Salt Bridges Do Not Stabilize Polyproline II Helices†

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ABSTRACT: Interactions between side chains, and in particular salt bridges, have been shown to be important in the stabilization of secondary structure. Here we investigate the contribution of a salt bridge formed between a lysine and a glutamate to the polyproline II (P_{II}) helical content of proline-rich peptides. Since this structure has precisely three residues per turn, charged residues spaced three residues apart are on the same side of the helix and are best situated to interact. By contrast, computer simulations show that charged residues spaced four residues apart are both too far apart to interact strongly and are oriented such that interactions are unlikely. We have measured the P_{II} content of peptides containing a lysine and glutamate pair spaced three or four residues apart using circular dichroism spectroscopy. Somewhat surprisingly we find that the P_{II} content is insensitive to both the spacing and the pH. These findings indicate that $i \rightarrow i + 3$ salt bridges do not stabilize the P_{II} helical conformation. The implications of these observations for both P_{II} helix formation and denatured protein conformations are discussed.

The left-handed polyproline II (P_{II}) helix has recently received much attention. It is believed to be the dominant conformation of proline-rich sequences (*1*) and is often involved in mediating protein−protein interactions (*2*−*4*). Thirty-five years ago, Tiffany and Krimm (*5*−*7*) hypothesized that denatured proteins possess significant P_{II} helix content. This hypothesis was based on the remarkable similarity between circular dichroism (CD) spectra of denatured proteins and homopolymers of proline which are known to form P_{II} helices. During the three decades following Tiffany and Krimm's hypothesis, a number of groups examined this hypothesis and concluded that it may well be true (*8*−*11*). More recently, there has been a burst of renewed interest in the hypothesis (*12*−*17*).

For the Tiffany and Krimm hypothesis to hold, the P_{II} helical conformation must be stabilized under denaturing conditions. There are two possible sources of stabilization. The first is some direct effect of the denaturant. The second source is the protein sequence. As is the case with other secondary structures, P_{II} helices could be stabilized by the propensities of individual residues to adopt the conformation, as well as interactions between residues within the structure. In this work we investigate the latter and, more specifically, the effect of salt bridges upon P_{II} helix formation.

The P_{II} helical conformation is characterized by repeating backbone (ϕ, ψ) dihedrals of around −75°, +145°, which result in three residues per turn of helix (Figure 1). The Cα−Cβ bond of each residue points perpendicularly out from the backbone, and the Cβ−Cβ distance for residues i and $i + 3$ is around 9 Å. Putting these together, side chains spaced $i \rightarrow i + 3$ are well situated to interact with one another should they be able to span the space between. When side chains are spaced $i \rightarrow i + 4$, however, they are no longer on the same side of the helix and are farther apart, making direct interactions highly unlikely. Therefore, if there are side chain interactions that stabilize the P_{II} helical conformation, they are likely to be between side chains spaced $i \rightarrow i + 3$.

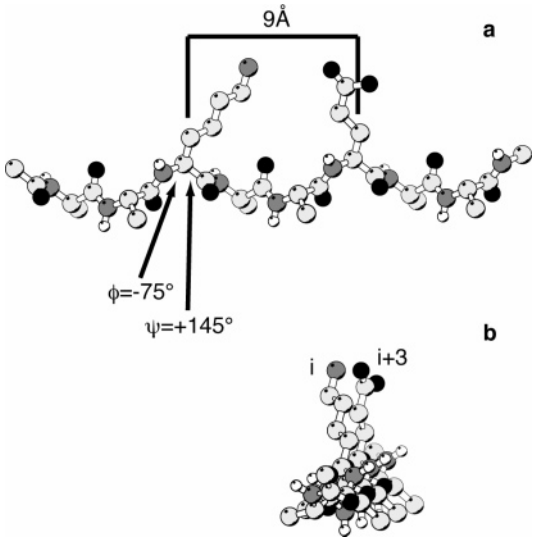

FIGURE 1: Alanine-based peptide in the P_{II} helical conformation. The peptide contains lysine and glutamate residues at a spacing of $i \rightarrow i + 3$. (a) View normal to the long axis. (b) View down the long axis. Figure generated using MOLSCRIPT (*18*).

Salt bridges are capable of stabilizing α-helices (*19*−*21*) and β-sheets (*22*). Such stabilization occurs when the oppositely charged side chains are located on the same side of an α-helix spaced at $i \rightarrow i + 3$ or $i \rightarrow i + 4$ or on the same face of a β-sheet with the side chains on adjacent

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FIGURE 2: Sequences of the four peptides used in this work. Two pairs are illustrated: (A) peptides KP2E and KP3E, in which the guest residues are separated by prolines, and (B) peptides KPAEP3 and KP3EPA, in which a flexible hinge alanine replaces one proline.

strands. Most of the charged residues possess long side chains, so an $i \rightarrow i + 3$ salt bridge is a potential stabilizing interaction in a P_{II} helix. We have chosen to investigate the effects of a lysine−glutamate salt bridge upon P_{II} helix formation by a proline-based peptide. The side chains of these residues can span the intervening distance when at a spacing of $i \rightarrow i + 3$ in a P_{II} helix (Figure 1). The proline-based peptides used have underlying P_{II} helix character and are incapable of α-helix formation. Simple Monte Carlo computer simulations show that such a salt bridge is possible at a spacing of $i \rightarrow i + 3$, but is highly unlikely at $i \rightarrow i + 4$. CD measurements of peptides containing a lysine−glutamate pair spaced at $i \rightarrow i + 3$ or $i \rightarrow i + 4$ indicate that any interactions between these side chains add negligible stability to the P_{II} helical structure. These findings imply that salt bridges are unlikely to be a stabilizing factor in P_{II} helix formation, both in proline-rich peptides and in denatured proteins.

MATERIALS AND METHODS

Peptide Sequence Design. The peptides used in this study employ a proline-based host peptide. Such host peptides are slightly longer versions of a host peptide we employed in earlier studies of the P_{II} helical conformation (*23*, *24*). The main impetus for using this system is that short oligomers of proline are known to form P_{II} helices (*7*, *25*), primarily as a result of steric interactions between prolyl rings (*26*). Another advantage to using a proline-based host system is that proline is the most soluble amino acid (*27*, *28*), so solubility of the peptides is not an issue. Finally, salt bridges between charged side chains at spacings of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ are generally believed to stabilize α-helices (*19*−*21*, *29*, *30*). Such interactions could compete with $i \rightarrow i + 3$ salt bridges in P_{II} helices. Using a proline peptide-based host system precludes α-helix formation and allows us to focus on P_{II} helix stabilization.

The two pairs of peptides used here are illustrated in Figure 2. Each pair consists of one peptide with the lysine and glutamate guests spaced $i \rightarrow i + 3$ and a control peptide with the guests spaced $i \rightarrow i + 4$. The residue composition of each peptide in a pair is identical. The peptides are blocked in order to remove potential electrostatic interactions involving the termini. The C-terminal tyrosine is included to allow for concentration determination using the method of Brandts and Kaplan (*31*). A glycine is placed between the terminal tyrosine and the remainder of the peptide in order to minimize electronic interactions that interfere with interpretation of CD spectra (*32*).

Each peptide possesses two stretches of three consecutive prolines (Figure 2). This is the minimal number of prolines required to form a P_{II} helix (*25*). Therefore, each of these peptides will possess P_{II} character that is independent of any possible interactions between the guest lysine and glutamate residues. An alanine has been inserted into the second pair of peptides in order to provide a more flexible "hinge" region between the guest residues when spaced $i \rightarrow i + 3$. Furthermore, in each case the guest residues are immediately followed in sequence by proline residues. It is known that proline sterically restricts the immediately preceding residue to the β-region of ϕ, ψ space (*33*). However, this restriction does not necessarily result in the residue preferentially occupying the P_{II} region (*26*). Despite the underlying P_{II} content and steric restriction of the guest residues, these peptides will allow for the detection of strong $i \rightarrow i + 3$ interactions between the lysine and glutamate residues.

Circular Dichroism Spectroscopy. Peptides were purchased from Peptidogenic Research and Co. (Livermore, CA). Peptides were purified to ≥95% purity via reverse-phase HPLC and had their identities confirmed using mass spectrometry. Stock solutions consist of the peptides dissolved in pH 7 buffer containing 5 mM potassium phosphate, 5 mM sodium fluoride, and 0.02% sodium azide as a preservative. Peptide concentrations were determined using the method of Brandts and Kaplan (*31*). Absorbance was measured using a 1.0 cm cuvette in a Beckman-Coulter DU640B spectrophotometer.

Circular dichroism (CD) spectra were measured using a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control device. Peptide solutions of concentrations 100−200 μM were placed in a 1 mm path length quartz cuvette, and spectra were measured at 0.5 nm resolution and a scan rate of 200 nm·min^{-1}. Reported spectra are averages of 30 scans, and errors in molar ellipticity are estimated to be around ±3%. The spectrum of each peptide was collected three times using three separately prepared solutions.

Monte Carlo Computer Simulations. Monte Carlo computer simulations of peptides were run to determine the feasibility of the lysine and glutamate side chains interacting. Since the goal was simply to determine whether the side chains could interact, simulations were performed using the hard sphere potential with atomic radii used previously (*34*). The backbone of each peptide was fixed in the P_{II} helical conformation with (ϕ, ψ) = (−75°, +145°). Metropolis sampling (*35*) was employed. Simulations were equilibrated for 10^3 cycles, with data collected every 100 cycles for the next 10^6 cycles. A cycle is the number of attempted moves set equal to the number of rotatable bonds in the peptide being simulated. Four peptides, with sequences Ac-Ala-Lys-$(Ala)_2$-Glu-Ala-NMe (peptide KA2E), Ac-Ala-Lys-$(Ala)_3$-Glu-Ala-NMe (KA3E), Ac-Ala-Glu-$(Ala)_2$-Lys-Ala-NMe (EA2K), and Ac-Ala-Glu-$(Ala)_3$-Lys-Ala-NMe (EA3K), were simulated. Identical data were obtained for sequences where all alanine residues were replaced by prolines (data not shown).

RESULTS

Monte Carlo Computer Simulations. The distributions of distances between the charged groups on the lysine and

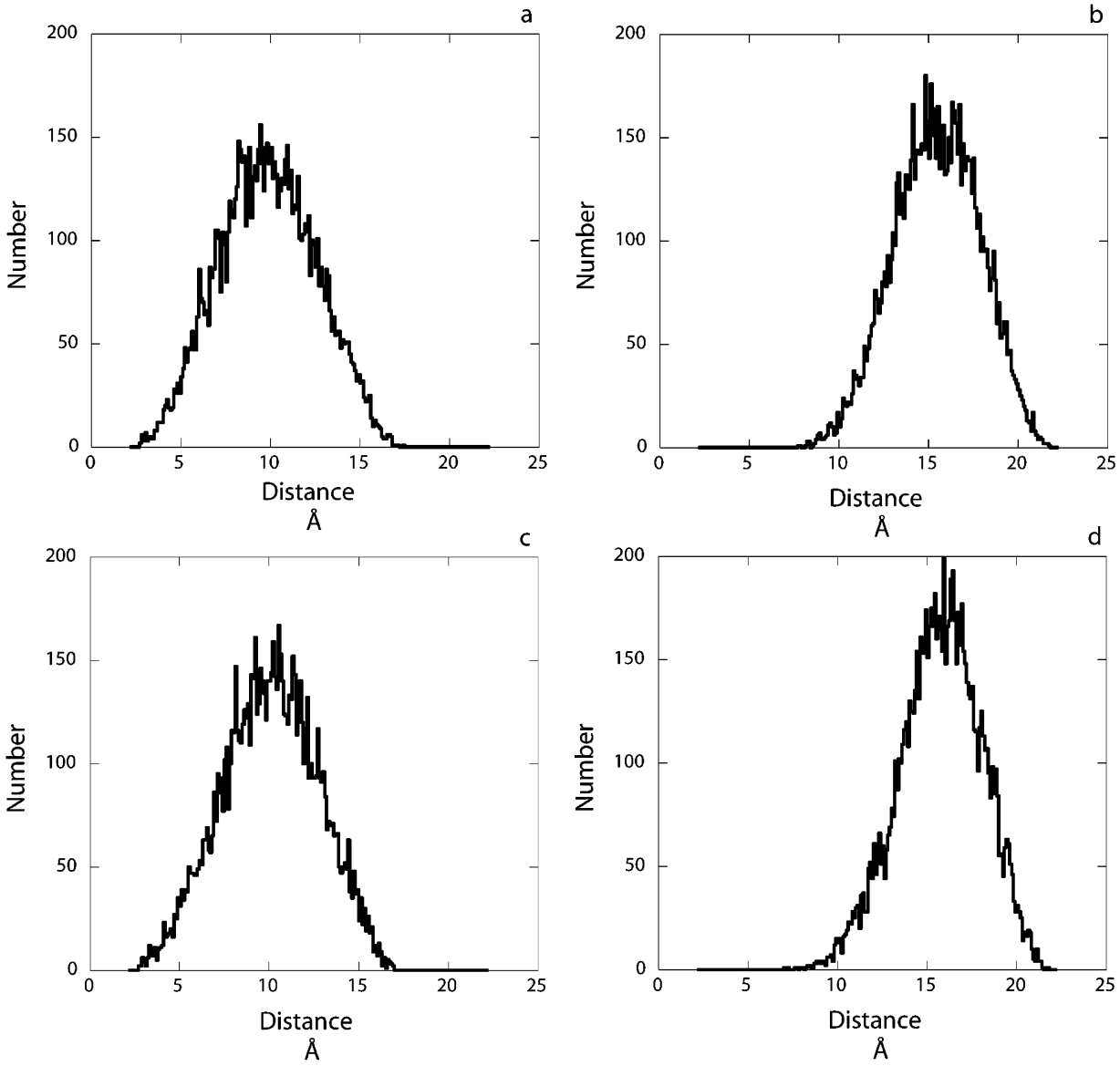

FIGURE 3: Distributions of lysine NZ to glutamate OE1 distances for peptides (a) KA2E, (b) KA3E, (c) EA2K, and (d) EA3K.

glutamate residues in peptides KA2E, KA3E, EA2K, and EA3K were determined from the simulation data and are plotted in Figure 3. In each case the distances analyzed are between the NZ atom of the lysine side chain and the OE1 of the glutamate. Within error the NZ−OE2 distances produce identical distributions (data not shown). Note that the simulations employed the hard sphere potential, so the distance distributions do not reflect what would occur were the side chains modeled with explicit charges. However, the data acquired using such a model will reflect what is feasible and will, therefore, tell us whether a salt bridge or hydrogen bond is possible between the lysine and glutamate guest residues.

As can be seen from Figure 3a,c, when spaced $i \rightarrow i + 3$, the lysine and glutamate residues can approach one another close enough to form not only a salt bridge but potentially also a hydrogen bond (∼3−4 Å). When spaced $i \rightarrow i + 4$, however, the two guests perhaps come just close enough for a water-mediated salt bridge interaction (∼7−8 Å). The simple hard sphere modeling would then indicate that $i \rightarrow i + 3$ salt bridge interactions in a P_{II} helix are quite possible whereas $i \rightarrow i + 4$ interactions that stabilize the P_{II} conformation are unlikely. Furthermore, the similarity of the distributions for KA2E (Figure 3a) and EA2K (Figure 3c) suggests that putative salt bridge interactions do not appear to be dependent upon the ordering of the lysine and glutamate residues within the sequence.

Circular Dichroism Spectroscopy. The CD spectra at pH 7 and a temperature of 5 °C for the four peptides examined are shown in Figure 4. It is clear that peptides KP2E and KP3E (Figure 4a) and peptides KPAEP3 and KP3EPA (Figure 4b) possess overlapping spectra. These spectra are all indicative of considerable P_{II} helical content, as would be expected given the high proline content of the peptides (*23*, *24*). Within experimental error each pair of peptides has the same CD signal. This indicates that each pair has the same P_{II} helical content. Peptides KPAEP3 and KP3EPA possess lower P_{II} helix contents, as judged by the shallower minima, than peptides KP2E and KP3E. This indicates that the alanines in KPAEP3 and KP3EPA are more flexible than the corresponding prolines in KP2E and KP3E. The alanine

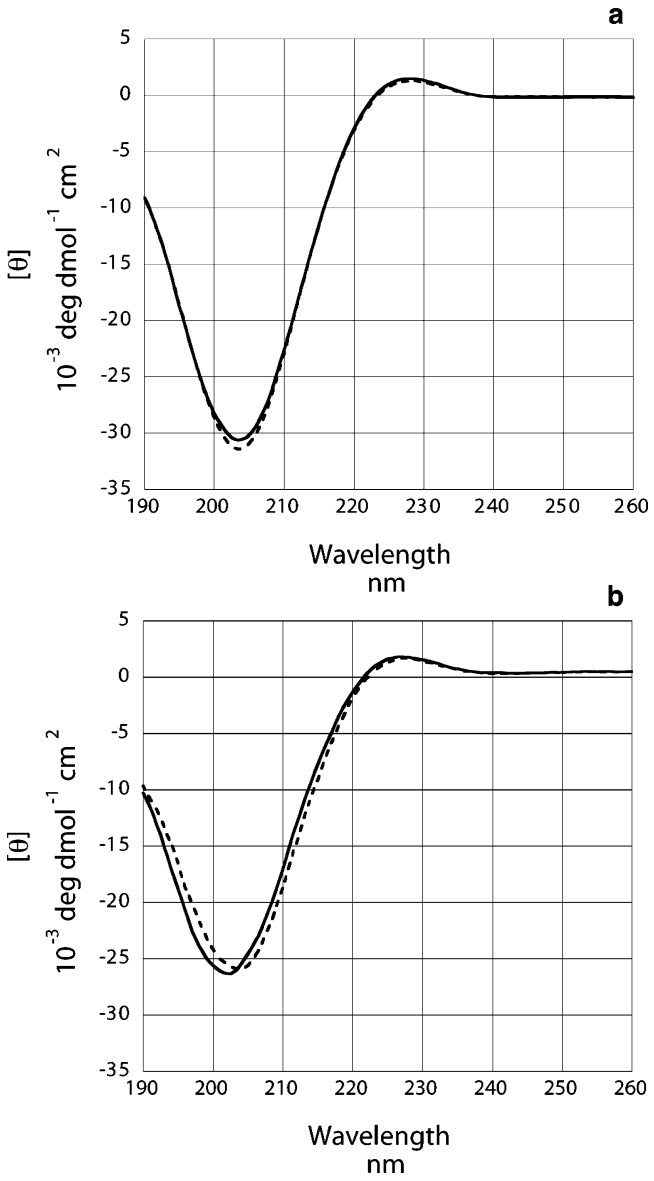

FIGURE 4: CD spectra collected at pH 7 and 5 °C for (a) peptides KP2E (solid line) and KP3E (dashed line) and (b) peptides KPAEP3 (solid line) and KP3EPA (dashed line).

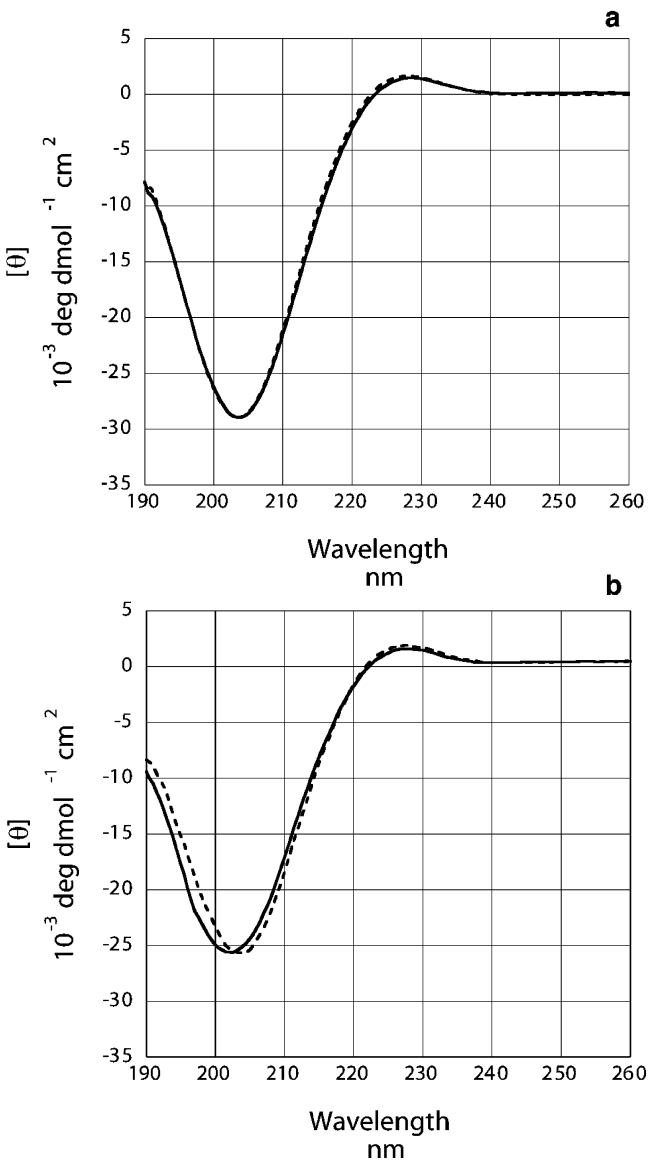

FIGURE 5: CD spectra collected at 5 °C and pH 2 for (a) peptides KP2E (solid line) and KP3E (dashed line) and (b) peptides KPAEP3 (solid line) and KP3EPA (dashed line).

in KPAEP3 does then act as a "flexible hinge" region as expected.

That peptides KP2E and KP3E have the same P_{II} content is perhaps not altogether surprising. The guest residues in each of these peptides are separated by proline residues, which will be predominantly P_{II} in nature. Furthermore, each guest residue is followed immediately by a proline which will restrict it to the β-region of ϕ, ψ space (*26*). Finally, both lysine and glutamate have reasonably high propensities to adopt the P_{II} helical conformation when in a proline-based host peptide (*24*). Taken in sum, these two peptides should possess considerable P_{II} character.

It is more surprising that peptides KPAEP3 and KP3EPA have almost identical spectra. The shift in the position of the minima (Figure 4) is most likely due to the electronic effects upon the spectra arising from the relative ordering of primary, secondary, and tertiary amines within the peptides (*8*, *36*). Although alanine is known to have a relatively high P_{II} propensity (*14*, *23*, *24*, *37*), it is certainly more flexible than a proline and should allow peptide KPAEP3 to adopt conformations not available to KP3EPA. The alanine does lower the overall per residue P_{II} content when the spectrum for KPAEP3 is compared to that of KP2E. It is clear from Figure 4 that the minimum in the spectrum for KPAEP3 is not as deep as that for KP2E. This minimum, in conjunction with the small maximum, is characteristic of the P_{II} conformation (*8*, *25*). The spectra in Figure 4 indicate that salt bridge interactions do not appear to add to the overall P_{II} content of these peptides.

To further investigate, the dependence of the CD spectra for the peptides on pH was determined. Spectra were collected at pH 2 (Figure 5) and pH 12 (Figure 6), where the glutamates and lysines, respectively, would be predominantly uncharged. As can be seen from Figures 5 and 6, within error peptides KP2E and KP3E behave identically at both low and high pH. The same is true for peptides KPAEP3 and KP3EPA. These data further indicate that salt bridge interactions do not contribute significantly to the stability of P_{II} helices in this host−guest system. Furthermore, the

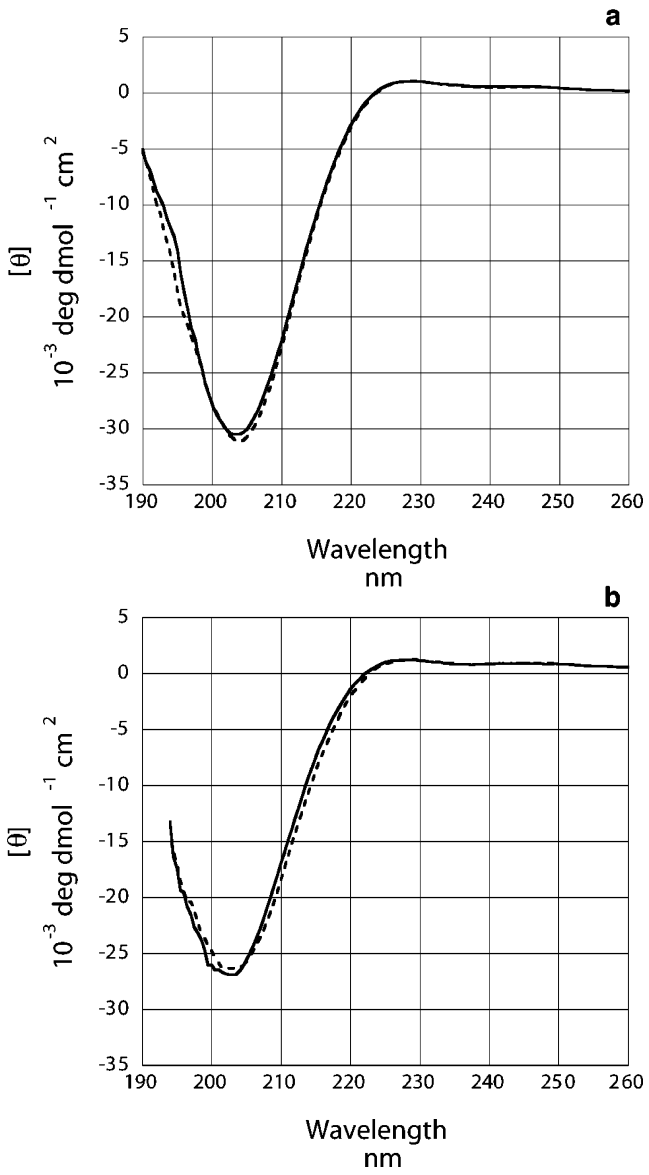

FIGURE 6: CD spectra collected at 5 °C and pH 12 for (a) peptides KP2E (solid line) and KP3E (dashed line) and (b) peptides KPAEP3 (solid line) and KP3EPA (dashed line).

data indicate that hydrogen bonds between the lysine and glutamate side chains, which could still form at pH 2 and 12, also do not contribute to P_{II} helix stability. Such hydrogen bonds could exist at the $i \rightarrow i + 3$ spacing in peptides KP2E and KPAEP3 but not at the lysine−glutamate $i \rightarrow i + 4$ spacing in peptides KP3E and KP3EPA.

DISCUSSION

With precisely three residues per turn, one could expect that P_{II} helices might be stabilized by interactions between side chains spaced $i \rightarrow i + 3$ (Figure 1). The Cβ atoms of side chains at this spacing are 9 Å apart, so any such interactions will probably only occur with pairs of residues possessing long side chains. Lysine and glutamate possess side chains that are sufficiently long to be able to interact in a P_{II} helix at a spacing of $i \rightarrow i + 3$, and such an interaction would most likely be in the form of a salt bridge, although a hydrogen bond is also a possibility. Monte Carlo computer simulations, using the hard sphere potential, of peptides with lysine and glutamate residues at $i \rightarrow i + 3$ indicate that such interactions are in fact feasible (Figure 3). Corresponding simulations with the lysine and glutamate residues at $i \rightarrow i + 4$ suggest that interactions that will stabilize the P_{II} conformation are unlikely at this spacing.

The experimental data tell a different story. It is clear from the CD spectra (Figure 4) of the four peptides examined that the putative lysine−glutamate bridge does not significantly stabilize the P_{II} helices formed by proline-based peptides. The data indicate that this is the case for the relatively rigid KP2E and KP3E peptides. It may be argued that the rigid P_{II}-like proline spacers between the lysine and glutamate residues in this pair of peptides keep the peptides in a P_{II} conformation that is unaffected by the presence or absence of a salt bridge, and so we would not see a difference in the spectra. However, although the lysine and glutamate residues are sterically restricted by immediately following prolines, this restriction is to the β-region of ϕ, ψ space, not necessarily to the P_{II} region. In addition, the prolines immediately preceding the lysine and glutamate residues are not sterically restricted to the P_{II} region.

The addition of an alanine residue in a position that is *not* followed by a proline (Figure 2) does not change the observation that a $i \rightarrow i + 3$ lysine−glutamate salt bridge does not stabilize the P_{II} helical structure of the peptides. The alanine is free to act as a somewhat flexible hinge, allowing the peptide to bend and bring the lysine and glutamate into closer proximity. An interesting observation is that this does not appear to happen. The spectra for the KPAEP3 and KP3EPA peptides are almost identical, indicating that the contributions of the alanines in these short peptides are similar. The conformational behavior of these alanines must therefore be similar. The P_{II} helix content of the KPAEP3 and KP3EPA peptides is lower than that of the KP2E and KP3E peptides (Figure 4), which can only arise as a result of the alanines in the former two peptides having a lower P_{II} content than the corresponding prolines in the latter two peptides. This is in keeping with our earlier measurements that indicate that proline is the best P_{II} helix former in a proline-based system and that alanine, while having a reasonably high propensity, is not nearly as good (*23*, *24*).

Each pair of peptides was found to respond identically to changes in pH (Figures 5 and 6). This is further indication that any salt bridge interactions have a negligle effect upon the measured P_{II} helix content. At pH 2 the glutamate side chains will be uncharged, while at pH 12 the lysine side chains will be uncharged. The CD spectra taken at pH 2 (Figure 5) are very similar to those obtained at pH 7 (Figure 4). There is perhaps a small loss of P_{II} helix content at the lower pH, but this is found in all four peptides, indicating that this is not due to a loss of stabilizing salt bridge interactions. The CD spectra collected at pH 12 (Figure 6) differ from those at pH 7, having smaller maxima around 228 nm and possibly a new positive band appearing around 240−250 nm. This is precisely the same behavior we had observed earlier in work on single charged guest residues in a proline-based host peptide (*24*) and is believed to be a property of the high-pH solution acting directly on the backbone rather than on the side chains. Since each pair of peptides behaves similarly at pH 12, it is clearly not related to the disruption of a stabilizing salt bridge interaction.

From the data presented here one can conclude that $i \rightarrow i + 3$ salt bridges, while certainly geometrically possible, do not significantly stabilize the P_{II} helices formed by the proline-based peptides examined in this work. By extension, one would then expect such interactions to be insignificant in the formation of P_{II} helices by proline-rich sequences. We would argue that our data indicate that such salt bridges are also unlikely to be stabilizing for P_{II} helices formed by sequences with low levels, or devoid, of proline. Our reasoning is as follows. First, the inclusion of an alanine hinge region did not affect the observed similarity between peptides with the lysine and glutamate pair spaced at $i \rightarrow i + 3$ and those spaced at $i \rightarrow i + 4$. Second, $i \rightarrow i + 3$ salt bridges can stabilize the α-helical conformation (*19*−*21*). Our proline-rich host peptides are incapable of forming α-helices; however, sequences devoid of proline would be capable of doing so. Given that we see no indication of salt bridges stabilizing the P_{II} helical conformation in proline-based peptides, it seems possible, if not likely, that such a spacing would in fact *destabilize* P_{II} helices in other sequences by stabilizing α-helical conformations.

It was suggested 35 years ago that denatured proteins have significant P_{II} helix content (*5*−*7*). If this hypothesis is true, there must be sequence-dependent factors that stabilize the P_{II} conformation. One such set of factors would be underlying propensities for individual residues to adopt this conformation. Recent work suggests that residues do indeed possess such propensities (*14*, *23*, *24*, *38*). In fact, significant data indicate that the polypeptide backbone has a high propensity to form P_{II} helices under conditions where the formation of more compact structures is disfavored (*12*, *14*, *37*, *38*). Another potential set of stabilizing factors for P_{II} helices would be interactions between side chains, particularly those spaced at $i \rightarrow i + 3$, which place the side chains on the same side of the helix (Figure 1). Our data indicate that salt bridges, possibly the strongest type of side chain interaction, do not in fact stabilize P_{II} helices. If side chain interactions do not stabilize P_{II} helices, then the formation of such helices in denatured proteins must be driven by the individual propensities of residues modulated by the effects of the denaturant.

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