Evidence for Many Unique Solution Structures for Chymotrypsin Inhibitor 2: A Thermodynamic Perspective Derived from vT-ESI-IMS-MS Measurements

Shannon A. Raab, Tarick J. El-Baba, Daniel W. Woodall, Wen Liu, Yang Liu, Zane Baird, David A. Hales, Arthur Laganowsky, David H. Russell, and David E. Clemmer

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ABSTRACT: Chymotrypsin inhibitor 2 (CI-2) is a classic model for two-state cooperative protein folding and is one of the most extensively studied systems. Alan Fersht, a pioneer in the field of structural biology, has studied the wild-type (wt) and over 100 mutant forms of CI-2 with traditional analytical and biochemical techniques. Here, we examine wt CI-2 and three mutant forms (A16G, K11A, L32A) to demonstrate the utility of variable-temperature (vT) electrospray ionization (ESI) paired with ion mobility spectrometry (IMS) and mass spectrometry (MS) to map the free energy folding landscape. As the solution temperature is increased, the abundance of each of the six ESI charge states for wt CI-2 and each mutant is found to vary independently. These results require that at least six unique types of CI-2 solution conformers are present. Ion mobility analysis reveals that within each charge state there are additional conformers having distinct solution temperature profiles. A model of the data at ~30 different temperatures for all four systems suggests the presence of 41 unique CI-2 solution conformations. A thermodynamic analysis of this system yields values of $\Delta C_p$ as well as $\Delta G$, $\Delta H$, and $\Delta S$ for each state at every temperature studied. Detailed energy landscapes derived from these data provide a rare glimpse into Anfinsen’s thermodynamic hypothesis and the process of thermal denaturation, normally thought of as a cooperative two-state transition involving the native state and unstructured denatured species. Specifically, as the temperature is varied, the entropies and enthalpies of different conformers undergo dramatic changes in magnitude and relative order to maintain the delicate balance associated with equilibrium.

INTRODUCTION

In their classic 1963 paper, “The Genetic Control of Tertiary Protein Structure: Studies With Model Systems”, Epstein, Goldberger, and Anfinsen argued that “According to [the thermodynamic] hypothesis, the particular conformation that a protein assumes, under any specific set of conditions, is the one that is thermodynamically the most stable.” Implicit in this statement is the idea that many different structures would be favored as one changes conditions that influence a protein’s stability (e.g., environmental factors such as temperature, solvent, and pH, as well as post-translational modifications, interactions with ligands, and formation of protein complexes). Computations support this. Unlike the simple solid-to-liquid phase transitions that occur upon destabilizing small-molecule crystals, denaturation of proteins involves distributions of structures. But, rarely is it possible to experimentally observe these higher-energy, low-abundance species even near the transition region for denaturation. Structural changes of polypeptide chains are observed as cooperative, two-state processes that occur when well-ordered, functional (native) structures become unstable—unfolding to produce distributions of non-native (denatured) states. And, very little is known about these non-native species.

In the work presented below, we use a variable-temperature (vT) electrospray ionization (ESI) source coupled with ion mobility spectrometry (IMS) and mass spectrometry (MS) techniques to investigate the thermal denaturation of a 64-residue truncated form of chymotrypsin inhibitor 2 (CI-2), a well-characterized, textbook model system that was developed by Fersht and his collaborators. In addition to wild-type (wt) CI-2, detailed studies were carried out on three mutants: a sequence in which a Gly residue is substituted for Ala16 (A16G), one having Ala substituted for Lys11 (K11A), and a mutant having Ala substituted for Leu32 (L32A). These data and analyses are provided in the Supporting Information (SI). The evaporative cooling phenomenon associated with ESI droplet shrinkage and ion formation “freezes out” populations of solution species, and in the absence of lubricating solvent, different antecedent states are trapped as ions, each with
distinct shape and charge, which can be investigated during
their short residence times in the spectrometer.\textsuperscript{25,23,24}

The truncated 64-residue form of CI-2 (Scheme 1) was
chosen for these vT-ESI-IMS-MS studies because the

![Scheme 1. Amino Acid Sequence (Top) and Cartoon
Representation (Bottom) of Truncated wt CI-2 (PDB:
2CI2), with Sites Chosen for Mutation in Bold](image)

transition between folded and denatured states is thoroughly
characterized.\textsuperscript{21} Crystallographic\textsuperscript{25} and solution nuclear
magnetic resonance\textsuperscript{26} studies show that under native
conditions CI-2 adopts an $\alpha/\beta$ configuration such that a
mixed parallel and anti-parallel $\beta$-sheet packs against a single $\alpha$
-helix to form the hydrophobic core.\textsuperscript{22} Differential scanning
calorimetry (DSC) and chemical denaturation measurements
are consistent with a simple process involving only two
states.\textsuperscript{21} Detailed studies of more than 100 mutant forms reveal
a weakly formed transition state, suggesting a nucleation–
condensation mechanism of folding where a structured nucleus
consolidates favorable long-range interactions to form the
native structure.\textsuperscript{27,28}

Because the truncated form of CI-2 is relatively small, it is
attractive for IMS-MS studies. Unlike larger proteins, small
peptides often show sharp, mobility-resolved peaks associated
with isolation of specific conformations.\textsuperscript{29–35} Mobility
measurements for larger proteins typically yield broad peaks,
\textsuperscript{36–38} indicating that many structures have similar cross
sections and thus are not resolved.\textsuperscript{39,40} We suspected that the
64-residue truncated CI-2 might allow similar conformer types
to be resolved, making it possible to discern many closely
related precursor and product species. As shown below, this is
the case. We find evidence for many discrete solution
structures. An analysis and model of these data provide evidence
that 41 structural transitions are involved in the
thermal denaturation of CI-2. From changes in the abundances
of these species with solution temperature, we extract detailed
thermochemistry ($\Delta G$, $\Delta H$, $\Delta S$, and $\Delta C_p$) for each state at 30
solution temperatures (from 22 to $80^\circ\text{C}$) over the range of the
melting transition. Many of the observed species exist in only
minute abundances. Because of the exceptional sensitivity and
dynamic range of IMS-MS techniques, it is straightforward to
quantify complex many-state solution equilibria where unique
conformers differ in abundances by over 4, or, in some cases,
even 5 orders of magnitude—far beyond the dynamic range
and detection limits of traditional calorimetric methods. The
ability to quantify species over such a wide range allows us to
determine a free energy landscape that includes species that are
much less stable than have ever been reported experimentally.
By definition, near each conformer’s melting temperature ($T_m$), its
enthalpy and entropy are balanced (i.e., $\Delta H \approx T \Delta S$). In
this paper, we show that above and below the $T_m$ of each,
this balance is harder to maintain, and slight offsets control the
populations of native and denatured states.

While these data provide a remarkable glimpse into the
intricate balance of populations of many species that appear
and disappear as a protein undergoes thermal denaturation, we
note that the issue of structural characterization of non-native
states remains elusive. But, the present data provide some clues
and opportunities for future studies. Collision cross sections
are a measure of the sizes of ions in the gas phase, and to the
extent that these can be computationally described and then
translated into solvated conformers, they provide a structural
constraint.\textsuperscript{41–44} Thermodynamic values provide additional
constraints that should be testable by theory. In addition to
insight about stability (from values of $\Delta H$) and conformer
accessibility (from $\Delta S$), values of $\Delta C_p$ can be associated with
differences in the solvent exposure of more or less polar side
chains,\textsuperscript{46} offering an additional constraint for characterizing
structures and structural changes.

## Experimental Section

**vT-ESI-IMS-MS Measurements.** Experiments were carried out
using a home-built 4-m long drift tube/time-of-flight (TOF) mass
spectrometer (Figure S1) which records nested IMS-MS data.\textsuperscript{47–49} A
detailed description of IMS-MS instrumentation is found else-
where.\textsuperscript{50–53} Briefly, ions generated by ESI enter the IMS-MS
instrument source through a narrow capillary and are stored in an
hourglass ion funnel\textsuperscript{144,145} until they are pulsed into a 4-m drift tube by
lowering an electrostatic gate for 100 $\mu$s. Protein ions traverse the drift
tube filled with a buffer gas (2.5 Torr He) under the influence of a
weak electric field ($\sim 12$ V-cm$^{-1}$). Protein structures are separated
based on size, shape, and charge.\textsuperscript{56} Drift times are converted to cross
sections as described in the SI. Ions exit the drift region and are
pulsed into an orthogonal two-stage reflectron geometry TOF mass
analyzer and are detected by microchannel plates. To induce thermal
denaturation, a custom-built temperature-controlled ESI source
similar to those described by our group\textsuperscript{5} and others\textsuperscript{56–58} was used
(Figure S2).

**Determination of Ensemble Melting Temperatures from
Analysis of Weighted Average of Charge State Distributions
Recorded at Varying Solution Temperatures.** Benesch and co-
workers have reported a simple means of determining melting
temperatures based on the changes in the ESI protein charge state
distribution as a function of solution temperature.\textsuperscript{59} Here, the
intensities of different charge states are used to compute the weighted
average charge state ($z$) determined at each solution temperature, as
described by eq 1:

\[
z = \frac{\sum_{i} z_i f_i}{\sum_{i} f_i}
\] (1)

where $i$ is the intensity of a mass spectrum peak having a charge state
of $z_i$. Melting/formation temperatures ($T_m$/$T_f$) are determined as the
midpoint of the transition according to a fit with a sigmoidal function
as described previously\textsuperscript{5} using the nonlinear curve fit tool and
the Boltzmann function in OriginPro shown in eq 2.
Thermodynamic Analysis by the Gibbs–Helmholtz Relationship. The Gibbs–Helmholtz equation is used to obtain thermodynamic values for CI-2. For this analysis, the abundance of each conformer family at each solution temperature is converted to a free energy value, $\Delta G$. The equilibrium constant $K_i(T)$ for each conformer family is calculated from the relative abundance of a conformer family at a specific solution temperature $[I_i(T)]$ with reference to the relative abundance of the reference state, product group 1 (see SI), at the same solution temperature $[P_1(T)]$, as shown by eq 3:

$$K_i(T) = \frac{[I_i(T)]}{[P_1(T)]}$$

The value for $\Delta G(T)$ is then calculated for each conformer group using eq 4:

$$\Delta G(T) = -RT \ln(K_i(T))$$

where $R$ is the gas constant, $T$ is the solution temperature, and $K_i(T)$ is the equilibrium constant for a conformer group as calculated using eq 3. Plots of $\Delta G$ versus solution temperature are generated for each conformer family, and a nonlinear least-squares optimization is used to fit each $\Delta G(T)$ plot to solve for values of enthalpy ($\Delta H$), entropy ($\Delta S$), and heat capacity ($\Delta C_p$) using the Gibbs–Helmholtz equation:

$$\Delta G(T) = \Delta H_R - T \Delta S_R + \Delta C_p \left( -\frac{T}{T_R} + T \ln \left( \frac{T}{T_R} \right) \right)$$

where $\Delta H_R$ is the enthalpy at the reference temperature $T_R$ (arbitrarily set at 323.15 K) and $\Delta S_R$ is the entropy at $T_R$.

RESULTS AND DISCUSSION

Analysis of Average ESI Charge State as a Function of Solution Temperature. The inset in Figure 1a shows representative mass spectra acquired for an aqueous solution of wt CI-2 (pH ≈ 2.6) at solution temperatures 28, 50, and 76 °C. At 28 °C, the wt mass spectrum is dominated by the $[M + 6H]^{6+}$ species at $m/z$ 1242 and the $+7$ charge state at $m/z$ 1065. This narrow distribution comprised of low-charge species is consistent with a compact structure having six to seven surface-exposed basic residues protonated. At 50 °C, the +6 charge state has markedly decreased in abundance and the MS distribution shifts to favor several signals at lower $m/z$ assigned as the $+8$ through $+11$ charge states. At 76 °C, a distribution centered around the +9 and +10 charge states emerges. This trend is observed for the mutant sequences as well, shown in Figure S3; however, K11A does not accommodate 11 charges at high temperatures, suggesting K13 is a prominent site for protonation for the unfolded structures of CI-2.

A shift in charge state with temperature signifies a structural change—as the protein elongates, basic core residues become exposed to the aqueous solvent and are protonated. For a discussion on the influence of solution conformation on ESI...
charge states, see the SI. A quantitative view of this is obtained by plotting the weighted average charge state as a function of solution temperature as shown in Figure 1a for the wt and each mutant. The $T_m$ for each sequence is obtained by fitting these data to a two-state transition as described by eq 2.

$T_m$ values provide an overall signature for stability such that a protein with a high $T_m$ is more stable than a protein with a lower $T_m$. Analysis of the wt data yields $T_m = 48.4 \pm 0.1 \degree C$, in agreement with $T_m$ values at similar pH reported from DSC analysis.21 See the Figure 1 caption for mutant $T_m$ values, and Table S1 for a comparison of mutant $\Delta \Delta G$ values obtained from IMS-MS measurements and traditional methods.21,27,28

Evidence for Differences in Conformation Types from the Temperature Dependence of Individual Charge States. The MS data are further analyzed by plotting the relative abundances of the individual charge states as a function of solution temperature as shown in Figure 1b. In the wt plot, the +6 charge state decays with increasing temperature at $T_m = 44.5 \pm 0.4 \degree C$, a value significantly lower than the overall $T_m$ obtained from the wt average charge analysis. Between 20 and 45 \degree C, the wt +7 increases in abundance before decaying at $T_m = 50.5 \pm 0.1 \degree C$. The +8 through +11 charge states increase distinctively with further increases in solution temperature, as shown in Figure 1b and Table S2. That the abundance of each of the six ESI charge states changes uniquely with solution temperature (i.e., $T_m$ and $T_f$ values, curve shapes) provides evidence for a minimum of six distinct conformation types. Often, the abundances of protein charge states appear as a statistical distribution, suggesting a single (or at most a few) antecedent solution conformation(s).62–64

The charge state behavior of the mutants with increasing solution temperature is also shown in Figure 1b and described...
in Table S2. The $T_m$ and $T_f$ for each transition have unique values, and the abundances of the charge states for each mutant vary. Therefore, while global structural transitions of the wt and mutant sequences appear similar, each sequence stabilizes a unique distribution of conformer types at each solution temperature during thermal denaturation.

**Overview of Mobility Distributions for wt, K11A, A16G, and L32A CI-2.** IMS distributions provide a unique opportunity to examine the effects of a single amino acid mutation on the structures of CI-2. Figure 2 shows mobility distributions for the observed charge states $+6$ through $+11$ at representative solution temperatures for wt CI-2 and the mutant data corrected with intrinsic size parameters. The $+6$ mobility distribution for wt at $26\,^\circ\mathrm{C}$ shows two main features at 1440 and 1520 Å$^2$. The feature centered about 1000 Å$^2$ is conserved throughout each sequence, but the feature at 1440 Å$^2$ is almost completely absent in the K11A dataset.

The $+7$ mobility distributions span from 900 and 1400 Å$^2$, which is significantly broader than the mobility distribution for the $+6$ charge state, implying that a large range of structures present as $+7$ ions. The $+7$ distribution includes a compact feature centered around 920 Å$^2$ and a small population of features from $\sim$920 to 970 Å$^2$. This distribution is not significantly altered by the A16G and L32A mutations; however, the K11A +6 mobility distribution depicts increased abundance of the elongated mobility features, a result that has been reproduced multiple times.

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the +6 charge state have different $T_m$ values at 47.2 ± 0.2 °C and $T_m = 43.9 ± 0.4$ °C, respectively. In this case, $N_1$ represents structures that are more stable than structures grouped in $N_2$. Analysis of the +8 and +9 conformer families show relative abundance plots that increase with temperature and subsequently decay at higher temperatures. This implies that structures that present as +8 and +9 ions are less abundant at higher temperatures where structures in the +10 and +11 charge states dominate.

The relative abundances of 10 representative conformers ($N_{19}, N_{20}, N_{21}, N_{22}, N_{23}, N_{24}, N_{25}, N_{26}, N_{27}, N_{28}$) resulting from the grouping analysis are shown in Figure 4 as a function of temperature across 4 orders of magnitude. The incredible sensitivity and dynamic range of vT-ESI-IMS-MS allows us to sample low-abundance structures that are often beyond the limit of detection for traditional techniques. This method has allowed us to provide evidence for 41 unique structures of wt CI-2 that each undergo unique two-state transitions with increasing solution temperature.

**Thermodynamic Analysis: Examples of $\Delta G$ at Solution Temperatures of 26, 46, and 74 °C for Each of the 41 Unique Solution Conformers of wt CI-2.** An explanation of our choice of a reference point for the thermodynamic analysis is provided in the SI. Values of $\Delta G$ for all 41 wt CI-2 conformer types with respect to conformer $P_1$ are derived at all solution temperatures, see Tables S5–S8. Figure 5 shows examples for three solution temperatures (26, 46, and 74 °C). It is interesting to plot values of $\Delta G$ against the measured cross sections for each conformer (within each charge state) as these plots resemble free energy landscapes. These landscapes provide a rare experimental opportunity to determine the relative energetics of many conformers; as shown below, the changes that are observed with increasing temperature offer insight about the driving forces involved in thermal denaturation.

At 26 °C (Figure 5a), precursors, observed as the abundant populations of the cross section distributions for the +6 and +7 charge states, have favorable free energies ranging from ca. −12 to 2 kJ·mol$^{-1}$. Products of melting, the scarcely populated species observed experimentally as the cross section distributions for the +8 through +11 charge states, have less favorable free energies (i.e., ca. 0 to 20 kJ·mol$^{-1}$). The large range (ca. 20–32 kJ·mol$^{-1}$) of free energies (from ca. −12 to 20 kJ·mol$^{-1}$) favor the more compact ions observed for the +6 and +7 charge states (ca. 700 to 1340 Å$^2$) and disfavor the higher +8 to +11 charge states which appear as more elongated ions (ca. 1100 to 1900 Å$^2$). At 46 °C (Figure 5b), near the $T_m$ the range of free energies (ca. 18 kJ·mol$^{-1}$; ca. −3 to 15 kJ·mol$^{-1}$) is compressed such that the landscape is much flatter than at 26 °C. This compression results from both the destabilization of the 10 precursor structures as well as an overall stabilization of the 31 denatured states. Because of this balance in stability, at 46 °C each of the 41 structures is easily detected experimentally. This provides experimental insight about this system near the $T_m$. A two-state model assumes that at the $T_m$ (where $\Delta G = 0$ kJ·mol$^{-1}$), 50% of the native state population has unfolded to any number of denatured states such that the population of native and denatured structures is equal. Our data reveal that a few precursor states and product states are primarily responsible for this balance; however, many structures are populated. Finally, at 74 °C (Figure 5c), the precursor conformers have high, unfavorable free energies and the product conformers become the favored signals.

**Thermodynamic Analysis: Contributions of Enthalpy, Entropy, and the Key Role of Solvent in This System.** As described above, the Gibbs–Helmholtz relationship (eq 5) describing $\Delta G$ as a function of temperature can be used to determine values of $\Delta H$, $\Delta S$, and $\Delta C_p$ for each solution state (see Figures S23–S25 for each Gibbs–Helmholtz fit). Figure 6a–c shows plots of $\Delta G$ (black), $\Delta H$ (blue), and $\Delta C_p$ (red).
on the same cross section scale for each charge state that was used in plotting $\Delta G$ at three representative temperatures. At first glance, one sees that at each temperature, the range of energies required to include all of the states that we have observed is far greater than was needed for the $\Delta G$ plot. On these scales, values of $\Delta G$ are so compressed (and near zero) that each free energy diagram appears relatively flat. This is because at equilibrium, $\Delta H$ and $T\Delta S$ must compensate for one another to establish a balance of thermodynamic factors associated with protein–protein, protein–solvent, and solvent–solvent interactions. Figure 6d–f illustrates this compensation by plotting values of $\Delta H$ versus $T\Delta S$ for each state. It is useful to consider how each quadrant of these plots is populated. Nearly all of the data points reside in the lower left or upper right quadrants because these are regions where $T\Delta S$ and $\Delta H$ compensate for one another. Tightly bound, energetically favorable states are disfavored by entropy (lower left quadrant) and dynamic conformations with favorable entropies are energetically unfavorable (upper right quadrant).

Figure 6. (a–c) Plots of $\Delta H$ in blue, $T\Delta S$ in red, and $\Delta G$ in black for each conformer family plotted on a cross section scale within each charge state at solution temperatures (a) 26, (b) 46, and (c) 74 °C. (d–f) Plots of entropy–enthalpy compensation ($\Delta H$ versus $T\Delta S$) for each state at the three temperatures where the color of each conformer family is denoted in Table S4. The line of balance indicates where $\Delta H = T\Delta S$ and $\Delta G = 0$. 
The large magnitudes of $\Delta H$ and $T\Delta S$ values and their respective temperature dependences are noteworthy. At low temperatures (26 °C, Figure 6a), the distribution of precursor states is highly favored enthalpically, with $\Delta H$ values ranging from ca. $-50$ to $-25$ kJ mol$^{-1}$ for the most abundant precursor species. Corresponding values of $T\Delta S$ for each of these species are extremely unfavorable. The distribution of native-like precursors is abundant because the magnitude of $\Delta H$ outweighs $T\Delta S$ in this region, as shown by Figure 6d in which many of the native-like precursor data points lie in the lower left quadrant and below the line of balance. At low temperatures, the low-abundance features that dominate at high temperatures (i.e., the product states) are energetically unfavorable, but more favorable values of $T\Delta S$ make such species observable.

At intermediate solution temperatures near the $T_m$ (Figure 6b), the difference in $T\Delta S$ and $\Delta H$ values for each state diminishes and all structures fall along the line of balance shown in Figure 6e. At higher solution temperatures (Figure 6c), values of $\Delta H$ for the precursor states decrease substantially. Values of $\Delta H$ for the most intense precursor state (the conformer at 900 Å$^2$ observed for the +6 charge state) decrease from $-30$ kJ mol$^{-1}$ at 26 °C to $-220$ kJ mol$^{-1}$ and $-520$ kJ mol$^{-1}$ at 46 and 74 °C, respectively. In fact, the energies of all 41 structures become more favorable with respect to $P_1$, as the solution temperature is increased. In the case of the precursor states, this tremendous energetic stabilization ($\Delta H$) is offset by an even larger unfavorable change in $T\Delta S$—disfavoring the precursor states. Nearly all of the product states are more favorable enthalpically and entropically at high temperatures. These states are ultimately favored entropically.

That an increase in temperature leads to an energetic stabilization of precursor states that decrease in abundance at high temperatures is somewhat paradoxical. At first glance one might expect precursor states to be enthalpically as well as entropically disfavored at high temperatures. To rationalize this, we begin by noting that this effect is also observed for product states. With this in mind, we imagined how changes in the solvent with increasing temperature might influence the stability of different structures. One physical property stands out. At low temperatures, water exists as a highly organized solvent with a dynamic and fluctuating hydrogen-bonded structure. This leads water to have an abnormally high dielectric constant ($\varepsilon = 80$ at 20 °C) making it exceptional as a solvent for shielding electrostatic interactions between solute molecules. As one expects, the entropy of water increases with increasing temperature—causing the network of hydrogen bonding interactions to become even more dynamic. In effect, the polarity of water decreases with increasing temperature and the less polar water has a substantially lower dielectric constant ($\varepsilon = 58$ at 90 °C). Thus, the increase in entropy of the aqueous solvent has two effects—as expected, this more dynamic state of water disfavors the precursor states entropically, making them less accessible. However, the decrease in shielding would make all of the electrostatic interactions associated with tightly folded states (i.e., zwitterionic, charge—charge interactions, hydrogen bonding between polar groups, and van der Waals interactions) more favorable—leading to a decrease in the enthalpies for highly structured states such as the precursors.

**Thermodynamic Analysis: Contributions of Heat Capacity.** An additional thermodynamic parameter obtained by the Gibbs–Helmholtz analysis is $\Delta C_p$ which has energetic contributions from internal interactions within the protein and external interactions between the protein and solvent. Ensemble protein unfolding is typically accompanied by a positive $\Delta C_p$ such that native conformers have small $C_p$ values which elongate to form structures that have large positive $C_p$ values. In other words, a solution of unfolded protein will require less heat to raise its solution temperature than a solution of unfolded protein at the same concentration. This is consistent with the behavior observed in Figure S22 for the overall melting transition obtained from the average charge state analysis for wt CI-2. The two-state transition can be fit over multiple temperature ranges. Figure S22a shows the Gibbs–Helmholtz fit from 297 to 345 K, and Figure S22b shows the data fit from 307 to 339 K. Both fits derive a positive change in heat capacity with temperature at either $\Delta C_p = 2.6 \pm 1.1$ kJ mol$^{-1}$ K$^{-1}$ (Figure S22a) or $\Delta C_p = 2.7 \pm 1.2$ kJ mol$^{-1}$ K$^{-1}$ (Figure S22b), both in good agreement with values obtained by DSC.21

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**Figure 7.** (a) Example Gibbs–Helmholtz fits for conformers $P_1$ and $N_1$. (b) $\Delta C_p$ as a function of charge state and collision cross section for each conformer family.
We have also used the Gibbs–Helmholtz analysis to calculate individual ΔC_p values for each of the 41 conformational families. In this way, we can analyze any changes in C_p for a single structure relative to reference P_1 as temperature is increased. Figure 7a shows example Gibbs–Helmholtz fits to temperature-dependent ΔG data for precursor state N_1 and product state P_2. The curvature in these data represents a non-zero value for ΔC_p, such that a concave-up curve as observed in the N_1 fit is attributed to a decrease in heat capacity with temperature and a concave-down curve signifies an increase in heat capacity with temperature as shown by the P_2 fit.

Figure 7b shows the ΔC_p of each conformational family with reference to P_1 on the same cross section scale as plots for ΔG, ΔH, and TΔS. The precursor states that comprise the +6 and +7 charge states show large negative values for ΔC_p such that there is an overall decrease in C_p with increasing temperature. The product states in the +8 distribution (centered around 1175 and 1300 Å^2) show a positive ΔC_p while the rest of the product states have slightly negative ΔC_p values between −5 and 0 kJ·K^−1·mol^−1. Each static configuration exhibits a non-zero heat capacity which indicates that the interactions between the structures and the changing solvent properties at high temperatures likely drive the signs and magnitude of ΔC_p.

As described above, the aqueous solvent at high temperatures becomes less polar as its dielectric constant drops. This means that exposed hydrophobic residues will interact more favorably with the solvent at high temperatures thus driving the ΔC_p negative for hydrophobic (i.e., product) structures. Tightly folded states with buried hydrophobic residues interact less-favorably with the solvent at high temperatures, causing an increase in C_p for folded structures. However, the intramolecular interactions at high temperatures become extraordinarily favorable according to values of ΔH, a phenomenon that likely offsets the slight decrease in favorable protein–solvent interactions. Thus, the strong temperature dependence of ΔH and ΔS for folded structures (i.e., large decreases in ΔH and the subsequent compensation of ΔS) at high temperatures is likely a result of the large negative values of ΔC_p.

In previous studies, Fu and Freire observed decreases in ΔC_p with increasing alcohol content. Similarly, the dielectric of the high-temperature aqueous environment in these studies resembles a 50:50 water:methanol mixture (e = 61 at 25 °C), further explaining why nearly all conformers exhibit a negative ΔC_p as the solvent becomes more nonpolar. A caveat to these interpretations is that one must consider the choice of reference state which also has (unmeasured) changing thermochemical properties with temperature. The ΔC_p values ultimately signify whether C_p for a given conformer is increasing or decreasing relative to the reference configuration, P_1. It may be the case that the reference structure is particularly hydrophobic which would drive ΔC_p for all other structures negative.

### CONCLUSIONS

Anfinset’s thermodynamic hypothesis—that “the particular configuration that a protein assumes, under any specific set of conditions, is the one that is thermodynamically the most stable”—suggests that many configurations should be favored upon changing environmental factors. However, rarely are these species observed experimentally. In this work, we took advantage of the exceptional sensitivity and dynamic range of vT-ESI-IMS-MS to examine the well-studied CI-2 system (and three nearly identical forms, each having a single amino acid substitution) at 30 solution temperatures (from 22 to 80 °C) with the aim of characterizing low-abundance “non-native” configurations. Consistent with this hypothesis, we presented an analysis and simple model of these data suggesting 41 distinct solution species that undergo structural transitions with unique temperature profiles. Ten of these appear to correspond to “native-like” precursor states. These are favored near room temperature and become unstable and less abundant as the solution temperature is increased beyond the melting temperature. From our model, we interpret 31 conformers that are favored at elevated temperatures as products of solution melting.

It is important to stress that our analysis depends on how the experimental charge states and IMS distributions are modeled. We believe that the approach presented above provides the most accurate representation of all four systems at all temperatures. An attractive feature of this model is that the peak positions and peak widths are fixed for all datasets. The only variable that changes in representing the datasets with this model is the abundance of different structures. That said, our model may overfit or underfit these data—suggesting more or fewer unique solution species than are actually present, respectively. It is also possible that as the resolving power of IMS techniques improve, we will find additional states that are obscured in our analysis. Regardless, the results provide a new experimental approach to the long-standing problem of measuring the complexity of structural transitions in proteins.

Within the model that is presented, it is interesting to consider the physical and chemical origins of these assignments. The temperature-dependent MS data, showing changes in the weighted average charge state distributions, provide information about the relative stabilities of each protein sequence as an overall melting temperature—in good agreement with DSC measurements and prior studies. A clue about the many-state nature of this system was that individual charge states varied independently with temperature, suggesting that structural changes result in exposure of new basic residues reflecting different types of conformations from solution. Because of the exceptional sensitivity and dynamic range of these techniques it is possible to quantify many additional species and equilibria from solution. Within each charge state, collision cross section distributions for the wt and each mutant form show additional features that also change uniquely with solution temperature.

A Gibbs–Helmholtz analysis was used to derive thermodynamic properties (ΔG, ΔH, ΔS, and ΔC_p) for each state. As the temperature is increased, all configurations become more stable enthalpically; however, unfavorable changes in entropy compensate in a way that prohibits formation of the stable folded structures at high temperatures. We propose that the increase in enthalpic stabilities at high temperatures arises because of changes in the solvent; at high temperatures, water is less ordered and the dielectric constant drops. This decrease in the ability of water to shield electrostatic interactions leads to an increase in all short-range stabilizing interactions (i.e., zwitterionic, hydrogen, and van der Waals bonding) within the protein conformer. At all temperatures, enthalpy and entropy are closely balanced (i.e., ΔH ≈ TΔS). Above and below the T_m of each, this balance is harder to maintain, and slight offsets control the populations of native and denatured states.

Although these data provide an experimental measure of the properties (charge, shape, ΔG, ΔH, ΔS, and ΔC_p) of many
conformations that exist during a structural transition, the issue of structural characterization of non-native states remains elusive. Advances in other types of spectroscopies may complement these measurements to provide details about non-native configurations in solution. In the meantime, these data are useful as structural constraints. As noted by many, collision cross sections are a measure of the shapes of protein conformations in the gas phase.\textsuperscript{5,7,9,10} Substantial progress in theory is making it possible to accurately model and calculate trial conformers for assignment of specific IMS peaks. Anhydrous structures may one day be computationally solvated to reveal their antecedent solution structures. The change in charge that is observed upon ionization under different solution conditions provides an additional constraint. Finally, the thermodynamic properties of these configurations should be testable by theory. Calculations of entropies and enthalpies for specific solution conditions provides an additional constraint. The authors declare no competing financial interest.

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