The Cold Denatured State Is Compact but Expands at Low Temperatures: Hydrodynamic Properties of the Cold Denatured State of the C-terminal Domain of L9

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A point mutation of a small globular protein, the C-terminal domain of L9 destabilizes the protein and leads to observable cold-denaturation at temperatures above zero. The cold denatured state is in slow exchange with the native state on the NMR time scale, and this allows the hydrodynamic properties of the cold unfolded state and the native state to be measured under identical conditions using pulsed-field gradient NMR diffusion measurements. This provides the first experimental measurement of the hydrodynamic properties of a cold unfolded protein and its folded form under identical conditions. Hydrodynamic radii of the cold-induced unfolded states were measured for a set of temperatures ranging from 2 °C to 25 °C at pD 6.6 in the absence of denaturant. The cold unfolded state is compact compared to the urea or acid unfolded state and a trend of increasing radii of hydration is observed as the temperature is lowered. These observations are confirmed by experiments on the same protein at pD 8.0, where it is more stable, in the presence of a modest concentration of urea. The expansion of the cold-denatured state at lower temperatures is consistent with the temperature dependence of hydrophobic interactions.

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Introduction

There has been considerable interest in the properties of unfolded proteins, both those unfolded under strongly denaturing conditions and those unfolded under mild native-like conditions.¹–⁷ Much of the motivation for the work has been provided by the realization that unfolded states are not classic random coils and instead can contain significant amounts of residual structure and can be relatively compact.¹²,⁸–²⁰

Abbreviations used: CTL9, the C-terminal domain of the ribosomal protein L9; CTL9-I98A, isoleucine to alanine point mutant of CTL9 at residue 98; kobs, the observed first-order rate constant measured in a stopped-flow experiment; ku, the rate constant for protein unfolding; PFG-NMR, pulsed-field gradient nuclear magnetic resonance; Rh, the radius of hydration; Tm, the midpoint of the high temperature thermal unfolding transition.

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It is well known that proteins can also be unfolded by cooling; so-called cold denaturation.²¹ Comparatively little structural characterization has been applied to these states although a number of detailed thermodynamic studies have been reported.²¹,²² In particular, it is not known how compact these states are and how their hydrodynamic properties compare to other unfolded states. The phenomenon of protein cold denaturation has been known for several decades, and the existence of such a cold-denatured state is predicted by the Gibbs–Helmholtz equation. Theoretically, cold denaturation should be a universal property for all proteins, reflecting interactions between water and protein molecules. Unfortunately, direct characterization of the cold induced unfolded state is generally hard to achieve in the absence of denaturant or extreme pH values because, for most proteins, the cold denaturation temperature is far below 0 °C. There have been multiple attempts to reach the cold-denatured state without strongly perturbing the environment. Studies have been reported which monitor cold denaturation in the presence of denaturant²¹,²³ and of super-cooled
proteins encapsulated in micelles. These studies have provided insight into the cold denatured state but do not directly probe the cold unfolded state under near native conditions. There are two ways to monitor cold denaturation under more native-like conditions: (1) in super-cooled water, which has been attempted without much success; and (2) moving the cold denaturation temperature to above 0 °C by modifying properties of the protein.

Here we explore the cold denatured state of the C-terminal domain of L9 (CTL9). CTL9 is a small mixed α–β protein whose thermal, denaturant induced and pH induced unfolding have been described (Figure 1). The cold denatured state of a point mutant of CTL9 can be populated under conditions where a significant population of the folded form is still present. The two states are in slow exchange in the NMR time scale allowing the hydrodynamic properties of each state to be measured by pulse-field gradient NMR diffusion measurements. NMR based diffusion measurements have been successfully applied to a range of folded and unfolded proteins and are a robust method for determining hydrodynamic properties.

Results

The I98A mutant of CTL9 undergoes cold denaturation at experimentally accessible temperatures

As part of a Φ-value analysis of the folding transition state of CTL9, we systematically examined the consequences of hydrophobic core mutations. Mutation of the buried residue I98 to alanine significantly destabilizes the protein but also allows cold denaturation to be observed above 0 °C. Figure 1 shows the position of residue I98. It is located in the second α-helix, and is part of the hydrophobic core, forming part of the interface between the helix and the central β-sheet. The mutant adopts the native fold as judged by the far-UV CD spectrum and the 1H-NMR spectrum (data not shown).

The stability of CTL9-I98A, like wild-type CTL9, strongly depends upon the pH, due to protonation of one or more of the histidine residues (Figure 1). The protein is more stable when these residues are deprotonated and neutral. Cold denaturation is not evident at pH 8.0, but is when the pH is lowered to pH 6.6 (Figure 2(a)). The addition of modest amounts of urea also makes cold denaturation observable at pH 8.0 (Figure 2(b)). Thermodynamic parameters for the cold-induced unfolding at both conditions are listed in Table 1. ΔC_p° was determined by fitting the unfolding curves to a two-state model. ΔC_p° is related to the difference in solvent accessible surface area between the native and denatured states. Thermodynamic analysis indicates that the temperature of cold denaturation increases as ΔH° at T_m decreases and ΔC_p° increases. Our data are consistent with the mutant having a larger ΔC_p° and a smaller ΔH°(T_m) value than wild-type. The unfolded state of CTL9 has been shown to be compact in the absence of denaturant, likely due to hydrophobic cluster formation. Truncation of the I98 side-chain to Ala might reduce the unfolded state hydrophobic clusters and result in a larger change in surface area upon unfolding and thus a larger ΔC_p°. The lower ΔH°(T_m) for I98A is likely a consequence of poorer native state packing caused by the mutation.

Hydrodynamic measurements reveal a compact cold-induced unfolded state that expands with decreasing temperature

Separate well-resolved resonances from the folded and cold unfolded state are observed in the NMR

Figure 1. Ribbon diagram of CTL9 prepared using MOLSCRIPT. The position of I98 is shown. The three histidine residues are labeled as are the N terminus and C terminus. The side-chain of Y126, whose resonance is followed in PFG-NMR experiments, is indicated.
spectrum of the mutant (Figure 3). The peaks are in slow exchange on the NMR time scale. This was directly confirmed by stopped-flow experiments at both room temperature and 15 °C (Figure 4). Experimental determined exchange rates are listed in Table 2. The observed exchange rate, \( k_{\text{obs}} = k_f + k_u \), is 6.5 s\(^{-1}\) at 25 °C in native buffer at pD 6.6. It decreases to 1.3 s\(^{-1}\) at 15 °C. Thus, a slower interconversion is expected at lower temperatures. This shows that the rate of interconversion is slow with respect to the diffusion time in the NMR experiment, 100 ms. This is critical because it means that the two forms of the protein can be treated as independent species. This assumption is clearly valid for the pD 6.6 sample at 15 °C and below, and is close to being satisfied at 25 °C. The same trend is observed for the sample in 1.4 M urea. An exchange rate of 2.0 s\(^{-1}\) at 25 °C and 0.5 s\(^{-1}\) at 15 °C is measured. Thus, the pD 8.0, 1.4 M urea sample is in slow exchange with respect to the diffusion time under all conditions studied. This allows the hydrodynamic properties of both the folded and unfolded forms to be measured under identical conditions by pulsed-field gradient (PFG)-NMR, and offers a unique opportunity to characterize the cold denatured state. We tested whether or not the cold unfolded state has molten globule-like properties by conducting ANS binding studies. Neither the pD 6.6 nor pD 8.0 1.4 M urea cold unfolded states

![Figure 2](image-url)  
Thermal-denaturation curve of the I98A mutant monitored by CD at 222 nm (a) at pD 6.6 without urea; (b) at pD 8.0 with 1.4 M deuterated urea. The continuous line represents the best fit with a variable \( \Delta C_p^° \) value. A 50 mM sodium phosphate, 100 mM NaCl in 100% \( ^2\)H\(_2\)O buffer was used for both experiments.

![Figure 3](image-url)  
(a) 1D \(^1\)H-NMR spectra of CTL9 mutant I98A at different temperatures in 100% \( ^2\)H\(_2\)O at pD 6.6 (uncorrected pH meter reading) in the absence of urea. (b) 1D \(^1\)H-NMR spectra of CTL9 mutant I98A at different temperatures in 100% \( ^2\)H\(_2\)O at pD 8.0 (uncorrected pH meter reading) in the presence of 1.4 M urea. Only the aromatic region is shown. Peaks due to Y126 in the folded state (F) and unfolded state (U) are labeled as is a resonance due to H106.

**Table 1.** Thermodynamic parameters for the unfolding of wild-type CTL9 and the mutant I98A under different solvent conditions derived from CD monitored thermal unfolding

<table>
<thead>
<tr>
<th>Condition</th>
<th>Wild-type</th>
<th>I98A mutant (no urea)</th>
<th>I98A mutant (1.4 M urea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pD 8.0</td>
<td>80.7</td>
<td>53.3</td>
<td>42.6</td>
</tr>
<tr>
<td>( \Delta H^°(T_m) ) (kcal mol(^{-1}))</td>
<td>69.0±2.4</td>
<td>42.9±0.6</td>
<td>26.6±1.2</td>
</tr>
<tr>
<td>( \Delta C_p^° ) (kcal mol(^{-1}) deg(^{-1}))</td>
<td>1.07±0.08*</td>
<td>1.44±0.02</td>
<td>1.30±0.05</td>
</tr>
</tbody>
</table>

* \( \Delta C_p^° \) for wild-type was determined by analysis of the Gibbs-Helmholzt plot as described.38

All measurements were performed in 100% \( ^2\)H\(_2\)O with 50 mM sodium phosphate and 100 mM NaCl. Quoted uncertainties are the standard error to the fit.
Cold Denaturation of CTL9

Figure 4. Chevron plots of the mutant I98A at pH 6.6 at 25 °C (open circles) and at 15 °C (filled circles). The continuous line represents the best fit to equation (4).

Table 2. Observed exchange rates of CTL9 I98A under different solvent conditions measured by stopped-flow fluorescence

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>pH 6.6 (no urea)</th>
<th>pH 8.0, 1.4 M urea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed k_{obs} (s⁻¹)</td>
<td></td>
</tr>
<tr>
<td>25 °C</td>
<td>6.5±0.01</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>15 °C</td>
<td>1.3±0.01</td>
<td>0.5±0.05</td>
</tr>
</tbody>
</table>

a Experiments were performed in 50 mM sodium phosphate, 100 mM NaCl buffer. Standard errors to the fits are given.
b Observed rate constants which were derived from fits to a double exponential, correspond to the sum of the folding and unfolding rates in 1.4 M urea. The standard errors for the determined folding and unfolding rates are less than 10%.

cold induced unfolded state is observed to expand with decreasing temperature, both for the pH 6.6 sample and for the sample at pH 8.0 in 1.4 M urea. At pH 6.6, 12 °C, the hydrodynamic radius of the cold unfolded state is 21.7 Å in the absence of urea, and this increases to 25.3 Å at 2 °C. At pH 8.0 1.4 M urea, the value of R_h of the cold unfolded state increases from 23.1 Å at 25 °C to 27.8 Å at 2 °C.

Table 3. Hydrodynamic radii for the native and cold denatured states of the I98A mutant of CTL9

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>pH 6.6 (no urea)</th>
<th>pH 8.0, 1.4 M urea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folded protein</td>
<td>Unfolded protein</td>
</tr>
<tr>
<td>2</td>
<td>18.7±1.3</td>
<td>25.3±1.5</td>
</tr>
<tr>
<td>4</td>
<td>18.0±1.3</td>
<td>22.1±1.5</td>
</tr>
<tr>
<td>7</td>
<td>17.3±1.3</td>
<td>21.7±1.5</td>
</tr>
<tr>
<td>12</td>
<td>17.2±1.3</td>
<td>17.0±1.5</td>
</tr>
<tr>
<td>25</td>
<td>17.2±1.3</td>
<td>17.0±1.5</td>
</tr>
</tbody>
</table>

All measurements were performed in 100% 2H2O. The experimental uncertainty is estimated to be ±5% based on repeated measurements.
a Uncorrected pH meter reading in 2H2O.
b The population of the native state is not large enough for reliable measurements.
c Not determined.
d The population of the unfolded state is not large enough for reliable measurements.

Discussion

Direct measurement of hydrodynamic radii for both unfolded and folded proteins allows comparison of the two states under identical conditions. As expected, the cold unfolded state is more expanded than the folded state. The cold induced unfolded state of the mutant is, however, compact compared to the denaturant induced or acid unfolded state of the wild-type. The unfolded state of the wild-type is expected to be even more compact under the same conditions than the mutant, since the mutation should attenuate the hydrophobic clusters in the denatured state. Our observation of a larger ΔC_p value for the mutant supports this conjecture.

The hydrodynamic radius of the unfolded state increases with decreasing temperature while the radius of hydration of the folded state remains relatively constant. It is known that the R_h of the unfolded state of wild-type is sensitive to pH and expands when the histidine residues are protonated, but we can rule out small changes in pH as a function of temperature as the cause of the significant expansion of the cold unfolded state, since we also observe an expansion of the pH 8.0 cold-denatured state. This pH is safely above the unfolded state pK_a values of the histidine residue. The expansion of the unfolded state due to cold denaturation is consistent with the temperature dependence of hydrophobicity, i.e. hydrophobic interactions are weaker at lower temperature.21,37
To the best of our knowledge, the data presented here are the first reported of the hydrodynamic properties of a cold unfolded state under conditions where the native state is also significantly populated. The two principle observations of this study are first that the cold denatured state is compact relative to the denaturant induced unfolded state and secondly that it expands as the temperature is lowered. The observation that the cold denatured state expands at lower temperature has obvious implications for cold denaturation studies of encapsulated proteins. Studies of the cold denaturation behavior of the CTL9 mutant under near physiological conditions present a promising model system for studying cooperativity in folding and protein solvent interactions.

**Material and Methods**

**Mutagenesis, protein expression and purification**

Primers were purchased from Operon and plasmids containing the primer were generated and amplified by PCR. The mutation was confirmed by DNA sequencing. Protein was overexpressed and purified as described. The purity was confirmed by reverse phase HPLC. Circular dichroism spectroscopy

Experiments were performed using an Aviv model 62A D8 spectrometer. Far-UV CD spectra were taken at different temperatures. For thermal denaturation experiments monitored at 222 nm, the protein concentration was 8–12 μM in 50 mM sodium phosphate and 100 mM sodium chloride, 100% 2H2O buffer. Experiments were carried out at either pD 6.6 without urea or pD 8.0 with 1.4 M urea (uncorrected pH value read directly from a pH meter). Thermal unfolding data were fit by assuming that the folded and unfolded baselines are a linear function of absolute temperature:

\[
\theta_d(T) = a_0 + b_0 T \\
\theta_u(T) = a_d + b_d T
\]

Gibbs–Helmholtz equation describes the temperature dependence of \(\Delta G_u\):

\[
\Delta G_u^o(T) = \Delta H^o(T_m)(1 - T/T_m) - \Delta C_p^o[(T_m - T) + T \ln(T/T_m)]
\]

where \(\Delta G_u^o(T)\) is the free energy of unfolding, \(T_m\) is the heat induced unfolding midpoint temperature, \(\Delta H^o(T_m)\) is the enthalpy change at \(T_m\), and \(\Delta C_p^o\) is the heat capacity change between the native and denatured states. Fits to individual thermal unfolding curves are fairly insensitive to the initial value of \(\Delta C_p^o\) for conventional unfolding experiments. However, if cold denaturation is observed, it is possible to obtain a precise value of \(\Delta C_p^o\) from the curve fits. \(T_m\), \(\Delta H^o(T_m)\) and \(\Delta C_p^o\) under each condition were determined by fitting the individual thermal unfolding curves to the following equation:

\[
\theta(T) = [(a_0 + b_0 T) + (a_d + b_d T) \exp(-\Delta G_u^o(T)/RT)]/[1 + \exp(-\Delta G_u^o(T)/RT)]
\]

**Pulse-field gradient NMR experiments**

Protein samples were prepared as described. NMR data were acquired on a Bruker 700MHz spectrometer. Pulse-field gradient NMR experiments were used to measure the diffusion coefficients of 198A at different temperatures. The resonances for residue Y126 in both the folded and unfolded states were followed. The linearity of the field gradient was pre-calibrated. The build-in pseudo-2D version of the PFG-NMR experiment developed by Bruker, diffusion-ordered spectroscopy (DOSY), was used for experiment setup and data processing. The diffusion delay, \(\Delta\), was 100 ms and the gradient pulse width, \(d\), was 6 ms. Gradient strengths were increased from 2% to 95% of the maximum strength in a linear fashion.

The diffusion coefficient of dioxane was obtained independently by repeating the same experiment on dioxane in buffer pD 6.6 or in 1.4 M urea (pD 8.0) in the absence of protein under exactly the same solvent conditions (data not shown). Results were compared with the diffusion coefficient of dioxane derived from samples containing protein at the same temperature. This allows the accuracy of the measurements to be estimated. Excellent consistency was observed for both protein samples in native buffer at pD 6.6 and in 1.4 M urea at pD 8.0, indicating that dioxane does not interact with the protein in the temperature range studied. The reported hydrodynamic radius of dioxane 2.12 Å was used for the calculation of the hydrodynamic radius of the protein, \(R_h\). Errors in the protein diffusion coefficient were estimated to be ±5% based on multiple measurements, so only differences greater than 10% in hydrodynamic radii were considered to be real.

**Stopped-flow fluorescence**

Stopped-flow fluorescence experiments were performed using an Applied Photophysics SX.18MV stopped-flow reaction analyzer equipped for asymmetric mixing at a ratio of 10:1 (v/v). The only tyrosine residue in the primary sequence of CTL9 was used as the fluorescent probe, with excitation at 276 nm and emission monitored at 305 nm. The folding measurements were initiated by an 11-fold dilution of the prepared completely denatured protein solution into lower concentrations of urea. The unfolding was initiated by an 11-fold dilution into higher concentrations of urea. Final protein concentrations were approximately 50–100 μM. The resulting curves at given urea concentrations were fit using a double exponential to obtain the first-order rate constants for each phase. The major phase was always used for analyses. The minor slow phase is believed to be due to proline isomerization. Each curve was an average of three to five individual shots. The chevon plots of \(\ln k_{obs}\) versus urea concentration were fit to the following equation:

\[
\ln k_{obs} = \ln k_0(H_2O)\exp[n_1[denaturant]/RT] + k_1(H_2O)\exp[n_2[denaturant]/RT]
\]
where $k_1(H_2O)$ and $k_2(H_2O)$ are the folding and unfolding rate constants in the absence of denaturant; $m_1$ and $m_2$ are constants that describe how $\ln k_1$ and $\ln k_2$ vary as a function of the concentration of denaturant. The urea concentration was determined by refractometry.

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References


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