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Stable Conformations of Tripeptides in Aqueous Solution Studied by UV Circular Dichroism Spectroscopy

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Abstract: Determination of the precise solution structure of peptides is of utmost importance to the understanding of protein folding and peptide drugs. Herein, we have measured the UV circular dichroism (UVCD) spectra of tri-alanine dissolved in D_2O, H_2O, and glycerol. The results clearly show the coexistence of a polyproline II or 3_1-helix and a somewhat disordered flat β-strand conformation, in complete agreement with recent predictions from spectroscopic data (Eker et al. *J. Am. Chem. Soc.* **2002**, *124*, 14 330−14 341). A thermodynamic analysis revealed that enthalpic contributions of about 11 and 17 kJ/mol stabilize polyproline II in D_2O and H_2O, respectively, but at room temperature they are counterbalanced by entropic contributions, which clearly favor the more disordered β-strand conformation. It is hypothesized that this delicate balance is the reason for the variety of structural propensities of amino acid residues in the absence of nonlocal interactions. The isotope effect yielding a higher occupation of polyproline II in H_2O with respect to D_2O strongly suggests that a hydrogen-bonding network involving the peptide and water molecules in the hydration shell plays a major role in stabilizing this conformation. The equilibrium between polyproline II and β-strand is practically maintained in glycerol, which suggests that glycerol can substitute water as stabilizing solvent for the polyproline II conformation. We also measured the UVCD spectra of tri-valine and tri-lysine (both at acidic pD) in D_2O and found them to adopt a flat β-strand and left-handed turn structure, respectively, in accordance with recent analyses of vibrational spectroscopy data. Generally, the present study adds substantial evidence to the notion that the so-called random coil state of peptides is much more structured than generally assumed.

Introduction

The details of the mechanism by which proteins fold and unfold were and remain one of the central yet unresolved problems of molecular biology.[1−14] Despite our excellent knowledge of the structure of more than a thousand proteins due to the application of X-ray crystallography and high-resolution NMR, much less is known about the pathways of protein folding. Generally, the investigation of protein folding is complicated by a complex multidimensional energy landscape.[15−18] One of the open questions in this context is how the native structure is encoded in the amino acid sequence.[19] In that regard, two issues are of particular importance. First, it is still unclear which of the properties of amino acid residues determine their contribution to the folding process. Dill mentions two general and not necessarily conflicting views in his review.[19] One of them emphasizes the importance of understanding backbone interactions, hydrogen bonding, and the (ϕ,ψ) propensity of amino acid residues.[20−23] The other one suggests that hydrophobic interactions between side chains, which also involve solvent mediated contact interactions, are essential for the folding process.[24] These two views are interrelated to another important issue, namely whether folding occurs hierarchically by first forming secondary structures and subsequently the tertiary structure of a protein.[22] Second, it is unclear to what

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(1) Epand, R. M; Scheraga, H. A. *Biochemistry* **1968**, *7*, 2864−2872.
(2) Taniuchi, H.; Anfinsen, C. B. *J. Biol. Chem.* **1969**, *244*, 3864−3875.
(3) Anfinsen, C. B. *Science* **1973**, *181*, 223−230.
(4) Hermans, J., Jr.; Puett, D. *Biopolymers* **1971**, *10*, 895−914.
(5) Kim, P. S.; Baldwin, R. L. *Annu. Rev. Biochem.* **1990**, *59*, 631−660.
(6) Matthwes, C. R. *Curr. Opin. Struct. Biol.* **1991**, *1*, 28−35.
(7) Davidson, E. R. *Chem. Rev.* **1993**, *93*, 2337.
(8) Ptitsyn, O. B *Curr. Opin. Struct. Biol.* **1995**, *5*, 74−78.
(9) Karplus, M.; Sali, A. *Curr. Opin. Struct. Biol.* **1995**, 5, 58−73.
(10) Fersht, A. R.; Serrano, L. *Curr. Opin. Struct. Biol.* **1997**, *7*, 3−9.
(11) Shakhnovich, E. I. *Curr. Opin. Struct. Biol.* **1997**, *7*, 29−40.
(12) Chan, H. S.; Dill, K. A. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 425−459.
(13) Lazaridis, T.; Karplus, M. *Science* **1997**, *278*, 1928−1931.
(14) Baldwin, R. L. *Nat. Struct. Biol.* **1999**, *6*, 814−817.
(15) Dobson, C. M.; Karplus, M. *Curr. Opin. Struct. Biol.* **1999**, *9*, 92−101.
(16) Karplus, M.; Shakhnovich, E. In *Protein Folding*; Creighton, T. E., Ed.; W. H. Freeman: New York, 1992; p 127.
(17) Bryngelson, J. D.; Onuchic, J. N.; Socci, N. D.; Wolynes, P. G. *Proteins Struct. Funct. Genet.* **1995**, *21*, 167−195.
(18) Becker, O. M *J. Mol. Struct (TEOCHEM)* **1997**, *507*, 398−399.
(19) Dill, K. A. *Protein Sci.* **1999**, *8*, 1166−1180.
(20) Honig, B.; Cohen, F. E. *Folding Design* **1996**, *1*, R17−R20.
(21) Aurora, R.; Creamer, T. P., Srinivasan, R. Rose, G. D. *J. Biol. Chem.* **1997**, *272*, 1413−1416.
(22) Baldwin, R. L.; Rose, G. D. *Trends Biochem. Sci.* **1999**, *24*, 77−83.
(23) Shi, Z.; Woody, R. W.; Kallenbach, N. R. *Adv. Protein Chem.* **2002**, 62, 163−240.
(24) Kauzmann, W. *Adv. Prot. Chem.* **1959**, *14*, 1−63.

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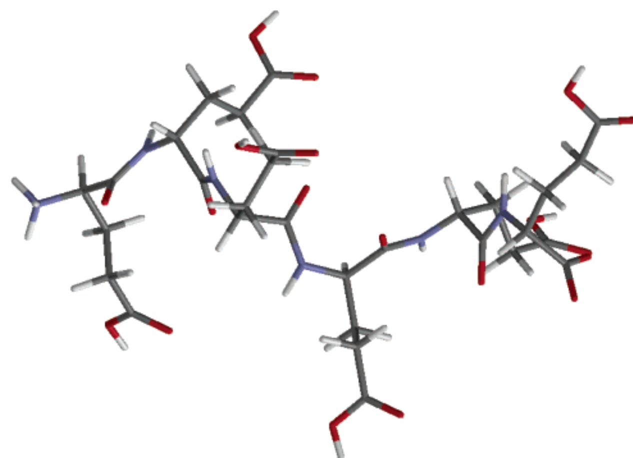
extent the initial unfolded state of a protein or peptide is a determinant of the folding process. If this were the case, then the (ϕ,ψ) propensities of amino acid residues would be important parameters. This issue is a subject of intense debates in the literature. Dill argues that since short peptides such as tri- and tetrapeptides exhibit large conformation entropy by sampling the allowed region of the Ramachandran plot, the (ϕ,ψ) propensity is not a key parameter in the absence of a protein context.[19] This view originates from Tanford's classical work[25] from which the unfolded state of peptides and protein segments emerged as very flexible entities, which fluctuate between different conformational minima on a very short time scale. A snapshot of an ensemble of such peptides is therefore thought to reveal a random distribution of coordinates (dihedral angles) within the sterically allowed region of the Ramachandran plot. This understanding, however, was questioned 35 years ago by Tiffany and Krimm,[26] who interpreted UV−CD-experiments as indicating that the coil state of poly-L-lysine is in reality an extended polyproline II (PPII) conformation (cf. Figure 1). Only at high temperatures a distinct fraction of the peptide sample decays into a more disordered random coil like state. This led these authors to hypothesize that the 'random coil′ state of peptides and proteins might exhibit a local order, which is describable as an extended, left-handed 3_1 helix. This hypothesis (referred to as the TK hypothesis) found support from other experiments,[27] but was also heavily contested by Mattice et al.[28,29] based on the fact that some blocked alanine based di-, tri and tetrapeptides exhibited very similar CD-signals as the polypeptides investigated by Tiffany and Krimm (cf. Figure 1).[26] On the basis of the general belief that the structures of these short peptides are random it was concluded that this also holds for the coil state of poly-L-lysine, in contrast to the TK hypothesis. After this debate in the 1970s, the hypothesis had not attracted serious consideration by researchers for nearly 15 years but the situation has significantly changed over the last 10 years, when ample evidence were provided by virtue of various spectroscopic and computational techniques that particularly peptides with alanine and lysine residues can adopt a PPII conformation in water.[30−41] In the absence of water, the PPII minimum seems to disappear in the energy landscape of the peptide.

If the coil states of peptides and proteins are indeed more structured, as suggested by the above results, then their conformations are certainly to a major extent determined by



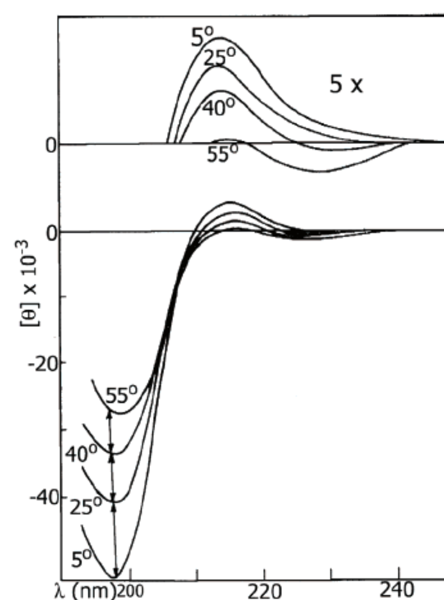


Figure 1. (a) Ball-and-stick model of a PPII conformation with six glutamic acid residues. (b) CD spectra of polyglutamic acid at the indicated temperatures as reported by Tiffany and Krimm.[32]

the intrinsic propensities of their amino acid residues in an aqueous environment. These can be explored by investigating small peptide fragments. For a long time they were believed to be structurally random, but this view has recently been substantially modified. We have developed an algorithm[42] by which we determined the structure of tripeptides from the amide I band shape in the respective isotropic and anisotropic Raman, FTIR, and VCD spectra.[38,43,44] With respect to the classical model system tri-alanine (AAA), we obtained two solutions. An analysis in terms of a one-state model yielded an extended

(25) Tandford, C. *Adv. Protein Chem.* **1968**, *23*, 121−282.
(26) Tiffany, M. L.; Krimm, S. *Biopolymers* **1968**, *6*, 1379−1382.
(27) Andries, J. C.; Anderson, J. M.; Walton, A. G. *Biopolymers* **1971**, *10*, 1049−1057.
(28) Mattice, W. L.; Riser, J. M.; Clark, D. S. *Biochemistry* **1976**, *15*, 4264−4272.
(29) Mattice, W. L. *Biopolymers* **1974**, *13*, 169−183.
(30) Dukor, R. K.; Keiderling, T. A. *Biopolymers* **1991**, *31*, 1747−1761.
(31) Tiffany, M. L.; Krimm, S. *Biopolymers* **1968**, *6*, 1767−1770.
(32) Tiffany, M. L.; Krimm, S. *Biopolymers* **1972**, *11*, 2309−2316.
(33) Park, S.-H.; Shalongo, W.; Stellwagen, E. *Protein Sci.* **1997**, *6*, 1694−1700.
(34) Shi, Z.; Olson, C. A.; Rose, G. D.; Baldwin, R. L.; Kallenbach, N. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 9190−9195.
(35) Rucker, A. L.; Creamer, T. P. *Protein Sci.* **2002**, *11*, 980−985.
(36) Poon, C.-D.; Samulski, E. T.; Weise, C. F.; Weisshaar, J. C. *J. Am. Chem. Soc.* **2000**, *122*, 5642−5643.
(37) Woutersen, S.; Hamm, P. *J. Phys. Chem B.* **2000**, *104*, 11 316−11 320.
(38) Schweitzer-Stenner, R.; Eker, F.; Huang, Q.; Griebenow, K. *J. Am. Chem. Soc.* **2001**, *123*, 9628−9633.
(39) Han W.-G.; Jalkanen, K. J.; Elstner, M.; Suhai, S. *J. Phys. Chem. B* **1998**, *101*, 2587−2602.
(40) Mu, Y.; Stock, G. *J. Phys. Chem. B.* **2002**, *106*, 5294−5301.
(41) Weise, C. F.; Weisshaar, J. C. *J. Phys. Chem. B.* **2003**, *107*, 3265−3277.
(42) Schweitzer-Stenner, R. *Biophys. J.* **2002**, *83*, 523−532.
(43) Eker, F.; Cao, X.; Nafie, L.; Schweitzer-Stenner, R. *J. Am. Chem. Soc* **2002**, *124*, 14 330−14 341.
(44) Eker, F.; Cao, X.; Nafie, L.; Huang, Q.; Schweitzer-Stenner, R. *J. Phys. Chem. B.* **2003**, *107*, 358−365.

β-helix like structure for all three protonation states. The conformation shows a larger ϕ-angle than a classical PPII structure, which was beforehand reported for the cationic state of AAA[38] as well as for the blocked alanine dipeptide,[43] based on vibrational and NMR spectroscopy. As an alternative we performed a two-state analysis which yielded nearly a 50:50 mixture of PPII ((ϕ,ψ) = −60°, 150°) and a flat β-strand conformation ((ϕ,ψ) = −160°, 150°) for AAA.[43] This result is in agreement with NMR-data and close to theoretical predictions by MD simulations.[40] Similar results were obtained for KAA, SAA, and AcAA.[44] On the contrary, VVV was found to be locked in a flat β-strand like conformation with ((ϕ,ψ) = −170°, 140°); a two state model appeared to be inconsistent with the experimental data. Another homopeptide, KKK, was found to exhibit a left-handed turn like structure and again, a significant population of other, significantly different conformers was ruled out based on the experimental data. In view of the relevance of these results for the understanding of the coil state of peptides and proteins, an independent check of them by another spectroscopic technique is highly desirable. In the present study we therefore measured the UV−CD spectra of cationic AAA, VVV and KKK in aqueous solution as a function of temperature. Moreover, we examined the temperature dependence of amide I in the respective FTIR-spectra. Finally, we measured the CD spectra of AAA dissolved in glycerol to investigate whether the solvent can to some extent serve as substitute for water. Our study can be considered as a final proof of our earlier results on the investigated tripeptides. In addition, we provide conclusive evidence that the CD-signal shown in Figure 1 is indeed diagnostic of a PPII structure. This is of relevance for the interpretation of the CD-spectra of many unfolded proteins reported in the literature, which are still interpreted as indicating a disordered, random coil state.

Material and Methods

Materials. L-alanyl-L-alanyl-L-alanine (AAA), L-vanyl-L-vanyl-L-valine (VVV), l-lysyl-L-lysyl-L-lysine (KKK) were purchased from Bachem Bioscience Inc. (>98% purity). AAA, VVV, and KKK were used without further purification. D_2O, NaOD, DCl, glycerol, and $NaClO_4$ were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All chemicals were of analytical grade.

Sample Preparation. For the CD measurements lyophilized AAA, VVV and KKK were disolved in 1 mL D_2O. The respective concentrations were 1mM (for AAA and VVV) and 3 mM (for KKK). Moreover, we carried out CD measurements of AAA disolved in H_2O and glycerol. For the latter, the peptide was first dissolved in D_2O at a pD of 1 and then lyophilized for 2 days. The resulting lyophilized powder was dissolved in neat glycerol. For the FTIR experiment on AAA we disolved 50 mg of the peptide in 1 mL D_2O, which corresponds to a concentration of 0.2 M.

The pH value of the respective solutions were adjusted by adding small aliquots of DCl/NaOD or HCl/NaOH to obtain the cationic, zwitterionic and anionic state of the peptides. The respective pD values were determined by utilizing the method of Glasoe and Long.[45]

CD Spectroscopy. Far-UV CD spectra (250−195 nm) were measured with an OLIS DSM-10 UV/Vis CD spectrophotometer in a 1.0-mm quartz cell with 2 nm resolution. The samples were placed in a nitrogen gas purged OLIS CD module. The temperature at the cuvette was controlled by means of a Peltier-type heating system (accuracy ± 1 °C). For each measurement, the sample in the cuvette was allowed to equilibrate for 5 min at the adjusted temperature prior to data acqusition. For all experiments reported in this paper $\Delta A(\lambda,T)$ was measured by increasing the temperature in increments of 5 °C. The integration time was set as a function of high volts to obtain an appropriate signal-to-noise ratio. The room temperature spectra were obtained by averaging 5 scans. The solvent reference spectra were used as baselines, which were automatically subtracted from the peptide CD spectra. For the final presentation in this paper, the original $\Delta A(\lambda,$T) spectra were converted to the $\Delta\epsilon(\lambda,$T) representation by using the above sample concentrations and the path length of the cuvette.

(45) Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188−193.

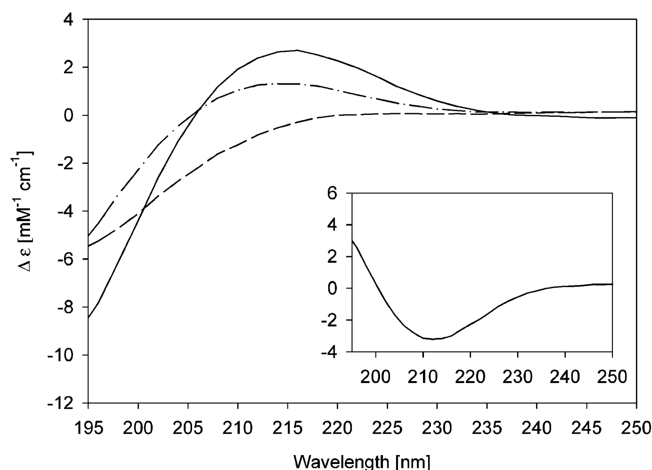


Figure 2. CD spectra of AAA in D_2O at cationic, zwitterionic and anionic pDs. (Solid line: pD 1.0, dash−dot-dot: pD 6.0, short dash: pD 12.0.) Inset: CD difference spectrum for AAA at pD 12.0 and pD 1.0.

FTIR Spectroscopy. FTIR spectra were measured with a Nicolet Magna-IR System 560 optical bench as described elsewhere.[46] A total of 256 scans at 2 cm^{-1} resolution using Happ-Ganzel apodization were averaged to obtain each spectrum. For all experiments, a liquid temperature cell equipped with CaF_2 windows and 6−15 μm thick Teflon spacers was used. A Spectra Tech digital temperature controller was used to adjust the temperature of the cell (±1 °C accuracy). Routinely, 5 min were allowed for thermal equlibrium to be reached prior to FTIR spectral acquisition. The optical bench was purged with dry air produced with a Parker laboratory gas generator to reduce interfering water vapor IR absorption. Each peptide sample was measured at least four times. Spectra were corrected for the solvent background in interactive manner using Nicolet OMNIC 3.1 software. All FTIR spectral curve-fitting was performed by using the program MULTIFIT.[47] The spectra between 1550 and 1800 cm^{-1}, which contains amide I′ were decomposed into Voigtian bands as described before.[38,43,44]

Results and Discussion.

Tri-Alanine in Aqueous Solution. The CD spectra of cationic (pD 1), zwitterionic (pD 6) and anionic (pD 12) AAA (0.3 mg/mL) in D_2O were measured. In what follows we interpret these and other spectra in terms of the basis spectra reported by Sreerama and Woody.[48] Figure 2 shows the CD spectra of AAA at room temperature at indicated pD values. The CD spectra of cationic and zwitterionic AAA exhibit a weak positive peak at 217 nm and a very sharp negative peak below 195 nm. In the absence of interfering signals from aromatic side chains, such a couplet is generally interpreted as indicative of a significant contribution from a PPII-like conformer (Figure 1).[30−35,49] Compared with the cationic state, the signals of the

(46) Griebenow, K.; Diaz, L. Y.; Santos, A. M.; Montañez, C. I.; Rodriquez, L.; Vidal, M.; Barletta, G. *J. Am. Chem. Soc* **1999**, *121*, 8157−8163.
(47) Jentzen, W.; Unger, E.; Karvounis, G.; Shelnutt, J. A.; Dreybrodt, W.; Schweitzer-Stenner, R. *J. Phys. Chem.* **1996**, *100*, 14 184−14 191.
(48) Sreerema, N.; Woody, R. W. *Biochemistry* **1994**, *33*, 10 022−10 025.

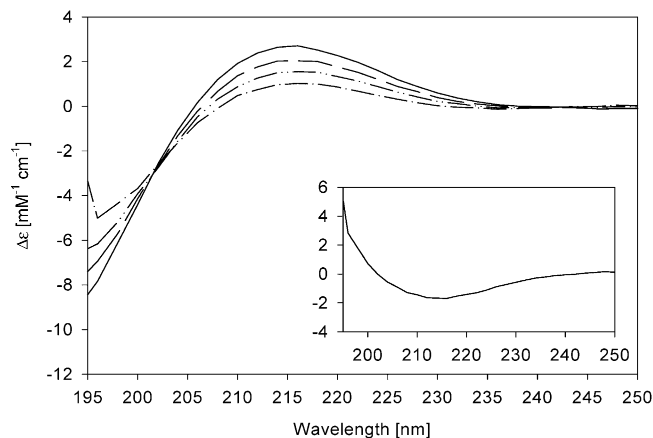


Figure 3. Temperature-dependent CD spectra of AAA in D_2O at pD 1.0. (Solid line: 20 °C, long dash: 40 °C, dash−dot−dot−dash: 60 °C, dash−dot−dash: 85 °C.) Inset: CD difference spectrum for AAA between 85 °C and 20 °C.

zwitterionic and anionic states are somewhat more pronounced. The difference between the spectrum of the anionic and cationic state shown as inset in Figure 2 is indicative of either a β-strand like or a so-called disordered conformation becoming dominant at higher temperatures.[34] Even the cationic signal is weaker than that obtained for an unblocked trans PPP as reported by Dukor and Keiderling.[30] Altogether, this suggests a mixture of β-strand (disordered) and PPII-conformers with a larger fraction of the former for the zwitterionic and anionic state. However, the spectra of the latter are somewhat difficult to interpret because of the presence of charge-transfer transitions from n and π-HOMOs of the carboxylate group into the π*-LUMOs of the C-terminal peptide.[50,51] Therefore, the investigation was confined to the cationic state.

Figure 3 shows the CD spectrum of cationic AAA measured at different temperatures between 20 °C and 85 °C. The difference between the spectra taken at 85 °C and 20 °C is depicted in the inset. It indicates that either a β-strand or a disordered conformer becomes more populated at the expense of the PPII-conformer at higher temperatures. Most important, a clear isodichroic point appears at 205 nm. This shows that the temperature dependence of the CD-spectrum can be explained in terms of a two-state transition between a PPII and a β-strand conformation, in full agreement with the result from a two-state analysis of Raman, IR, and VCD data.[43] This mutual corroboration of CD and vibrational spectroscopy data provides on one hand conclusive evidence for the assignment of the CD-signal in Figure 1 to a PPII conformation and on the hand proves that two extended conformers of AAA coexist in aqueous solution in accordance with results from MD simulations.[40] Hence, Mattice's[29] criticism of the TK-hypothesis was based on the wrong assumption that tripeptides cannot adopt a well-defined structure in solution.

It is noteworthy in this context that CD-measurements of somewhat longer polypeptides yielded results very similar to those reported above. Temperature-dependent CD measurements of poly-L-lysine by Tiffany and Krimm[26,30] also clearly depict an isodichroic point reflecting the coexistence of two conforma-

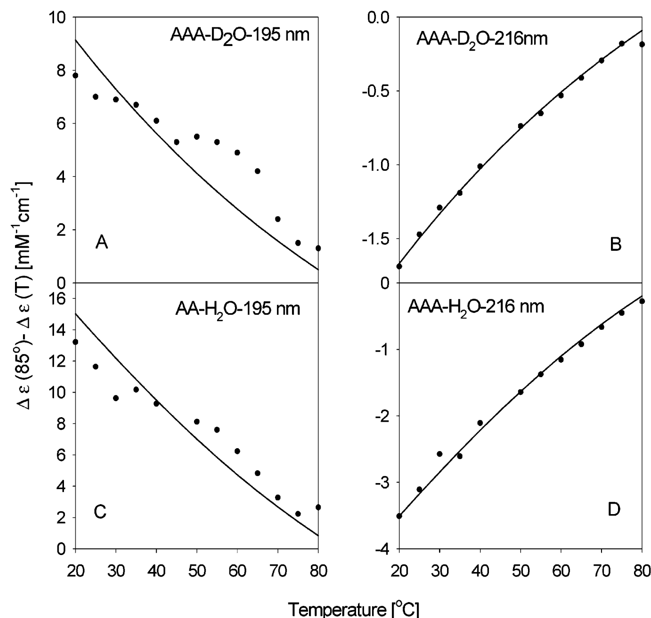


Figure 4. Difference circular dichroism plot of AAA in D_2O(A, B) and in H_2O (C, D) between 85 °C and at indicated temperatures obtained at negative and positive peak maximums. The solid line resulted from global fitting as described in the text.

tions. Rucker and Creamer obtained a PPII signal for a seven lysine residues containing peptide.[35] More recently, Shi et al.[34] analyzed the seven-residue alanine peptide $AcXX(A)_7OOamide$ (X: diaminobutyric acid, O: ornithine) by NMR and CD spectroscopy and found again a transition between PPII (stabilized at low temperature) and a β-strand structure (stabilized at high temperature).

The current understanding of the PPII structure invokes a hydrogen bonding network between the peptides and water molecules of the hydration shell as the most relevant stabilization factor.[34,36,39,44,52,53] Indeed, we could show that AcAA switches to a β-strand like structure in DMSO.[44] If hydrogen bonding to water plays indeed a major role in stabilizing PPII, we shall obtain a larger PPII contribution to the CD signal for AAA dissolved in H_2O[54] because deuteration destabilizes hydrogen bonding. Indeed, our experiment clearly revealed a more pronounced couplet for AAA in H_2O indicative of a substantial stabilization of PPII in H_2O (data not shown). This corroborates the notion that this conformation is mostly stabilized by hydrogen bonding to water.

We carried out a thermodynamic analysis of the temperature dependence of $\Delta(\Delta\epsilon)$ by adopting the following strategy. First, we calculated the difference $\Delta(\Delta\epsilon) = \Delta\epsilon(85\ °C) - \Delta\epsilon(T)$ as a function of temperature for 195 and 216 nm. The results are shown in Figure 4 for AAA in D_2O and H_2O. Assuming a thermodynamic equilibrium between the two states, elementary statistical mechanics yields the expression

$$\Delta(\Delta\epsilon)(T) = \frac{\Delta\epsilon_\beta e^{-\Delta H/RT_0+\Delta S/R} + \Delta\epsilon_{PPII}}{Z(T_0)} - \frac{\Delta\epsilon_\beta e^{-\Delta H/RT+\Delta S/R} + \Delta\epsilon_{\mathrm{PPII}}}{Z(T)} \quad (1)$$

(49) Silligardi, G.; Drake, A. F. *Biopolymers* **1995**, *37*, 281−292.
(50) Pajcini, V.; Chen, X. G.; Bormett, W.; Geib, S. J.; Asher, S. A *J. Am. Chem. Soc.* **1996**, *118*, 9716−9726.
(51) Sieler, G.; Schweitzer-Stenner, R.; Holtz, J. S. W.; Pajcini, V.; Asher, S. A. *J. Phys. Chem. B* **1999**, *103*, 372−384.
(52) Adzubei, A. A.; Stenberg M. J. E. *Prot. Sci.* **1994**, *3*, 2395−2410.
(53) Barron, L. D.; Hecht, L.; Wilson, G. *Biochemistry* **1997**, *36*, 13 143−13 147.

Table 1. Thermodynamic and Optical Parameters of AAA Obtained from the Analysis of the Temperature Dependence of $\Delta(\Delta\epsilon)$ as Illustrated in Figures 4 and 7

	D_2O 195 nm	D_2O 216 nm	H_2O 195 nm	H_2O 216 nm	glycerol 195 nm	glycerol 220 nm
ΔH[kJ/mol]	11 ± 1	11 ± 1	17 ± 2	17 ± 2	13 ± 2	13 ± 2
ΔS[J/K·mol]	40 ± 2	40 ± 2	56 ± 3	56 ± 3	50 ± 4	50 ± 2
$\Delta\epsilon_{PPII}$ [mM^{-1} cm^{-1}]	−32	4.5	−35	6	−33	6
$\Delta\epsilon_{\beta}$ [mM^{-1} cm^{-1}]	15	−5	15	−5	13	−6

where $\Delta\epsilon_{\beta}$, $\Delta\epsilon_{PPII}$ are the intrinsic $\Delta\epsilon$ values of β-strand and PPII conformer at the respective wavelength, $\Delta H = H_{\beta} - H_{PPII}$ and $\Delta S = S_{\beta} - S_{PPII}$ are the enthalpy and entropy differences between the two conformers, R is the gas constant, and T the temperature in Kelvin. T_0 is the reference temperature (358 K in our case), $Z(T)$ and $Z(T_0)$ are the partition sums at T and T_0, respectively. We first fitted eq 1 to the respective $\Delta(\Delta\epsilon)$ (T) values taken at 216 nm because the corresponding data are much less scattered. This position corresponds to the maximal $\Delta\epsilon$ signal and is generally utilized for determining the PPII content of proteins and peptides.[48] To avoid correlation effects we estimated the $\Delta\epsilon_{\beta}$, $\Delta\epsilon_{PPII}$ from the basis spectra of Sreerama and Woody[48] and allowed only small variations to fine-tune the fits. As expected different ΔH and ΔS but nearly identical values for $\Delta\epsilon_{\beta}$, $\Delta\epsilon_{PPII}$ were obtained for AAA in H_2O and D_2O. The values for ΔH and ΔS and the respective $\Delta\epsilon_{\beta}$, $\Delta\epsilon_{PPII}$-values estimated from ref 48 were then employed to calculate the $\Delta(\Delta\epsilon)$ (T) for $\lambda = 195$ nm. Small variations of $\Delta\epsilon_{\beta}$, $\Delta\epsilon_{PPII}$ were subsequently allowed to fine-tune the fits to somewhat scattered data. This self-consistent analysis eventually yielded the fitting parameters listed in Table 1. The reproduction of the experimental data is satisfactory.

As mentioned above it is difficult to discriminate between β-strand and disordered structures by means of the basis spectra reported by Sreerama and Woody.[48] Our two-state analysis of Raman, IR and VCD data indicates a flat β-strand. One might still argue, however, that the obtained dihedral angles ($(\phi,\psi) = -160°, 150°$) represent a heterogeneous ensemble of various structures in the β-strand region of the Ramachandran plot. As shown below our thermodynamic data argue in favor of this view. However, other observations argue against a large conformational heterogeneity with respect to the central dihedral angles, which involves a large region of the Ramachandran space. First, we showed earlier that the inhomogeneous broadening of IR and Raman lines of tripeptides is comparable with that observed for the respective dipeptides. Nearly half of this broadening stems from hydrogen bonding with water rather than from conformational heterogeneity.[44] Second, we would not expect to obtain an isodichroic point in the CD spectra if the "high temperature" conformer was substantially heterogeneous. A third argument can be obtained from the temperature dependence of amide I in the IR-spectra. If the "high temperature" conformer is indeed well represented by the coordinates reported by Eker et al.[44] it follows that the intensity ratio $R_{IR} = I_{IR}^{-}/I_{IR}^{+}$ increases with increasing temperature since our theoretical model predicts $R_{\beta} > R_{PPII}$ for the intrinsic IR-intensity ratios of two coexisting conformers[42] (I_{IR}^{+} and I_{IR}^{-} are the IR intensities of the bands assignable to the in-phase and out-of-phase combination of the two coupled amide I modes which appear at around 1673 and 1648 cm^{-1}, respectively). If, however, the "high temperature" conformer is highly heterogeneous a significant temperature dependence is unlikely because conformational heterogeneity gives rise to a broad distribution of (excitonic) mixing coefficients and angles between the amide I transition dipole moments and thus to a corresponding distribution of intensity ratios (cf. the contour plots in refs 37, 38, and 44). The upper panel in Figure 5 shows the amide I IR-profile of AAA in D_2O at different temperatures between 0 °C and 75 °C, whereas the lower panel depicts the difference of the spectra taken at 75 °C and 25 °C. These data indeed suggest that R_{IR} increases with rising temperature. This is confirmed by a spectral analysis of the results (Figure 6). R_{IR} increases from 1.48 at 5 °C temperatures to about 1.6 at 75 °C. Due to correlations between the free spectral parameters, the R_{IR} values exhibit some scattering, which, however, cannot obfuscate the result.

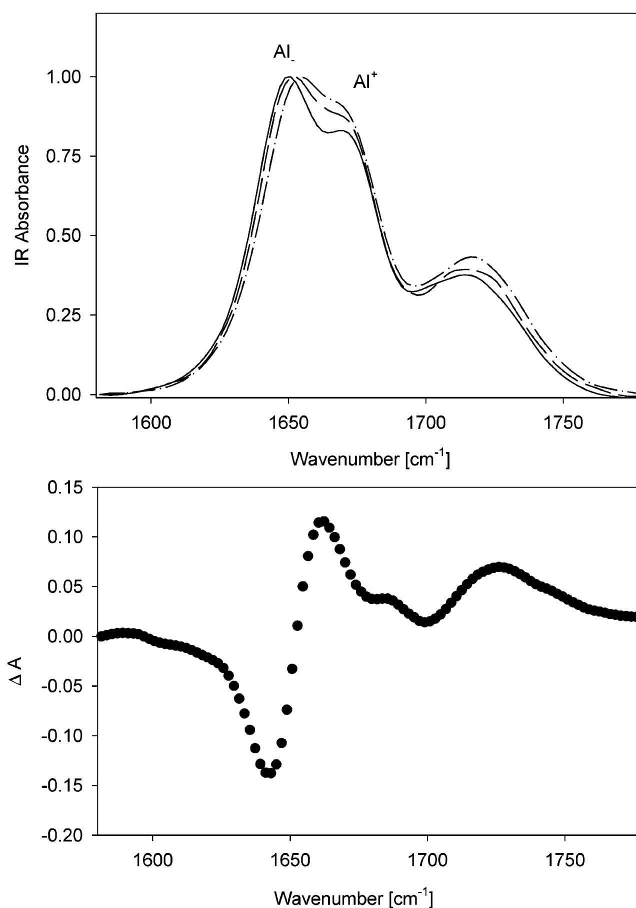


Figure 5. Upper panel: Temperature-dependent FTIR spectra of AAA in D_2O at pD 1.0 between 1580 and 1780 cm^{-1}. (Solid line: 25 °C, medium dash: 40 °C, dash−dot: 75 °C.) Lower panel: Difference FTIR spectra of AAA between 75 °C and 25 °C.

If the above analysis of the CD-data is correct it shall be possible to reproduce the $R_{IR}(T)$ data by means of the thermodynamic parameters obtained for AAA in D_2O. Assuming identical intrinsic oscillator strengths of the two coupled amide I modes[42] R_{IR} can be calculated by employing

$$R_{IR}(T) = R_{\beta} \cdot \frac{e^{-\Delta H/RT + \Delta S/R} + \left(1 + \frac{1}{R_{\beta}}\right)\left(1 + \frac{1}{R_{PPII}}\right)^{-1}}{e^{-\Delta H/RT + \Delta S/R} + \frac{(1 + R_{\beta})}{(1 + R_{PPII})}} \tag{2}$$

(54) Shi, Z.; Olson, C. A.; Kallenbach, N. R.; Sosnick, T. R. *J. Am. Chem. Soc.* **2002**, *124*, 13 994−13 995.

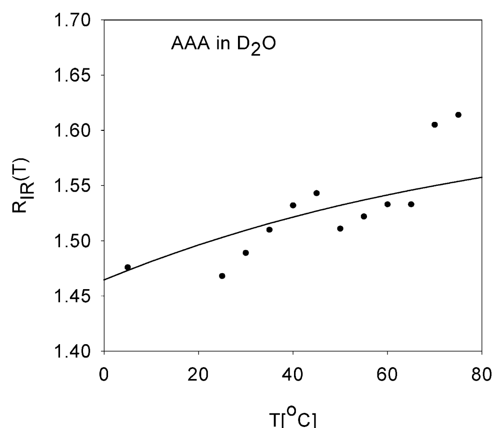


Figure 6. Temperature-dependent regression plot for Amide I_1/Amide I_2 intensity ratio of AAA at cationic pD.

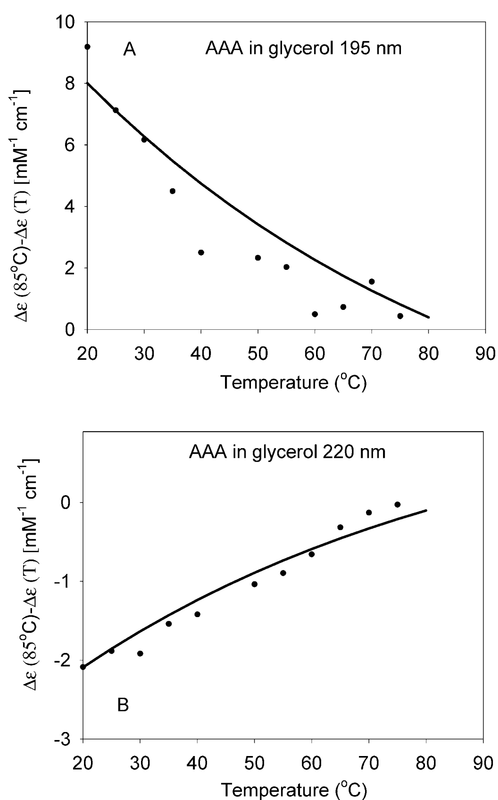


Figure 7. Difference circular dichroism plot of AAA in glycerol (A, B) between 85 °C and at indicated temperatures obtained at negative and positive peak maximums. The solid line resulted from global fitting as described in the text.

where $R_\beta = 1.7$ and $R_{PPII} = 1.2$ are the IR-intensity ratios for the two conformers as inferred from the two-state analysis of the IR and Raman data described earlier.[43] The calculated (not fitted) solid line in Figure 7 demonstrates that IR and CD data can consistently be reproduced by the same thermodynamic parameters of the two-state model.

The thermodynamic parameters deserve some further comments. Apparently, the enthalpic difference between the conformers is larger than one would intuitively expect, i.e., the PPII conformer is stabilized by 11 kJ/mol in D_2O and even 17 kJ/mol in H_2O with respect to the flat β-strand conformer. The different values obtained for the two solvents underscore the notion that the hydration shell plays a major role in stabilizing PPII.[36,39,40,44] A recent DFT calculation on a blocked di-alanine peptide hydrogen bonded to water yielded a enthalpy difference of ca. 8 kJ/mol.[39] This is somewhat lower than our values, but these calculations certainly do not account for the entire hydration shell of the peptide, which can be expected to contribute mostly to enthalpic stabilization of PPII. From their NMR-experiments on the seven alanine residue containing peptide Shi et al.[34] estimated that the enthalpy difference between the respective PPPII and β-strand conformations lies between 12 and 45 kJ/mol (in H_2O with 10% D_2O). That corresponds well to our results. Apparently, if the enthalpy were the dominating contribution to the Gibbs free energy difference between the two conformers, then the β-strand would remain nearly unpopulated at room temperature. However, our data show that the latter is stabilized by a substantial entropic contribution to the free energy, i.e., 12.1 and 16.8 kJ/mol for D_2O and H_2O at 300 K, respectively. Thus, the β-strand conformation is slightly favored by 1.1 kJ/mol in D_2O. The isotopic effect ($D_2O \Leftrightarrow H_2O$ exchange) brings about a stabilization free energy of 1.9 kJ/mol for the PPII structure.

Presently, we have no clear explanation of why the strand conformer is so much favored entropically. Previously, Klibanov and Griebenow[57] stated that β-sheet secondary structure binds less water than helical structures. Such an effect should be even more pronounced for PPII than for α helices. Thus, switching from PII to a β-sheet conformation could lead to a release of bound water from the peptide backbone and this should subsequently increase the entropy of water. For this reason, we suspect a higher disorder of the hydration shell, but it cannot be excluded that the flat β-strand conformer is structurally more disordered than the PPII conformer within the limitation discussed above. Such a heterogeneity will be restricted to a distinct region in the upper left corner of the Ramachandran plot as illustrated in Figure 6 of the paper of Shi et al.[34] In general terms, their and our data suggest that that conformational transition from PPII to β-strand is mostly driven by entropy. We therefore hypothesize that a high PPII propensity of an amino acid residue (like A) reflects the capability to provide sufficient enthalpy to the PPII conformation that it can compensate the overall entropic advantage of a (more heterogeneous) β-strand like conformation.

Tri-Alanine Dissolved in Glycerol. If the hydration shell is indeed significantly involved in stabilizing the PPII conformation, then the question arises whether this could also be accomplished by glycerol, which has been shown to stabilize the native structure of proteins.[55,56] The reason for this behavior is currently poorly understood. To shed some more light on this issue we have also measured the CD spectra of AAA in glycerol at various temperatures and observed that the mixture of PPII and β-strand is practically preserved in glycerol (data not shown). The temperature dependency of the respective $\Delta(\Delta\epsilon) = \Delta\epsilon(85\ °C) - \Delta\epsilon(T)$ value is depicted in Figure 7. Again, we used eq 1 for a thermodynamic analysis of the results (Table 1). Corresponding data sets could be fitted with the same thermodynamic parameters but data scattering for the $\Delta(\Delta\epsilon)$ values of the respective lower wavelengths gives rise to some

(55) Griebenow, K.; Klibanov, A. M. *J. Am. Chem. Soc.* **1996**, *47*, 11 695−11 700.
(56) Knubovets, T.; Osterhout, J. J.; Connolly, P. J.; Klibanov, A. M. *Proc. Natl. Acad. Sci.* **1999**, *96*, 1262−1267.
(57) Griebenow, K.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10 969−10 976.

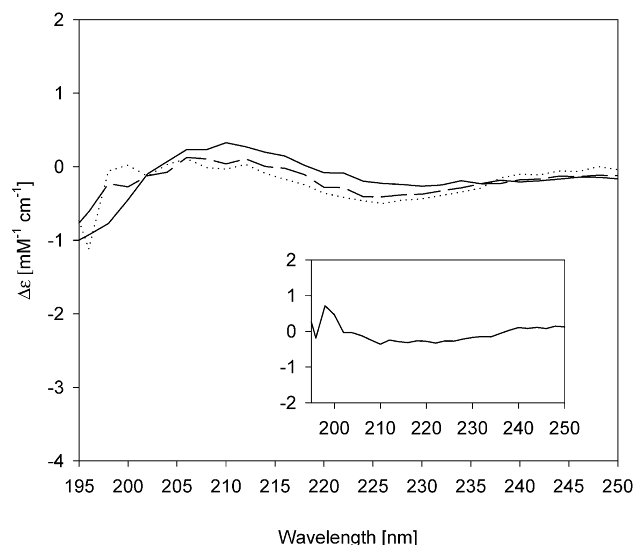


Figure 8. Temperature-dependent CD spectra of VVV in D_2O at pD 1.0. (Solid line: 20 °C, medium dash: 40 °C, dotted: 65 °C.) Inset: CD difference spectrum for VVV between 65 °C and 20 °C.

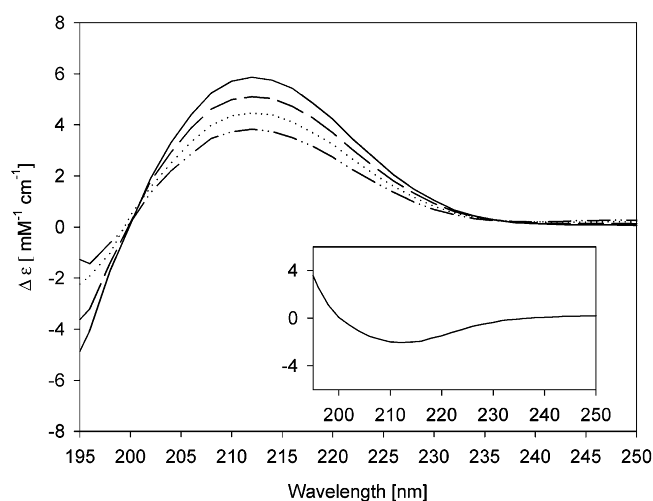


Figure 9. Temperature-dependent CD spectra of KKK in D_2O at pD 1.0. (Solid line: 20 °C, medium dash: 40 °C, dotted: 60 °C, dash−dot−dot: 85 °C.) Inset: CD difference spectrum for KKK between 85 °C and 20 °C.

uncertainty. This, notwithstanding the obtained parameters, clearly indicates that compared to D_2O glycerol increases the enthalpic and entropic differences between the two conformers. The net effect is a slightly larger free energy stabilization of the flat β-strand at 300 K by 2 kJ/mol (1.1 kJ/mol in D_2O).

Tri-Valine and Tri-Lysine in D_2O. As a further check of the structure analysis reported in previous papers we have also measured the CD spectra of VVV and KKK at pD 1 as a function of temperature. The results are shown in Figures 8 and 9. VVV exhibits a very weak CD signal, which is practically temperature independent. A comparison with the basis spectra of Sreerama and Woody[48] suggests an mixture of about 10% PPII (inferred from the small negative signal at 195 nm) and 90% (disordered) β-strand at room temperature. Apparently, the PPII contribution disappears at higher temperatures. This is in satisfactory agreement with our earlier analysis,[43] which indicated a predominance of a flat β-strand like conformation. This result is in line with the generally assumed high sheet propensity of valine residues.

Our recent analysis of the vibrational spectra of KKK at acidic pD surprisingly revealed a left-handed turn structure as the dominant conformation.[42] On the basis of the experimental data, it was concluded that the population of significantly different conformers must be negligible. The CD spectra in Figure 9 exhibit a nearly symmetric couplet (negative peak at 190 nm, a positive one at 210 nm), which cannot be rationalized as weighted superposition of PPII and β-strand signals. Such a signal is also not found in the reference spectra reported by Sreerama and Woody.[48] However, it pretty much looks like a reversed signal of the right-handed turn spectrum reported by these authors. This is what one would expect for the predicted left-handed turn. Thus, the CD-spectrum again supports the earlier analysis of the vibrational spectroscopy data. The difference spectrum in the inset suggests a small admixture of a (disordered) β-strand conformer.

Conclusions

In a series of papers we have recently introduced a method by which the "secondary" structure of tripeptides in solution can be determined from their Raman, IR, and VCD data.[38,43,44] The analyses reveal that in contrast to common belief, even small peptide fragments adopt stable structures. In view of the importance of these results and their potential relevance for the understanding of the so-called "random coil" state of peptides and proteins an independent check is highly warranted. This has been fully accomplished in the present study by measuring the UVCD spectra of AAA, VVV, and KKK at various temperatures. Besides the general confirmation of the earlier analysis we could now finally confirm the two state model for tri-alanine in water, namely the coexistence of PPII and (disordered) β-strand conformers. A thorough and self-consistent thermodynamic analysis of the CD data revealed that comparatively large enthalpic contributions favoring PPII are counterbalanced by entropic contributions at room temperature, which yield rather small free energy differences between these conformers. This suggests that the structural propensities of amino acid residues might be related to an easily changeable balance of enthalpic and entropic contributions to the free energy. The stabilization of PPII in H_2O (compared with D_2O) supports the emerging view that a hydrogen-bonding network involving the peptide and the water molecules in the hydration shell is mainly responsible for the formation of PPII conformers.

The mutual correspondence between the present CD data and the results of our recent analysis of vibrational spectra data of AAA provides conclusive evidence that the CD-signal in Figure 1 is indeed indicative of ordered PPII structure, in accordance with what is assumed in the studies of Keiderling, Woody, Creamer, Kallenbach, and their respective associates.[30,34,35,48] In view of the fact that such a PPII signal appears in most CD spectra of proteins subjected to denaturing reagents, a general biological relevance of this conformation becomes apparent, as suggested by the TK hypothesis.

Finally, this and our earlier studies[43,44] point to a principal new understanding of the so-called random coil state. Rather than sampling the entire allowed regions of the Ramachandran space, the individual residues exist in one or two conformers depending on their respective propensity. Alanine and most likely also glycine spend a substantial amount of time in the PPII conformation and can therefore be used as substitutes of

proline in peptides, which exhibits a natural PPII structure.[57] On the contrary, valine does not exhibit any preference for PPII. We hypothesize that in the absence of any Coulomb interactions between charged side chains the structure of an unfolded polypeptide is determined by the intrinsic propensities of its amino acid residue. Some of them (like alanine) fluctuate between PPII and an extended β-strand, others (like valine and most likely proline) are locked into one of these conformations. This view agrees with results of conformational analyses by Pappu and associates[59,60] and is still in line with Flory's isolated-pair hypothesis,[61] but it assumes much less conformational entropy and, thus, more conformational restriction for a polypeptide than the conventional picture of the unfolded state.[19]

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(58) Ferreon, J. C.; Hilser, V. J. *Protein Sci.* **2003**, *12*, 447−457.
(59) Pappu, R. V.; Strinivasan, R.; Rose, G. D. *Proc. Natl. Acad. Sci. U.S.A.* *97*, 12 565−12 570.
(60) Pappu, R. V.; Rose, G. D. *Protein. Sci.* **2002**, *11*, 2437−2455.
(61) Flory, P. J. *Statistical Mechanics of Chain Molecules*; Hanser Publishers: Munich, 1969; Chapter 7.