Protein Cold Denaturation as Seen From the Solvent

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Abstract: Unlike most ordered molecular systems, globular proteins exhibit a temperature of maximum stability, implying that the structure can be disrupted by cooling. This cold denaturation phenomenon is usually linked to the temperature-dependent hydrophobic driving force for protein folding. Yet, despite the key role played by protein—water interactions, hydration changes during cold denaturation have not been investigated experimentally. Here, we use water—$^{17}$O spin relaxation to monitor the hydration dynamics of the proteins BPTI, ubiquitin, apomyoglobin, and $\beta$-lactoglobulin in aqueous solution from room temperature down to $-35^\circ$C. To access this temperature range without ice formation, we contained the protein solution in nonperturbing picoliter emulsion droplets. Among the four proteins, only the destabilized apomyoglobin was observed to cold denature. Ubiquitin was found to be thermodynamically stable at least down to $-32^\circ$C, whereas $\beta$-lactoglobulin is expected to be unstable below $-5^\circ$C but remains kinetically trapped in the native state. When destabilized by 4 M urea, $\beta$-lactoglobulin cold denatures at 10°C, as found previously by other methods. As seen from the solvent, the cold-denatured states of apomyoglobin in water and $\beta$-lactoglobulin in 4 M urea are relatively compact and are better described as solvent-penetrated than as unfolded. This finding challenges the popular analogy between cold denaturation and the anomalous low-temperature increase in aqueous solubility of nonpolar molecules. Our results also suggest that the reported cold denaturation at $-20^\circ$C of ubiquitin encapsulated in reverse micelles is caused by the low water content rather than by the low temperature.

1. Introduction

Under physiological conditions, most natural polypeptides adopt a unique, biologically active conformation. The high cooperativity of this folding process greatly simplifies the analysis of protein stability, allowing many single-domain proteins to be described in terms of a two-state equilibrium between a native (N) and a denatured (D) state.1,2 Ordered molecular systems are usually more stable at low temperatures, where thermal fluctuations are suppressed, but native proteins tend to be most stable at a temperature, $T^*$, near room temperature.3–5 As a consequence, the D-state population can be increased either by heating (for $T > T^*$) or by cooling (for $T < T^*$). At sufficiently high or low temperatures, the native protein is thus denatured, meaning that the D state is more populated than the N state. These two ways of disrupting the native protein conformation are known as heat denaturation and cold denaturation, respectively.6

The term cold denaturation usually refers to the process whereby a protein is denatured by reducing the temperature. However, equilibrium thermodynamics deals with states, and the properties of a particular cold-denatured state must be independent of the path by which it was reached. According to the standard two-state model, there is only one D state with continuously (noncooperatively) variable properties. The D state can be said to be cold-denatured whenever it has lower enthalpy than the N state, so that its population increases on cooling. Cold denaturation is phenomenologically linked to the large increase of the isobaric heat capacity on denaturation, responsible for the dome-shaped free-energy function, $\Delta G (T) = G_D(T) - G_N(T)$.6–7 While still somewhat controversial,8 the large $\Delta C_p$ has long been attributed principally to the hydration of nonpolar side-chains that become solvent-exposed upon disruption of the protein’s hydrophobic core.9–11 In the conventional view, there is thus a close analogy between cold denaturation of proteins and cold swelling of hydrophobic polymers or the anomalous low-temperature enhancement of the solubility of nonpolar organic solutes in water.12,13 Statistical—mechanical studies of the cold-denaturation mechanism are generally based on this assumption.14–22

(4) Hawley, S. A. Biochemistry 1971, 10, 2436–2442.
The degree of perturbation of the protein from left to right in this chart. The approach used here is indicated by the shaded boxes.

For a two-state protein, the cold-denaturation temperature, \( T_{cd} \), can be estimated by extrapolating the thermodynamic parameters that characterize heat denaturation.\(^{3-5}\) The predicted \( T_{cd} \) is typically 20 K or more below the equilibrium freezing point of water. To observe cold denaturation, it is thus necessary to raise \( T_{cd} \) (by destabilizing N or stabilizing D) and/or to prevent the solvent from freezing (Figure 1). To date, most cold-denaturation studies have employed modified proteins, non-physiological solvents, or high pressure. In the earliest studies of cold-denaturation studies, the D state was stabilized by urea,\(^{23-26}\) which, along with guanidinium chloride, remains the most popular strategy for making cold denaturation experimentally accessible.\(^{27-36}\) More recently, application of hydrostatic pressure has been used to raise \( T_{cd} \).\(^{37-43}\) Cosolvents and elevated pressure both have the additional desirable effect of depressing the equilibrium freezing point of the solvent.

To understand the role of (hydrophobic) hydration for the stability and folding of proteins under physiological conditions, we need to study proteins in their natural aqueous environment, unperturbed by cosolvents, high pressure, or confinement/dehydration. Cold denaturation without solvent perturbations has been studied with proteins destabilized by charge perturbations (usually at reduced pH), amino acid replacements, or truncation of the polypeptide chain.\(^{44-55}\)

If the solvent water can be maintained in a supercooled state, a wider temperature range becomes available for cold-denaturation studies. Using capillaries\(^{44}\) or specially designed sample cells,\(^{51}\) temperatures down to \(-20^\circ\text{C}\) have been reached. By subdividing the protein solution into picoliter droplets suspended in an inert nonpolar carrier fluid in a water-in-oil emulsion, protein solutions can be studied at least down to \(-35^\circ\text{C}\), where the rate of homogeneous ice nucleation becomes the limiting factor.\(^{36-58}\) Emulsion samples interfere with most scattering and optical spectroscopic measurements, but they were used early on to study cold denaturation at subzero temperatures via UV absorbance.\(^{44,59}\)

Here, we use the emulsion approach and NMR relaxation measurements to detect cold denaturation and to characterize the hydration of the cold-denatured state in aqueous protein solutions down to \(-35^\circ\text{C}\).

Cold denaturation is often taken as evidence for hydrophobic stabilization of native proteins. Yet, to our knowledge, the hydration of cold-denatured proteins has not been studied. As one of few techniques that can selectively monitor the water molecules in a protein solution, water-\(^{17}\)O magnetic relaxation dispersion (MRD) has been used to characterize the single-molecule water dynamics in the hydration layer of native and non-native proteins.\(^{60,61}\) \(^{17}\)O MRD studies have thus been

**Figure 1.** Different experimental strategies for observing cold denaturation.
Table 1. Results Derived from Fits to $^{17}$O MRD Data

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<th>protein</th>
<th>solvent</th>
<th>pH</th>
<th># data</th>
<th>$\nu_1$</th>
<th>$\nu_2$</th>
<th>$E_{20}$ (kJ mol$^{-1}$)</th>
<th>$E_{20}$ (kJ mol$^{-1}$)</th>
<th>$N_0/N_m$</th>
<th>$T_{d,0}$(°C)</th>
<th>$\Delta T_{d,0}$(°C)</th>
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<td>30(4)</td>
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$^{a}$Uncertainty in last digit given within parentheses. Parameter values within square brackets were frozen in the fit. $^{b}$Fit to restricted data set, consisting of MRD profiles at 19.4, 10.2, and -1.1 °C.

reported of proteins denatured by urea,63 guanidinium chloride,64 low pH,64 and heat.65 Here, we describe the first $^{17}$O MRD study of cold-denatured proteins. We have investigated four proteins that are popular model systems for protein folding studies: bovine pancreatic trypsin inhibitor (BPTI), mammalian ubiquitin (mUb), equine apomyoglobin (aMb), and bovine $\beta$-lactoglobulin (bLg). The first two of these proteins do not cold denature under the conditions examined here and therefore serve as models for native proteins at low temperatures.61

2. Materials and Methods

2.1. Sample Preparation. Bovine pancreatic trypsin inhibitor (BPTI, batch 9104, 97% purity by HPLC) from Bayer HealthCare AG (Wuppertal, Germany) was exhaustively dialyzed to remove residual salt. Mammalian ubiquitin (mUb) was expressed in Escherichia coli and was purified to >99% as described.66 Apomyoglobin (aMb) was prepared from equine myoglobin (Sigma, M-0630, > 95% purity) by heme extraction with 2-butanol at pH 2.67 followed by exhaustive dialysis at 8 °C. The residual absorbance in the 409 nm Soret band indicated <1% heme occupancy. Bovine $\beta$-lactoglobulin (bLg) isoform A (Sigma, L-7880) was purified by anion exchange and size-exclusion chromatography, followed by dialysis.68 Protein solutions were prepared by dissolving the purified lyophilized protein in $^{17}$O-enriched H$_2$O or (to allow $^2$H MRD measurements) a mixture of $^{17}$O-enriched H$_2$O and D$_2$O (99.9 atom % $^2$H, low paramagnetic content, CIL). The bLg samples at neutral pH also contained 0.02% sodium azide. After adjusting pH to the desired value, the solution was centrifuged to remove any aggregates. A small fraction of each MRD sample was subjected to complete amino acid analysis to determine the protein concentration with ~1% accuracy. The $^{17}$O relaxation enhancement produced by the protein is inversely proportional to the water/protein mole ratio $N_0$, which can be calculated from the protein concentration. The bLg solutions in 4 M urea were prepared by adjusting the required mass of dry urea (>99%, pro analysis grade, Merck) to the aqueous protein solution. pH was measured at room temperature and is quoted without H/D isotope correction.

Relaxation measurements at temperatures below the equilibrium freezing point of the solvent were performed on emulsion samples,56 prepared by mixing ~1 mL protein solution with an equal volume of n-heptane (>99%, HPLC grade, Sigma) containing 3% (w/w) of the nonionic emulsifier sorbitan tristearate (Sigma). A sufficiently stable water-in-oil emulsion was obtained by mixing the two solutions with the aid of two 5 mL syringes connected via a 0.56 mm i.d. nozzle (Hamilton) and pressing the mixture through the nozzle ~40 times. In a typical aqueous droplet of 10 µm diameter, only 0.3% of the protein molecules in the solution are within 5 nm of the interface. Furthermore, polyols (like the sorbitan headgroup of the emulsifier) are preferentially excluded from protein surfaces,69 so the protein should not interact strongly with the interface. Indeed, no effect of the interface could be detected in control experiments where $^{17}$O MRD profiles were recorded (at room temperature) from the same protein solution before and after incorporation in emulsion droplets (part a of Figure 3 and part a of Figure 6). Relevant sample characteristics are summarized in Table S1 of the Supporting Information.

2.2. Spin Relaxation Measurements. The relaxation rate, $R_1$, of the water-$^{17}$O longitudinal magnetization was measured either as a function of temperature, $T$, at a fixed $^{17}$O resonance frequency, $\nu_0 = 81.3$ MHz (corresponding to 600 MHz $^1$H frequency), or as a function of resonance frequency at a fixed temperature. The results of these two types of measurement series will be referred to as temperature profiles and MRD profiles, respectively. $R_1$ was determined from a three-parameter fit to the single-exponential magnetization curve obtained with the inversion recovery pulse sequence with 20 delay times in nonmonotonic order and a sufficient number of transients to obtain a signal-to-noise ratio of >100. Acoustic ringing, which interferes at low fields (<0.3 T) and low temperatures (where $T_1$ is short), was suppressed using filters or a modified 90° pulse70 in the inversion recovery sequence. The ice signal from the small fraction of frozen water droplets is broadened beyond detection and does not affect the $R_1$ measurement. The small scatter in the inversion recovery fits indicates that freezing does not occur to a significant extent during relaxation measurements at subzero temperatures. This was the case even at the lowest temperature (−35 °C) accessed here.

Most of the $^{17}$O MRD profiles reported here extend from 0.57 or 2.2 MHz up to 67.8 or 81.3 MHz. Frequencies <11 MHz were accessed with field-variable iron-core magnets (Drusch EAR-35N or GMW 3474–140) interfaced to Tecmag (Apollo or Discovery) consoles, whereas higher frequencies were provided by conventional superconducting magnets interfaced to commercial NMR spectrometers (Bruker Avance DMX 100 and 200, Varian Unity Plus 360, 500 and 600). At each temperature and frequency, we also measured the relaxation rate, $R_1$, of a reference sample containing the solvent present in the corresponding protein solution. All samples were equilibrated at the measurement temperature (see sect. 4.5), which was regulated with a precooled stream of dry air and was constant within 0.3 K for the iron-core magnets at the lowest temperatures. On the basis of the scatter in the frequency-independent $R_1$, the accuracy in $R_1$ ranged from <0.5% in temperature profiles to 1–2% in low-temperature MRD profiles. Data matrices $R_0(\nu_0,T)$ comprising 39–82 $R_1$ values from several MRD profiles and one $T$ profile


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were analyzed by global fits using the Marquardt–Levenberg nonlinear least-squares algorithm.

3. Results

3.1. Protein Hydration from Water $^{17}$O MRD Data. The dependence of the water–$^{17}$O spin relaxation rate, $R_1(\omega_0,T)$, on resonance frequency, $\omega_0$, and temperature, $T$, provides information about the number of water molecules that interact directly with the protein and about their rotational correlation times. In a protein solution, $R_1(\omega_0,T)$ exceeds the relaxation rate, $R_1^H$, for the bulk solvent because water molecules that interact with the protein rotate more slowly. The dynamically perturbed water molecules can be divided in two classes. A small number, $N_H$, of internal water molecules are buried in cavities with residence time $t_1 > \sim 10$ ns at room temperature. A much larger number, $N_{HN}$, of more mobile water molecules interact with the external surface of the protein, constituting the hydration layer. The hydration number $N_H$ can be estimated by dividing the solvent-accessible surface area of the protein with the effective surface area, 10.75 Å$^2$, occupied by one water molecule. For proteins$^{61}$ as well as for peptides and small organic solutes,$^{71}$ this simple procedure yields $N_H$ values in excellent agreement with results from MD simulations with cutoff distances based on radial distribution functions.

We present the relaxation data in the form of an apparent dynamic perturbation factor (ADPF), defined as

$$\xi(\omega_0,T) \equiv 1 + \frac{N_{HN}}{N_{HN}} \left[ \frac{R_1(\omega_0,T) - R_1(0)}{R_1(0)} \right]$$

The ADFP is obtained directly from the measured quantities $R_1(\omega_0,T)$, $R_1(0)$, and $N_W$ (the water/protein mole ratio in the sample) and the hydration number $N_{HN}$ calculated from the known structure of the native protein (Table S1 of the Supporting Information). By normalizing the $R_1$ data in this way, we remove the effect of protein concentration and most of the effects of protein size and solvent composition. Furthermore, in the limit $\omega_0 = 0$ (or when all correlation times are short compared to $1/\omega_0$), the ADFP reduces to the true (rather than apparent) DPF, $\xi(T) \equiv \langle T \rangle / \tau_0$, which relates hydration-water dynamics to bulk-water dynamics in an essentially model- and method-independent way.$^{61}$

For a native protein (i.e., in the absence of cold denaturation), the ADFP is a sum of two contributions (section S1 of the Supporting Information). The internal-water contribution (colored red in the following figures) is negligibly small at low temperatures or high frequencies. Under conditions where the internal-water contribution is non-negligible, it can be accurately estimated from the known protein tumbling time and the residence time and order parameter of the internal water molecules (section S2 of the Supporting Information). The ADFP is usually dominated by the contribution (colored blue in the following figures) from the $N_{HN}$ water molecules in the hydration layer. The rotational correlation times of these water molecules range from picoseconds to nanoseconds (at room temperature) and can be modeled by a power-law distribution$^{61}$ (section S1 of the Supporting Information). The two parameters in this model, the power-law exponent, $\nu_H$, and the activation energy, $E_{HN}$, of water rotation at the short-correlation-time end of the distribution, can be determined from a fit to a temperature profile $R_1(\omega_0,T)$ at a fixed high frequency and extending to sufficiently low temperatures (in the supercooled regime).$^{61}$

The extension of the model to the case of coexisting native (N) and denatured (D) protein molecules in the solution is straightforward (section S1 of the Supporting Information). The ADFP now contains a third contribution (colored yellow in the following figures), associated with the $N_{HD}$ water molecules that are in contact with the denatured protein. At temperatures where the protein is fully denatured, the model contains three parameters: $N_{HN}$, $\nu_D$, and $E_{HD}$. To analyze $^{17}$O relaxation data at temperatures where the N and D forms coexist, we model their relative populations in terms of two parameters: the cold denaturation temperature, $T_{cd}$, and the transition width, $\Delta T_{cd}$ (section S1 of the Supporting Information). To determine the model parameters, we perform global fits to large sets of $R_1(\omega_0,T)$ data, comprising a temperature profile (extending far into the supercooled regime) at a high fixed frequency as well as two or more dispersion profiles at fixed temperatures. In most cases, the number of free parameters can be reduced by physically motivated approximations (see below).

3.2. BPTI. At the temperatures examined here (down to $\sim 35$ °C), BPTI is expected to remain fully folded (Discussion). The temperature-dependent water–$^{17}$O relaxation data for BPTI (part a of Figure 2) thus serve as a baseline for the more complex situations encountered with proteins that do cold denature. The internal-water contribution to the ADFP (section S2 of the Supporting Information) is negligibly small at low temperatures, where the protein tumbling rate is much smaller than the high $^{17}$O resonance frequency (81.3 MHz) of these measurements.

![Figure 2](image-url) Temperature dependence of the water–$^{17}$O ADFP at 81.3 MHz for (a) BPTI (sample 1) and (b) mUb (sample 2). The curves were obtained by fitting the two model parameters $\nu_H$ and $E_{HN}$ (Table 1) to the data (filled circles). The computed ADFP contributions from internal water (red) and hydration water (blue) are indicated.
The hydration-layer contribution to the ADPF exhibits a broad maximum near \(-5^\circ C\), where \(\xi(\omega_0, T) \approx 4\). As discussed in detail elsewhere,\(^{61}\) this maximum reflects a crossover of the (temperature-dependent) activation energies of water rotation in the hydration layer and in the bulk solvent, with the latter having the largest activation energy at low temperatures. The true hydration-layer DPF, \(\xi_H(T)\), which can be calculated\(^{61}\) from the parameters \(\nu_H\) and \(E_{HN}\) (Table 1), exhibits a similar maximum near \(-10^\circ C\), where \(\xi_H \approx 4.8\). Because the distribution of correlation times in the hydration layer is very broad, slow hydration water molecules (say, with \(\tau_H > 0.1\) ns at \(20^\circ C\)) contribute significantly to the DPF even though they are few in number (0.5%). Accordingly, the maximum DPF for the 90 or 50% most mobile hydration water molecules drops to 2.4 and 1.6, respectively. These partial DPF values represent the dominant generic part of the hydration layer and are similar to the DPF values obtained for small peptides and other organic solutes.\(^{71}\)

### 3.3. Ubiquitin

On the basis of previous work (Discussion), we expect \(T_H\) to be around \(-50^\circ C\) (at pH 5.0) so that cold denaturation of mUb should not occur in our temperature range. This prediction is confirmed by the very similar temperature profiles that we observe for mUb and BPTI (Figure 2). The slightly larger ADPF for BPTI at the highest temperatures is fully accounted for by the larger internal-water contribution for BPTI (section S2 of the Supporting Information). The fits to the data in Figure 2 show that the hydration dynamics parameters \(\nu_H\) and \(E_{HN}\) are virtually identical for mUb and BPTI (Table 1), as expected if both proteins remain fully folded at all investigated temperatures. We therefore conclude that mUb remains fully folded at least down to \(-32^\circ C\).

### 3.4. Apomyoglobin

Removal of the heme group causes partial unfolding of myoglobin, with a 20% reduction in \(\alpha\)-helix content.\(^{49}\) The consequent stability reduction has allowed cold denaturation of aMb to be observed (by far-UV circular dichroism) not far below 0 \(^\circ C\).\(^{49,51}\) The temperature profile of aMb (part c of Figure 3) does indeed differ qualitatively from those of BPTI and mUb (Figure 2), but these data alone do not contain sufficient information to determine the additional model parameters associated with the cold-denatured (D) state. We therefore acquired MRD profiles at \(27^\circ C\), where aMb is fully native (part a of Figure 3), and at \(-20^\circ C\), where the ADPF is dominated by the D state (part b of Figure 3). At \(27^\circ C\), measurements were performed directly on the aMb solution as well as on the emulsion sample made from the same solution. As seen from part a of Figure 3, incorporation of the protein solution into micrometer-sized emulsion droplets has no significant effect on the measured \(^{17}\)O relaxation. The negligible influence of the droplet interface has previously been demonstrated also for BPTI.\(^{72}\) In the global fit to the 41 data points in Figure 3, we constrained \(E_{HN}\) to the same value (27 kJ mol\(^{-1}\)) as found for BPTI and mUb (Table 1). This parameter describes the most mobile part of the hydration layer, which should be similar for all globular proteins.\(^{61}\) For the same reason, we expect \(E_{HH}\) to be similar to \(E_{HN}\). Accordingly, we imposed the constraint \(E_{HD} = E_{HN}\).

The N-state exponent obtained from the global fit, \(\nu_N = 2.06\), is slightly smaller than for BPTI and mUb, corresponding to a somewhat wider distribution of correlation times, that is, a larger proportion of relatively long correlation times. This feature may be associated with the large solvent-filled heme cavity and the disordered F helix in aMb.\(^{73}\) The D state has an even broader distribution, with \(\nu_D = 1.72\), as expected if the cold-denatured protein is penetrated by water. To illustrate the significance of these power-law exponents, we can calculate the fraction of hydration water molecules with correlation times \(\tau_H\) longer than, say, 0.1 ns at \(20^\circ C\).\(^{61}\) This fraction is 1.5% for the N state and 5.6% for the D state of aMb (and 0.5% for BPTI and mUb).

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For the cold-denaturation transition, the fit yields $T_{cd} = -16 \pm 1^\circ C$ and $\Delta T_{cd} = 14 \pm 2^\circ C$. Cold denaturation is expected to increase the solvent-accessible area and thereby the number of water molecules in contact with the protein. However, our analysis indicates that this number actually decreases: $N_{HN}/N_{IN} = 0.88 \pm 0.03$. To rationalize this unexpected result, we recall that aMb has an exceptionally low packing density: disordered and relatively mobile water molecules are expected to penetrate the large heme cavity and at least some of the several smaller cavities. Presumably, cold denaturation causes these cavities to collapse, thus effectively reducing the solvent-exposed surface area of the protein. At the same time, other water molecules penetrate the structurally disordered protein. Although cold denaturation reduces the total solvent exposure slightly, it increases the number of strongly perturbed water molecules. Furthermore, because the transition width cannot be determined from the fit with useful accuracy, we fix it to a value, $\Delta T_{cd} = 0.1$ ns (at $20^\circ C$) increases from 11 in the N state to 35 in the D state.

3.5. β-Lactoglobulin at pH 2.6. Bovine β-lactoglobulin is predominantly dimeric at neutral pH, but at pH 2.6 and in salt-free solvent, as in samples 5–9 (Table S1 of the Supporting Information), the dimer fraction is $<10\%$. The dimer interface buries only 6% of the solvent-accessible area (with a correspondingly small reduction of $N_{IN}$; Table S1 of the Supporting Information) so a few percent dimer should not significantly affect the water-$^{17}$O ADPF.

The global fit comprises 39 data points, including a temperature profile (part d of Figure 4), MRD profiles at 19.4, 10.2, and $-1.1^\circ C$ (parts a–c of Figure 4) and partial MRD profiles at $-20$ and $-25^\circ C$ (Table S1 of the Supporting Information). The data, extending down to $-35^\circ C$, show no sign of cold denaturation. The hydration parameters, $\nu_N = 2.17$ and $E_{IN} = 27.6$ kJ mol$^{-1}$, do not differ significantly from what was obtained from a previous fit to the temperature profile alone (with slightly different internal-water parameters). Furthermore, these hydration parameter values are very similar to those obtained for BPTI and mUb (Table 1), which do not cold denature above $-35^\circ C$ (Figure 2). We therefore conclude that bLg in water at pH 2.6 does not cold denature above $-35^\circ C$. This conclusion is apparently at odds with predictions based on calorimetric and CD studies (section S3 of the Supporting Information), but it can be explained by the slow folding kinetics of bLg at low temperatures (Discussion).

To examine bLg under conditions where it is sure to cold denature (on the experimental time scale), we recorded $^{17}$O MRD profiles of bLg at pH 2.6 and 4.0 M urea at six temperatures from 19.4 to $-30^\circ C$ (Figure 5). Under these conditions, bLg is completely dissociated into monomers. According to optical rotation measurements, the cold-denaturation temperature of bLg at pH 3.0 and 4.0 M urea is $T_{cd} = 7^\circ C$. Differential scanning calorimetry at pH 2.0 and 4.0 M urea yields $T_{cd} = 4^\circ C$ on cooling and $T_{cd} = 13^\circ C$ on heating at a scan rate of 1 $K$ min$^{-1}$. Our sample should thus be fully native at 19.4 $^\circ C$ and fully cold-denatured at subzero temperatures.

In the global fit to the six MRD profiles (47 data points), we freeze $E_{IN}$ to the same value as that in the absence of urea. This simplification is justified by the negligible effect of urea on water dynamics in its hydration shell ($\xi_H = 1.00 \pm 0.02$). Furthermore, because the transition width cannot be determined from the fit with useful accuracy, we fix it to a value, $\Delta T_{cd} = 0.1$ ns (at $20^\circ C$) increases from 11 in the N state to 35 in the D state.

**Figure 4.** Variation of the water-$^{17}$O ADPF for bLg at pH 2.6 with frequency (sample 5) at (a) $19.4^\circ C$, (b) $10.2^\circ C$, (c) $-1.1^\circ C$, and (d) with temperature (sample 7) at 81.3 MHz. The solid curves were obtained from a global fit to all data (filled circles), including MRD data at $-20$ and $-25^\circ C$ (sample 6, Table S1 of the Supporting Information). The resulting parameter values are given in Table 1. The computed ADPF contributions from internal (red) and hydration (blue) water are indicated.


Figure 5. Variation of the water\(^{17}\)O ADPF for bLg at pH 2.6 and 4.0 M urea with frequency (sample 8) at (a) 19.4 °C, (b) 10.2 °C, (c) -1.1 °C, and (d) with temperature at 81.3 MHz. The solid curves were obtained from a global fit to all 6 MRD profiles. The resulting parameter values are given in Table 1. The computed ADPF contributions from internal water (red) and N-state (blue) or D-state (yellow) hydration water are indicated. In panel (d), the contributions from D-state hydration water (yellow triangles) and N- plus D-state hydration water (blue squares) are also indicated.

10 °C, consistent with the calorimetric cold-denaturation enthalpy (cf. eq S7 of the Supporting Information).\(^{29}\) The data and fitted curves are shown in Figure 5 for the three highest temperatures, which span the cold-denaturation transition. The MRD profiles at the three lowest temperatures closely resemble the one at -1.1 °C, but the ADPF increases slightly with decreasing temperature. This trend is evident in part d of Figure 5, where we have reconstructed the temperature profile from the six fitted ADPF values at 81.3 MHz. (We use fitted values because the highest measurement frequency was 67.8 MHz at the 3 lowest temperatures.)

Unlike all other temperature profiles reported here, the one in part d of Figure 5 does not show the ADPF dropping sharply at low temperatures. The drastic reduction of the ADPF seen in the other cases at temperatures around -30 °C is produced by the very strong (super-Arrhenius) slowing down of molecular rotation (and, hence, of \(R_2\)) in bulk water at these temperatures.\(^{77,78}\) In 4 M urea, \(R_2\) has a weaker temperature dependence, thereby shifting the drop in the ADPF to attainably low temperatures. In the analysis of the \(^{17}\)O relaxation data, we have taken into account the (modest) increases in solvent viscosity produced by the urea cosolvent (section S1 of the Supporting Information). Furthermore, we use the solvent-exchange model\(^{79,80}\) to describe the preferential solvation of the protein by urea (section S4 of the Supporting Information).

The ADPF data demonstrate a gradual loss of the internal-water contribution between 19.4 and -1.1 °C (parts a–c of Figure 5), as expected if the protein unfolds on cooling. In contrast, in the absence of urea, the internal-water contribution is fully retained at -1.1 °C (parts a–c of Figure 4) and at lower temperatures. The global fit in Figure 5 yields \(T_{cd} = 10.0 \pm 0.3 °C\), in excellent agreement with previous spectroscopic\(^{26}\) and calorimetric\(^{29,30}\) results (Discussion). The exponents \(\nu_0 = 2.07\) and \(\nu_0 = 2.21\) differ little from the value 2.17 obtained in the absence of urea, indicating that the fraction hydration water molecules with relatively long correlation times is similar in the three cases. The result \(N_{H2}^{\text{H2O}}/N_{H2}^{\text{H2O}} = 1.3 \pm 0.1\) means that the number of water molecules interacting directly with the protein is 30% larger (675 versus 525) in the cold-denatured state than in the native state in the presence of 4 M urea. The increase in solvent-accessible area will be larger than 30% if the D state has a higher urea affinity than the N state or if water molecules interact simultaneously with different parts of the polypeptide chain (solvation layer overlap).

3.6. \(\beta\)-Lactoglobulin at pH 7.2. The native conformation of bLg is more stable at pH 2–3 than at neutral pH. For example, the urea denaturation midpoint concentration at 20 °C is 4.7 M at pH 3.0 but only 4.1 M at pH 7.0,\(^{51}\) and the cold-denaturation temperature \(T_{cd}\) in the presence of 4 M urea increases from 7 °C at pH 3.0\(^{26}\) to 25 °C at pH 7.0,\(^{36}\) A (rather long) linear extrapolation from the range 3–5 M urea led to the prediction that \(T_{cd} = -14 °C\) in the absence of urea at pH 7.0.\(^{36}\) It therefore seemed likely that cold denaturation of bLg at pH 7 should be observable with the emulsion technique used here. The unusual pH dependence of bLg stability is all the more remarkable.
because bLg is stabilized by dimer formation at neutral pH. On the basis of reported dimer association constants,82,83 we expect that 90–95% of the protein in our samples 10–12 exists in dimeric form. Hydrophobically stabilized protein oligomers should dissociate on cooling for the same reason that proteins unfold at low temperatures. However, the small dimer interface in bLg is predominantly polar, and studies by isothermal titration calorimetry indicate that the dimerization constant increases slightly on cooling, at least down to 15 °C.83 The dimerization enthalpy may become positive at lower temperatures, but the available data suggest that the dimer fraction in our samples is unlikely to vary by more than a few percent in the investigated temperature range.

The global fit comprises 82 data points (Figure 6), including MRD profiles at 27, 2, −5, −15, and −25 °C and a temperature profile at 81.3 MHz. (The shape of the 2 and −15 °C profiles, which are not displayed in Figure 6, are intermediate between the ones shown and the fit quality is similar.) In contrast to aMb and bLg in 4 M urea, the data in Figure 6 do not provide any qualitative indications of cold denaturation. However, attempts to fit a one-state model (with temperature-independent protein structure) to the data were unsuccessful, producing systematic deviations well outside the experimental uncertainty. Furthermore, a comparison of the bLg data at pH 2.6 (Figure 4) and pH 7.2 (Figure 6) reveals substantial quantitative differences. At the highest examined temperature, the MRD profiles are similar, the main difference stemming from the different correlation times of the two long-lived internal water molecules (section S2 of the Supporting Information). However, at low temperatures, both the low- and high-frequency ADPFs are much larger at neutral pH than at pH 2.6. Thus, at −1 MHz and −25 °C, the ADPF is 3 times larger at pH 7.2, and at 81.3 MHz and −35 °C, the ADPF is a factor 1.7 larger at pH 7.2. This large difference in the temperature dependence of hydration dynamics at pH 2.6 and 7.2 cannot be explained by the dimeric state per se (or by dimer dissociation). Rather, it indicates a temperature-dependent variation in protein structure.

As seen from Figure 6, the two-state model accounts well for the rather large data set at pH 7. In the global fit, we fixed $E_{\text{HN}}$ to the value (27.6 kJ mol$^{-1}$) obtained at pH 2.6. Furthermore, because the parameters $E_{\text{HD}}$ and $N_{\text{HD}}/N_{\text{HN}}$ turned out to be strongly coupled in the fit, we imposed the physically motivated (section 3.4) constraint $E_{\text{HD}} = E_{\text{HN}}$. Although the data can be described by a two-state model, the parameter values (Table 1) do not suggest a cooperative unfolding transition. The transition temperature, $T_{\text{cd}} = -36 ± 7 ^\circ \text{C}$, is 22 °C lower than expected from extrapolation of cold denaturation in urea solvent (above). Furthermore, the transition is very broad, $\Delta T_{\text{cd}} = 30 ± 4$ °C, and does not seem to increase the solvent exposure significantly. The D-state exponent, $\nu_D = 1.56 ± 0.05$, is even lower than for cold-denatured aMb. As for aMb, we can use the N- and D-state exponents to compute the fraction of hydration water molecules with correlation times $\tau_H$ longer than 0.1 ns at 20 °C.65 This fraction increases from 1.8% (or 13 water molecules) for the N state to 10% (or 64 ± 17 water molecules) for the D state. Similarly, we find that the D state contains 16 ± 4 water molecules with $\tau_H > 1$ ns at 20 °C. Taken together, these results suggest that cooling induces not a cooperative unfolding but a more gradual loosening of the structure, with


![Figure 6](image-url)
4. Discussion

4.1. BPTI and Ubiquitin. As long as its three disulfide bonds are intact, BPTI is an exceptionally stable protein with $T_{cd} = 104.5 \, ^{\circ}C$ and $\Delta H_{cd} = 312 \, kJ \, mol^{-1}$ at pH 4.9. Using these parameters and linear or quadratic fits to the measured temperature dependence of $C_p$ for the N and D states, respectively, we find from the standard two-state thermodynamic relations that BPTI is most stable (maximum N-state population) at $-22 \, ^{\circ}C$, where $\Delta G = 50 \, kJ \, mol^{-1}$. Further, we obtain a cold-denaturation temperature, $T_{cd}$, of $-112 \, ^{\circ}C$. With water as the solvent, wildtype BPTI is thus not expected to cold denature under any experimentally attainable conditions. Consistent with this prediction, high-resolution $^1$H NMR spectroscopy indicates that the structure of BPTI (at pH 3.5) is virtually independent of temperature between $+30$ and $-16 \, ^{\circ}C$. Because we can be confident that BPTI remains fully folded at the temperatures examined here (down to $-35 \, ^{\circ}C$), the high-frequency temperature profile $R_{(\omega_0,T)}$ in part a of Figure 2 reflects the low-temperature hydration behavior of a native protein.

Mammalian ubiquitin lacks disulfide bonds, but the heat-denaturation temperature is still high, $T_{cd} = 90 \, ^{\circ}C$ at pH 4.0. In contrast to BPTI, mUb can be denatured by guanidinium chloride (GdmCl) at 25 $^{\circ}C$, with $C_{1/2} = 3.86 \, M$ at pH 4.0 and with little or no residual secondary structure in the D state. Cold denaturation of mUb above 0 $^{\circ}C$ has been observed in the presence of 3–4 M GdmCl and extrapolation to zero GdmCl concentration yields $T_{cd} = -45 \, ^{\circ}C$ at pH 4.0. Because the stability of mUb increases with pH, $T_{cd}$ should be even lower in our sample at pH 5.0. There are also reports of cold denaturation of mUb at high pressure, $p \geq 2 \, kbar$. A two-state thermodynamic analysis of NMR spectral intensities for mUb at pH 4.5 and 2 kbar yielded $T_{cd} = -12 \, ^{\circ}C$. At this pressure, the maximum stability of mUb, $\Delta G (31 \, ^{\circ}C) = 8.6 \, kJ \, mol^{-1}$, is only one-third of that at atmospheric pressure. Cold denaturation of mUb at 2 kbar is reported to be highly cooperative, and the D state at $-21 \, ^{\circ}C$ appears to be almost fully unfolded. It has also been shown that a highly destabilized hydrophobic-core mutant (V26G), with $\Delta G (25 \, ^{\circ}C) = 5.9 \, kJ \, mol^{-1}$, undergoes two-state cold denaturation with $T_{cd} = -12 \, ^{\circ}C$ (at pH 5.9). In summary, these studies show that mUb can undergo cooperative cold denaturation to a highly unfolded state, but only in the presence of an additional, strongly destabilizing perturbation. Under physiological solvent conditions, we expect $T_{cd}$ to be around $-50 \, ^{\circ}C$ (at pH 5.0). Indeed, high-resolution NMR spectra indicate that the structure of mUb at pH 5.9 is virtually unchanged between $+25$ and $-15 \, ^{\circ}C$. Our finding that mUb remains fully folded at least down to $-32 \, ^{\circ}C$ is fully consistent with all of the above cited results.

4.2. Cold Denaturation of Ubiquitin in Reverse Micelles. In contrast to our findings, mUb (at pH 5, as in our sample) has been shown by high-resolution NMR spectroscopy to be partly cold-denatured at $-20 \, ^{\circ}C$. In these studies, temperatures down to $-30 \, ^{\circ}C$ were accessed without ice formation by encapsulating the protein in reverse micelles, surfactant-covered nanometer-sized water droplets immersed in a nonpolar alkane solvent. The volume of the encapsulated protein–water droplet is some 10 orders of magnitude smaller than the aqueous droplets in our emulsions. Whereas the droplet interface has a negligible effect on our emulsified protein solutions (part a of Figure 3 and part a of Figure 6), the water in reverse micelles is substantially perturbed by the surfactant-covered interface (as well as by the protein). In emulsion droplets, water is in a metastable state at subzero temperatures. This state survives because of the absence of templates for heterogeneous nucleation and because the barrier to homogeneous nucleation is high. In reverse micelles, on the other hand, water remains liquid at subzero temperatures because the equilibrium freezing point is depressed by water–surfactant and water–protein interactions.

In reverse micellar systems, the equilibrium micelle size distribution and the macroscopic phase equilibrium are established by exchange processes involving the transient fusion and fission of the aqueous nanodroplets. If this equilibration process could be arrested, the water in a protein-loaded micelle would probably be unfreezable, forming instead a glass at very low temperatures. So far, the discussion of the possible effects of the reverse micelle on the observed cold denaturation has focused on Coulomb interactions, and it has been shown that modest variations of the net charge of the protein (by pH variation) or of the reverse micelle interface (by varying the surfactant composition) have only minor effects on the cold denaturation of mUb. However, the principal perturbation in a reverse micelle is likely to be a direct consequence of the low water content. Even subtle changes in the thermodynamic properties of the solvent water can have major effects on protein stability.

Under certain conditions, a reverse micelle solution is a thermodynamically stable phase, sometimes called a microemulsion. However, at low temperatures, the microemulsion phase separates into an essentially pure water phase and an alkane-rich phase with dehydrated reverse micelles. Low-temperature equilibrium studies are therefore feasible only at low water content, typically less than what is required for the primary hydration shells of the bulky surfactant headgroups and the associated counterions. As a further complication, reverse micelles are dynamic entities that change their size and shape in response to external conditions. Therefore, the usual geometry-based estimate for the size of the (assumed spherical) aqueous core of the reverse micelle is not applicable under the conditions used in cold-denaturation studies. In the absence of protein, the reverse micelles tend to change from spherical to rodlike shape.

as they are dehydrated at low temperatures.\textsuperscript{95} In the presence of protein, also at room temperature, water is redistributed between droplets that contain protein and those that do not. The amount of water surrounding the encapsulated protein can thus not be estimated from the water/surfactant ratio, as is commonly done.

The marginal stability of native proteins is a result of near cancelation of strong but opposing thermodynamic forces, the largest of which are the conformational entropy of the polypeptide chain and the free energy of hydration of exposed nonpolar side-chains.\textsuperscript{10} These two dominant driving forces are both altered by encapsulation of the protein in a reverse micelle. The geometric confinement limits the configurational space available to the polypeptide chain, thereby destabilizing the D state.\textsuperscript{100} Limitations in the amount of water and in its configurational freedom (because it is already perturbed by the reverse micelle interface) weaken the hydrophobic effect, thereby stabilizing the D state. Heat-denaturation studies of encapsulated proteins as a function of (total) water content reveal both of these effects and show that the latter is the dominant one at the water contents used in the cold-denaturation studies.\textsuperscript{101,102} Moreover, the heat-denaturation enthalpy is substantially reduced in reverse micelles,\textsuperscript{101,102} implying that encapsulation makes unfolding less cooperative, as observed for cold denaturation in reverse micelles.\textsuperscript{89,91,92,103}

Reverse micelles have been promoted as a method for studying cold denaturation under native conditions,\textsuperscript{89,91,92} without the need for an adjunct perturbation such as urea. In our view, this claim is not justified. Our finding that mUb in supercooled water remains fully folded at least down to \(-32\) °C indicates that the previously reported\textsuperscript{89–92} cold denaturation of mUb in reverse micelles is induced by the low water content rather than by the low temperature per se. Our low-temperature results for mUb as well as studies of heat denaturation of other proteins in reverse micelles\textsuperscript{101,102} indicate that encapsulation destabilizes the protein substantially. Furthermore, unlike the addition of denaturant, this perturbation cannot easily be extrapolated to zero. (The micrometer-sized emulsion droplets used in our work may be regarded as the limit of negligible solvent perturbation.) If encapsulation in a reverse micelle (of low, but unknown, water content) did not significantly affect the stability of the protein, then also the heat-denaturation temperature, \(T_{cd}\) for the encapsulated protein should be the same as for the protein in aqueous solution. This critical control experiment has apparently not been carried out for mUb. However, for several other proteins, it has been shown that encapsulation can depress \(T_{cd}\) by 20–40 °C.\textsuperscript{101}

4.3. Aponomyoglobin. Circular dichroism studies of equine aMb at pH 5.9 indicate a broad cold-denaturation transition with \(\Delta T_{cd} \approx 20\) °C and midpoint \(T_{cd} \approx -6\) °C.\textsuperscript{89,94} Our \(^{17}\)O MRD data yield a significantly lower cold-denaturation temperature, \(T_{cd} \approx -16 \pm 1\) °C. An acceptable fit to our data cannot be obtained if \(T_{cd}\) is fixed to \(-6\) °C. Because a low-temperature baseline was not evident in the CD temperature profiles,\textsuperscript{95,96} it is conceivable that a lower \(T_{cd}\) would have been obtained had the CD measurements been extended to lower temperatures. Alternatively, the different \(T_{cd}\) values deduced from measurements sensitive to secondary structure and hydration may be taken to indicate that cold denaturation of aMb does not occur with full cooperativity, as assumed in the simple two-state model.

What is the structure of cold denatured aMb? If the polypeptide chain were completely unfolded and fully exposed to solvent, we would expect a DPF of \(\sim 2\) or less, as for small peptides.\textsuperscript{71} However, at temperatures near \(-30\) °C where the D state dominates (part c of Figure 3), the ADPF \(\xi_{BD}\) is in the range 4.5–6.0 (eq S12 in the Supporting Information), demonstrating that cold-denatured aMb is far from fully unfolded. Rather, the large ADPF for cold denatured aMb, which is about twice as large as that for native BPTI and mUb at the same temperature (Figure 2), suggests a relatively compact structure penetrated by water molecules (perhaps bridging nearby polypeptide segments) that are more strongly dynamically perturbed than most water molecules in the hydration layer of native proteins. This scenario can account for the broad distribution of hydration water correlation times (as indicated by the small \(\tau_{D}\) exponent) for cold denatured aMb, and it is consistent with the indication from CD measurements\textsuperscript{93} that the G and H helices are largely intact in cold denatured aMb. Cold denaturation has also been demonstrated for charge-destabilized (low pH) metmyoglobin\textsuperscript{45} and apomyoglobin,\textsuperscript{39,46} including cold denaturation of the molten–globule intermediate of apoMb.\textsuperscript{47,49} The compactness of these cold denatured states depends strongly on pH.\textsuperscript{47}

4.4. \(\beta\)-Lactoglobulin in 4 M Urea. Like most other proteins, bLg is unfolded by urea. At 20 °C and pH 3.0, the urea denaturation midpoint concentration, \(C_{1/2}\), has been determined to 5.1 M (150 mM ionic strength)\textsuperscript{26} or 4.7 M (50 mM ionic strength).\textsuperscript{81} At this temperature, \(\partial C_{1/2}/\partial T > 0\),\textsuperscript{81} implying that the denaturation entropy is negative.\textsuperscript{103} Traditionally, the same low-entropy D state is referred to as urea-denatured when it is obtained by increasing the urea concentration and as cold-denatured when it is produced by cooling. At urea concentrations somewhat below \(C_{1/2}\), where the protein is highly destabilized but remains predominantly in the native state, further destabilization by cooling can induce unfolding even at ambient temperature. Because of this convenience, cold denaturation of bLg has mostly been studied in the presence of \(\sim 4\) M urea.

The modest increase of \(N_{H}\) on cold denaturation of bLg in 4 M urea indicates that the D state is not extensively unfolded. This conclusion is consistent with small-angle X-ray scattering (SAXS), CD, and heteronuclear NMR studies, indicating that cold-denatured bLg in 4 M urea at pH 2.5 is relatively compact and exhibits some residual structure, including a native-like \(\beta\)-hairpin stabilized by one of the two disulfide bonds.\textsuperscript{35} According to the SAXS measurements, the radius of gyration, \(R_g\), increases by 9% on addition of 4 M urea at 25 °C, by a further 15% on reducing the temperature to 0 °C, and by a further 45% on increasing the urea concentration from 4 to 8 M at 0 °C.\textsuperscript{35} The 15% increase of \(R_g\) on cold denaturation corresponds to a 52% increase of the effective protein volume, or 11,700 Å\(^3\) of internal solvent. Because the protein is preferentially solvated by urea, the fraction of this volume occupied by urea molecules must be larger than the bulk urea volume fraction at 4 M, which is 18%. We thus estimate that \(\sim 300\) water molecules penetrate the denatured protein. This

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  \item \textsuperscript{89} Zhou, H.-X.; Dill, K. A. Biochemistry 2001, 40, 11289–11293.
  \item \textsuperscript{91} Battistel, E.; Luisi, P. L.; Rialdi, G. J. Phys. Chem. 1988, 92, 6680–6685.
  \item \textsuperscript{92} Shastry, M. C. R.; Eftink, M. R. Biochemistry 1996, 35, 4094–4101.
  \item \textsuperscript{103} Scharnagl, C.; Reif, M.; Friedrich, J. Biochim. Biophys. Acta 2005, 1749, 187–213.
\end{itemize}
estimate does not clearly distinguish internal from external hydration, but it suggests that the SAXS and MRD results are compatible.

The failure of the two-state model to describe cold denaturation of bLg is clearly indicated by the large difference between the calorimetric and van’t Hoff denaturation enthalpies. Another observation pointing to a more complex process is the finding that the far-UV ellipticity shows no sign of leveling off even at −10 °C, although, according to the two-state model, the transition should be complete at 0 °C. The gradual loss of residual structure on cooling below the main transition, indicated by the ellipticity data, is likely to be accompanied by a gradual expansion of the, initially rather compact, cold-denatured protein. An increase of $N_{\text{HD}}$ as the protein expands on further cooling below $T_{\text{cd}}$ can explain the anomalously large apparent activation energy $E_{\text{HD}}$ (Table 1). At high frequencies, the D-state contribution to $R_1$ is proportional to $N_{\text{HD}}$ ($\tau_{\text{HD}}$). Even if $\langle \tau_{\text{HD}} \rangle$ has the same temperature dependence as $\langle \tau_{\text{RN}} \rangle$ (as we expect), a temperature dependence in $N_{\text{HD}}$, if not explicitly accounted for by the model, will give rise to an apparent activation energy $E_{\text{HD}}$ that differs from $E_{\text{RN}}$. The finding that $E_{\text{HD}} > E_{\text{RN}}$ implies that $N_{\text{HD}}$ increases on cooling. That the large $E_{\text{HD}}$ is an artifact of imposing the two-state model (with temperature-independent D-state structure) is also suggested by the result of a fit to a restricted data set including only the MRD profiles at the three highest temperatures (the ones shown in Figure 5). In this fit, which is of slightly better quality than the fit to all six profiles, there is no significant difference between $E_{\text{HD}}$ and $E_{\text{RN}}$ (Table 1).

### 4.5. Kinetics of Cold Denaturation

In analyzing the $^{17}O$ relaxation data, we have tacitly assumed that the measurements were carried out on samples containing an equilibrium ensemble of protein molecules. We thus assumed that the protein conformations present in the sample are given by the Boltzmann distribution. However, because the rate of protein unfolding varies strongly with temperature, cold denaturation may be too slow to be observed on the experimental time scale, even though the D state is more stable than the N state. In other words, the protein may be kinetically trapped in the N state at low temperatures. To observe cold denaturation, the sample equilibration time, $t_{\text{eq}}$, must be much shorter than the conformational relaxation time, $\tau_{\text{rel}}$, defined as

$$\tau_{\text{rel}}(T) = \frac{1}{k_u(T) + k_d(T)}$$

where $k_u$ and $k_d$ are the folding and unfolding rate constants (for a two-state protein). For the temperature profiles, the samples were equilibrated 25–30 min at the measurement temperature and kept at 4 °C for at least 2 h between measurements at different temperatures. For the MRD profiles, the samples were maintained at the fixed temperature for several hours while performing relaxation measurements at the lower magnetic fields. Thus, $t_{\text{eq}} \approx 0.5–3$ h.

The fact that we do observe cold denaturation in the temperature profile of aMb (part c of Figure 3) shows that, at least for this protein, $\tau_{\text{rel}} \ll 1$ h even at −20 °C. This conclusion is consistent with the CD observation of aMb cold denaturation on cooling from +20 to −9 °C at a rate of 0.5 K min$^{-1}$. The question that we must now address is whether also mUb and bLg, for which we do not observe cold denaturation, would unfold sufficiently rapidly at low temperatures. A quantitative analysis (section S5 of the Supporting Information) indicates that the N state of mUb is not kinetically trapped but is actually thermodynamically stable down to −32 °C, whereas bLg may be kinetically trapped in the N state (on our experimental time scale) if $T_{\text{cd}} < 0$ °C.

In an attempt to extend the experimental time scale, we measured the water−$^{17}O$ $R_1$ in emulsion samples (≈1 mM bLg, pH 2.6) that were continuously maintained at different fixed temperatures between 0 and −30 °C. These measurements were performed at a fixed low frequency (2.6 MHz), where unfolding should be manifested as a substantial reduction in $R_1$ due to the loss of the long-lived internal water molecules. On the basis of these results (section S6 of the Supporting Information), we can conclude that $T_{\text{cd}} < −5$ °C for bLg in water at pH 2.6. If $T_{\text{cd}}$ were ≥ −5 °C, we would have seen the cold denaturation. If $T_{\text{cd}}$ is lower, bLg will be kinetically trapped in the N state on our experimental time scale. Because dimeric bLg at pH 7.2 is not likely to have drastically faster (un)folding kinetics than the monomer at pH 2.6, we can also conclude that the structural changes indicated at low temperatures (with an apparent $T_{\text{cd}}$ of −36 °C) do not correspond to complete cold denaturation.

## 5. Conclusions

The molecular basis of the generic stability maximum of soluble globular proteins and the associated phenomenon of cold denaturation is not fully understood, but hydration effects are generally thought to play a key role. Yet, changes in protein hydration on cold denaturation have not been investigated experimentally. Here, we have addressed this fundamental issue by using water−$^{17}O$ spin relaxation to monitor the hydration of four proteins from room temperature down to −35 °C. Such low temperatures could be reached without ice formation by containing the protein solution in picoliter emulsion droplets with $\sim 10^6$ protein molecules. Control experiments demonstrated that containment does not influence the system or the measurements. The turbidity and inhomogeneous magnetic susceptibility of emulsions severely limit the number of physical techniques that can be applied to study the contained protein solution. Optical spectroscopy studies of emulsified protein solutions have been reported, but it is not clear that the observed changes in UV absorbance reflect cold denaturation rather than subunit dissociation or localized structural changes.

Few, if any, proteins have been shown to cold denature under otherwise native conditions. Although most, or all, proteins are expected, on thermodynamic grounds, to cold denature in water, freezing of the solvent or slow unfolding kinetics usually prevent observation of the transition. In accordance with this experience, none of the three unmodified proteins examined here (BPTI, mUb, and bLg) was found to cold denature in water above −32 °C. For BPTI and mUb, the native state is expected to remain stable down to this temperature, whereas, for bLg, cold denaturation is too slow to be observed on the time scale set by the limited lifetime of the supercooled emulsion droplets. For bLg at pH 2.6, our data indicate that the cold denaturation temperature is lower than −5 °C, which is consistent with the available thermodynamic data on this protein. For dimeric bLg at pH 7.2, we infer hydration changes with a likely structural origin, but they are not characteristic of cold denaturation. Our finding that mUb remains folded down to −32

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°C is consistent with a cold-denaturation temperature of −45 °C, as predicted by extrapolation of data on the urea-assisted cold denaturation of mUb. Furthermore, our results suggest that the cold denaturation observed at −20 °C for mUb encapsulated in reverse micelles is induced by the low water content rather than by the low temperature per se. The one protein for which we did observe cold denaturation in water is apomyoglobin, which is destabilized by heme removal. Our ability to directly monitor hydration changes and to access lower temperatures than hitherto indicates that cold denaturation of aMb is not a simple two-state transition.

As expected from previous studies, we find that bLg cold denatures at 10 °C in 4 M urea. Our data indicate that the cold-denatured protein is relatively compact, in agreement with SAXS data, and that it expands on further cooling. For both bLg and aMb, the cold-denatured protein is thus better described as solvent-penetrated than as unfolded. If this turns out to be a general feature of cold-denatured proteins, then the widely accepted analogy between cold denaturation and the aqueous solubility of small nonpolar molecules is misleading. This conclusion follows because water penetration of the hydrophobic core and transfer of nonpolar side-chains into water have opposite thermodynamic signatures: low temperature facilitates transfer of nonpolar molecules into water but not transfer of water into nonpolar media.

The results presented here and elsewhere show that the hydration layer of native proteins, with the exception of a small fraction of secluded hydration sites, is indistinguishable form the hydration shell of a small organic solute. In contrast, even an extensively unfolded, random-coil-like protein is expected to be hydrated in an essentially different way from a fully extended polypeptide chain or a solution of small molecules. Even a random-coil-like protein is likely to contain regions where water molecules bridge polypeptide segments with strong conformational preferences and relatively slow dynamics. The more compact cold-denatured state may contain strings and sheets of water molecules embedded in, and interacting strongly with, the disordered protein. The thermodynamic and kinetic properties of such water molecules are likely to differ substantially from those of water in the hydration shell of a small molecule surrounded by bulk water.

The view outlined here is opposite to the conventional one, where the native protein has unusual hydration properties and the denatured protein is hydrated like a collection of small molecules. So far, theoretical studies of the mechanism of cold denaturation have either used oversimplified models, or considered only the N state. It seems clear, however, that a full understanding of cold denaturation will require further experimental characterization of the structure and hydration of cold-denatured proteins.

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Supporting Information Available: Spin relaxation data, equations, and figures; table of samples investigated by 17O MRD. This material is available free of charge via the Internet at http://pubs.acs.org.

Supporting Information

Protein cold denaturation as seen from the solvent

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S1. Water $^{17}$O Spin Relaxation in Solutions of Coexisting Native and Denatured Proteins

The water-$^{17}$O spin relaxation rate, $R_1(\omega_0, T)$, in a solution containing only native protein at a concentration specified by the water/protein mole ratio $N_w$ can be expressed as:

$$R_1(\omega_0, T) = \left(1 - \frac{N_H + N_I}{N_w}\right)R_0^0(T) + \frac{N_H}{N_w} \left[R_0^H(\omega_0, T) + \frac{N_I}{N_w} \left[\tau_1(T) + 1/R_1^I(\omega_0, T)\right]^{-1}\right]$$  \hspace{1cm} (S1)

where $R_0^0(T)$ is the $^{17}$O spin relaxation rate for a solvent-only reference sample and the angular brackets signify averages over all sites in each hydration class. Water exchange between the hydration layer (H) and bulk solvent is assumed to be fast compared to the intrinsic relaxation rate $R_1^H$, but this need not be the case for internal water molecules (I). The intrinsic relaxation rate for internal water molecules is given by:

$$R_1^I(\omega_0, T) = \omega_{Q1}^2 \left[0.2 J_I(\omega_0, T) + 0.8 J_I(2\omega_0, T)\right]$$  \hspace{1cm} (S2)

where $\omega_{Q1} = 7.60 \times 10^6$ s$^{-1}$ is the $^{17}$O nuclear quadrupole frequency$^1$ and the spectral density function $J_I(\omega, T)$ is the cosine transform of the time correlation function.

$$G_I(t, T) = S_I^2 \exp\left[-\left(\frac{1}{\tau_p(T)} + \frac{1}{\tau_t(T)}\right)t\right]$$  \hspace{1cm} (S3)

Here, $S_I$ is the orientational order parameter for a particular internal hydration site with mean residence time $\tau_s$, and $\tau_p = 1/(6D_R)$ is the tumbling time of the protein. The rotational diffusion coefficient, $D_R$, in H$_2$O at 20 °C is given in Table S1 for the investigated proteins.$^3$ To obtain the tumbling time at other temperatures, we use the scaling relation $\tau_p(T) \propto \eta(T)/T$ with the viscosity $\eta$ appropriate for the water isotope composition in the solvent.$^4$ The viscosity of 4 M urea...
solution does not appear to have been reported at subzero temperatures, but the ratio, $\eta/\eta_0$, of the viscosities of 4 M urea and H$_2$O varies only weakly with temperature, increasing from 1.16 at 5 °C to 1.26 at 70 °C, so it can be extrapolated to subzero temperatures with some confidence. As seen from eqs (S1) and (S3), $R_1$ is independent of $\tau$ as long as $\tau << 1/R_1^1$.

The intrinsic relaxation rate, $R_{1H}$, for a water molecule in the hydration layer is modeled in the same way as $R_1^I$, except that we set $S_H = 1$ and interpret $\tau_H$ as the local water rotational correlation time (water exchange and rotation occur on the same time scale in the hydration layer). Whereas the internal-water average in eq (S1) is computed by explicitly summing over each internal hydration site (or group of 2–3 similar sites), the hydration-layer average involves hundreds of sites and must therefore be described by a continuous distribution of correlation times $\tau_H$. This distribution is wide, ranging from picoseconds to nanoseconds at room temperature, and it is highly skewed towards the short-$\tau_H$ end. MD simulations indicate that it can be represented by a power law:

$$f(\tau_H, T) = C(T) \tau_H^{-\nu}$$  \hspace{1cm} (S4)

with exponent $\nu$ and normalization constant $C$. The lower and upper limits of the power-law distribution are identified, at a reference temperature of 20 °C, with the bulk-solvent correlation time, $\tau_0$, and the protein tumbling time, $\tau_p$, respectively. Because the distribution is highly skewed towards shorter $\tau_H$, the upper limit is unimportant and the only additional parameter needed to complete the model is the Arrhenius activation energy, $E_H^-$, for the most mobile hydration water molecules. The average $\langle R_{1H}(\omega_0, T) \rangle$ in eq (S1) can be obtained in analytical form only in special cases (such as for $\nu = 2$), and it is therefore computed by numerical integration over the distribution in eq (S4).

For a protein that does not cold denature in the investigated temperature range and for which the internal-water parameters are known, as is the case for BPTI and ubiquitin, the two adjustable parameters ($\nu$ and $E_H^-$) can be determined from a fit to a temperature profile $R_1(\omega_0, T)$ at a fixed high frequency and extending to sufficiently low temperatures (so that the hydration-layer contribution is dominant). For a protein that undergoes cold denaturation, the model must be extended. We describe cold denaturation as a two-state equilibrium, $N \rightleftharpoons D$. The fraction native protein, $x_N$, is then related to the free energy difference, $\Delta G = G_D - G_N$, as
\[ x_N(T) = \left\{ 1 + \exp\left[ -\frac{\Delta G(T)}{k_B T} \right] \right\}^{-1} \]  

(S5)

The temperature dependence of \( x_N(T) \) can be approximately described by two parameters: the cold-denaturation midpoint temperature, \( T_{cd} \), and the width, \( \Delta T_{cd} \), of the cold-denaturation transition. These parameters are defined through the relations

\[ x_N(T_{cd}) \equiv \frac{1}{2} \]  

(S6a)

\[ \frac{1}{\Delta T_{cd}} \equiv \frac{dx_N}{dT} \bigg|_{T=T_{cd}} \]  

(S6b)

The cold-denaturation enthalpy, \( \Delta H_{cd} = H_D(T_{cd}) - H_N(T_{cd}) \) is related to the width parameter as

\[ \Delta H_{cd} = -\frac{4 k_B T_{cd}^2}{\Delta T_{cd}} \]  

(S7)

By integrating the Gibbs-Helmholtz identity and expanding \( \Delta C_p \) in powers of \( T \), we obtain an expansion of \( \Delta G \) in powers of the reduced temperature, \( \nu \equiv (T - T_{cd})/T_{cd} \),

\[ \Delta G(\nu) = -\Delta H_{cd} \nu + O(\nu^2) \]  

(S8)

We only need to model \( x_N(T) \) accurately in the transition region, where \( \nu \ll 1 \) so that the power series (S8) can be truncated after the leading term. We then obtain with eqs (S5) and (S7):

\[ x_N(T) = \left\{ 1 + \exp\left[ \frac{4T_{cd}}{\Delta T_{cd}} \left( \frac{T_{cd}}{T} - 1 \right) \right] \right\}^{-1} \]  

(S9)

In the case of two coexisting protein species (N and D), eq (S1) is generalized to

\[ R_1(\omega_0, T) = \left[ 1 - \frac{x_N (N_{HN} + N_{IN}) + (1 - x_N) N_{HD}}{N_W} \right] R_1^0(T) + \frac{x_N N_{HN}}{N_W} \langle R_1^{HN}(\omega_0, T) \rangle \]

\[ + \frac{(1 - x_N) N_{HD}}{N_W} \langle R_1^{HD}(\omega_0, T) \rangle + \frac{x_N N_{IN}}{N_W} \left[ \tau_i + 1/\tau_i^{IN}(\omega_0, T) \right]^{-1} \]  

(S10)

where we have assumed that the cold-denatured state has no long-lived internal hydration sites. In other words, water molecules that penetrate the denatured protein are treated as part of the hydration layer, which is described by a power-law correlation time distribution as in eq (S4), but with different exponent, \( \nu_D \), and activation energy, \( E_{HD}^- \), than for the native protein (\( \nu_N \), \( E_{HN}^- \)).
$E_{\text{HN}}$). Whereas $N_{\text{HN}}$ is calculated from the solvent-accessible surface area of the native protein (Table S1), the number, $N_{\text{HD}}$, of water molecules in contact with the denatured protein is a free parameter.

We present the relaxation data as the apparent dynamic perturbation factor (ADPF), defined as

$$
\xi(\omega_0, T) \equiv 1 + \frac{N_W}{N_{\text{HN}}} \left[ \frac{R_i(\omega_0, T) - R_i^0(T)}{R_i^0(T)} \right]
$$

(S11)

The ADPF is obtained directly from the measured quantities $R_i(\omega_0, T)$, $R_i^0(T)$, and $N_W$ and from the ‘hydration number’ $N_{\text{HN}}$ calculated from the native protein structure (Table S1). By normalizing the data in this way, we remove the effect of protein concentration and most of the effects of protein size and solvent composition. Furthermore, in the limit $\omega_b = 0$ (or when all correlation times are short compared to $1/\omega_b$), the ADPF reduces to the true (rather than apparent) DPF, $\xi(T) \equiv \langle \tau \rangle / \tau_0$, which relates hydration-water dynamics to bulk-water dynamics in an essentially model- and method-independent way. It follows from eqs (S10) and (S11), that the ADPF can be decomposed into contributions from internal water molecules, the N-state hydration layer and the D-state ‘hydration layer’ as

$$
\xi(\omega_0, T) = x_N(T) \frac{N_{\text{IN}}}{N_{\text{HN}}} \left[ \xi_{\text{IN}}(\omega_0, T) - 1 \right] + x_N(T) \xi_{\text{HN}}(\omega_0, T)
$$

$$
+ [1 - x_N(T)] \left\{ 1 + \frac{N_{\text{HD}}}{N_{\text{HN}}} \left[ \xi_{\text{HD}}(\omega_0, T) - 1 \right] \right\}
$$

(S12)

with the partial ADPFs defined as

$$
\xi_{\text{IN}}(\omega_0, T) = \left\langle \left[ R_i^0(T) \tau_i + R_i^0(\omega_0) / R_i^{\text{IN}}(\omega_0, T) \right] \right\rangle^{-1}
$$

(S13a)

$$
\xi_{\text{HN}}(\omega_0, T) = \frac{\langle R_i^{\text{HN}}(\omega_0, T) \rangle}{R_i^0(T)}
$$

(S13b)

$$
\xi_{\text{HD}}(\omega_0, T) = \frac{\langle R_i^{\text{HD}}(\omega_0, T) \rangle}{R_i^0(T)}
$$

(S13c)

In the figures of the main paper, the contributions to the total ADPF, $\xi(\omega_0, T)$, from the three terms in eq (S12) are colored red, blue and yellow, respectively.
S2. Contribution to the $^{17}$O Spin Relaxation Rate from Internal Water Molecules

S2.1. BPTI and Ubiquitin. BPTI contains four internal water molecules, but one of them exchanges too slowly to contribute to $R_1$ in the temperature range investigated here.\textsuperscript{9} The parameters of the other three internal water molecules have been determined by MRD studies of rotationally immobilized proteins\textsuperscript{8} and were fixed to these values in the fit of Fig. 2a ($\tau = 6 \mu$s for two and $\tau = 90$ ns for one water molecule at 27 °C, with average activation energy 40 kJ mol$^{-1}$ and average order parameter $S_I = 0.95$). Ubiquitin has only one internal water molecule ($\tau = 23$ ns at 27 °C with activation energy 35 kJ mol$^{-1}$ and $S_I = 0.60$).\textsuperscript{8}

S2.2. Apomyoglobin. The MRD profile at 27 °C (Fig. 3a) is more extended than expected for a single internal-water residence time. This is also evident from an earlier $^{17}$O MRD profile for aMb (in D$_2$O solution and at slightly higher protein concentration).\textsuperscript{10} Both MRD profiles indicate that aMb contains one internal water molecule with a residence time much longer than the protein tumbling time, $\tau_p = 9$ ns (at 27 °C), but shorter than the intrinsic $^{17}$O relaxation time of 2.4 μs and ~ 3 internal water molecules with a residence time of ~ 2 ns (at 27 °C). In analyzing the data in Fig. 3, we fix the long residence time to $\tau_{i1} = 200$ ns with an activation energy of 40 kJ mol$^{-1}$ (as for BPTI). The precise value of $\tau_{i1}$ is unimportant as long as $\tau_p \ll \tau_{i1} \ll 1/R_1$, so that $\tau_{i1}$ does not influence $R_1$ (see sect. S1). Furthermore, we treat the short residence time, $\tau_{i2}$, for the 3 other internal water molecules and the common order parameter, $S_I$, as adjustable parameters. The fit yields $\tau_{i2} = 1.7 \pm 0.4$ ns and $S_I = 0.89 \pm 0.04$, in the expected range. Because the internal-water contribution to the ADPF is small in the temperature range where the D state is populated (cf the red areas in Fig. 3b,c), the internal-water parameters need not be accurately known to extract information about cold denaturation.

S2.3. β-Lactoglobulin. The β-barrel fold of bLg encloses a large (315 Å$^3$) internal cavity, known as the calyx, that binds fatty acids and other nonpolar molecules.\textsuperscript{11} Being lined almost exclusively by aliphatic carbon atoms, the calyx is completely empty (free from water) in the apo form present in our samples.\textsuperscript{12} Crystal structures,\textsuperscript{11,13} MD simulations\textsuperscript{12} and $^{17}$O MRD data\textsuperscript{12,14} indicate that bLg contains 2 – 3 internal water molecules (buried in small cavities distinct from the calyx) with residence times of ~ 20 ns (at 27 °C)\textsuperscript{12} as well as several water molecules, presumably located in surface pockets, with residence times of a few ns. In analyzing the ADPF data at pH 2.6, we assume that bLg contains 2 internal water molecules with a residence time $\tau_{i1}$
= 20 ns (frozen in the fit) and 3 additional water molecules with adjustable residence time $\tau_{12}$. The fit yields $\tau_{12} = 2.9 \pm 0.7$ ns (at 27 °C), a common activation energy of $48 \pm 4$ kJ mol$^{-1}$, and a common order parameter $S_I = 0.89 \pm 0.03$. Because all internal water molecules are in the fast-exchange limit, the ADPF depends on the number of internal water molecules and their order parameter only through the combination $N_I S_I^2$ which equals 1.6 and 2.4 for the two groups of internal water molecules. It may be noted that we explicitly include internal water molecules with residence times shorter than the upper bound ($\tau_p = 9$ ns at 27 °C) of the power-law distribution (see sect. S1). This overlap of the internal-water and hydration-layer classes should be regarded as a correction to the long-$\tau_{fi}$ tail of the power-law distribution, which, for bLg, only predicts 0.45 water molecules with $\tau_{fi} > 1$ ns (at 20 °C).

A comparison of Figs. 4a and 5a shows that 4 M urea has little effect on the internal-water contribution (red area) to the ADPF. The only visible difference is a slight stretching of the dispersion in Fig. 5a, which can be explained if urea binding slows down the conformational fluctuations that govern the exchange of the most long-lived internal water molecules.$^{15}$ In the global fit, we thus assume that these two water molecules have residence times much longer than the tumbling time of bLg. Specifically, we set $\tau_{1i} = 200$ ns (at 27 °C), but the precise value does not influence the fit (see above). The internal-water activation energy is frozen to the same value as in the absence of urea ($48$ kJ mol$^{-1}$). The fit then yields $\tau_{12} = 1.6 \pm 0.6$ ns and $S_I = 0.79 \pm 0.04$, not far from the values obtained in the absence of urea.

As at pH 2.6, we assign 2 long-lived internal water molecules to bLg at pH 7.2 and the activation energy for water exchange is fixed at the same value as found from the pH 2.6 fit. Because of additional secluded hydration sites at the periphery of the dimer interface,$^{12,16}$ we assign to the dimer five (as compared to three in the monomer) water molecules (per bLg molecule) with residence times of a few nanoseconds. The fit yields $\tau_{1i} = 60 \pm 10$ ns, $\tau_{12} = 2.8 \pm 0.5$ ns (at 27 °C) and $S_I = 0.76 \pm 0.03$. The threefold increase of $\tau_{1i}$ from the monomer value can be attributed to constraints on conformational fluctuations in the dimer and the 15 % smaller root-mean-square order parameter $S_I$ is reasonable since the seven internal water molecules per protein molecule in the dimer should be less constrained on average than the five internal water molecules in the monomer.
S3. The Cold Denaturation Temperature of β-Lactoglobulin in Water

In the absence of denaturant and at pH 2.0, bLg heat denatures at $T_{hd} = 78 ^\circ C$ with $\Delta H_{hd} = 312 \text{ kJ mol}^{-1}$ and $\Delta C_p = 5.6 \text{ kJ K}^{-1} \text{ mol}^{-1}$.\textsuperscript{17,18} Inserted into the standard two-state thermodynamic relations, these values (ignoring any temperature dependence in $\Delta C_p$) yield $T_{cd} = –22.6 ^\circ C$. This prediction of the cold-denaturation temperature is likely to be too low for several reasons. First, the thermodynamic parameters used to predict $T_{cd}$ were determined at pH 2.0 and in the presence of 100 mM KCl,\textsuperscript{17,18} whereas we study bLg at pH 2.6 and in the absence of salt or buffer. The net charge of bLg ($Z \approx +20$) changes very little between pH 2.0 and 2.6, but 100 mM salt should preferentially stabilize the N state of the highly charged protein. Thus, we expect a higher $T_{cd}$ under our salt-free solutions. Second, $\Delta C_p$ is usually not constant, but tends to increase below $T_{hd}$ and exhibit a broad maximum between $T_{cd}$ and $T_{hd}$.\textsuperscript{19} If we assume that the relative temperature dependence of $\Delta C_p$ is the same as for ubiquitin, then the bLg parameters yield $T_{cd} = –2.2 ^\circ C$. Third, studies of the heat denaturation of bLg have produced a range of $\Delta C_p$ values, often larger than 5.6 kJ K$^{-1}$ mol$^{-1}$.\textsuperscript{20} A larger $\Delta C_p$ would raise $T_{cd}$ further. Fourth, at least in the presence of urea, cold denaturation of bLg is not a simple two-state process, but appears to involve a substantially populated intermediate state.\textsuperscript{17,18,21,22} An equilibrium intermediate would raise the apparent $T_{cd}$. In summary, the equilibrium cold denaturation midpoint temperature, $T_{cd}$, of bLg in water at pH 2.6 should be significantly higher than –22.6 °C.

S4. Water-Urea Exchange Equilibrium in the Hydration Layer

In analysing the data for bLg in 4 M urea, we must recognize that urea partly displaces water at the protein surface. We thus express the number of hydration water molecules for the N state as

$$N_{HN} = \theta_N N_{HN}^0 = \frac{N_{HN}^0}{1 + K_N a_U^c/a_W^x} \quad (S14)$$

where $N_{HN}^0$ is the hydration number in the absence of urea and $\theta_N$ is the fraction of the surface occupied by water in the presence of urea. In the last form of eq (S14), we use the solvent-exchange model\textsuperscript{23,24} to express $\theta_N$ in terms of the urea binding constant $K_N$ (to the surface of the native protein), the urea activity (molarity scale), $a_U^c = 3.67$ M and the water activity (mole fraction scale), $a_W^x = 0.926$ in 4.0 M aqueous urea.\textsuperscript{25} From previous studies,\textsuperscript{25,28} we expect that $K_N = 0.05 – 0.20$ M$^{-1}$. The $^{17}$O relaxation data do not allow us to determine the binding constant,
so we fix it to the plausible value $K_N = 0.10 \text{ M}^{-1}$, corresponding to $\theta_N = 0.716$. For the cold-denatured state, $N_{HD}$ is given by an expression analogous to eq (S14).

In the global fit, increasing $K_N$ from 0.05 to 0.20 M$^{-1}$ decreases the number of water molecules in the N-state solvation layer from 613 to 410. With fewer water molecules in the solvation layer, a larger dynamical perturbation (larger $\xi_H$) is needed to account for the experimental data. However, the model parameters are either insensitive to $K_N$ variations (for example, $T_{cd} = 10.0 \pm 0.3 \text{ °C}$ for any $K_N$ value in the range $0.05 - 0.20 \text{ M}^{-1}$) or vary in a trivial way. Thus, with increasing $K_N$, the parameter $N_{HD}/N_{HN}$ increases in proportion to $1/N$, meaning that the number of D-state hydration water molecules remains constant at $N_{HD} = 675 \pm 50$.

**S5. Kinetics of Cold Denaturation for Ubiquitin and β-Lactoglobulin**

Mammalian ubiquitin folds on the millisecond time scale at room temperature, with $k_f = 1500 \text{ s}^{-1}$ at 25 °C and pH 5.0. To estimate the relaxation time $\tau_{\text{rel}}$ in eq (2) at low temperatures, we note that $k_u / k_i = \exp[-\Delta G(T)/(k_B T)]$. The denaturation free energy, $\Delta G$, is obtained from the usual two-state thermodynamic relations with $T_{\text{hd}} = 90 \text{ °C}$, $\Delta H_{\text{hd}} = 298 \text{ kJ mol}^{-1}$ and temperature-dependent $\Delta C_p$. Further, we write $k_u = k_0 \exp[-\Delta G_u^0(T)/(k_B T)]$ with the elementary rate constant, $k_0 = 10^7 \text{ s}^{-1}$, taken to be independent of temperature. (A tenfold variation of $k_0$ changes $\tau_{\text{rel}}$ by less than a factor 2.) The unfolding activation energy is given a weak quadratic temperature dependence, $\Delta G_u'(T) = \Delta G_u^0(298) - \sigma (T - 298)^2$ with $\sigma = 10^{-3}$, to introduce the typical slight curvature in the Arrhenius plot of $\ln k_u$ versus $1/T$. In this way, we obtain $\tau_{\text{rel}} = 14 \text{ min at } -30 \text{ °C}$. However, the $\Delta G(T)$ function obtained from the calorimetric data predicts a cold-denaturation temperature, $T_{cd} = -22 \text{ °C}$, that is substantially higher than the value, $T_{cd} = -45 \text{ °C}$, obtained from direct observation of (part of) the cold denaturation in the presence of denaturant and extrapolation to zero GdmCl concentration. We therefore added a linear correction to $\Delta G(T)$ to make $T_{cd} = -45 \text{ °C}$ without affecting the experimentally determined $T_{\text{hd}}$. With this corrected $\Delta G(T)$ function, we find $\tau_{\text{rel}} = 2 \text{ min at } -30 \text{ °C}$. This estimate of $\tau_{\text{rel}}$ is crude, but it is 1 – 2 orders of magnitude shorter than the experimental equilibration time, $\tau_{\text{eq}}$. We therefore conclude that the N state of mUb is not kinetically trapped, but is actually thermodynamically stable down to –32 °C.

Bovine β-lactoglobulin has much slower folding kinetics than aMb or mUb. In the presence of 4 M urea at pH 2, the $C_p$ peak occurs 9 °C higher on heating than on cooling at a
scan rate of 1 K min\(^{-1}\). This hysteresis indicates that \(\tau_{\text{rlx}}\) is in the range 1 – 10 min at \(T_{\text{cd}}\) (\(~ 10 ^\circ\text{C}\) ), five orders of magnitude longer than for mUb\(^{29,34}\) but still shorter than \(\tau_{\text{eq}}\) in our experiments (Fig. 5). To estimate \(\tau_{\text{rlx}}\) at subzero temperatures in the absence of urea, we proceed as for mUb (see above). Despite indications of a more complex folding behavior for bLg, we compute \(\Delta G(T)\) from the two-state model with \(T_{\text{hd}} = 78 ^\circ\text{C}, \Delta H_{\text{hd}} = 312 \text{ kJ mol}^{-1}\) and constant \(\Delta C_p = 5.6 \text{ kJ K}^{-1}\) mol\(^{-1}\).\(^{17,18}\) (These parameters yield \(T_{\text{cd}} = -22.6 ^\circ\text{C}\) in the two-state model.) The rate constants \(k_f\) and \(k_u\) have been determined at 15.5 °C and pH 2.8 at several urea concentrations, \(C_U > 4 \text{ M}\).\(^{35}\) The logarithm of these rate constants depends linearly on \(C_U\), as expected,\(^{31}\) and extrapolation to \(C_U = 0\) yields \(k_f = 4.3 \times 10^{-2} \text{ s}^{-1}\) and \(k_u = 4.2 \times 10^{-8} \text{ s}^{-1}\). The equilibrium constant obtained from the ratio of these rate constants corresponds to \(\Delta G = 21.7 \text{ kJ mol}^{-1}\), not far from the value 22.5 kJ mol\(^{-1}\) computed at 15.5 °C from the calorimetric data.\(^{17,18}\) Taking \(k_0 = 10^7 \text{ s}^{-1}\) and \(\sigma = 10^{-3}\), as for mUb, we obtain \(\tau_{\text{rlx}} = [12 \text{ min, 59 min, 6.4 h, 22 d, 237 d}]\) at \(T = [0, -5, -10, -20, -30] ^\circ\text{C}\), respectively. If \(\Delta G\) is linearly adjusted to raise \(T_{\text{cd}}\) by 10 °C to –12.4 °C (as for mUb), we find \(\tau_{\text{rlx}} = [23 \text{ min, 2.3 h, 15 h, 4.8 d, 11 d}]\) at the same temperatures. These estimates indicate that, on our experimental time scale, bLg may be kinetically trapped in the N state if \(T_{\text{cd}} < 0 ^\circ\text{C}\).

**S6. Time-dependent \(^{17}\text{O} \) Spin Relaxation Measurements for \(\beta\)-Lactoglobulin**

We measured the water-\(^{17}\text{O} \) \(R_1\) in emulsion samples (\(~ 1 \text{ mM bLg, pH 2.6}\) that were continuously maintained at different fixed temperatures between 0 and –30 °C. These measurements were performed at a fixed low frequency (2.6 MHz), where unfolding should be manifested as a substantial reduction in \(R_1\) due to the loss of the long-lived internal water molecules. At subzero temperatures, the experimental time scale is limited by water freezing by two different mechanisms. First, at moderate supercooling, droplet coalescence causes liquid droplets to freeze by heterogeneous nucleation when they come into contact with (the initially very small fraction of) frozen droplets. Second, the rate of homogeneous nucleation increases strongly on cooling,\(^{36}\) leading to rapid freezing of all droplets at temperatures just below –35 °C. The fraction frozen water in the sample can be monitored \textit{in situ} via the integrated intensity of the \(^{17}\text{O} \) resonance. For the emulsion samples used here, we thus found a practical time limit of \(~ 10 \text{ h at –20 °C and ~ 1 h at –30 °C (Fig. S1a)}\). Within these limits, and up to \(~ 100 \text{ h at 0 and –5 °C, we found no significant variations in } R_1 \text{ (Fig. S1b)}\).
**Figure S1.** (a) Fraction frozen water in an emulsion sample maintained continuously at the indicated temperature (in °C). (b) Relative variation of water-$^{17}$O $R_i$ at 2.6 MHz for emulsion samples of bLg (pH 2.6) maintained continuously at the indicated temperature (in °C). The −30 °C data represent two separate runs. The error bars are 1% at −30 °C and 0.5% at the other temperatures. The ordinate ranges from 0.97 to 1.03 in each of the four panels.
Table S1. Samples investigated by $^{17}$O MRD.

<table>
<thead>
<tr>
<th>ID #</th>
<th>Protein</th>
<th>Solvent$^a$</th>
<th>pH $^b$</th>
<th>$10^{-3} \times N_w$</th>
<th>T (°C)</th>
<th>$v_0$ (MHz)</th>
<th>$N_{HN}$</th>
<th>$D_R^0$ (μs$^{-1}$) $^c$</th>
</tr>
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<td>1</td>
<td>BPTI</td>
<td>H$_2$O/emu</td>
<td>5.2</td>
<td>5.59</td>
<td>[–34.7, +14.9]</td>
<td>81.3</td>
<td>380</td>
<td>42.5</td>
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<td>2</td>
<td>mUb</td>
<td>H$_2$O/emu</td>
<td>5.0</td>
<td>10.5</td>
<td>[–31.9, +14.9]</td>
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<td>443</td>
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<td>aMb</td>
<td>H$_2$O</td>
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<td>34.7</td>
<td>+27</td>
<td>[2.2, 67.8]</td>
<td>719</td>
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<tr>
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<td>aMb</td>
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<td>5.8</td>
<td>34.7</td>
<td>–20 &amp; +27</td>
<td>[2.2, 67.8]</td>
<td>719</td>
<td>15.6</td>
</tr>
<tr>
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<td>5.8</td>
<td>63.0</td>
<td>[–31.9, +14.9]</td>
<td>81.3</td>
<td>719</td>
<td>15.6</td>
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<td>HDO</td>
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<td>70.6</td>
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<td>15.2</td>
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<td>–20 &amp; +27</td>
<td>[2.2, 6.1]</td>
<td>735</td>
<td>15.2</td>
</tr>
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<td>7</td>
<td>bLg</td>
<td>H$_2$O/emu</td>
<td>2.7</td>
<td>55.7</td>
<td>[–34.7, +14.9]</td>
<td>81.3</td>
<td>735</td>
<td>15.2</td>
</tr>
<tr>
<td>8</td>
<td>bLg</td>
<td>urea/HDO</td>
<td>2.6</td>
<td>70.6</td>
<td>–1.1, +10.2 &amp; +19.4</td>
<td>[1.3, 81.3]</td>
<td>526$^d$</td>
<td>15.2</td>
</tr>
<tr>
<td>9</td>
<td>bLg</td>
<td>urea/H$_2$O/emu</td>
<td>2.6</td>
<td>48.4</td>
<td>–20, –25 &amp; –30</td>
<td>[2.2, 67.8]</td>
<td>526$^d$</td>
<td>15.2</td>
</tr>
<tr>
<td>10a</td>
<td>bLg</td>
<td>HDO</td>
<td>7.2</td>
<td>65.4</td>
<td>+27</td>
<td>[0.57, 81.3]</td>
<td>691$^e$</td>
<td>6.72</td>
</tr>
<tr>
<td>10b</td>
<td>bLg</td>
<td>HDO/emu</td>
<td>7.2</td>
<td>65.4</td>
<td>–5, +2 &amp; +27</td>
<td>[0.57, 81.3]</td>
<td>691$^e$</td>
<td>6.72</td>
</tr>
<tr>
<td>11</td>
<td>bLg</td>
<td>HDO/emu</td>
<td>7.2</td>
<td>62.9</td>
<td>–15</td>
<td>[0.57, 81.3]</td>
<td>691$^e$</td>
<td>6.72</td>
</tr>
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<td>12</td>
<td>bLg</td>
<td>H$_2$O/emu</td>
<td>7.0</td>
<td>60.1</td>
<td>–25 &amp; [–34.7, +14.9]</td>
<td>[0.57, 81.3]</td>
<td>691$^e$</td>
<td>6.72</td>
</tr>
</tbody>
</table>

$^a$ ‘HDO’ refers to H$_2$O/D$_2$O mixtures with 50 or 52 atom% $^2$H; ‘urea’ to 4 M aqueous urea; ‘emu’ to emulsified protein solutions.

$^b$ Uncorrected for H/D isotope effect (in HDO solvent).

$^c$ Rotational diffusion coefficient of native protein in H$_2$O at 20 °C.

$^d$ Corrected for urea binding with $K_N = 0.10$ M$^{-1}$ (see text).

$^e$ Corrected for dimerization (see text).
References


