Eur Biophys J (2006) 35: 363–366
DOI 10.1007/s00249-005-0028-4

BIOPHYSICS LETTER

Thomas Szyperski · Jeffrey L. Mills · Dieter Perl
Jochen Balbach

Combined NMR-observation of cold denaturation in supercooled water and heat denaturation enables accurate measurement of ΔC_p of protein unfolding

Received: 27 July 2005 / Revised: 23 September 2005 / Accepted: 28 September 2005 / Published online: 21 October 2005

Abstract Cold and heat denaturation of the double mutant Arg 3 → Glu/Leu 66 → Glu of cold shock protein Csp of *Bacillus caldolyticus* was monitored using 1D ^{1}H NMR spectroscopy in the temperature range from −12°C in supercooled water up to +70°C. The fraction of unfolded protein, f_u, was determined as a function of the temperature. The data characterizing the unfolding transitions could be consistently interpreted in the framework of two-state models: cold and heat denaturation temperatures were determined to be −11°C and 39°C, respectively. A joint fit to both cold and heat transition data enabled the accurate spectroscopic determination of the heat capacity difference between native and denatured state, ΔC_p of unfolding. The approach described in this letter, or a variant thereof, is generally applicable and promises to be of value for routine studies of protein folding.

T. Szyperski (✉) · J. L. Mills
Department of Chemistry, The State University of New York, Buffalo, NY 14260, USA
E-mail: szypersk@chem.buffalo.edu
Tel.: +1-716-6456800
Fax: +1-716-6456963

Present address: J. L. Mills
E633B School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

Present address: D. Perl
Biotechnology Development, Novartis Pharma AG, CH-4002 Basel, Switzerland

J. Balbach · D. Perl
Laboratorium für Biochemie, Universität Bayreuth, 95440 Bayreuth, Germany

Present address: J. Balbach
Fachbereich Physik, Fachgruppe Biophysik, Martin-Luther-Universität Halle-Wittenberg, 06099 Halle (Saale), Germany

Keywords Protein cold denaturation · Cold shock protein · Protein folding thermodynamics · Supercooled water

Introduction

The accurate thermodynamic characterization of protein stability (Jaenicke 2000) is of central importance for unraveling the relation between amino acid sequence and tertiary fold of a polypeptide chain. Despite advances in recent years (Buchner and Kiefhaber 2005), our current understanding of protein folding pathways is far from enabling us to predict protein structures. In fact, the entirety of structure prediction methods we have at hand today is based on previously solved experimental atomic resolution structures (Rost et al. 2003). One might argue that the complexity of the folding problem is at least comparable to the structural complexity of proteins (Szyperski 2002, 2005). In turn, this suggests that a 'semi-empirical solution' of the protein folding problem in the framework of structural genomics is required (Montelione 2001; Szyperski 2002). Irrespective of the open questions regarding the nature of the solution to the protein folding problem, efficient methodology is needed to thermodynamically characterize the folding process. Such studies are invaluable complements for both a putative algorithmic and/or a semi-empirical solution of the folding problem, and they are pivotal for future rational protein design.

Thermodynamics of protein (un)folding can be studied at increased temperatures ('heat denaturation') or at very low temperatures ('cold denaturation'). For reversibly unfolding proteins, heat denaturation can be readily assessed by various experimental techniques and has been studied in detail for a larger number of systems. Cold denaturation, in contrast, can be expected for most proteins to occur at temperatures well below the freezing point of water at 0°C and 1 atm (Privalov 1990). Various approaches have been developed to overcome the obstacle of studying a system at temperatures where the

solvent's thermodynamically most stable state is the solid state. Those include the use of cryosolvents (Franks 1995), the application of high pressure (Jonas 2002), the generation of supercooled aqueous solutions in water/oil emulsions (Franks 1982, 1995), or the addition of chaotrops (Agashe and Udgaonkar 1995; Katou et al. 2001). All these approaches, however, are beset by the possibility that the folding reaction is not proceeding in an unperturbed aqueous solution. For example, cryosolvents can artificially stabilize native proteins (Jacob et al. 1997) and high pressure evidently impacts on the cold denaturation of proteins (e.g., Kitahara et al. 2001; Mills and Szyperski 2002), while denaturants and/or chaotrops interact differently with cold and heat denatured state (Griko and Privalov 1992). Alternatively, one may destabilize the protein by a change in pH (Privalov 1986), or generate cold-sensitive mutants (Chen and Schellman 1989) to enable observation of cold denaturation.

Recently, the feasibility of NMR-based structural biology in supercooled water was demonstrated (Skalicky et al. 2000, 2001; Mills and Szyperski 2002): the use of capillary tubes (Poppe and van Halbeek 1994) allows one to supercool aqueous solutions of biological macromolecules, which are placed in the vibration-free large magnets of high-field NMR spectrometers, down to temperatures around −15°C for long periods of time. Here we explore the use of NMR spectroscopy in supercooled water for studying the cold denaturation of proteins. Specifically, we show that such studies pave the way to accurately determine the heat capacity difference between native and denatured state, ΔC_p, when combined with monitoring the heat denaturation transition. Since ΔC_p is an essential thermodynamic parameter for characterizing the subtle balance of driving forces leading to the distinct spatial fold of a protein, the protocol presented here can be expected to be of broader interest.

Materials and methods

For the present study, we focused on the Arg 3 → Glu/Leu 66 → Glu double mutant of cold shock protein Csp from *Bacillus caldolyticus*, a small β-sheet protein comprising 66 residues (Müller et al. 2000). Thermodynamic parameters derived by use of circular dicroism (CD) spectroscopy (Perl and Schmid 2001) from the heat denaturation transition predict that cold denaturation occurs around −26°C, suggesting that the onset of the cold denaturation can be observed at temperatures down to ∼ −15°C (which can be routinely achieved when using capillary tubes).

1D ^{1}H NMR spectra were recorded for a 0.1 mM Csp solution (85% H_2O/15% D_2O; 20 mM sodium cacodylate/HCl; pH 7.0) filled in nine 1.0 mm OD capillary tubes as was described (Skalicky et al. 2000, 2001; Mills and Szyperski 2002). The cacodylate buffer ensures that pH shifts with temperature can be neglected (Deutscher 1990). The spectra were recorded on a Varian INOVA 500 spectrometer and the water NMR line was suppressed using the Watergate scheme (Piotto et al. 1992). Between 256 (T = 70°C) and 4,096 transients (T = −12°C) were accumulated, yielding a total measurement time of about 40 h.

Figure 1 shows the high-field region (which comprises methyl group resonances) of 1D ^{1}H NMR spectra recorded for the R3E/L66E-Csp capillary sample between 70°C and −12°C. The spectral region annotated as 'integral' contains solely signals arising from the native, folded protein. As described in the following, the integral $I(T)$ of this region allows one to measure the fraction of native protein, $f_n(T)$, as a function of the temperature, T, and enables one to determine the heat capacity difference between native and denatured state, ΔC_p.

Previous CD measurements (Perl and Schmid 2001) indicated that even at the temperature of maximal stability, T_{max}∼25°C, a few percent of the Csp protein remain unfolded, that is, $f_n(T_{max}) < 1$. After normal-

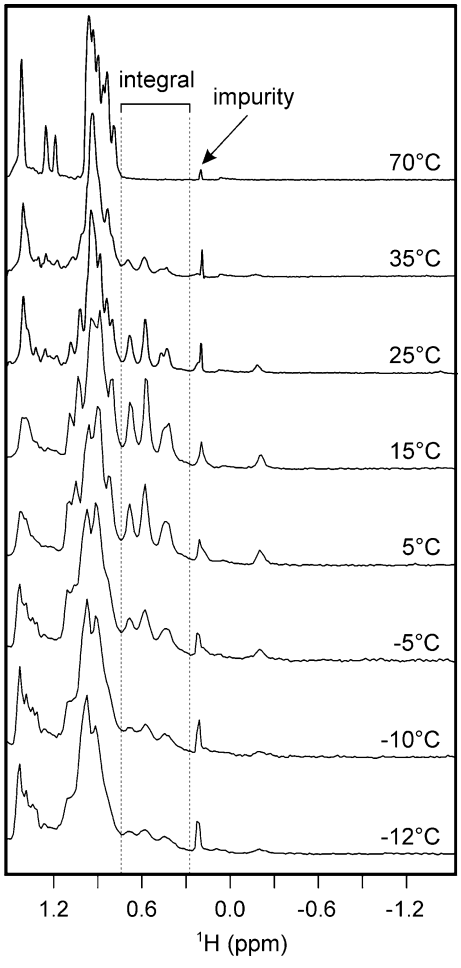

Fig. 1 High-field region of 1D ^{1}H NMR spectra recorded for the Arg 3 → Glu/Leu 66 → Glu double mutant of cold shock protein Csp from *Bacillus caldolyticus* at various temperatures. ^{1}H chemical shifts are relative to 2,2-dimethyl-2-silapentane5-sulfonate (DSS)

ization with the total intensity of the aliphatic region, $I_{tot}^{ali}(T)$, one obtains

$$f_n(T) = \frac{\left[\left(\frac{I(T)}{I_{tot}^{ali}(T)}\right) \cdot f_n(T_{max})\right]}{\left(\frac{I(T_{max})}{I_{tot}^{ali}(T_{max})}\right)} \quad (1)$$

with $f_u(T) = 1 - f_n(T)$ representing the fraction of unfolded protein. Considering that the equilibrium constant of unfolding is defined as

$$K_u(T) = \frac{f_u(T)}{f_n(T)} \quad (2)$$

one has with $f_u(T) = K_u(T)/[1 + K_u(T)]$ and

$$K_u(T) = \exp[-\Delta G_u(T)/RT] \Leftrightarrow \Delta G_u(T) = -RT \ln K_u(T) \quad (3)$$

that

$$f_u(T) = \frac{\exp[-\Delta G_u(T)/RT]}{1 + \exp[-\Delta G_u(T)/RT]} \quad (4)$$

where $\Delta G_u(T)$ denotes the Gibbs free energy of unfolding at a given T, and R represents the universal gas constant. Provided that the molar heat capacity difference between native and denatured state, ΔC_p, is temperature independent, the free energy difference is given (Privalov and Gill 1999) by

$$\Delta G_u(T) = \Delta H_u(T_h) \cdot \left(\frac{T_h - T}{T_h}\right) - \Delta C_p \left[T_h - T + T \cdot \ln\left(\frac{T}{T_h}\right)\right], \quad (5)$$

where T_h indicates the temperature at the mid-point of heat denaturation. Substitution of Eq. 5 into Eq. 4 yields f_u as a function of T, ΔH_u, T_h and ΔC_p. A least-squares fit of to experimental f_u-values then allows one to obtain $\Delta H_u(T_h)$ and ΔC_p. To obtain $f_n(T_{max})$ of Eq. 1, a grid search for minimal χ^2 yields the optimal value for $f_n(T_{max})$.

Results

Comparison of 1D ^{1}H NMR spectra (Fig. 1) recorded for Csp mutant Arg 3 → Glu/Leu 66 → Glu shows that both protein cold denaturation in supercooled water and heat denaturation are registered with the same NMR sample. $f_u(T)$-values (Fig. 2) obtained from the NMR spectra reveal that at −12°C more than 50% of the protein chains are unfolded. Moreover, a spectrum recorded at 25°C after the partial denaturation in supercooled water (data not shown) proved that the cold denaturation of the Csp mutant is reversible. The function $f_u(T, \Delta H_u, T_h, \Delta C_p)$ (Eqs. 4, 5) was fitted to experimental $f_u(T)$-values (solid line in Fig. 2) assuming 'mirror image thermodynamics' for cold and heat denaturation. This yielded with $f_n(T_{max}) = 0.92$:

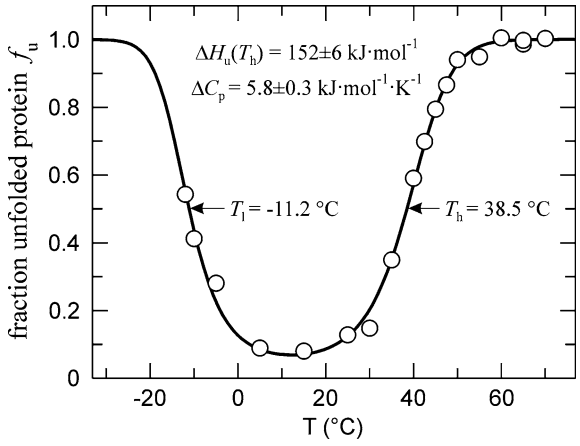

Fig. 2 Fractions of unfolded Arg 3 → Glu/Leu 66 → Glu double mutant of cold shock protein Csp, f_u, registered by 1D ^{1}H NMR spectroscopy (Fig. 1) versus temperature, T. The *solid line* indicates a fit (Eqs. 4, 5) to the plotted data points using the program GraFit 4.0. The values thus obtained for T_l, T_h, $\Delta H_u(T_h)$, and ΔC_p are indicated

$T_l = -11 \pm 0.5$°C, $T_h = 39 \pm 0.5$°C, $\Delta H_u(T_h) = 152 \pm 6$ kJ mol^{-1}, and $\Delta C_p = 5.8 \pm 0.3$ kJ mol^{-1} K^{-1}, where T_l defines the temperature at the mid-point of the cold denaturation transition. The goodness of the fit reveals that both high and low temperature data are well represented by the fitted curve. This can also be visualized (Fig. 3) by comparing the temperature dependence of $\Delta G_u(T)$ in Eq. 5 with $\Delta G_u(T)$ values calculated from $f_u(T)$ (Eqs. 2, 3); the curvature of $\Delta G_u(T)$ is rather well defined when having experimental data over a temperature range of >80°C covering both transitions.

The comparison of our thermodynamic parameters with those previously derived from CD heat denaturation data is impeded by the fact that the two studies were performed with different buffers and ionic strength,

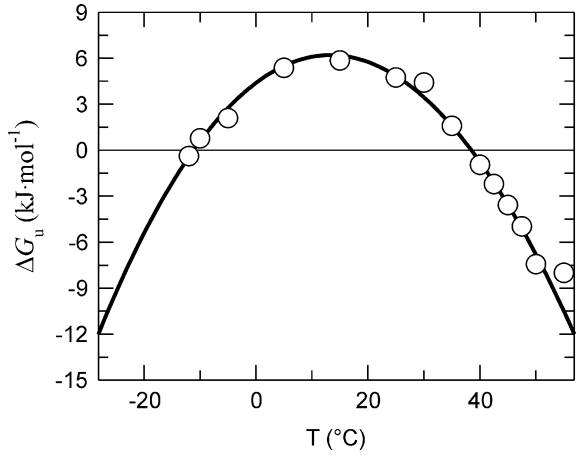

Fig. 3 Temperature dependence of the Gibbs free energy of unfolding, $\Delta G_u(T)$. The *circles* represent ΔG_u(T) derived from the NMR data (Fig. 1), and the *solid line* was calculated from Eq. 5 using the thermodynamic parameters derived from the joint fitting of cold and heat transition data

which modulate the thermodynamic stability of Arg 3 → Glu/Leu 66 → Glu Csp (Perl and Schmid 2001). Importantly, however, the previous determination of $T_h = 44.6 \pm 0.5$°C and $\Delta H_u(T_h) = 154 \pm 1$ kJ mol^{-1} *relied* on the assumption that $\Delta C_p = 4$ kJ mol^{-1} K^{-1} to obtain a high-quality fit to a curve representing a two-state model. The comparison with our parameters (Fig. 2) suggests that a reliable spectroscopic determination of ΔC_p may well depend on the joint characterization of heat and cold denaturation transitions. Possibly as a consequence of the comparably inaccurate ΔC_p-value, T_l was underestimated by ~15°C. In contrast to ΔC_p, about the same value was obtained for $\Delta H_u(T_h)$, indicating that this value is less sensitive to inaccuracies of ΔC_p-value.

Conclusions

Our study demonstrates that joint characterization of cold and heat denaturation transitions by NMR enables one to accurately measure an essential thermodynamic parameter of protein stability, that is, the difference in heat capacity of folded and unfolded state, ΔC_p. The advantage of studying cold denaturation has long been recognized (Privalov et al. 1986). However, only samples kept in an essentially vibration-free environment (which is required to retard formation of nucleation sites for freezing) such as heavy magnets of highest-field NMR spectrometers allows one to preserve supercooled aqueous solutions without adding chemicals or applying high pressure for an extended period of time. Moreover, most unfolded proteins do not exhibit methyl proton resonances upfield to 0.7 ppm, so that the approach introduced here can be expected to be quite generally applicable. An advantage of studying cold denaturation is certainly due to the reduced likelihood to encounter aggregation often preventing reversible unfolding. Since novel atomic resolution insights into the phenomenon of protein folding is also desirable, we thus expect that NMR in supercooled water shall contribute to characterizing the solution structure of folding intermediates.

Acknowledgements This work was supported by a Research Innovation Award of the Research Corporation (to T.S.) and the National Science Foundation (MCB 0416899 to T.S.), grants of the Deutsche Forschungsgemeinschaft (DFG 1821/2-1 and DFG 1821/3-1 to J. B.), and INTAS 2001-2347 (to J.B.). We thank M. Zeeb and F. X. Schmid for helpful discussions.

References

Agashe VR, Udgaonkar JB (1995) Biochemistry 34:3286–3299
Buchner J, Kiefhaber T (2005) Protein folding handbook. Wiley-VCH, Weinheim
Chen BL, Schellman JA (1989) Biochemistry 28:685–691
Deutscher MP, Simon MI, Abelson JN (1990) Guide to protein purification. In: Deutscher MP (ed) Methods in enzymology, vol 182, Academic, London, pp 24–37
Franks F (1982) Water: a comprehensive treatise. Plenum, New York
Franks F (1995) Adv Protein Chem 46:106–139
Griko YV, Privalov PL (1992) Biochemistry 31:8810–8815
Jacob M, Schindler T, Balbach J, Schmid FX (1997) Proc Natl Acad Sci USA 94:5622–5627
Jaenicke R (2000) J Biotechnol 79:193–203
Jonas J (2002) Biochim Biophys Acta 1595:145–159
Katou H, Hoshino M, Kamikubo H, Batt CA, Goto Y (2001) J Mol Biol 310:471–484
Kitahara R, Yamada H, Akasaka K (2001) Biochemistry 40:13556–13563
Mills JL, Szyperski T (2002) J Biomol NMR 23:63–67
Montelione GT (2001) Proc Natl Acad Sci USA 98:13488–13489
Müller U, Perl D, Schmid FX, Heinemann U (2000) J Mol Biol 297:975–988
Perl D, Schmid FX (2001) J Mol Biol 313:343–357
Piotto M, Saudek V, Sklenar V (1992) J Biomol NMR 6:661–665
Poppe L, van Halbeek H (1994) Nature Struc Biol 1:215–216
Privalov PL (1990) Crit Rev Biochem Mol Biol 25:281–305
Privalov PL, Gill SJ (1990) Adv Prot Chem 39:191–234
Privalov PL, Griko YV, Venyaminov S, Kutyshenko VP (1986) J Mol Biol 190:487–498
Rost B, Liu J, Przybyski D, Nair R, Wrzeszczynski KO, Bigelow H, Ofran Y (2003) In: Gasteiger J, Engel T (eds) Chemoinformatics—from data to knowledge. Wiley, New York, pp 1789–1811
Skalicky JJ, Sukumaran DK, Mills JL, Szyperski T (2000) J Am Chem Soc 122:3230–3231
Skalicky JJ, Mills JL, Sharma S, Szyperski T (2001) J Am Chem Soc 123:388–397
Szyperski T (2002) Nachr Chem 50:1128–1131
Szyperski T (2005) Protein NMR spectroscopy. In: Encyclopedia of molecular cell biology and molecular medicine. Wiley-CH, Weinheim