Osmotically Induced Helix-Coil Transition in Poly(Glutamic Acid)

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ABSTRACT Protein folding and conformational changes are influenced by protein-water interactions and, as such, the energetics of protein function are necessarily linked to water activity. Here, we have chosen the helix-coil transition in poly(glutamic acid) as a model system to investigate the importance of hydration to protein structure by using the osmotic stress method combined with circular dichroism spectroscopy. Osmotic stress is applied using poly(ethylene glycol), molecular weight of 400, as the osmolyte. The energetics of the helix-coil transition under applied osmotic stress allows us to calculate the change in the number of preferentially included water molecules per residue accompanying the thermally induced conformational change. We find that osmotic stress raises the helix-coil transition temperature by favoring the more compact α-helical state over the more hydrated coil state. The contribution of other forces to α-helix stability also are explored by varying pH and studying a random copolymer, poly(glutamic acid-*r*-alanine). In this article, we clearly show the influence of osmotic pressure on the peptide folding equilibrium. Our results suggest that to study protein folding in vitro, the osmotic pressure, in addition to pH and salt concentration, should be controlled to better approximate the crowded environment inside cells.

INTRODUCTION

To survive changes in their aqueous environment, bacteria have to sense and actively counteract external osmotic pressure (1–3). For this, protein conformational changes have to be coupled to their hydration. Modulating biomolecular systems through the addition of osmolytes that vary in chemistry and size can provide a better understanding of the interactions responsible for their stability and function, which, it is important to note, include the role of water (4–14). An instructive way to view osmolyte effects on biological systems is through the osmotic stress method (15,16). This approach incorporates the various interactions from an added osmolyte while affording the measurement of forces between biopolymers and hydration changes accompanying reactions. The osmolyte size and chemical nature determine its exclusion from biomolecular surfaces, thereby acting osmotically on these regions and favoring more dehydrated states.

The importance of hydration to protein conformational change previously has been investigated by osmotic stress. Using osmolytes, like poly(ethylene glycol) (PEG), that are excluded from the pores and cavities of interest, large water volume changes associated with ion channel closure (17,18), the generation of coagulation factor Xa (19), heparin binding to antithrombrin (20), and glucose binding to hexokinase (21,22) have been measured. For hexokinase, it was shown that glucose affinity of the enzyme increases dramatically with lowered water activity and ~326 water molecules are released upon substrate binding. This is many more waters than can be accounted for by closing the glucose-binding pocket, and indicates that a large protein conformational change accompanies binding. Most interesting was that in the absence of glucose, osmotic pressure alone was sufficient to cause this conformational change. This work of dehydration was found to be on the order of one k_BT per hexokinase molecule, suggesting that thermal fluctuations allow the protein sufficient conformational flexibility to explore a number of different hydration states that would not be observed in the crystal structure. NMR measurements of water exchange also suggest that there are a number of different hydration states accessible to a protein (23). Osmotically induced conformational changes in the absence of ligand also have been observed for *Scapharca* dimeric hemoglobin (24), certain ''star'' sequence complexes of the restriction endonuclease *Eco*RI with DNA (12), and the BtuB bacterial transport protein (25,26).

Here, we investigate, at a fundamental level, the role of water in protein structure by studying the helix-coil transition in poly(glutamic acid) (PGlu) using osmotic stress. From about pH 6 and higher, PGlu is natively a random coil where electrostatic repulsion forces between negatively charged glutamate side groups favor a less compact state with higher conformational entropy. The random-coil conformation is also characterized by a higher degree of hydration. Osmotic pressure (Π) is applied by the addition of PEG with a molecular weight of 400, which lowers the activity of water in solution and makes the energy of interaction with the water molecules more costly for PGlu. This results in PGlu adopting the entropically lower α-helical state to gain the energy of

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intramolecular hydrogen-bonding interactions and require less water for stability (Fig. 1).

Since the PGlu α-helix has a well-defined repeat structure, we can calculate the change in hydration/residue involved in the thermal denaturation of the α-helix. All of the results here are for a single osmolyte, PEG 400, so that we may focus on differences in α-helix stability that occur as the pH and poly(amino acid) composition are varied. PEG exclusion varies predominately by size (27), but its osmotic pressure effects become independent of size once equally excluded from all regions of the surface being explored (22). Our measurements provide a more quantitative basis for thinking about the involvement of water in protein folding and stability, which should be useful for protein structure prediction incorporating water-mediated interactions (28,29).

MATERIALS AND METHODS

Materials

Poly-L-(glutamic acid) (M_n 54,400 and 61,200 g/mol) and poly-L-(glutamic acid-*r*-alanine) (M_n 30,000 g/mol), with a 42% molar percentage of alanine residues, were purchased from Sigma-Aldrich (St. Louis, MO). Poly(ethylene glycol), with a relative molecular weight M_r of 380–420 g/mol (Ph. Eur. grade), was purchased from Fluka (Buchs, Switzerland). All chemicals were used without further purification.

Circular dichroism measurements

Aqueous solutions of 1 mM (of residues) PGlu, 10 mM buffer (sodium acetate for pH 5.0, sodium phosphate for pH 5.8–7.0), 1 mM Na_2EDTA, and 0.5 M NaCl were prepared using MilliQ grade water. A random copolymer of glutamic acid and alanine, poly-L-(glutamic acid-*r*-alanine), with a 42% molar percentage of alanine residues (P(Glu-*r*-Ala)), was also used at the same concentration and with the same buffer and salt conditions. Osmotic stress was applied by adding poly(ethylene glycol) with molecular weight of 400 (PEG 400) (mass/mass) and allowing 4 days for equilibration. The osmotic pressure of the aqueous PEG 400 solutions, and the temperature dependence, were determined separately by sedimentation equilibrium ultracentrifugation (Optima XL-I, Beckman Instruments, Fullerton, CA) (30). Osmotic pressures of PEG 400 solutions >55 wt % ($\Pi > 20$ MPa) were estimated by extrapolating Eq. 14 in Stanley and Strey (30). The fraction of α-helical PGlu segments, f_h, was calculated by circular dichroism (CD) spectropolarimetry (J-715, Jasco, Tokyo, Japan) measurements as $f_h = [(\theta)_{222} - (\theta)_c]/[(\theta)_h - (\theta)_c]$, where the measured mean residue ellipticity at 222 nm, $(\theta)_{222}$, was normalized by the mean residue ellipticity for a completely random-coil, $(\theta)_c$, and α-helical, $(\theta)_h$, system at the same wavelength and temperature. The concentration of poly(amino acid) relative to the total amount of solution (water and PEG 400) was calculated to normalize the CD signal in determining (θ).

For the CD experiments, a quartz cuvette with a 2-mm pathlength was used. Wavelength scans were performed at 20°C from 260 to 195 nm using a step resolution of 0.2 nm, speed of 20 nm/min, 50 mdeg sensitivity, and four accumulations. Temperature scans were performed at 222 nm from 4 to 65°C at a rate of 1°C/min, resolution of 0.1°C, and one accumulation. All other settings were the same as those used for the wavelength scans.

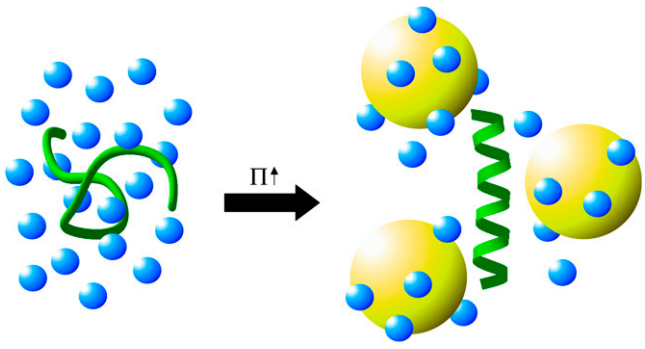

FIGURE 1 Osmotically induced transition from the random-coil to the α-helical state in PGlu. As osmotic pressure is applied, the activity of water in solution is lowered. Since the random-coil state relies on hydration for stability, this results in PGlu adopting the entropically lower α-helical state to gain the energy of intramolecular hydrogen-bonding interactions and thus requiring less water for stability. The dimensions of the α-helix (pitch of 5.4 Å, radius of 3 Å), water molecules (radius of 1.5 Å), and osmolyte molecules (radius of gyration R_g = 8.1 Å for PEG 400, from Bhat and Timasheff (27)) are drawn to scale for comparison.

Solvent-accessible surface area calculations

The solvent-accessible surface (SAS) area of PGlu and P(Glu-*r*-Ala) molecules with 20, 40, and 60 residues and having a fully extended (all-*trans* configuration), to approximate the random-coil state, or an ideal α-helical backbone conformation, were performed using the MSMS program (31) with a probe sphere radius of 1.5 Å to approximate a water molecule. The coordinates for the extended and α-helical conformations were generated using the RIBOSOME program (32), where all glutamate side groups were set to an extended conformation using torsion angles of $\chi_0 = -122.5°$, $\chi_1 = 180°$, and $\chi_2 = 180°$. For P(Glu-*r*-Ala), χ_0 was set to $-122°$. The slope of SAS area versus number of residues for each state yields the SAS area/residue.

RESULTS

Circular dichroism spectra of PGlu at pH 5.8 show the transition from a random-coil to α-helical conformation with increasing PEG 400 concentration (Fig. 2 *a*). There is an isodichroic point at ~205 nm, which is indicative of a simple two-state transition. The signal at 222 nm is the most sensitive to this transition, where the change in $(\theta)_{222}$ with PEG 400 concentration is observed to be sigmoidal (Fig. 2 *b*). Fraction helicity, f_h, is calculated from $(\theta)_{222}$ as described in Materials and Methods.

The importance of electrostatic interactions in the helix-coil transition of PGlu is seen by the increase in osmotic pressure required to induce the α-helical state as the solution pH is increased from pH 5.0–7.0 (Fig. 3). At a constant temperature of 20°C, the helix-coil transition point ($f_h = 0.5$) is raised from $\Pi = 1.3$ MPa at pH 5.0 to $\Pi = 21$ MPa at pH 6.0. In fact, at pH 7.0, it requires $\Pi > 100$ MPa to reach the transition point. It also should be noted that the transition for PGlu at pH 5.0 and 6.0 can be considered two-state, where the CD spectra have a clear isodichroic point over all osmotic pressures measured (e.g., Fig. 2 *a*). For PGlu at pH 7.0, the same isodichroic point is present up to about $\Pi = 40$ MPa, but slightly shifts toward smaller wavelengths for higher osmotic pressures (not shown). In this case, an intermediate conformation may be forming, or it is possible that either, or both, the initial (random-coil) and final (α-helical) states are

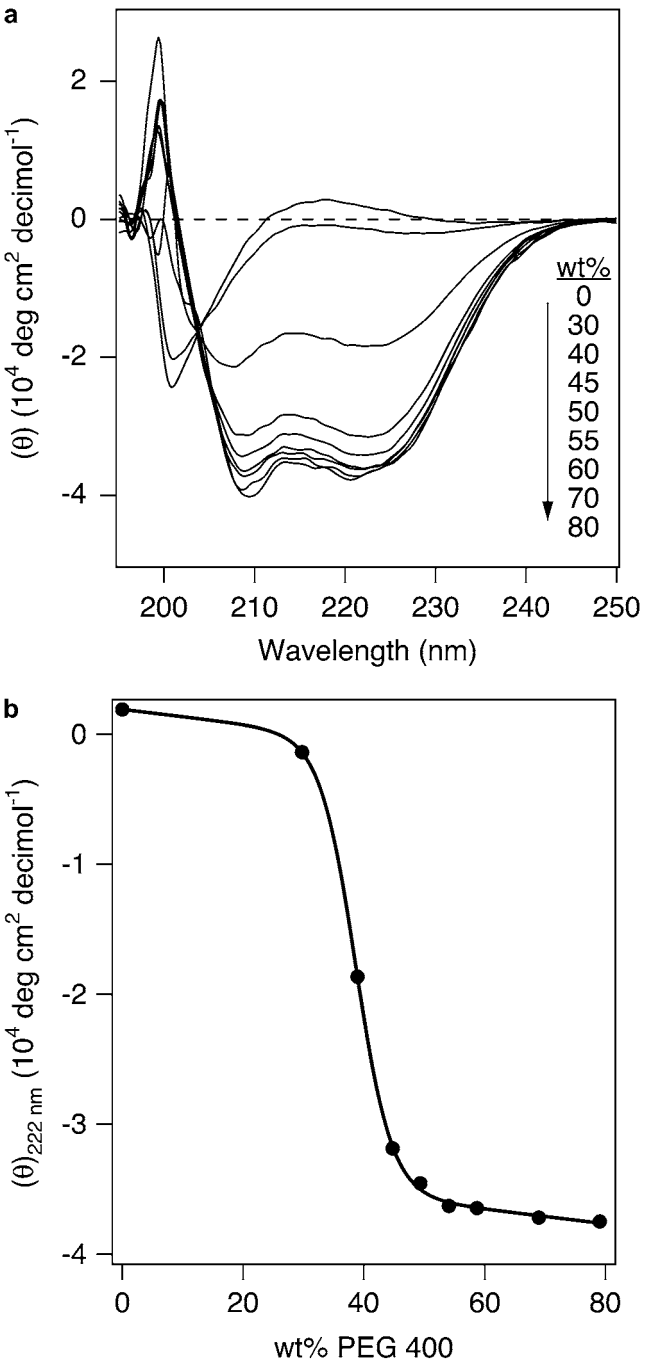

FIGURE 2 (*a*) CD spectra for PGlu (M_n 61,200 g/mol) at pH 5.8 show the transition from random coil to α-helix as PEG 400 concentration is increased from 0 to 80 wt %. (*b*) The CD signal at 222 nm shows this transition to be sigmoidal with PEG 400 concentration.

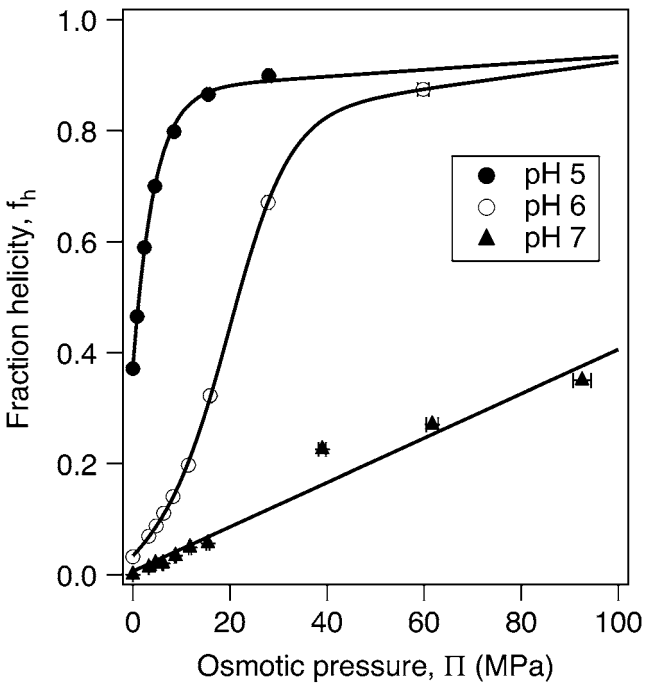

FIGURE 3 Effect of pH on the PGlu helix-coil transition under applied osmotic stress. Fraction helicity (f_h) increases with increasing osmotic pressure (Π), as shown for PGlu (M_n 54,400 g/mol) at pH 5.0, 6.0, and 7.0 at 20°C. Lines drawn are to guide the eye.

perturbed above $\Pi = 40$ MPa such that they are not the same conformations as in the absence of osmotic stress. This would complicate any detailed interpretation of PGlu at pH 7.0. However, the ability to induce the α-helical state by osmotic stress, rather than lowering pH, provides a method of studying the helix-coil transition under conditions where electrostatic interactions are significant.

To ensure that the PGlu helix-coil transition is thermally reversible, a series of heating and cooling CD scans were performed for pH 5.0 with no added PEG 400 (Fig. 4). PGlu in the presence of PEG 400 also demonstrated thermal reversibility over the temperature range of study (not shown). Then, by inducing the α-helical state with osmotic stress and following the helix-coil transition as a function of temperature, it was found that added osmolyte stabilizes the compact α-helical conformation by raising the denaturation (melting) temperature (T_m) (Fig. 5). Increases in the PGlu T_m up to 80°C with 60 wt % PEG 400 at pH 5.8 were observed when no helicity was found at this pH in the absence of any osmolyte. In comparison, $T_m = 5$°C at pH 5.0 with no osmolyte present, thus indicating the significant thermal stabilization by PEG 400. This increase in thermal stability is attributed to the higher cost of water molecules for PGlu due to the applied osmotic stress.

Using the osmotic stress analog of the Clapeyron equation,

$$\Delta\overline{S}\frac{dT_m}{d\Pi_m} = \nu_w\Delta\overline{N_w}, \tag{1}$$

the change in the number of preferentially included water molecules/residue ($\Delta\overline{N_w}$) associated with the helix-coil transition can be calculated, where $\Delta\overline{S}$ is the change in entropy/residue, Π_m is the transition osmotic pressure, and ν_w is the molecular volume of water (~30 Å^3). Invoking the Ising model for infinite chains, $\Delta\overline{S}$ can be calculated:

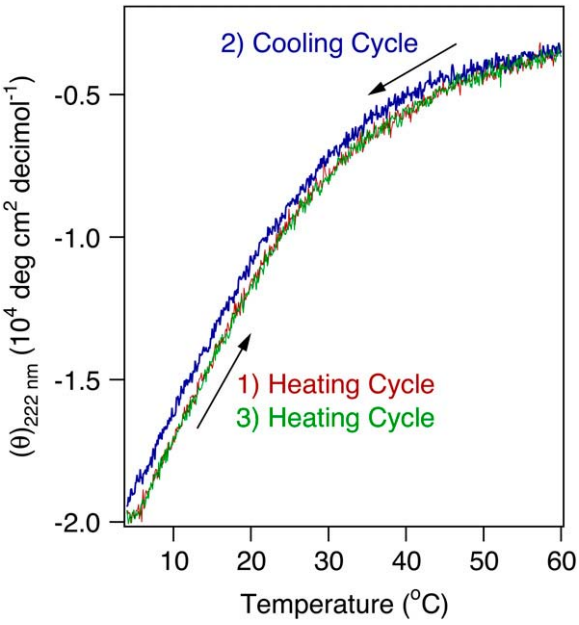

FIGURE 4 CD heating and cooling scans for PGlu (M_n 54,400 g/mol) at pH 5.0 monitored at 222 nm. The scans demonstrate that the helix-coil transition in PGlu is thermally reversible.

$$\Delta\overline{S} = -4k_B T_m \sqrt{\sigma}\left(\frac{df_h}{dT}\right)\Big|_{f_h=0.5}, \qquad (2)$$

where k_B is the Boltzmann constant, σ is the cooperativity parameter and df_h/dT is evaluated at the transition point (33).

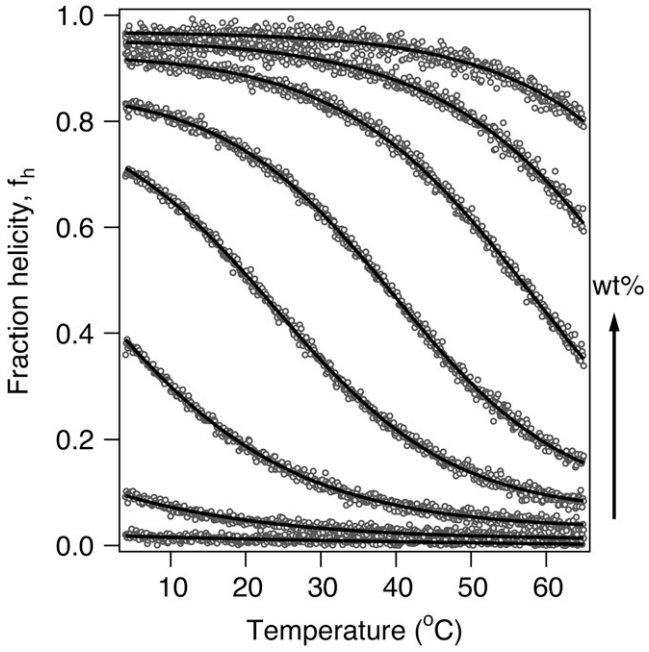

FIGURE 5 Thermal denaturation of the osmotically induced PGlu α-helix at pH 5.8. PGlu (M_n 61,200 g/mol) is induced to an α-helix by the addition of 25–60 wt % (in increments of 5 wt %) PEG 400. The presence of the osmolyte is seen to stabilize the α-helical state by increasing the temperature at which it melts back to the random coil (T_m). Lines drawn are sigmoidal fits to the curves.

We take $\sigma = 3 \times 10^{-3}$ for PGlu and P(Glu-*r*-Ala), as determined experimentally by Snipp et al. for high-molecular-weight PGlu and leucine-glutamic acid copolymers in aqueous solution and found to be independent of NaCl concentration (34). It is easiest to evaluate the slope df_h/dT in the absence of osmolyte, so that a correction for the temperature dependence of the osmotic pressure is not needed (30). For PGlu at pH 5.0 and $\Pi = 0$ MPa ($T_m = 5$°C), the slope $df_h/dT = -1.4 \times 10^{-2}$ K^{-1}, giving $\Delta\overline{S} = 1k_B$. Similar $\Delta\overline{S}$ values were calculated for the coil-to-helix transition in poly(alanine) using calorimetric measurements (−2.2 cal mol^{-1} deg^{-1}) (35) and molecular dynamics simulations (−2.11 cal mol^{-1} deg^{-1}) (36), where both give an entropy loss of 1.1 k_B during α-helix formation. It is still important to note that by employing the $\Delta\overline{S}$ value calculated for PGlu at pH 5.0 for the other conditions, we likely are making a simplification that may affect comparisons between these conditions. For the remainder of this analysis, we will assume that $\Delta\overline{S}$ is constant over the temperature range investigated.

The thermal energy required to melt an α-helical segment to a random coil ($T_m\Delta\overline{S}$) is $k_B T_m$, and its variation with Π_m is shown in Fig. 6 for PGlu at pH 5.0 and 5.8, and a 58:42 glutamic acid/alanine random copolymer, P(Glu-*r*-Ala), at pH 6.0. Since osmotic pressure data are available between 0 and 15 MPa and 10 and 40°C (30), the helix-coil transition data obtained around these ranges were used in calculating $\Delta\overline{N_w}$ to avoid error in extrapolating to higher osmotic pressures. The linear dependence of $k_B T_m$ on Π_m for each of these cases indicates that $\Delta\overline{N_w}$ is a constant across the range measured. Although we focus our description on the change in the number of associated water molecules to describe our results, it can be equivalently considered as a change in the

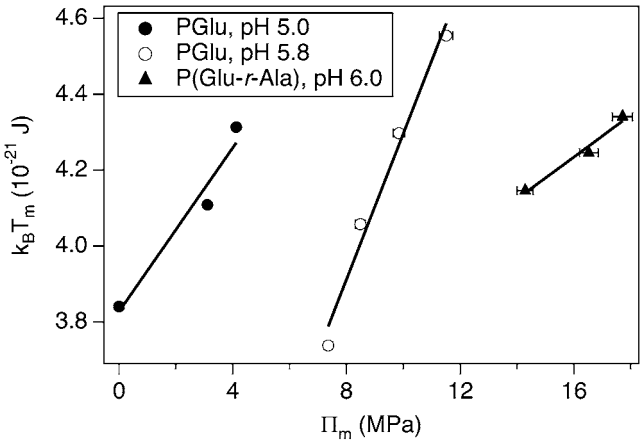

FIGURE 6 The change in thermal energy required to melt the α-helix ($k_B T_m$) with transition osmotic pressure (Π_m) for PGlu at pH 5.0 and 5.8 (M_n 54,400 and 61,200 g/mol, respectively) and P(Glu-*r*-Ala) at pH 6.0. The osmolyte is PEG 400. The slopes of these curves yield the change in water volume/residue ($\overline{V_w}$) that accompanies the thermal transition. Using the molecular volume of water ($\nu_w \approx 30$ Å^3), the change in the number of osmolyte-excluding water molecules/ residue ($\Delta\overline{N_w}$) for PGlu and P(Glu-*r*-Ala) upon melting can be calculated.

preferential interaction coefficient between the osmolyte and the two poly(amino acid) states. This means $\Delta\overline{N_w}$ is sensitive to the extent of osmolyte exclusion from (or water preferentially associated with) the individual poly(amino acid) random-coil and α-helical conformations.

The calculated $\Delta\overline{N_w}$ values for PGlu and P(Glu-*r*-Ala) are given in Table 1. For PGlu, it was found that $\Delta\overline{N_w} = 3.6 \pm 0.8$ water molecules at pH 5.0 and $\Delta\overline{N_w} = 8.1 \pm 0.6$ water molecules at pH 5.8. This shows, for PEG 400 as the osmolyte, the change in the number of osmolyte-excluding water molecules that accompanies the thermally induced PGlu-α-helix-to-random-coil transition. For P(Glu-*r*-Ala) at pH 6.0, $\Delta\overline{N_w} = 1.8 \pm 0.3$ water molecules, which may be less than the $\Delta\overline{N_w}$ values for PGlu due to the slightly hydrophobic nature of the alanine residues. An additional 5–8 MPa are required to induce the α-helix in P(Glu-*r*-Ala), compared to PGlu at a similar pH and the same temperature, even though the effective degree of ionization of the copolymer is only ~60% that of PGlu. In addition, the helical propensity of alanine is also higher than that of glutamic acid (37).

The traditional description of protein stability in the presence of destabilizing and stabilizing osmolytes has been in the context of the modulation of the free energy change with molar concentration of osmolyte, c_{osm}, which is the so-called *m*-value (38,39). Analogously, we can take the thermal energy needed to melt a mole of α-helical residues, $(T_m\Delta\overline{S})N_A = RT_m$, where N_A is Avogadro's number and R is the gas constant, to calculate $m\text{-}value = -RdT_m/dc_{osm}$ over the same range as for the data shown in Fig. 6. This gives *m*-value $= -80 \pm 20$ cal mol^{-1} M^{-1} ($c_{osm} = 0$–1 M) for PGlu at pH 5.0, -280 ± 10 cal mol^{-1} M^{-1} ($c_{osm} = 0.6$–1.6 M) for PGlu at pH 5.8, and -170 ± 20 cal mol^{-1} M^{-1} ($c_{osm} =$ 1.3–1.5 M) for P(Glu-*r*-Ala) at pH 6.0. Others have related the *m*-value for protein unfolding to changes in solvent-accessible surface area (40,41).

It is instructive to calculate the overall osmotic work/residue required to induce the α-helix, $\Delta\overline{W} = \Pi_m\Delta\overline{V_w}$, at a given temperature, where $\Delta\overline{V_w} = \nu_w\Delta\overline{N_w}$. The data for all three conditions: PGlu at pH 5.0, PGlu at pH 5.8, and P(Glu-*r*-Ala) at pH 6.0 span $T = 30$°C, and at this temperature, $\Delta\overline{W} \approx 0.1k_BT, 0.5k_BT$, and $0.2\ k_BT$, respectively, for the three conditions. Note that both $\Delta\overline{N_w}$ and $\Delta\overline{W}$ values are relatively small (Table 1) but quickly become significant when summed over many residues (e.g., the cooperative length, $1 + \sigma^{-1/2} = 19$ residues). The overall importance of these results is that they begin to reveal the role of electrostatic and hydration forces for the helix-coil transition in poly(amino acid)s.

TABLE 1 Changes in hydration and work per residue for the helix-coil transition

Sample	$\Delta\overline{N_w}$	$\Delta\overline{W}$ at 30°C (k_BT)
PGlu, pH 5.0	3.6 ± 0.8	0.1
PGlu, pH 5.8	8.1 ± 0.6*	0.5*
P(Glu-*r*-Ala), pH 6.0	1.8 ± 0.3	0.2

*At a constant temperature of 20°C, PGlu at pH 5.8 yields $\Delta\overline{N_w} = -5.1 \pm 0.6$ and $\Delta\overline{W} \approx 0.3k_BT$ (from data in Fig. 5). Calculating from the data in Fig. 6, $\Delta\overline{W} \approx 0.5k_BT$ for PGlu at pH 5.8 and 20°C.

To compare with our experimental values for $\Delta\overline{N_w}$, we calculated the difference in SAS area between the random coil (approximated by an all-*trans* extended configuration) and α-helix using the MSMS program (31). For PGlu, this results in 159.7 Å^2 and 115.4 Å^2/residue for the extended and α-helical conformations, respectively. With the surface area of a water molecule of ~9 Å^2, the difference in SAS area for PGlu gives $\Delta\overline{N_w} = 4.9$ water molecules. This value is in close agreement with $\Delta\overline{N_w}$ determined experimentally for PGlu at pH 5.0, and suggests that it is the first hydration layer being probed by PEG 400 in these osmotic stress experiments. The difference in SAS area of the copolymer P(Glu-*r*-Ala) yields $\Delta\overline{N_w} = 4.6$ water molecules with slightly smaller SAS areas/residue (138.7 Å^2 and 97.7 Å^2 for the extended and α-helical conformations, respectively). The $\Delta\overline{N_w}$ value obtained from experiment is lower than the value calculated from the SAS area change, which possibly could be the result of the hydrophobicity introduced within the copolymer by the alanine residues.

DISCUSSION

The ability to probe protein-water interactions presents a formidable challenge that often can only be studied indirectly (42). Volumetric (43) and vapor pressure osmometry (9) studies of hydration demonstrate that these techniques are useful probes of water about a protein structure. Osmotic stress measurements on protein-DNA complexes (12–14) and adamantane binding to cyclodextrin (10) have been particularly insightful by showing the involvement of water in structure and how it is related to reactions and molecular recognition. With an appreciation for incorporating water into protein structure prediction (28,29), we performed osmotic stress experiments to interrogate the PGlu helix-coil conformational transition and measure the accompanying hydration changes for varying pH and poly(amino acid) composition. The helix-coil transition in PGlu represents a model system for α-helix formation occurring during protein folding. By using osmotic stress to induce the α-helical state, the influence of competing electrostatic interactions can be measured. Also, the increase in T_m for the PGlu α-helix with PEG 400 addition (Fig. 5) demonstrates how osmotic stress increases thermal stability, where lowered water activity favors the more compact α-helical state over the more hydrated coil state. Similar thermal stabilizing effects have been shown for proteins in the presence of glycine, sarcosine, *N*, *N*-dimethylglycine, and glycine betaine (6), and chemical stabilization has been observed with trimethylamine *N*-oxide (7,8).

For calculating the preferential hydration change accompanying the helix-coil transition, $\Delta\overline{N_w}$, we use the osmotic stress analog of the Clapeyron equation (Eq. 2) and assume

that $\Delta\overline{S}$ is constant over the temperature range investigated. Typically, in a thermodynamic analysis, it is better to make the approximation that the enthalpy, rather than the entropy, is constant with temperature. The equation connecting the enthalpy/residue, $\Delta\overline{H}$, and $\Delta\overline{N_w}$ is

$$\frac{d(1/T_m)}{d\ln a_w} = \frac{k_B\Delta\overline{N_w}}{\Delta\overline{H}}, \quad (3)$$

where a_w is the water activity. Another concern is a possible temperature dependence of $\Delta\overline{N_w}$, which may become relevant over a larger temperature range. The linearity observed in the experimental data in Fig. 6 indicates that the temperature range is sufficiently narrow that both $\Delta\overline{S}$ and $\Delta\overline{N_w}$ can be taken as constants, and this is reasonable for the comparative analysis we wish to make between the results. The experimental difficulty in determining $\Delta\overline{H}$ and $\Delta\overline{S}$ for each osmotic pressure measured is that the osmotic pressure is not constant with temperature. This affects the slope of the thermal denaturation curves shown in Fig. 5. In principle, if enough denaturation curves are obtained to construct interpolated curves at a constant osmotic pressure, then both $\Delta\overline{H}$ and $\Delta\overline{S}$, and any temperature dependence, could be directly measured.

Higher osmotic pressures are required to induce and thermally stabilize the PGlu α-helical state for increasing pH, as illustrated in Figs. 3 and 6, respectively. The calculations for PGlu at pH 5.0 ($\Delta\overline{N_w} = 3.6 \pm 0.8, \Delta\overline{W} \approx 0.1k_BT$) and pH 5.8 ($\Delta\overline{N_w} = 8.1 \pm 0.6, \Delta\overline{W} \approx 0.5k_BT$) may also suggest that the preferential hydration change and osmotic work are greater for higher pH. However, because of the simplification of applying the $\Delta\overline{S}$ value calculated for PGlu at pH 5.0 to the pH 5.8 case, these differences may be within the error of this assumption. To compare, we can calculate $\Delta\overline{N_w}$ for the osmotically induced coil-to-helix transition at constant $T =$ 20°C for PGlu at pH 5.8 using the data in Fig. 5. Using the relation

$$\frac{d\ln K}{d\Pi} = -\frac{\nu_w\Delta\overline{N_w}}{\sqrt{\sigma}k_BT}, \quad (4)$$

where $K = f_h/(1 - f_h)$, and the points around $f_h = 0.5$, yields $\Delta\overline{N_w} = -5.1 \pm 0.6$. The corresponding osmotic pressure, $\Pi_m = 8.9$ MPa, gives $\Delta\overline{W} \approx 0.3k_BT$ ($T = 20$°C). At 20°C, thermal denaturation measurements in Fig. 6 give a calculated $\Delta\overline{W} \approx 0.5k_BT$ for PGlu at pH 5.8. Therefore, it seems $\Delta\overline{N_w}$ and $\Delta\overline{W}$ for PGlu at pH 5.8 are possibly only slightly greater than, or about the same as, the values for PGlu at pH 5.0. Based on NMR measurements, estimates of seven water molecules/glutamate group and two water molecules/glutamic acid group have been reported (42). A shift in pK_a (of ~4.4 for an isolated glutamic acid residue) could account for a greater $\Delta\overline{N_w}$ at the higher pH. Such pK_a effects were directly measured by Sukhishvili and Granick for PGlu (44). Based on this reasoning, the additional osmotic work needed to achieve the α-helical state at pH 5.8 includes the energy necessary to overcome the electrostatic repulsion forces between charged glutamate side groups by causing a shift in the pK_a of PGlu. However, to make better quantitative comparisons for probing electrostatic effects, it would be best to perform similar osmotic stress experiments varying salt concentration in addition to pH.

Our SAS area calculations represent the extreme case for the helix-coil transition by using a fully extended conformation to approximate the random-coil state (see Materials and Methods). The calculated difference in SAS area for PGlu gives $\Delta\overline{N_w} = 4.9$ water molecules, which is close to $\Delta\overline{N_w}$ determined experimentally for the thermal denaturation of PGlu at pH 5.0 and the osmotically induced coil-to-helix transition in PGlu at pH 5.8. This suggests that the approximation of the random coil by a fully extended conformation is reasonable here on a local, per-residue, length scale. It also indicates that PGlu preferential hydration by PEG 400 is sensitive to the change in hydration occurring within the first hydration layer of PGlu for the helix-coil transition. However, since only $\Delta\overline{N_w}$ is measured with our osmotic stress experiments, the preferential hydration of each PGlu state by PEG 400 is not known.

The copolymer P(Glu-*r*-Ala) has an experimentally calculated $\Delta\overline{N_w} = 1.8 \pm 0.3$ at pH 6.0, which is slightly lower than the value anticipated from the change in SAS area ($\Delta\overline{N_w} = 4.6$). One possibility is that the extended conformation used to approximate the random-coil state for the SAS area calculation is unreasonable for P(Glu-*r*-Ala). Alanine substitution within the copolymer reduces intramolecular electrostatic repulsion interactions between glutamate residues and provides favorable hydrophobic interactions between alanine residues. Both of these effects will lead to a more compact random coil and thus could explain the lower $\Delta\overline{N_w}$ found experimentally. Also, P(Glu-*r*-Ala) seems to require less osmotic work to stabilize the α-helical state compared with PGlu at a similar pH, both at 30°C. However, as discussed previously, the simplification of using the same $\Delta\overline{S}$ value as calculated for PGlu at pH 5.0 may mean that these differences are within the error of this assumption. It does appear, though, that these results begin to elucidate the interdependence between intramolecular interactions and hydration forces for the helix-coil transition in poly(glutamic acid).

CONCLUSIONS

We have quantified the significance of hydration in the thermal denaturation of the PGlu and P(Glu-*r*-Ala) α-helix using the osmotic stress method and PEG 400 as the osmolyte. The results for PGlu compared well to the calculated change in solvent-accessible surface area between the random-coil and α-helical states. We have demonstrated the ability to use osmotic stress combined with circular dichroism spectroscopy to measure hydration changes occurring at a per-residue level. This is possible because of the well de-

fined repeat structure of the α-helix, but it certainly can be extended to investigate hydration changes accompanying other conformational transitions involving random-coil, α-helix, and β-sheet structures. A specific example where osmotic stress studies analogous to the one we describe here could lend insight is the random-coil to β-spiral inverse temperature transition of elastinlike peptides (45,46), where water release is implicated in the mechanism of this transition. These results also provide a basis for further study of the helix-coil transition in poly(amino acid)s using osmotic stress, and it is anticipated that protein structure prediction incorporating water-mediated interactions (28,29) can take advantage of these measurements. Overall, these findings illustrate the important role that water plays in protein structure and function.

For the future, our experiments may offer a possible mechanism for sensing osmotic pressure in biology. As we mentioned in the Introduction, bacteria have to sense osmotic pressure for their survival (1–3). One might speculate that, similar to our experiments, a membrane channel could be opened or closed by using partially charged random-coil peptide chains reaching into the extracellular space. Opening or closing is then triggered by folding of one or more peptide chains into higher-order structures. Our suggestion is to investigate sequences of potential candidates for osmosensing in bacteria for such partially charged chains. Another possible impact of our work is for the in vitro study of protein folding (47). Our results show that osmotic pressure significantly affects the protein-folding equilibrium. We therefore suggest that to study protein folding in vitro, the osmotic pressure, in addition to pH and salt concentration, should be controlled to better approximate the crowded environment inside cells.

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