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Cold Denaturation and Aggregation: A Comparative NMR Study of Titin I28 in Bulk and in a Confined Environment

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Recent studies have shown the importance of characterizing the unfolding of mesophilic proteins at temperatures not only above but also below room temperature.[1] Access to the cold denatured state was originally allowed only by severe destabilization induced by chemical and physical agents, i.e., extreme pH values, chemical denaturants, cryosolvents, and very high pressures or by a combination of destabilizing agents with *ad hoc* point mutations. However, there is no a priori reason for not having natural cases, albeit unusual, in which a mesophilic protein is, under specific conditions, so unstable to have an accessible low temperature melting point: a paradigmatic example is the iron binding protein Yfh1, whose cold denaturation occurs when in an ion free form, at temperatures close to 0 °C and at physiological conditions, without added denaturants.[2] Only determination of the full stability curve of Yfh1 could show that well-known denaturants like alcohols, at low concentrations, *stabilize* proteins.[1] Another important instance for which measuring both transitions, and thus determining the full stability curve, could be decisive is when the high temperature transition point might be difficult or impossible to determine.

Here, we exploited the properties of another protein with limited stability, the domain module of the multidomain muscle protein titin I28, to study by NMR a case in which the strong interference between thermal unfolding and aggregation limits the information obtainable from the high temperature transition.[3] We studied the stability curve of I28 by NMR, both in bulk aqueous solution and using conditions that can influence aggregation significantly, i.e., a medium containing polyethylene glycol (PEG) and polyacrylamide gel (PAG), thus combining confinement with crowding. This approach is also an alternative to the use of bundles of capillaries successfully employed in the past[4] to create supercooled water, but has the advantage of overcoming some of the difficulties intrinsic in handling capillaries, such as the risk of breakages of the microtubes. The internal structure of the gel may be thought of as a collection of thin capillaries, and the irregular structure of the cavities may favor attainment of low temperatures without risking crystallization in some of the capillaries.[5] Our work, besides yielding a second case of unbiased cold denaturation,[2] offered us the opportunity to investigate the differences between low and high temperature unfolded states, the influence of crowding conditions on protein stability, and the use of neutral gels to reach subzero temperatures. By comparing the unfolding process of I28 in bulk and in the gel, we could demonstrate that characterization of the entire stability curves allows accurate determination of the thermodynamic parameters of unfolding even when the protein has a strong tendency to aggregate.

^{15}N-labeled titin I28 was expressed in *E. coli* BL21 [DE3] pLysS, essentially as described in the literature.[6] An exploratory study showed that I28, although not undergoing a complete cold denaturation at detectable temperatures, begins to unfold between room temperature and 0 °C. Polyacrylamide gels were prepared by mixing the appropriate amount of acrylamide and water to yield several average cavity dimensions, dried at 37 °C, and left to swell in a polyethylene glycol (PEG) (Sigma-Aldrich) solution until the original size was fully recovered. When ready, the gels were dried for a second time before the final swelling, with 400 μL of the protein solution, inside a Teflon liner (NewEra, NJ, USA) that could prevent damage of the NMR tube due to accidental water crystallization at very low temperatures.

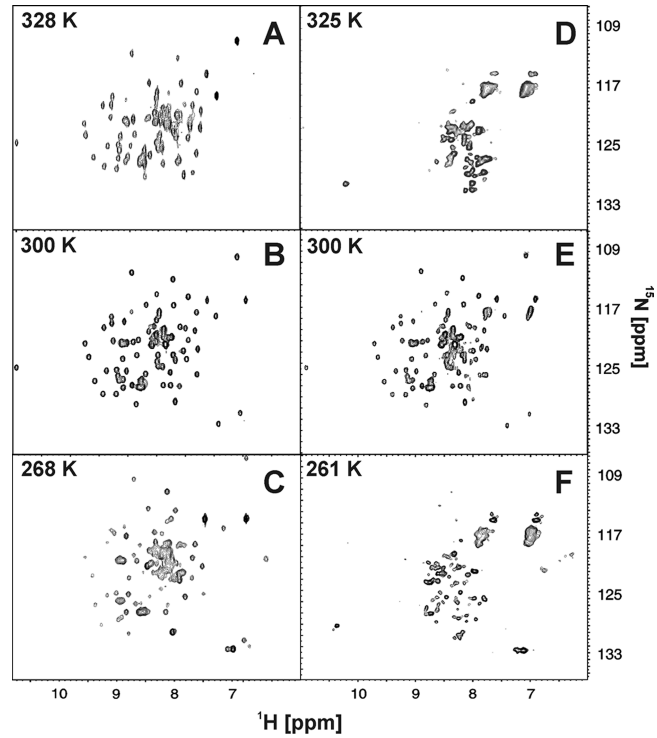

Figure 1. Representative ^{15}N−^{1}H HSQCs of titin I28 in bulk and in crowding and confined conditions. (A−C) 25 mM TRIS pH 7.2 (left); (D−F) confined environment created by the combination of 25 mM TRIS, 5% PAG, and 10% PEG2000 at pH 7.2 (right). Three representative temperatures were chosen for either sample.

Figure 1A−C shows a comparison of ^{15}N-HSQC NMR spectra of titin I28 at three different temperatures. It is clear that both raising (Figure 1A) and lowering (Figure 1C) the temperature with respect to room temperature causes a uniform decrease in the peak volumes, consistent with an unfolding transition. However, the two transitions are not equally reversible. When starting from the lowest attainable temperature, the ensuing heating process does restore the full intensity

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of room temperature protein signals indicating a full recovery of the initial fold. On the contrary, heating the sample to temperatures higher than 328 K leads to precipitation and subsequent cooling back to room temperature does not restore the folded state.

The best combination of confining and crowding agents was that of 5% PAG and 10% PEG2000. The presence of PEG, added to slow down crystallization of water, can still be considered compatible with unbiased environmental conditions[2] because PEG is neither a denaturing agent nor a strong stabilizer of native structure.[7] The corresponding spectra at room temperature, in bulk (B) and in gel (E), are very similar, apart from a few extra peaks from PAG, confirming that it is possible to use the confined environment to study unfolding processes, starting from the natural fold. However, the simultaneous presence of both PAG and PEG determines crowding and confinement conditions that favor aggregation of the protein. The spectrum of Figure 1F corresponds to a complete unfolding of I28 at low temperature, but the high temperature spectrum (Figure 1D) indicates that aggregation, already observed in bulk, is further favored by confinement and crowding. This is not too surprising because confining environments are known to greatly favor aggregation phenomena more than the stabilization of compact, folded structures.[8]

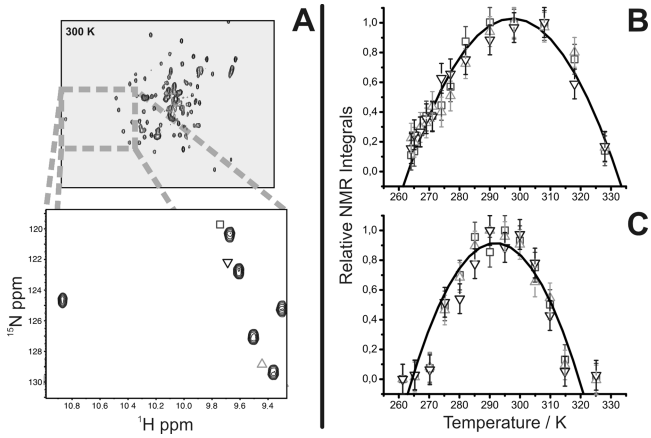

Figure 2. Quantitative evaluation of NMR data. (A) HSQC spectrum of I28 in 25 mM TRIS pH 7.2; the inset shows an enlargement of the low field portion of the spectrum with the three well isolated peaks chosen for the intensity measurements.. (B) Variation of the peak volumes as a function of temperature in bulk 25 mM TRIS pH 7.2. (C) Variation of the peak volumes as a function of temperature in 25 mM TRIS, 5% PAG, and 10% PEG2000 at pH 7.2. Peak volumes were normalized at each temperature by comparison to the volumes of side chain peaks.

NMR data, both in bulk and in the crowded environment, could be used for a direct evaluation of the thermodynamic parameters of both cold and heated denaturation of I28. Figure 2 shows that by monitoring the volumes of four isolated NH resonances it is possible to follow the thermal stability over the entire range, covering both the high and low temperature unfolding. Fitting the curves with the Becktel−Schellman equation[9] we calculated the thermodynamic parameters reported in Table 1. The thermodynamic parameters in bulk are consistent with those of Yfh1, in agreement with the similar dimensions of the two proteins, in spite of their difference in folding. The most interesting aspect of these data is the fact that, whereas the low transition temperatures coincide for bulk solution and gel, the high temperature transitions are appreciably different. In addition, the value of ΔC_p observed for I28 in gel is much higher than expected for a protein of ~100 residues.[10] The simplest interpretation is that the confined environment accelerates aggregation at high temperatures and thus the lower T_m reflects the combination of two competing phenomena: unfolding and aggregation. This view is consistent with the higher values of ΔC_p and ΔH_m that are typical of higher ΔASA.[10]

Table 1. Thermodynamic Parameters of I28

	TRIS/pH 7.2	PAG/PEG/pH 7.2
T_m/K	318	310
$\Delta H_m°$/kcal mol^{-1}	38.2	46.5
$\Delta S_m°$/cal mol^{-1} K^{-1}	120	150
ΔC_p/kcal mol^{-1} K^{-1}	1.77	2.7
T_c/K	275	275
$\Delta H_c°$/kcal mol^{-1}	−38.2	−46.5
$\Delta S_c°$/cal mol^{-1} K^{-1}	120	150

It has been proposed that the low-temperature unfolded state is intrinsically different from the corresponding state at high temperature.[11] Comparison between our results in bulk and in a confined environment could suggest an intrinsic difference between the cold and warm denatured states of I28. However, the apparent differences may be explained more simply by considering how atomic forces have a different temperature dependence. Aggregation involving hydrophobic surfaces is more relevant at high temperatures than at low temperatures. It was known that I28 has a hydrophobically driven tendency to aggregate,[3] and we did observe the effect of this tendency in the unfolding transitions: cold denaturation is completely reversible whereas the high temperature transition is irreversible. The use of crowding conditions enhanced this tendency at high temperature, while having no effect at low temperature. Crowding is in fact known to favor aggregation phenomena even though it could also stabilize the more compact folded conformation with respect to the unfolded one. The effect of crowding on aggregation phenomena is known to be much larger than that on conformations.[8]

The most important result of our study is that access to cold denaturation is the best way to characterize the thermodynamics and stability of proteins, especially in cases in which high temperature unfolding competes with other phenomena. Direct observation of cold denaturation, and the consequent reliable determination of the whole stability curve, may be the only way to assess the thermal stability of proteins that have a high tendency to aggregate at high temperatures. Last but not least, the use of a gel to reach temperatures well below 0 °C shows that it is possible to study supercooled solutions of proteins in a way akin to the use of bunches of capillaries, but simpler and safer.

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