Polyprolines form the well-known all-cis, right-, and all-trans left-handed helices (PPI and PPII, respectively), as shown in Scheme 1.\textsuperscript{10} These structures are sensitive to environment.\textsuperscript{2,11}

\textbf{Scheme 1.} Two Views of All-cis PPI with Backbone Torsion angles of φ = −83°, ψ = 158°, and ω = 0° (top) and All-trans PPII with φ = −78°, ψ = 149°, and ω = 180° (bottom) helices of Pro7\textsuperscript{a}

\textsuperscript{a}An indication of length and width dimensions and charge stabilization of the macro dipole of PPI is also provided. See ref 10 for details.

The tightly folded PPI configuration excludes solvent from the peptide backbone and is favored in nonpolar solutions such as propanol. Upon immersion in water, each cis-configured peptide bond flips to a trans orientation, leading to the PPI helix that is stabilized by interactions of the exposed backbone carbonyls with the polar solvent; a configuration also found in denatured and intrinsically disordered sequences.\textsuperscript{12} Below, we show that the PPI → PPII transition for Pro7 occurs by two mechanisms: a primary, exothermic process involving a proton transfer that proceeds through a PPI-like transition state, and a far less-efficient, entropically driven, endothermic conformational change. The intimate coupling of the protonation and configurational change leads to an unexpectedly slow rate of protonation; in fact, the slowest protonation event ever reported. The transfer of protons in biological systems is key to a range of physiological processes from photosynthetic reaction centers, in which electron-coupled proton uptake and release are involved in multiple reaction cycles,\textsuperscript{13} to structural changes that
Proton conduction in the F0 domain of H+-ATPase proceeds via a transition of Pro7 in 40:59.5:0.5 1-propanol:H2O:HOAc (v:v:v) at 296 K. Distributions obtained by electrospraying 1-propanol and H2O are shown in Scheme 1. When Pro7 is electrospayed from pure water (PPII@water), a single peak at m/z = 349.70, assigned to the [M + 2H]+ ion, is observed (Figure 1). The cross section for this ion, Ω = 205.0 ± 1.5 Å2, is substantially greater than Ω(PPI). Molecular modeling and cross section calculations indicate that Ω = 205 Å2 corresponds to a PPII conformation that has one proton located at the N–H terminus and another on a backbone N atom associated with the seventh, C-terminal pyrrolidine ring group (See Supporting Information, SI).

As we studied this system, it appeared that the conformation change and proton-transfer events were not instantaneous, so we investigated the kinetics. We began by incubating Pro7 in PrOH at defined temperatures for 72 h to ensure a PPI starting structure. Kinetics measurements of the proton-transfer process associated with reaction 1:

\[
PPI^{+}\text{PrOH} + H_2O^{+} \rightarrow \text{PPIII}^{2+}\text{PrOH}/aq + H_2O
\]

were initiated by rapid dilution of the PPI@PrOH stock solution to a final composition of 6 μM Pro7 in 60:40 water:propanol and (0.01 to 1%) acetic acid (PrOH/aq). Figure 1 shows that 2 min after dilution, ions formed from electrospraying the Pro7@PrOH/aq solution appear at the same position in the nested IM-MS distribution, indicating that PPI is initially retained. This is a remarkable result. Addition of a second proton to PPI@PrOH/aq to form the PPII2+ product is extremely slow, ∼10^4 times slower than the slowest reported proton-transfer process.22

At longer reaction times (8 min), a small peak corresponding to the proton-transfer product PPII2+@PrOH/aq is observed; this species becomes the largest feature after ∼30 min. There is no evidence for intermediates. Proton transfer appears to be coupled with the conformational change in a cooperative two-state process. Continued monitoring shows that at very long times (∼100 min), a new peak corresponding to a singly protonated Pro7 is observed. These ions have Ω = 178.8 ± 1.1 Å², consistent with the previously reported value, Ω = 177.4 Å², assigned to a compact, globular PPII configuration that arises from reaction 2,

\[
PPI^{+}\text{PrOH} \rightarrow \text{PPII}^{+}\text{PrOH}/aq
\]

when the all-trans PPIII@aq geometry collapses upon desolvation.19 In the absence of protonation the configurational change is less efficient. The observation that reactions 1 and 2 occur on such different time scales is an indication that these are solution-phase phenomena and not an outcome of the electrospray process.

We examine the kinetics and thermodynamics in more detail to understand how protonation is regulated. Figure 2 shows abundance profiles over time of the species in Figure 1 obtained at 278, 298, and 303 K. Analysis of these kinetics (see SI) shows that the PPI+ precursor decays following a unimolecular pseudo-first-order rate law upon formation of the PPII+ product. This is the case over a 3–12 μM range of Pro7 concentrations; thus, there is no evidence that multimer formation restricts the proton-transfer rate. Studies at acetic acid concentrations (from 0.01 to 1%) show that the transition is independent of the proton concentration (i.e., zeroth order).

**Figure 1.** Cross section distributions showing the PPI → PPII transition of Pro7 in 40:59.5:0.5 1-propanol:H2O:HOAc (v:v:v) at 296 K. Distributions obtained by electrospraying 1-propanol and H2O are shown at the bottom. The slight offset of the [PPI + H]+ and [PPII + 2H]+ baselines is shown to clarify that these distributions are extracted by integrating the ion intensities for ions having different m/z values. The dashed insets show blow ups of low-abundance species, and arrows are used to show the assignment of Pro7 conformations, where the dark dot indicates the charge site.
The less-efficient reaction 2 is also independent of the acid concentration and shows no evidence of multimer formation.

By fitting the $T = 301$, 296, and 278 K data sets shown in Figure 2, rate constants for reaction 1 of $k = 100 \pm 17$, 53 $\pm$ 3, and $6.1 \pm 0.8 \times 10^{-5}$ s$^{-1}$, respectively, are obtained. These values (along with others measured at $T = 288$ and 283 K (see SI) are converted to Arrhenius plots (Figure 2) yielding transition-state thermochemistry of $\Delta G^\ddagger = 91 \pm 3$ and $\Delta H^\ddagger = 84 \pm 9$ kJ.mol$^{-1}$, and $\Delta S^\ddagger = -23 \pm 31$ J.mol$^{-1}$.K$^{-1}$. These values fall within a range of transition-state thermochemistry for stepwise cis $\rightarrow$ trans conversion measured for Pro13 in the absence of proton transfer, reported elsewhere.\(^{18}\)

Temperature-dependent abundance measurements (shown in the SI) recorded when the system reaches equilibrium (i.e., long reaction times where abundances are independent of reaction time) allow us to determine the overall reaction thermochemistry. A key advantage of the IM-MS measurement is that the large dynamic range makes it possible to determine equilibrium distributions even when abundances for different species differ by many orders of magnitude. From these data, we generate the Van’t Hoff plots, also shown in Figure 2, and determine: $\Delta G$ and $\Delta H = -20 \pm 19$ and $-75 \pm 14$ kJ.mol$^{-1}$, respectively, and $\Delta S = -188 \pm 48$ J.mol$^{-1}$.K$^{-1}$ for reaction 1; and $\Delta G$ and $\Delta H = -9 \pm 20$ and $64 \pm 14$ kJ.mol$^{-1}$, respectively, and $\Delta S = 247 \pm 50$ J.mol$^{-1}$.K$^{-1}$ for reaction 2. Finally, although protonation of [PPII + H]$^+$ to form [PPII + 2H]$^{2+}$ by reaction 3

$$\text{PPII}^+ + \text{H}_2\text{O}^+ \rightarrow \text{PPII}^{2+} + \text{H}_2\text{O}^+$$

is not observed experimentally, its thermochemistry ($\Delta G$ and $\Delta H = -10 \pm 29$ and $-139 \pm 20$ kJ.mol$^{-1}$, respectively, and $\Delta S = -435 \pm 70$ J.mol$^{-1}$.K$^{-1}$) can be derived from the values measured for reactions 1 and 2.

A summary of this thermochemistry is shown in Figure 3. The free energy plot of $\Delta G$ shows that PPI$^+$ $\rightarrow$ PPII$^+$ is favorable with and without the additional protonation. The plots of $\Delta H$ и $\Delta S$ show that these processes are favored for different reasons. Reaction 2 is endothermic, driven instead by a large increase in the entropy of the system associated with the PPI $\rightarrow$ PPIII configurational change. In contrast, when an
additional protonation accompanies the conformational change, as in reaction 1, the process is exothermic, and this energy covers the cost of ordering the system, $\Delta S = -188 \pm 48 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. The overall entropic cost associated with forming the PPI12+ product is much greater than what is required to reach the transition state for reaction 1, where $\Delta S^T = -23 \pm 31 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. This provides the clue that the critical step in regulating proton transfer (i.e., the transition state) involves protonation of the cis-configured PPI' helix, rather than a solvated PPI1 helix. The doubly protonated PPI2+ transition state then reconfigures to the PPI12+ product, which is an energetically favorable, far-more-ordered state of the system.

Addison insight about how proton-transfer is regulated comes from considering the thermochimistry for reaction 3, the protonation of PPI2+ to form PPI12+. Since the oligomer has already adopted the all-trans PPII configuration, one might expect additional protonation to be more straightforward than protonation of PPI'. The derived thermochimistry shows that protonation of PPI' reaction 3 to form PPI12+ is highly exothermic ($\Delta H = -139 \pm 20 \text{ kJ} \cdot \text{mol}^{-1}$), and our thermochimistry suggests a favorable free energy ($\Delta G = -10 \pm 29 \text{ kJ} \cdot \text{mol}^{-1}$). But, the drastic differences in appearance times for the PPI12+ and PPI' (Figure 3) lead us to note that there is no direct evidence for reaction 3 experimentally. Presumably this is because of the enormous entropic cost associated with the second protonation, $\Delta S = -435 \pm 70 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. While the dearth of direct evidence for reaction 3 does not rule out the direct interconversion of PPI12+↔PPI2+, the differences in entropy suggest that equilibrium may involve a more complex process, such as PPI12+ ↔ [PPI' ↔ PPI' ↔ PPI12+ ↔ PPI12+]. In this way, the entropy of the system also regulates proton transfer.

With an understanding of the critical importance of entropy in regulating this proton transfer, we cannot resist speculating about the nature of the PPI-like transition state that regulates such a slow second protonation event. One might consider two extremes: Does the second proton in this system add directly to the C-terminal portion of the PPI-like peptidic, or might the protonation event proceed from approach of H2O from the N-terminal side? The latter is especially intriguing. In such a case, Coulombic interactions through the solvent as the second proton approaches could force the existing helix-stabilizing hydronium into the pore of the PPI helix. Examination of the PPI-helix pore (Scheme 1) shows that the pore cavity is large enough to accommodate an interior hydronium ion (having a diameter of ~3–4 Å) and the channel appears to be hydrophilic. Thus, as the second hydronium approaches, the initial hydronium may effectively extrude a water wire as it is forced into the helical pore. Such a mechanism may provide insight into how proton transfer occurs in larger systems.

**ASSOCIATED CONTENT**

**Supporting Information**
Experimental procedures, Tables S1 and S2, and Figures S1–S5. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04287.

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The authors declare no competing financial interest.

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