Cold Denaturation of Monomeric Peptide Helices

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From the perspective of thermodynamics, the stable conformation of globular proteins represents a delicate compromise between very large opposing entropy and enthalpy terms with the hydrophobic effect playing a significant role.[1] Cold denaturation studies, typically carried out with the addition of limited quantities of denaturants[2] or at nonoptimal pH[3] to reduce the enthalpic advantage of the folded state, have had a pivotal role in elucidating the thermodynamics of protein folding. Cold denaturation is expected for any macroscopic two-state unfolding when the unfolded state has a larger heat capacity; proteins with significant burial of fatty residues typically display ΔCp values of 60−70 (JK^{-1})/residue (res). Cold denaturation has been observed for molten globule states of proteins[4] and for helical peptide oligomers;[5] however, it has never been observed for a monomeric helical peptide.[6] Peptide helices display very broad melting transitions both calorimetrically and by circular dichroism (CD) and this precludes a direct measurement of ΔCp.[9] Calculations of the ΔCp expected for the unfolding of an alanine-rich helix range from −27 to +8.3 (JK^{-1})/res (based on the estimated changes in apolar versus polar surface area).[10] The largest ΔCp derived from experimental data for a peptide helix is +19 (JK^{-1})/res[6c]. We now report that dramatic cold denaturation can be observed in aqueous hexafluoroisopropanol (HFIP) for both amphiphilic helices and alanine-rich helices with zero hydrophobic moment. The phenomenon is illustrated with data collected for the four peptides shown here (the extent the helix in fluoroalcohol-rich media is indicated by underlining).

YGG-3X AcYGGKAXAAKAXAAKAXAAKNH$_2$

X = S, V, or T

sCT(8-32) VLGKLSQELHKLQTYPRTNTGSGTPNH$_2$

The CD traces recorded at the low- and high-temperature limit and at the temperature which displays maximal helicity for two of the peptides in 8 vol % HFIP appear in Figure 1. These spectra are remarkably similar to those reported[11] in a seminal study of cold denaturation using staphylococcal nuclease; the similarity extends to the observation of a shift in the "isodichroic" for denaturation to the red for cold denaturation versus melting.[12] To date we have observed, for more than 20 peptides, cold denaturation of helicity induced upon addition of HFIP (but not trifluoroethanol, TFE). These peptides are demonstrably monomeric[13] and display normal fluoroalcohol (FA) titration curves at constant temperature, with increasing helicity, as measured by $-[\theta]_{221}$, up to the point where a leveling effect occurs.[14] The manner in which FA increases helicity and the basis for the leveling effect remain a matter of contention.[14a,d−f,15] For the systems which we have examined, the coil → helix transition is in all cases sharper with HFIP (rather than TFE), particularly at subambient temperatures. The requisite for the observation of helix cold denaturation appears to be a sequence with modest helical propensity in water which is titrated to $\Delta G \approx 0$ at 4−10 vol % HFIP medium composition.

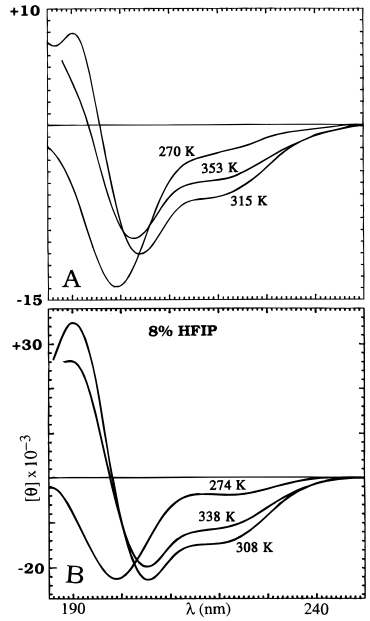

Figure 1. CD traces recorded during thermal studies of sCT(8-32) (50 μM, panel A) and YGG-3V (90 μM, panel B) in 8% (v/v) aqueous HFIP. The temperatures are indicated on the traces. All CD spectral values for peptides are expressed in units of amide bond molar ellipticity (deg·cm^2/amide dmol) based on the backbone amide bond count. Peptide concentrations were measured by UV spectroscopy of stock solutions in water, $\epsilon_{274}-\epsilon_{310} = 1405$. Aliquots of the stock solutions were added to previously prepared mixtures of HFIP and buffered aqueous medium (10 mM acetate, pH 4.2 ± 0.2).

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(6) (a) Several instances of a decrease in $-[\theta]_{221}$ have been reported for peptides displaying little or no helicity; this has been rationalized[7] on the basis of the thermal dependence of the CD for the coil state.[8] (b) A partially folded icosamer has been reported to display a broader distribution of end-to-end distances on cooling (Beals, J. M.; Haas, E.; Krausz, S.; Scheraga, H. A. *Biochemistry* **1991**, *30*, 7680−7692). (c) The thermal dependence of ^{13}C═O NMR resonances in a salt bridge stabilized alanine helix suggested ΔCp values as large as +19 (JK^{-1})/res (Shalongo, W.; Dugad, L.; Stellwagen, E. *J. Am. Chem. Soc.* **1994**, *116*, 2500−2507).
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(12) The absence of a single isodichroic point for the two limbs of a thermal two-state transition reflects the thermal dependence of the shape of the CD spectrum for the random coil state.[6a,8]
(13) The thermal dependence of $[\theta]$ for YGG-3V and -3T and sCT(8-32) in aqueous HFIP is concentration independent (5−90 μM). For other peptides, concentration independent cold denaturation has been observed over a much larger range of concentration (5 μM to 1.6 mM).
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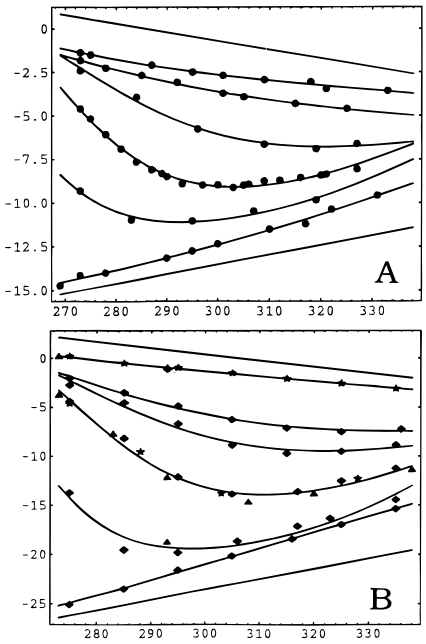

Figure 2. Plots of $[\theta]_{221} \times 10^{-3}$ versus temperature (K) at different volume percent HFIP for two peptides. The lines passing through points correspond to three parameter fits using eqs 1 and 2. In both panels, the top and bottom lines are, respectively, the coil and helical state base line employed to fit all runs. We employ the thermal dependence of $[\theta]_{221}$ in buffer with added Gdn·HCl ($\geq$7 M) as the coil base line. We approximate the helix base line as a line that is approached by the $[\theta]_{221}$ values at 25% HFIP as the temperature decreases. Panel A, 50 μM sCT(8-32): the fitted runs correspond, in descending order, to aqueous buffer alone and with HFIP added to 6, 8, 10, 12, and 25 vol %. The coil and helix base lines are given by $[\theta]^{C} = +650 - 50 \cdot T(°C)$ and $[\theta]^{H} = -15\,000 + 55 \cdot T(°C)$. Panel B, YGG-3V at three concentrations (5 μM, ★; 50 μM, ◆; 90 μM, ▲): the fitted runs correspond, in descending order, to aqueous buffer alone and with HFIP added to 6, 7, 8, 10, and 20 vol %. The coil and helix base lines are given by $[\theta]^{C} = +2125 - 64 \cdot T(°C)$ and $[\theta]^{H} = -26\,400 + 105 \cdot T(°C)$.

The complete $[\theta]_{221}$ temperature profiles for two of the peptides at varying levels of added HFIP appear in Figure 2. We are unable to fit this data using the polypeptide/FA binding model[14d] for rationalizing increasing helicity upon FA addition. Rather, we view the shift toward the helical state upon addition of FA as a reflection of decreased solvation of the exposed amide units of the coil state[14a,f] and the curvature in the thermal plots as the result of a solvophobic effect reflecting a positive ΔCp. The free energy of unfolding for an admittedly inappropriate macroscopic two-state model can be derived (with the folded, helical state as the reference state) from the CD data as

$$\Delta G(T) = -RT \ln[([\theta]^{obs} - [\theta]^{H})/([\theta]^{C} - [\theta]^{obs})] \quad (1)$$

Approximate thermodynamic parameters can then be extracted using eq 2:

$$\Delta G(T) = \Delta H^* - T\Delta S^* + \Delta Cp[T - T^* - T \ln(T/T^*)]^{1a} \quad (2)$$

Alternatively, the Lifson−Roig helix/coil model[16] can be employed with a temperature dependence introduced into the propagation parameter (w, a microscopic equilibrium constant for the states of individual residues).

$$RT \ln w = + \Delta H° - T\Delta S° + \Delta Cp[T - T° - T \ln(T/T°)] \quad (3)$$

The curves through the experimental points in Figure 2 are those obtained using the macroscopic two-state model of unfolding, eq 2 fitting for three variables (ΔH^*, ΔS^*, and ΔCp) with T^* set to 300 K which is within 10° of the temperature of maximal helicity (T_s) at 8−12% HFIP. The resulting ΔCp values depend on the concentration of HFIP, reaching values as large as +130 (JK^{-1})/helical-residue[17] in 10 vol % HFIP. Extrapolating to higher temperatures does not yield medium independent values for ΔH or ΔS.[18]

Since the helix/coil transition of a peptide cannot be assumed to be a macroscopic two-state equilibrium, we also employed eq 3 ($T° = 92$ °C) to derive ΔCp values for YGG-3V.[19] This model gave fits equally as good as those of eq 2, see supporting information. A temperature dependent w was employed only for the residues in the helical segment.[17] Once again, ΔCp values are maximal at at 8−10% HFIP and somewhat larger than those derived from the cooperative two-state model (+140 $\pm$ 10 JK^{-1} versus 110 $\pm$ 10 JK^{-1}) and the derived conformational entropies (21 $\pm$ 2 JK^{-1}) are in accord with expectations.

A comparison of the thermal behavior within the YGG-3X series provides support for our contention that the cold denaturation observed is indeed a solvophobic effect associated with differential exposure of nonpolar surface. YGG-3T displays unfolding upon cooling in 10% HFIP that is as dramatic as that displayed by YGG-3V; while YGG-3S fails to unfold on cooling and displays facile thermal fraying at all HFIP/H_2O ratios (supporting information). If $\Delta\Delta$Cp(S→T) is attributed to a single exposed methyl group in each of three threonine side chains, the unfolding ΔCp due to methyl exposure is greater than 200 JK^{-1}. In any case, the data requires an enhanced solvophobic effect in the presence of limited amount of HFIP and supports the hypothesis that fluoroalcohol addition favors peptide helices by a medium effect that destabilizes the coil state[14f] rather than stabilizing interactions between the helix and the fluoroalcohol.[14d] While the ΔCp for helix unfolding is smaller in water (and aqueous TFE),[20] helix formation may still represent a significant portion of the hydrophobic driving force for the formation of early protein folding intermediates. These results are also further evidence that helix formation, by itself, may play a significant role in the stabilization of the folded states of some proteins.[21]

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Supporting Information Available: Tabulation of derived parameters for the thermal denaturation of YGG-3V and sCT(8-32), comparisons of fits of YGG-3V using the macroscopic two-state versus a modified Lifson−Roig model, and figures showing the thermal dependence of $[\theta]_{221}$ in aqueous HFIP for YGG-3S and YGG-3T (3 pages). See any current masthead page for ordering and Internet access instructions.

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(17) In the peptides studied, there are residues at both ends of the sequence which remain "random coil" in the folded state. These should not contribute to the thermodynamic parameters for unfolding. The residues that are helical in the folded state are shown on the peptide sequences.

(18) The absence of a single convergence temperature could be attributed to the inappropriateness of the model, a temperature-dependent ΔCp, or to a wider difference in the entropy and enthalpy convergence temperatures in these media. At high temperature, the nonideal behavior of aqueous solutions of both proteins and hydrocarbons disappears (105−125 °C in water, see: Privalov, P. L. *Adv. Protein Chem.* **1979**, *33*, 167−241. Baldwin, R. L. *Proc. Nat. Acad. Sci. U.S.A.* **1986**, *83*, 8069−8072. Makhatadze, G. I.; Privalov, P. L. *Protein Sci.* **1996**, *5*, 507−510). Other studies[10a] have established that the experimental convergence temperatures for ΔS and ΔH can differ, and there is no basis for assuming that a similar temperature would apply to aqueous HFIP medium.

(19) CD data were converted to fractional helicities, and a modified Lifson−Roig model was employed to derive the w value for the $(KAXAA)_3$ segment at each temperature. Three parameter (ΔCp, $\Delta H°$ and $\Delta S°$) fits to eq 3 were performed at each medium composition with an alternative fit with $\Delta S°$ restricted to values greater than +15 (JK^{-1})/res.

(20) Our search of physicochemical studies of alcohol/water mixtures has located only one measure by which HFIP is distinguished from both TFE and iPrOH: HFIP/water mixtures display a large negative excess molar enthalpy of mixing at all mole fractions (Denda, M.; Touhara, H.; Nakanishi, K. *J. Chem. Thermodyn.* **1987**, *19*, 539−542).

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