studies relied on techniques that operated at resolutions so fuzzy to hardly enable accurate measurement of the differences.

Further experimental studies on proteins under pressure were performed in the 1980s mainly in the laboratory of Hui Bon Hoa. Protein high-pressure NMR spectroscopy continued in the 1990s initially by Jonas and then was expanded into a crucial methodology by several other authors. All of these researchers consistently realized that thermodynamics requires that, in pressure-induced unfolding, the volume of the unfolded species must be smaller than that of the corresponding folded species.

This conclusion was supported by Dave and Gruebele in an elegant paper on protein unfolding in which the authors say: “High pressure unfolding of proteins is equally puzzling at a first glance. We tend to think of proteins as larger once they unfold, but Le Châtelier’s principle states the system should go to a smaller volume when pressure is increased. That is what actually happens: unfolded proteins have a smaller molar volume than folded proteins.” Interestingly, however, the incipit of this seminal paper states that “[…] pressure denaturation of proteins exhibits some unusual and interesting characteristics. It has been observed, for example, that while very high pressures (7500 atm) invariably produce protein denaturation, moderate pressures (1000 atm) may stabilize the native form, thereby increasing the temperature required for heat denaturation.”

This important distinction adds up to our understanding of the effects of the compactness occurring at high pressure.

Further information on the poorly known influence of pressure on protein stability may come from studies on marginally stable proteins. We have recently studied, by tryptophan fluorescence, the influence of pressure on a marginally stable protein (YhH1), whose cold and heat transitions can be observed above water freezing. This protein showed practically no lag time and a remarkable sensitivity to pressure: at 293 K, pressures around 10 MPa are sufficient to cause 50% of unfolding. We plan to extend this study by an NMR study at moderate pressure.

The importance of volume, as illustrated above when introducing two families of volumes in unfolded proteins, is crucial to understand recent papers on pressure unfolded proteins. An excellent paper on pressure-related folding and unfolding assumed that the unfolded species is always of smaller volume. This paper described a detailed pressure-induced study of folding and unfolding of a ubiquitin mutant. However, for clarification, in this case, the authors look at pressure jumps rather than a static steady state pressure increase. Using a pressure-sensitized mutant of ubiquitin, the authors demonstrated that rapidly switching the pressure within an NMR sample allows the study of the unfolded protein under native conditions. According to these authors, an abrupt drop in pressure is the most benign physical stress, allowing the protein to be examined under native-like conditions. However, they claim that because of the pressure-denatured state, secondary chemical shifts show no significant correlation with those of the folded state and are close to random-coil chemical shift values; the pressure-induced unfolded state is consistent with a dynamically highly disordered state similar to that of the wild-type protein denatured in 8 M urea.

In another study, Ramanujam et al. measured the translational diffusion and hydrodynamic radius ($R_h$) of a ubiquitin mutant by pulsed-field gradient NMR spectroscopy and reached the conclusion that the $R_h$ of the fully pressure-denatured state is essentially indistinguishable from the urea-denatured state.

A more recent paper on a mutant of the L9 protein (CTL9), which undergoes cold denaturation at temperatures above freezing, stated that “the structural properties of pressure-unfolded states of proteins do not differ substantially from those of unfolded states at atmospheric pressure and are, if anything, more, rather than less, expanded than their atmospheric state counterparts”. This conclusion was based on the comparison...
between X-ray scattering studies and high-pressure diffusion ordered NMR experiments.

These dissenting voices suggest that there may be some confusion which may come from the tendency to consider the unfolded state caused by chemical denaturants as "the" reference unfolded state. This view should be abandoned. Chemical denaturants are very useful in physicochemical studies but do not represent a physiologically relevant condition and embody a very specific mechanism. We simply use the same term—unfolded state—to describe completely different physical states.

### THERMAL UNFOLDING

Thermal unfolding has been the main topic in unfolding studies for years. For many decades these studies examined only heat unfolding, but in the past 30 years there was an increasing number of cold denaturation studies. There are several indications that the cold denatured state at low temperature is different from that at high temperature. However, none of the spectroscopic methods used to monitor unfolding has given much decisive information on the nature of the unfolded status. The conformational landscape of unfolded proteins is challenging and only accessible to a few solution state techniques. In the past, most researchers used small angle scattering (SAXS) techniques to investigate the nature of folded states. Small-angle X-ray scattering (SAXS) in particular allows accurate studies of the size and shape of proteins. Early on, Kataoka et al. demonstrated that measurements of the radius of gyration of folded globular proteins by SAXS were consistent with the crystallographic structures. For practical reasons, most of the SAXS studies on unfolded ensembles were not performed on heat denatured proteins but on proteins denatured by chemical agents, much easier to manipulate at ambient temperature in high concentrated samples. A detailed SAXS study on many proteins unfolded by chemical denaturants contradicted the (then) widespread opinion that residual structure persisted even in highly denatured proteins. Kohn et al., working on 28 chemically denatured proteins, showed that, like for synthetic polymers, their $R_g$ scale with polymer length, $N$.

More recently, using a combination of FRET, SAXS, and dynamic light scattering (DLS) on two proteins, it was shown that SAXS data can yield a radius of gyration compatible with residual contents of secondary structure, even in unfolding studies using chemical denaturants.

An NMR study on a protein that exhibits both low and high temperature unfolding above water freezing (Yfh1) showed that both unfolded forms of this protein, at low and high temperatures, are expanded with respect to the folded species. In the meantime, FRET studies came to prominence. A FRET study on the same protein studied by Adrov et al. confirmed that both the unfolded forms are expanded as compared to the folded state, albeit to a different extent.

Recently, the antinomy between extended and collapsed structures under physiological conditions has been well summarized by Riback et al. These authors chose to study intrinsically disordered proteins (IDPs) as reliable analogues of denatured state ensembles (DSEs) of an unfolded protein. Both species are almost completely expanded in solutions containing elevated concentrations of chemical denaturant, but collapse to more compact structures in aqueous solutions closer to physiological conditions. Most of the evidence in favor of more compact conformations in aqueous solutions comes from FRET studies, whereas SAS did not suggest similar compaction.

It is important to notice that the two techniques yield different length parameters: end-to-end distance from FRET and radius of gyration from SAS data. Riback et al. developed a new method to determine the main conformational parameters of IDPs from SAXS measurement and applied it to DSEs corresponding to well-folded proteins. Their results suggested that the DSEs of most proteins are expanded in water. The authors explained the discrepancy between SAXS and FRET data on the basis of the necessary addition in FRET of fluorophores. The hydrophobic character of the fluorophores might be responsible for chain compaction, contributing decisively to FRET signal changes. These conclusions were challenged by two groups. Best et al. observed that, indeed, while some very hydrophobic FRET fluorophores can favor compaction in solutions, the most commonly used fluorophores are hydrophilic and yield results in good agreement with SAXS. Fuertes et al. commented that they demonstrated experimentally that the dyes are not the source of systematic errors. Even more importantly, these authors reminded that the parameters measured by FRET and SAXS, $R_g$ and $R_e$ respectively, are two related but genuinely distinct measures of IDP conformations. The two measures provide complementary but distinct views of the dimensions of IDPs in aqueous solutions without chemical denaturants. Their results suggest a sequence-specific decoupling between the end-to-end distances ($R_e$) and the radii of gyration ($R_g$).

### HOW CROWDING CONTRIBUDES TO UNFOLDING

Unfolding is sensitive to the difference in volume between folded and unfolded species, not only when the hydrostatic pressure is directly changed (pressure unfolding) but also at ambient pressure when the solution is "crowded". During the past decades it became obvious that the environment in most biochemical studies on proteins, i.e. dilute solutions, is drastically different from that typical of cells, which contain high concentrations of other macromolecules in addition to the protein examined. It was said that in most cells, like in E. coli, macromolecules induced a volume occupancy of 30% to 40%.

Accordingly, the environment inside the cell was defined "crowded". The effect exerted on a protein by the concomitant presence of other macromolecules, albeit not related to actual cellular environments, was not totally unknown. Ogston described crowding effects, but he was not the first one because several years before Asakura and Oosawa formulated a theory concerning very similar effects. Minton introduced explicitly the concept of crowding, but for several years there were not many followers. The number of papers probing the influence of crowding on protein stability increased quickly only after Minton’s publication of an elegant simple model to explain the importance of excluded volume for protein stability.

According to this model, the effect of the excluded volume could lead to a large increase of the melting temperature ($T_m$).

This expectation was based on the estimation of the volume of the unfolded state. According to Minton, the unfolded state of a protein could be viewed as a sphere enveloping a Brownian walk, with a radius corresponding to the radius of gyration. In the same period there was an alternative view according to which the unfolded status could be depicted as a pearl-necklace chain. The difference was substantial because in the Minton model there was a great increase of volume in going from the folded to
the unfolded state whereas in the other model there was a decrease of volume. However, the view of the unfolded state as a pearl necklace is contradicted by significant experimental data at atmospheric pressure. In other unfolding experiments most people assume (or measure) volumes for unfolded states larger than that of the folded state. It is certainly true that several experimental studies have recently shown that the unfolded form of a protein at 1 bar is definitely larger than the folded one.\textsuperscript{2,3,30-36} The same conclusion has been reached for most IDPs which can be considered bona fide unfolded states.\textsuperscript{35-37,38}

Most people embraced the Minton model and performed experiments in crowded solutions that could confirm typical increases of $T_m$ between 5 and 20 °C,\textsuperscript{39} however, many studies showed only a modest increase in unfolding temperature.\textsuperscript{40}

To explain the discrepancy between these results and theoretical predictions, several laboratories emphasized the importance of weak interactions.\textsuperscript{41-45} In many examples, high concentrations of a macromolecular crowder led to either a decrease or an increase of the stability of a protein, owing to specific interactions. These results, albeit not denying that the unfolded form has a higher volume than the folded one, led to the conclusion that crowding can be enthalpically rather than entropically driven: traditionally, crowding was solely associated with the excluded volume effect. More recently, researchers have started mentioning the importance of weak interactions (quinary interactions).\textsuperscript{54} This perspective has enriched our understanding of the factors that contribute to the “real” environment inside cells. However, there might have been some confusion between understanding the effect of crowding and reproducing the real environment of proteins in the cell.\textsuperscript{55} The concepts of studies in crowded solutions and that of studies in “in vivo” conditions are rather different and should not be mixed.

The antithesis entropy vs enthalpy does not clarify the contribution of crowding to protein stability. Although it is difficult to study systems in which enthalpic effects are totally missing, it is better, when possible, to study entropic and enthalpic effects separately to evaluate their relative importance. A study by Alfano et al. (2017), before studying the effect of synthetic crowders on the stability of a frataxin protein, checked if crowding generally leads to a positive $\Delta T_m$ but with values much smaller than those predicted.\textsuperscript{55} In conclusion, many crowding studies favor a larger volume of the unfolded species under (quasi)-physiological conditions.

**CONCLUSIONS**

In many papers dealing with the nature of the conformational ensemble that characterizes the unfolded state, there is a tendency to regard the “true” unfolded state as one without any structure, i.e., not only without tertiary structure but also without any secondary one. This state corresponds well to an idealized tendency to regard the “true” unfolded state as one without any ensemble that characterizes the unfolded state, there is a

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It is not if we view *in vitro* studies as a means to understand what happens *in vivo*. From this point of view chemical denaturation is largely an artifact. Summarizing the findings of unfolding studies at ambient temperature and at high pressure it is possible to say that different studies at ambient pressure view the unfolded state in two ways, either slightly expanded (in solutions close to physiological conditions) or with a smaller volume than that of the folded state when forcing unfolding through the use of very highly concentrated chemical denaturants. On the other hand, nearly all studies at high pressure consider the unfolded state (or “all unfolded states”) as lower volume than that of the folded state. This view does not take into account the two families of unfolded states defined by molar volume and structural criteria.

How can we reconcile two opposing views on the volume of the unfolded state?

A possible explanation, at least with regard to pressure unfolding, is that at the beginning of the pressure induced unfolding (i.e., during the “lag time”, when the pressure is initially close to the ambient one) the unfolded state has a larger volume that rapidly shrinks to a compressed volume when the pressure is increased. During the lag time, which is long for most proteins, the unfolded state retains a smaller volume, but nothing happens to the equilibrium between folded and unfolded forms that should be in favor of the unfolded form, simply for kinetic reasons. In other words, the unfolding begins only when the pressure is very high (>$2$ kbar) because the persistence of a folded state is kinetically favorable. We thus argue that each condition has its own peculiarities that mainly reflect the physical forces acting under a specific condition.

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**Notes**

The authors declare no competing financial interest.

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