J|A|C|S
ARTICLES
Published on Web 02/11/2004

The Conformation of Tetraalanine in Water Determined by Polarized Raman, FT-IR, and VCD Spectroscopy

Reinhard Schweitzer-Stenner,*,† Fatma Eker,‡,§ Kai Griebenow,‡ Xiaolin Cao,|| and Laurence A. Nafie||

Contribution from the Department of Chemistry, Drexel University, Philadelphia, 32nd and Chestnut Streets, Pennsylvania 19104, Departments of Chemistry and Biology, University of Puerto Rico, Río Piedras Campus, San Juan, Puerto Rico 00931, and Department of Chemistry, Syracuse University, Syracuse, New York 13244

Received November 5, 2003; E-mail: rschweitzer-stenner@drexel.edu

Abstract: The present article reports the conformation of cationic tetraalanine in aqueous solution. The determination of the dihedral angles of the two central amino acid residues was achieved by analyzing the amide I′ band profile in the respective polarized visible Raman, Fourier transform-IR, and vibrational circular dichroism (VCD) spectra by means of a novel algorithm which utilizes the excitonic coupling between the amide I modes of nearest neighbor and second nearest peptide groups. It is an extension of a recently developed theory (Schweitzer-Stenner, R. *Biophys. J.*, **2002**, *83*, 523−532). UV electronic circular dichroism (ECD) spectra of the peptides were used to validate the results of the structure analysis. The analyses yielded the dihedral angles (ϕ_{12}, ψ_{12}) = (−70°, 155°) and (ϕ_{23}, ψ_{23}) = (−80°, 145°). The obtained values are very close to the Ramachandran coordinates of the polyproline II helix (PPII). The data suggest that this is the conformation predominantly adopted by the peptide at room temperature. This notion was corroborated by the corresponding electronic circular dichroism spectrum. Tetraalanine exhibits a higher propensity for PPII than trialanine for which a 50:50 mixture of polyproline II and an extended β-strand-like conformation was obtained from recent spectroscopic studies (Eker et al., *J. Am. Chem. Soc.* **2002**, *124,* 14330−14341*).* The temperature dependence of the CD spectra rule out that any cooperativity is involved in the strand ⇔PPII transition. This led to the conclusion that solvent−peptide interactions give rise to the observed PPII stability. Our result can be utilized to understand why the denaturation of helix-forming peptides generally yields a PPII rather than a heterogeneous random conformation.

Introduction

Alanine-based model peptides have been extensively studied by experimental and theoretical means to obtain the parameters which determine the folding and unfolding of α-helices.[1−8] These and other studies have shown over the last 15 years that even short alanine-containing peptides primed with charged side chains such as lysine or arginine can adopt an α-helical conformation in solution, provided that the number of amino acid residues exceeds a certain threshold value.[9] This finding was at variance with one of the central dogmas of protein folding, namely, that short peptide segments are unable to fold in solution due to the lack of stabilizing nonlocal tertiary interactions.[10] Time-resolved spectroscopic studies revealed that the helix ⇔ coil transition of the Baldwin peptides proceeds on a time scale of 10^{-7} s.[4,5] Thermal denaturation experiments indicate that their transition is noncooperative. The forces stabilizing the helix conformation are still poorly understood, in particular those concerning the role of the solvent.[11−13]

Kinetic and thermodynamic data of the helix ⇔ coil transition are generally interpreted in terms of the Zimm−Bragg[14] or the equivalent Lifson−Roig model,[15] which assume that each peptide residue can exist in two states corresponding to an α-helical and a so-called random coil conformation. The latter comprises nearly all conformations assignable to the sterically allowed region of the Ramachandran plot, which are considered nearly iso-energetic. This theoretical concept implies that the

† Drexel University.
‡ Department of Chemistry, University of Puerto Rico.
§ Department of Biology, University of Puerto Rico.
|| Syracuse University.

(1) Scholtz, J. M.; Baldwin, R. L. *Annu. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 95.
(2) Vila, J.; Williams, R. L.; Grant, J. A.; Wójcik, J.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7821.
(3) Williams, L.; Kather, K.; Kemp, D. S. *J. Am. Chem. Soc.* **1998**, *120*, 11033.
(4) Gilmashin, R.; Williams, S.; Callender, R. H.; Woodruf, W. H.; Dyer, R. B. *Biochemistry* **1997**, *36*, 15006.
(5) Lednev, I. K.; Karnoup, A. S.; Sparrow, M. C.; Asher, S. A. *J. Am. Chem. Soc.* **1999**, *121*, 8074.
(6) Vila, J. A.; Ripolli, D. R.; Scheraga, H. A. *Biopolymers* **2001**, *58*, 235.
(7) Silva, R. A. G. D.; Kubelka, J.; Bour, P.; Decatur, S. M.; Keiderling, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8318.
(8) Shi, Z.; Olson, C. A.; Bell, A. J.; Kallenbach, N. R. *Biopolymers* **2001**, *60*, 366.
(9) Park, S. H.; Shalongo, W.; Stellwagen, E. *Protein Sci.* **1997**, *6*, 1694.
(10) Marqusee, S.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8898.
(11) Garcia, A. E.; Sabonmatsu, K. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2782.
(12) Spek, E. J.; Olson, C. A.; Shi, Z. S.; Kallenbach, N. R. *J. Am. Chem. Soc.* **1999**, *121*, 5571.
(13) Ghosh, T.; Garde, S.; Garcia, A. E. *Biophys. J.* **2003**, *85*, 3187.
(14) Zimm, B. H.; Bragg, J. K. *J. Chem. Phys.* **1959**, *31*, 476.
(15) Lifson, S.; Roig, A. *J. Chem. Phys.* **1991**, *34*, 1963.

10.1021/ja039452c CCC: $27.50

coil state carries a lot of entropy. However, most recent experimental and theoretical studies on various types on alanine-based peptides have lead to a modification of this view. NMR and CD experiments on a seven-alanine-containing peptide indicate that this polymer adopts a very regular polyproline II (PPII) helix-like structure rather than a random coil conformation.[16] The canonical PPII is a left-handed 3_1-helix with an axial translation of 3.2 Å composed of three residues per turn.[17] The electronic circular dichroism (ECD) spectra of longer thermally unfolded alanine-based peptides reported by Lednev et al.[5] resemble those found for PPII conformations.[18,19] Blanch et al. used Raman optical acitivity measurements to identify PPII as the main conformation of a thermally "denaturated" alanine and lysine-containing peptide of 21 residues.[20] In previous studies, it has been found that even tripeptides with alanine as central residue can adopt a PPII structure.[21,22] A combined analysis of Raman, IR, vibrational circular dichroism (VCD), and ECD spectra of trialanine revealed a 50:50 mixture of PPII ((ϕ, ψ) = (−60°, 150°) and an extended ((ϕ, ψ) = (−165°, 150°)) β-strand-like conformation at room temperature.[23,24] Taken together, these data suggest the following picture: The alanine residue can exist in three, rather than two, conformations, namely helical (h), PPII (p), and extended β-strand (e_β). For tripeptides, only p and e_β are significantly populated. Recent molecular dynamics calculations suggest that the p-conformation becomes more stabilized with an increasing number of residues.[25,26] Above a certain threshold, even the helical conformation becomes populated at room temperature. Unfolding by denaturants populates p rather than e_β.[16] Taken together, ample evidence suggests that at room temperature, the so-called "random coil" state of peptides and proteins is much more ordered than generally assumed in that it exhibits a substantial fraction of polyproline II.[19] This notion is corroborated by a detailed analysis of the f-angle distribution in disordered segments of proteins by Avbelji and Baldwin,[27] which were shown to be inconsistent with the random coil model of Brant and Flory[28] in that the latter significantly underestimated the population of PPII.

In the present study, we determined the secondary structure of tetraalanine (Figure 1) in water by combining polarized Raman, FTIR, VCD, and ECD spectroscopy. We focus on cationic tetraalanine because its spectra are easier to analyze than those of the anionic state, but the VCD spectrum of the latter is used to confirm the result of our analysis. In the zwitterionic state, a substantial fraction of the peptide precipitates at the concentrations required for study. The article serves two purposes. First, we introduce an extended version of the recently developed algorithm by virtue of which the amide I band in the Raman, IR, and VCD spectra of tripeptides is analyzed to determine the dihedral angles of the central amino acid residue. The current formalism accounts for excitonic coupling between nearest and second-nearest amide I modes. It can thus be considered as a building block for the analysis of delocalized amide I states of longer non-α-helical peptides.[29] Second, by comparing the structure of tetra- and trialanine, we are able to decide whether the stability of the PPII conformation does indeed increase with the number of residues as predicted[25,26] and whether such an enhancement effect is due to cooperativity. Thus, our results contribute to the understanding of the unfolded state of peptides and the initial state of helix ⇔ coil transitions.

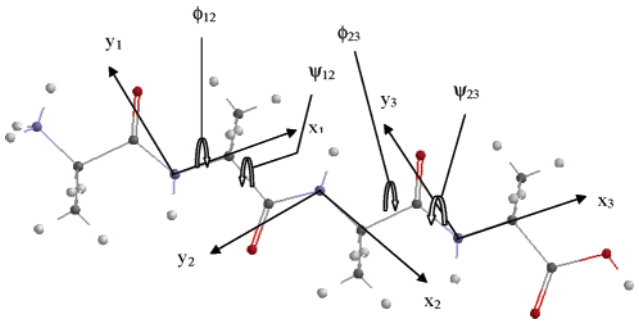

Figure 1. Planar structure of tetraalanine (ϕ = 180°, ψ = 180°). The coordinate systems $S_1(x_1, y_1, z_1)$, $S_2(x_2, y_2, z_2)$, and $S_3(x_3, y_3, z_3)$ were used to express the Raman tensors of the individual, uncoupled amide I modes and their transition dipole moments (the z-components have been omitted for the sake of clarity; they all point out of the drawing plane). Oxygen atoms are drawn in red, nitrogen atoms in blue, carbon atoms in dark gray, and hydrogen atoms in light gray. The structure was obtained by using the program TITAN from Wavefunction, Inc.

Theory

Calculation of the Raman Tensor. The theory used to obtain the dihedral angles of tripeptides from the amide I′ bands in their visible Raman and IR spectra has been described in detail elsewhere.[23,30,31] In what follows, we extend this formalism in that we derive a three-oscillator model to describe the mixing between the three amide I modes of a tetrapeptide by transition dipole and through bond coupling.[29] The corresponding excitonic states are written as:

$$\begin{aligned} |\tilde{\chi}_1\rangle &= a_{11}|\chi_1\rangle + a_{12}|\chi_2\rangle + a_{13}|\chi_3\rangle \\ |\tilde{\chi}_2\rangle &= a_{21}|\chi_1\rangle + a_{22}|\chi_2\rangle + a_{23}|\chi_3\rangle \\ |\tilde{\chi}_3\rangle &= a_{31}|\chi_1\rangle + a_{32}|\chi_2\rangle + a_{33}|\chi_3\rangle \end{aligned} \quad (1)$$

where $|\chi_1\rangle$, $|\chi_2\rangle$, and $|\chi_3\rangle$ are the vibrational eigenfunctions of the uncoupled amide I modes. Orthonormalization requires that the mixing coefficients are related by:

$$1 = \sum_i a_{ij}^2 = \sum_j a_{ij}^2 \quad (2)$$

The coefficients can be obtained from the solution of the time-independent Schrödinger equation, the Hamiltonian of which is written in the matrix representation as:

(16) Shi, Z.; Olson, C.; Rose, G.; Baldwin, R.; Kallenbach, N. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 9190.
(17) Cowan, P. M.; McGavin, S. *Nature* **1955**, *176*, 501.
(18) Woody, R. W. *Adv. Biophys. Chem.* **1992**, *2*, 37.
(19) Shi, Z.; Woody, R. W.; Kallenbach, N. R. *Adv. Protein Chem.* **2002**, *62*, 163.
(20) Blanch, E. W.; Morozova-Roche, L. A.; Cochran D. A. E.; Doig, A. J.; Hecht, L.; Barron, L. D. *J. Mol. Biol.* **2000**, *301*, 553.
(21) Woutersen, S.; Hamm, P. *J. Phys. Chem. B* **2000**, *104*, 11316.
(22) Schweitzer-Stenner, R.; Eker, F.; Huang, Q.; Griebenow, K. *J. Am. Chem. Soc.* **2001**, *123*, 9628.
(23) Eker, F.; Cao, X.; Nafie, L.; Schweitzer-Stenner, R. *J. Am. Chem. Soc.* **2002**, *124*, 14330.
(24) Eker, F.; Griebenow, K.; Schweitzer-Stenner, R. *J. Am. Chem. Soc.* **2003**, *125*, 8178.
(25) Gnanakaran, S.; Garcia, A. E. *J. Phys. Chem. B* **2003**, *107*, 12555.
(26) Garcia, A. E. *Polymers* **2004**, *45*, 669.
(27) Avbelj, F.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5742.
(28) Brant, D. A.; Flory, P. J. J. *J. Am. Chem. Soc.* **1965**, *87*, 2791.
(29) Torii, H.; Tasumi, M. J. *Raman Spectrosc.* **1998**, *29*, 81.
(30) Schweitzer-Stenner, R. *Biophys. J.* **2002**, *83*, 523.
(31) Schweitzer-Stenner, R.; Eker, F.; Griebenow, K.; Cao, X.; Nafie, L. A. *Biopolymers* (*Peptide Science*) **2003**, *71*, 558.

$$H = \begin{pmatrix} \nu_1 & \Delta_{12} & \Delta_{13} \\ \Delta_{12} & \nu_2 & \Delta_{23} \\ \Delta_{13} & \Delta_{23} & \tilde{\nu}_3 \end{pmatrix} \quad (3)$$

The diagonal elements $\tilde{\nu}_j$, $j = 1, 2, 3$, are the wavenumber values of the unmixed oscillators; and Δ_{ij} ($i,j = 1, 2, 3$) are the energies of excitonic coupling between the amide I modes. All energies are expressed in units of cm^{-1}. The diagonalization of eq 3 yields the eigenenergies of the three excitonic states, i.e., Ω_1, Ω_2, and Ω_3 and the coefficients of eq 1. The latter are subsequently used to calculate the Raman tensor of the excitonic states:

$$\begin{aligned} \hat{\alpha}'_1 &= a_{11}\hat{\alpha}_1 + a_{12}\hat{\alpha}_2 + a_{13}\hat{\alpha}_3 \\ \hat{\alpha}'_2 &= a_{21}\hat{\alpha}_1 + a_{22}\hat{\alpha}_2 + a_{23}\hat{\alpha}_3 \\ \hat{\alpha}'_3 &= a_{13}\hat{\alpha}_1 + a_{23}\hat{\alpha}_2 + a_{33}\hat{\alpha}_3 \end{aligned} \quad (4)$$

where $\hat{\alpha}_1$, $\hat{\alpha}_2$, and $\hat{\alpha}_3$ are the amide I Raman tensors of the three peptide groups. To calculate $\hat{\alpha}'_k$ ($k = 1, 2, 3$), all $\hat{\alpha}_k$ have to be expressed with respect to the same coordinate system. Thus, the Raman tensors of the excitonic states become dependent on the mutual orientation of the peptide groups. As in our earlier studies, we select the C-terminal peptide group as our reference system with the respective nitrogen atom as the origin of the coordinate system. The *x*-axis of the coordinate system coincides with the NC_α bond, which is also the rotational axis for the dihedral angle ϕ (Figure 1). The *y*-axis is (nearly) coplanar with the peptide group, and *z* is the out-of-plane coordinate. The small deviation from coplanarity (1.1°) results from the fact that the NC_α bond is not exactly coplanar with the peptide plane.[30] We assign $\hat{\alpha}_1$, $\hat{\alpha}_2$, and $\hat{\alpha}_3$ to the N-terminal, central, and C-terminal peptides, respectively, and rotate the first $\hat{\alpha}_1$ from the coordinate systems S_1 (N-terminal) into S_3 (C-terminal) by the following matrix operations:

$$\begin{aligned} \hat{\alpha}_1(S_2) = R^T(\omega_{12})(R^T(\psi_{12})(R^T(\xi_{12})(R^T(\phi'_{12})\hat{\alpha}_1(S_1)R(\phi'_{12})) \\ R(\xi_{12}))R(\psi_{12}))R(\omega_{12}) \\ \hat{\alpha}_1(S_3) = R^T(\omega_{23})(R^T(\psi_{23})(R^T(\xi_{23})(R^T(\phi'_{23})\hat{\alpha}_1(S_2)R(\phi'_{23})) \\ R(\xi_{23}))R(\psi_{23}))R(\omega_{23}) \end{aligned} \quad (5)$$

which can be understood as follows. First, S_1 has to be rotated by an angle $\phi_{12}' = \phi_{12} - \pi$. Subsequently, a rotation by ξ_{12} in the *xy*-plane is necessary so that the *y*-coordinate coincides with the $C_\alpha C$ bond, which is the rotational axis for ψ_{12}. ξ_{12} is the angle formed by the y_1-axis and the $C_\alpha C$ bond. Next, the system is rotated by the dihedral angle ψ_{12}. The fourth step involves the rotation by an angle ω_{12}, which is formed by the $C_\alpha C$ bond and the y_2-axis. This rotation causes the *x*-axis to coincide with the NC_α bond. Subsequently, a similar sequence of rotations around the angles ϕ_{23}, ξ_{23}, ψ_{23}, and ω_{23} has to be carried out to obtain $\hat{\alpha}_1$ in the coordinate system S_3. The same transformation has to be carried out to obtain $\hat{\alpha}_2$ in S_3. ω and ξ can be obtained from textbooks on peptide structure with 96 and 20°, respectively.[32]

The tensors calculated by means of eqs 4 and 5 can be used to calculate the isotropic and anisotropic scattering for the *i*th excitonic states:

$$\beta'^2_{si} = \frac{1}{9}(Tr\hat{\alpha}'_i)^2$$

$$\begin{aligned} \gamma'^2_{\text{aniso},i} = \frac{1}{2}[(\alpha'_{xx,i} - \alpha'_{yy,i})^2 + (\alpha'_{yy,i} - \alpha'_{zz,i})^2 + \\ (\alpha'_{zz,i} - \alpha'_{xx,i})^2] + \frac{3}{4}[(\alpha'_{xy,i} + \alpha'_{yx,i})^2 + (\alpha'_{yz,i} + \alpha'_{zy,i})^2 + \\ (\alpha'_{zx,i} + \alpha'_{xz,i})^2] \end{aligned} \quad (6)$$

Equation 6 can be used to calculate the intensity ratios of the amide I bands in the isotropic and anisotropic Raman spectrum of tetrapeptides:

$$R_{\text{iso},ii'} = \beta^2_{s,i}/\beta^2_{s,i'} \quad (7a)$$

$$R_{\text{aniso},ii'} = \gamma^2_{\text{aniso},i}/\gamma^2_{\text{aniso},i'} \quad (7b)$$

as functions of the mixing parameters a_{ij} and the dihedral angles $\phi_{ii'}$ and $\psi_{ii'}$.

Calculation of Infrared Intensity Ratios. We start with the assumption that the orientation of the amide I′ transition dipole moment is identical for all peptide groups. Each dipole moment can therefore be described by:

$$\vec{\mu}_j = \begin{pmatrix} \mu_{0j}\cdot\cos\vartheta \\ \mu_{0j}\cdot\sin\vartheta \\ 0 \end{pmatrix} \quad (8)$$

where *j* denotes the peptide group, μ_{0j} is the amount of the respective amide I′ transition dipole moment, and ϑ is the orientational angle between the dipole vector and the *x*-axis of the *j*th coordinate system. It should be noted that the dipole moment is in reality the first derivative of the peptide's electric dipole moment with respect to the normal coordinate of the amide I′ mode. For the sake of simplicity, we will use the term "transition dipole moment" throughout the article, in line with the literature.

To calculate the transition dipole moments of the excitonic states, $\vec{\mu}_1$ and $\vec{\mu}_2$ of the N-terminal and central peptide group have to be transformed into the coordinate system S_3 of the C-terminal peptide:

$$\begin{aligned} \vec{\mu}_1(S_3) = R(\omega_{23})R(\psi_{23})R(\xi_{23})R(\phi'_{23})R(\omega_{12})R(\psi_{12})R(\xi_{12}) \\ R(\phi'_{12})\vec{\mu}_1(S_1) \\ \vec{\mu}_2(S_3) = R(\omega_{23})R(\psi_{23})R(\xi_{23})R(\phi'_{23})\vec{\mu}_2(S_2) \end{aligned} \quad (9)$$

Equation 1 can then be used to describe the dipole moments of the excitonic states:

$$\begin{aligned} \vec{\mu}'_1 &= a_{11}\vec{\mu}_1 + a_{12}\vec{\mu}_2 + a_{13}\vec{\mu}_3 \\ \vec{\mu}'_2 &= a_{21}\vec{\mu}_1 + a_{22}\vec{\mu}_2 + a_{23}\vec{\mu}_3 \\ \vec{\mu}'_3 &= a_{31}\vec{\mu}_1 + a_{32}\vec{\mu}_2 + a_{33}\vec{\mu}_3 \end{aligned} \quad (10)$$

Now, the intensity ratios of the amide I′ bands in the IR spectrum of a tetrapeptide can be calculated by using:

$$R_{\text{IR},ii'} = \frac{\vec{\mu}'^2_i}{\vec{\mu}'^2_{i'}} \quad (11)$$

as a function of the mixing parameters a_{ij} and the dihedral angles $\phi_{ii'}$ and $\psi_{ii'}$.

(32) Schulz, G. E.; Schirmer, R. H. *Principles of Protein Structure*; Springer: Heidelberg, Germany, 1978; p 18.

Calculation of the VCD Spectrum. The original VCD model of Holzwarth and Chabay[33] considered only two oscillators with identical eigenenergies. We have recently expanded and generalized this model to account for the coupling of two nonisoenergetic oscillators and for the possibility that at least one of the amide I modes displays some intrinsic rotational strength, which has been found to be the case for C-terminal peptide groups.[23] In what follows, we derive a general model for the excitonic states of three interacting amide I′ modes. It follows from the work of Holzwarth and Chabay that the rotational strength of the *i*th mode ($i = 1, 2, 3$) can be written as:

$$R_i = \{\mathrm{Im}\}\left[\sum_{j=1}^{3} a_{ij}\vec{\mu}_j \cdot \left(\sum_{j=1}^{3} a_{ij}\vec{m}_j - \frac{i\pi}{2}(\tilde{\nu}_{12}\vec{\mathrm{T}}_{12}\cdot(a_{i1}\vec{\mu}_1 - a_{i2}\vec{\mu}_2) + \tilde{\nu}_{13}\vec{\mathrm{T}}_{13}\cdot(a_{i1}\vec{\mu}_1 - a_{i3}\vec{\mu}_3) + \tilde{\nu}_{23}\vec{\mathrm{T}}_{23}\cdot(a_{i3}\vec{\mu}_2 - a_{i2}\vec{\mu}_3))\right)\right] \quad (12)$$

where $\vec{\mu}_j$ ($j = 1, 2, 3$) is the above introduced electronic transition dipole moment, $\vec{m}_j$ is the corresponding magnetic transition dipole moment of the *j*th oscillator, $\vec{\mathrm{T}}_{ij}$ is the distance vector between oscillator *i* and *j*, and $\tilde{\nu}_{ij}$ is their average wavenumber. A straightforward calculation converts eq 12 into:

$$\begin{aligned} R_i = \{\mathrm{Im}\}\Bigg[& \Big(\sum_{j=1}^{3} a_{ij}^{\,2}\vec{\mu}_j\cdot\vec{\mathrm{m}}_j\Big) + \Big(\sum_{\substack{i,j=1\\ i\neq j}}^{3} a_i a_j \vec{\mu}_i\cdot\vec{\mathrm{m}}_j\Big) \\ & - \frac{i\pi\tilde{\nu}_{12}\vec{T}_{12}}{2}\cdot\{a_{i2}a_{i1}(\vec{\mu}_2\cdot\vec{\mu}_1) + a_{i3}a_{i1}(\vec{\mu}_3\cdot\vec{\mu}_1) - a_{i1}a_{i2}(\vec{\mu}_1\cdot\vec{\mu}_2) - a_{i3}a_{i2}(\vec{\mu}_3\cdot\vec{\mu}_2)\} \\ & - \frac{i\pi\tilde{\nu}_{13}\vec{T}_{13}}{2}\cdot\{a_{i2}a_{i1}(\vec{\mu}_2\cdot\vec{\mu}_1) + a_{i3}a_{i1}(\vec{\mu}_3\cdot\vec{\mu}_1) - a_{i1}a_{i3}(\vec{\mu}_1\cdot\vec{\mu}_3) - a_{i2}a_{i3}(\vec{\mu}_2\cdot\vec{\mu}_3)\} \\ & - \frac{i\pi\tilde{\nu}_{23}\vec{T}_{23}}{2}\cdot\{a_{i1}a_{i2}(\vec{\mu}_1\cdot\vec{\mu}_2) + a_{i3}a_{i2}(\vec{\mu}_3\cdot\vec{\mu}_2) - a_{i1}a_{i3}(\vec{\mu}_1\cdot\vec{\mu}_3) - a_{i2}a_{i3}(\vec{\mu}_2\cdot\vec{\mu}_3)\}\Bigg] \end{aligned} \quad (13)$$

The VCD signal $\Delta\epsilon$ (in units of $\mathrm{cm}^{-1}\,\mathrm{M}^{-1}$) is calculated as a superposition of three Voigtian profiles:

$$\Delta\epsilon = \frac{\tilde{\nu}_0}{2.3\times10^{-39}}\cdot\sum_{j=1}^{3}\left[\frac{R_j}{\sigma_j\sqrt{2\pi}}\int_{\Omega_j-\delta\tilde{\nu}}^{\Omega_j+\delta\tilde{\nu}}\frac{\Gamma_j/\pi}{((\tilde{\nu}-\Omega'_j)^2+\Gamma_j^2)}\exp\left(\frac{-(\Omega'_j-\Omega_j)^2}{2\sigma_j^2}\right)d\Omega'_j\right] \quad (14)$$

where Γ_j and σ_j are the Lorentzian and Gaussian halfwidths attributed to the *j*th band. $\tilde{\nu}_0$ is the first moment of the entire amide I′ band profile.

Materials and Methods

Materials. L-Alanyl-L-alanyl-L-alanyl-L-alanine (A_4) was purchased from Bachem Bioscience Inc. (>98% purity) and used without further purification. $NaClO_4$ was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All chemicals were of analytical grade. The peptides were dissolved in D_2O at a concentration of 0.15 M for FTIR, Raman, and VCD and 1 mM for ECD spectroscopy. The pD of the solutions was adjusted by adding small aliquots of DCl to obtain the cationic state of the peptides. The pD values were determined by utilizing the method of Glasoe and Long[34] to correct the values obtained from pH electrode measurements. For the Raman experiments, the 934 cm^{-1} Raman band of 0.1 M $NaClO_4$ was used as an internal standard.[35]

Methods. Spectroscopies. We used the same equipment and experimental setups described in earlier publications.[23] The Raman spectra were obtained with the 442-nm (70 mW) excitation from a HeCd laser (model IK 4601R-E, Kimmon Electric, U.S.A.). The VCD instrumental description is the same as for the previous articles.[23]

Spectral Analysis. All IR and Raman spectra were analyzed using the program MULTIFIT.[36] They were normalized to the internal standard, i.e., the ClO_4^- band at 934 cm^{-1}. To eliminate solvent contributions, we measured the solvent reference spectra for both polarizations, which were then subtracted from the corresponding peptide spectra. The intensities of the normalized polarized Raman bands were derived from their band areas. These and the corresponding IR spectrum were self-consistently analyzed in that they were fitted with a set of identical frequencies, halfwidths, and band profiles. The isotropic and anisotropic Raman intensities and the depolarization ratios ρ were calculated as:

$$\begin{aligned} I_{\mathrm{iso}} &= I_x - \frac{4}{3}I_y \\ I_{\mathrm{ansio}} &= I_y \\ \rho &= \frac{I_x}{I_y} \end{aligned} \quad (15)$$

It should be mentioned that, in principle, I_{aniso} should be written as $2.33\cdot I_y$. As discussed in our earlier articles,[23] we prefer to identify it with I_y in the depicted figures so that the polarization properties of different lines can be better inferred.

Results and Discussion.

Experimental Results and Spectral Decomposition. Figure 2 depicts the amide I′ region of the IR, isotropic and anisotropic Raman, and the VCD spectrum of cationic A_4 in D_2O. As expected, a self-consistent spectral decomposition of the amide I′ band profile yielded three Voigtian bands at 1643 (AI'_1), 1656 (AI'_2), and 1674 cm^{-1} (AI'_3). Their spectral parameters are listed in Table 1. The Lorentzian halfwidths of all three bands were fixed to 11 cm^{-1} to achieve consistency with the lifetime of the excited vibrational states observed for trialanine.[21] The Gaussian halfwidths were very similar. The bands differed in terms of their depolarization ratios, i.e., $\rho_1 = 0.42 \pm 0.5$, $\rho_2 = 0.16 \pm 0.03$, and $\rho_3 = 0.11 \pm 0.02$. Corresponding intensity ratios $R(1,2) = I(AI_1/AI_2)$ and $R(2,3) = I(AI_2/AI_3)$ of the IR and Raman spectra were also different, thus indicating substantial excitonic coupling between amide I′ modes. In general terms, the VCD signal can be considered as diagnostic of a left-handed, extended structure. A comparison with the corresponding VCD spectra of cationic A_2 and A_3 suggests that the asymmetry of the couplet was due to a magnetic transition dipole moments associated with the C-terminal peptide group.[23]

Analysis of Raman and IR Spectra. As in our earlier study on A_3 and related peptides we assumed that the unperturbed amide modes of the three peptide groups had the same oscillator

(33) Holzwarth, G.; Chabay, I. *J. Chem. Phys.* **1972**, *57*, 1632.
(34) Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188.
(35) Sieler, G.; Schweitzer-Stenner, R. *J. Am. Chem. Soc.* **1997**, *119*, 1720.
(36) Jentzen, W.; Unger, E.; Karvounis, G.; Shelnutt, J. A.; Dreybrodt, W.; Schweitzer-Stenner, R. *J. Phys. Chem.* **1996**, *100*, 14184.

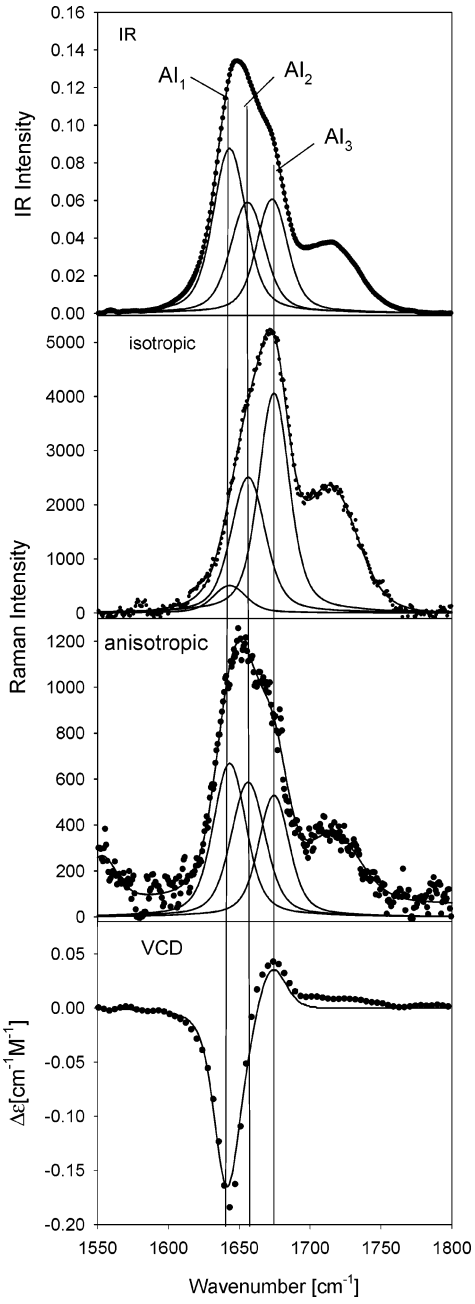

Figure 2. Amide I′ region of the FTIR, isotropic Raman, anisotropic Raman, and VCD spectra of L-alanyl-L-alanyl-L-alanyl-L-alanine in D_2O measured at pD 1. The Raman spectra were recorded with 442-nm excitation (laser power: 70 mW, slid width: 100 μm) The line profiles in the IR and Raman spectra result from a global spectral decomposition as described in the Materials and Methods section. The solid lines in the VCD spectra result from a calculation based on the dihedral angles obtained from the analysis of the amide I band intensities as described in the article.

strength and Raman cross section. Neglecting out-of-plane contributions, the Raman tensor is written as:

$$\hat{\alpha} = \begin{pmatrix} a & c & 0 \\ c & b & 0 \\ 0 & 0 & 0 \end{pmatrix} \tag{16}$$

As in reference 30, we assumed $b = 1$ as a reference value. Thus, we express a and c in units of b.

The first step of our analysis utilized the intensity ratios $R_{iso}(1,2)$ and $R_{iso}(2,3)$ of the amide I′ bands in the isotropic Raman spectrum to determine the quantum mechanical mixing factors a_{ij} and the wavenumbers $\tilde{\nu}_j$ of the unperturbed amide I′ modes. To this end, we inserted guess values for $\tilde{\nu}_j$ and Δ_{ij} in the Hamiltonian described by eq 3. For the excitonic coupling parameters we used the coupling parameter $\Delta = 5.2$ cm^{-1} obtained for cationic A_3[23,37] as a starting point for Δ_{12} and Δ_{23}. The MATHLAB software was employed to diagonalize the Hamiltonian described by eq 3. The obtained coefficients a_{ij} were used to calculate the Raman tensor of the excitonic states by means of eqs 4 and 5 and subsequently the intensity ratios $R_{iso}(1,2)$ and $R_{iso}(2,3)$ by eqs 6 and 7a. These values and the obtained eigenenergies were compared with the respective experimental values. The optimal reproduction of the latter required a slight increase of the coupling parameters ($\Delta_{12} = \Delta_{23} = 6.5$ cm^{-1}). The fine-tuning was achieved by considering a small negative contribution from 1- to 3-coupling, i.e., $\Delta_{13} = -2$ cm^{-1}. The calculated R_{iso} values and coupling and mixing coefficients are listed in Table 2. In the second step, we calculated the intensity ratios $R_{aniso}(1,2)$, $R_{ansio}(2,3)$ and $R_{IR}(1,2)$, $R_{IR}(2,3)$ of the amide I′ bands in the anisotropic and IR spectrum and their depolarization ratios as a function of ψ and ϕ and compared the results with the respective experimental values. This yielded a solution, which is visualized in Figure 3. It depicts the ϕ_{23} dependence of the intensity ratios and depolarization ratios for $(\phi_{12}, \psi_{12}) = (-70°, 155°)$ and $\psi_{23} = 145°$. Apparently, the respective experimental values visualized by horizontal dashed lines can all be reproduced in the limit of their

Table 1. Spectral Parameters and Raman Tensors of the Amide I′ Mode of L-Alanyl-L-alanyl-L-alanyl-L-alanine (A_4) Measured at pD 1 in D_2O

parameter	A_4	parameter	A_4
Ω_{A1_1} [cm^{-1}][a]	1643	$R_{iso}(1,2)$	0.2
Ω_{A1_2} [cm^{-1}][a]	1657	$R_{iso}(2,3)$	0.66
Ω_{A1_3} [cm^{-1}][a]	1674	$R_{aniso}(1,2)$	1.12
Γ_{L_1} [cm^{-1}][b]	11	$R_{aniso}(2,3)$	1.15
Γ_{L_2} [cm^{-1}][b]	11	$R_{IR}(1,2)$	1.42
Γ_{L_3} [cm^{-1}][b]	11	$R_{IR}(2,3)$	1.05
Γ_{G_1} [cm^{-1}][c]	21	ϕ_{12} [deg]	−70
Γ_{G_2} [cm^{-1}][c]	22.6	ψ_{12} [deg]	155
Γ_{G_3} [cm^{-1}][c]	19.0	ϕ_{23} [deg]	−80
ρ_1	0.49	ψ_{23} [deg]	145
ρ_2	0.18		
ρ_3	0.11		

[a] Wavenumber position. [b] Lorentzian halfwidth of the Voigtian profile. [c] Gaussian halfwidth of the Voigtian profile.

Table 2. Mixing Parameters and Intensity Ratios for Isotropic Raman Scattering Obtained by Diagonalizing the Hamiltonian of Eq 3, for Which We Assumed the Listed Wavenumbers of Uncoupled Amide I′ Modes Obtained from the Diagonalization of the Hamiltonian Eq 3, for Which We Assumed the Listed $\tilde{\nu}_j$ and Δ_{ij} values

parameters		parameters	
a_{11}	0.86	$R_{iso}(1,2)$	0.26
a_{12}	−0.49	$R_{iso}(2,3)$	0.69
a_{13}	0.035	$\tilde{\nu}_1$ [cm^{-1}]	1647
a_{21}	−0.47	$\tilde{\nu}_2$ [cm^{-1}]	1657
a_{22}	0.82	$\tilde{\nu}_3$ [cm^{-1}]	1675
a_{23}	0.31	Δ_{12} [cm^{-1}]	6.5
a_{31}	0.15	Δ_{23} [cm^{-1}]	6.5
a_{32}	0.26	Δ_{13} [cm^{-1}]	−2.0
a_{33}	0.95		

(37) Eker, F.; Cao, X.; Nafie, L.; Huang, Q.; Schweitzer-Stenner, R. *J. Phys. Chem.* **2003**, *107*, 358.

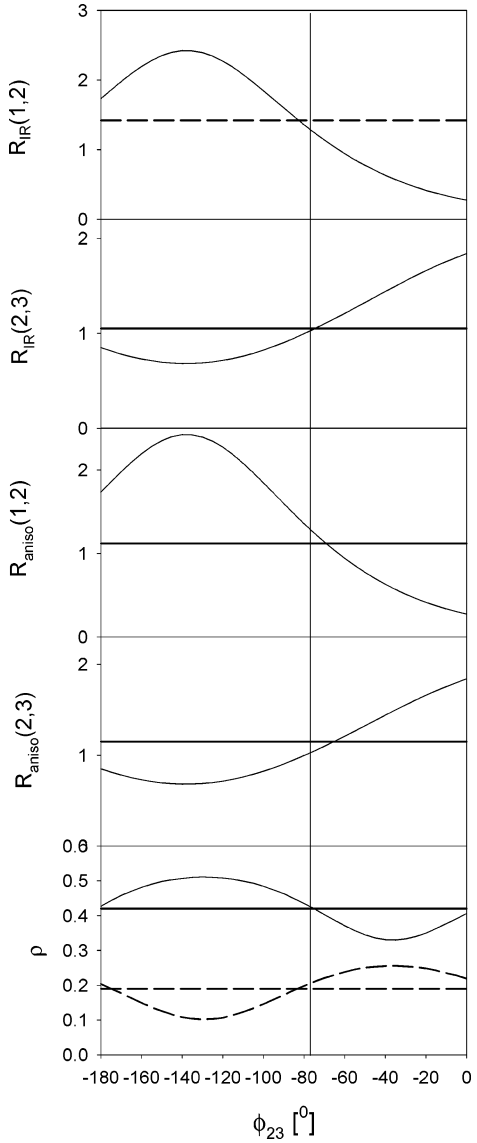

Figure 3. $R_{IR}(1,2)$, $R_{IR}(2,3)$, $R_{aniso}(1,2)$, $R_{aniso}(2,3)$, ρ_1, and ρ_2 calculated as functions of the dihedral angle ϕ_{23} for $\phi_{12} = -70°$, $\psi_{12} = 155°$, and $\psi_{23} = 145°$ by using the algorithm described in the Theory section and the Raman tensors derived in the Results and Discussion section. The horizontal dashed lines depict the respective experimental values. The solid vertical line labels the ϕ_{23} values forwhich all experimental values could be reproduced in the limit of their accuracy. For ρ_3, we calculated a nearly ϕ_{23} independent value of 0.012, which is close to the corresponding experimental value of 0.11.

experimental accuracy by $\phi_{23} = -80 \pm 10°$ (vertical line). We searched the entire conformational space related to the upper left square of the Ramachandran plot for an alternative solution without obtaining a result of the same degree of consistency. We disregarded the lower part of the Ramachandran plot from our search, because the VCD signal in Figure 2 clearly indicates a conformation with a left-handed chirality.

Calculation of the VCD Couplet. We employed the outlined theoretical approach to model the obtained VCD signals of the amide I′ in the lower panel of Figure 2 as follows. The transition dipole moment (2.0 esu·cm) for a single amide I′ mode was determined from the IR absorption spectra as described by Nafie et al.[38] The vector products of the transition dipole moments were calculated using the dihedral angles obtained from the IR and Raman spectra. To this end, we transformed all dipole

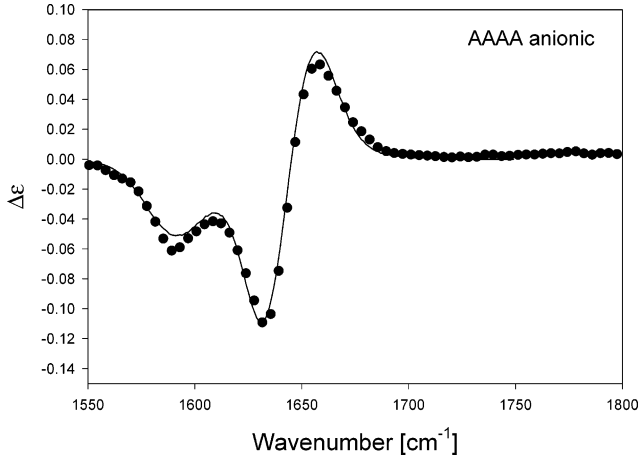

Figure 4. VCD spectrum of anionic L-alanyl-L-alanyl-L-alanyl-L-alanine measured at pD 12 between 1550 and 1800 cm^{-1}. The amide I′ couplet corresponds to the amide I bands at 1633 and 1656 cm^{-1}. The solid line results from the analysis described in the text.

vectors into the coordinate system of the C-terminal peptide by use of eq 9. The distance vector $\vec{T}_{ij}$ points from the carbon of the ith peptide to that of the $i + 1$th peptide. For the calculation of the couplet's shape, we first utilized the Lorentzian and Gaussian halfwidths obtained from the IR and Raman spectra. In accordance with earlier results, we considered a magnetic transition moment for the C-terminal peptide.[23] Amount and orientation were used as free parameters. We obtained a satisfactory reproduction of the experimental signal with $|\vec{m}_3| = 2.3 \times 10^{-23}$ esu·cm and an angle of 73° between $\vec{m}_3$ and $\vec{\mu}_3$. This is very close to what we obtained earlier for cationic trialanine ($|\vec{m}| = 2.3 \times 10^{-23}$ esu·cm and an angle of 87°).[23] However, this calculation overestimated the Gaussian halfwidth of the negative signal at the position of AI′$_1$. To obtain a good agreement shown in Figure 2, we had to reduce the Gaussian halfwidth for this band to 14 cm^{-1}. This result prompted us to revisit the IR and Raman spectra by trying to obtain a fit with this Gaussian halfwidth. This rendered impossible for all spectra. We interpreted the discrepancy as indication that the conformational heterogeneity probed by the Gaussian part of the Raman and IR band was different from that monitored by VCD.

One might argue that the necessity to invoke a magnetic moment for the C-terminal band makes the proof of our structure analysis less convincing since two unknown parameters are involved. Indeed, we are normally using the VCD spectrum of the zwitterionic species for our analysis because the corresponding couplet is normally nearly symmetric. Since this is not possible for tetraalanine for reasons given above we have employed the amide I′ VCD signal of the anionic species for an additional check. As shown in Figure 4, it is very pronounced and is much less asymmetric than the respective couplet of the cationic species. Unfortunately, a reliable spectral composition of the IR and Raman spectra is difficult because of the much stronger overlap of the three amide I′ bands. To circumvent this difficulty we proceeded as follow. By comparison of the isotropic, anisotropic, and IR spectra, we identified three amide I′ bands at 1633, 1642, and 1656 cm^{-1}. From the vibrational spectra of anionic dialanine we inferred that the 1633 cm^{-1} band

(38) Nafie, L.; Dukor, R. K.; Freedman, T. B. In *Handbook of Vibrational Spectroscopy*; Chalmers, J. M., Griffiths, P. R., Eds.; Wiley: Chichester, U.K., 2002.

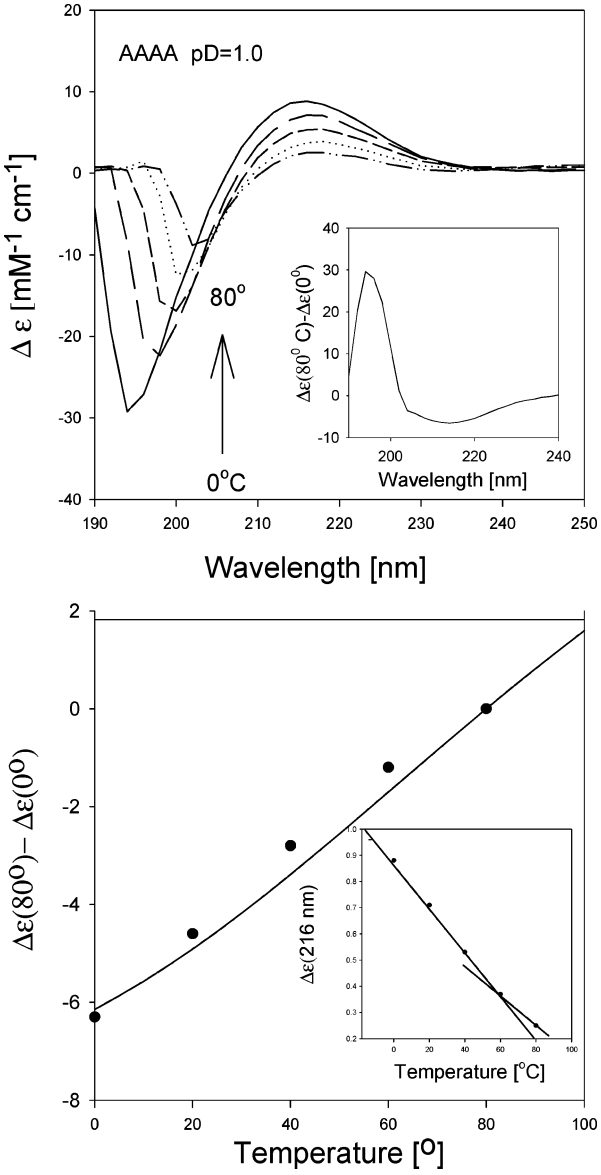

Figure 5. (a) UV−ECD spectra of L-alanyl-L-alanyl-L-alanyl-L-alanine at pD 1 measured at different temperatures between 0 and 80 °C. The inset figure displays the difference spectrum $\Delta\epsilon$ (80°) − $\Delta\epsilon$ (0°). (b) Temperature dependence of $\Delta\epsilon$ (216 nm, 80 °C) − $\Delta\epsilon$ (216 nm, 0 °C). The solid line results from a thermodynamic analysis described in the text. The inset figure displays the temperature dependence of $\Delta\epsilon$ (216 nm). The solid lines were inserted to visualize the inflection point of a biphasic transition.

was assignable to the N-terminal peptide (of course excitonically coupled to the other modes). The vibrational spectra of cationic tetraalanine suggested that the bands at 1642 cm^{-1} and 1656 cm^{-1} were assignable to the C-terminal and central peptide group. The correct assignment was crucial for the evaluation of the IR, Raman, and VCD spectra. We assumed that the coupling parameters were not affected by the protonation of the terminal groups, in accordance with our earlier findings for tripeptides.[23] Hence, we used these parameters and guess values for the wavenumbers of the unperturbed amide I′ modes to diagonalize the excitonic Hamiltonian (eq 3). Subsequently, we slightly changed the wavenumbers to reproduce the experimental wavenumbers of the amide I′ bands. Thus, we obtained mixing parameters which we employed to calculate the VCD couplet in Figure 5. The VCD signal of the antisymmetric COO^- stretch at 1590 cm^{-1} was heuristically accounted for by an appropriate

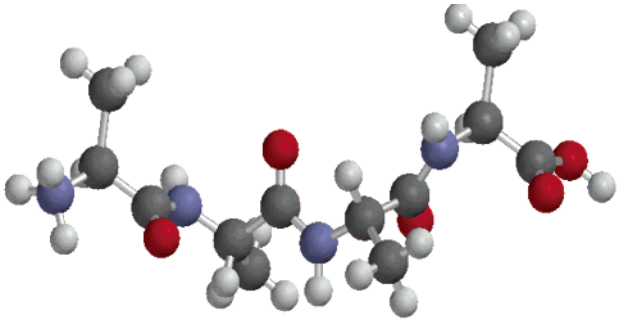

Figure 6. PPII conformation of cationic tetraalanine in water as obtained from the analysis of the amide I′ band in the IR, Raman, and VCD spectrum. The structure was obtained by using the program TITAN from Wavefunction, Inc.

Gaussian profile. A perfect reproduction of the positive signal at 1656 cm^{-1} was obtained with (ϕ_{12}, ψ_{12}) = (−60°, 160°) and (ϕ_{23}, ψ_{23}) = (−60°, 160°). Because of the asymmetry of the couplet the negative signal was slightly underestimated. This discrepancy could be eliminated by allowing a small magnetic moment ($m_1 = 5 \times 10^{-24}$ esu·cm, solid line in Figure 4). For the calculation, we used the bandwidths obtained from the Raman and IR spectra of tetraalanine. This time it was not necessary to reduce one of these bandwidths. Altogether, the analysis of the amide I′ VCD signal of anionic tetraalanine fully corroborated the notion that PPII was the dominant conformation (Figure 6).

As an additional check, we calculated the VCD spectra for slightly different orientations of the transition dipole moment with respect to their coordinate system. Variations of ±5° were found to have an insignificant influence on the VCD signal (changes of ±10%). We also calculated the VCD signal for an extended β-strand ((ϕ_{12}, ψ_{12}) = (−160°, 150°) and (ϕ_{23}, ψ_{23}) = (−160°, 150°)[22] for both the cationic and anionic species and obtained very weak signals. For cationic tetraalanine, we obtained a triplet with very small negative signals at Ω_1 and Ω_3 and a somewhat stronger signal at Ω_2. This bore no similarity with the experimental couplet.

Solution Structure of Tetraalanine. The dihedral angles obtained from the above analysis were very close to that of the canonical PPII conformation, i.e., (ϕ, ψ) = (−78°, 146°).[39] This observation was in contrast with our earlier results for trialanine for which we obtained (ϕ, ψ) = (−123°, 173°), which was then found to be an average conformation reflecting a 50:50 mixture of PPII and a very extended β-strand-like conformation. The present data show that the equilibrium between these two conformations was significantly shifted toward PPII in tetraalanine. The obtained values for the dihedral angles suggest that only a small fraction of the β-strand-like conformation is populated at room temperature.

ECD Spectra of Tetraalanine. Figure 5a shows the temperature dependence of the ECD spectra of cationic A_4 measured between 0 and 80 °C. The inset figure displays the difference spectrum obtained by subtracting the 0 °C from the 80 °C spectrum. The spectrum obtained at 0 °C appears somewhat blue-shifted and does not share the dichroic point formed by the remaining spectra. Irrespective of this observation, the spectra are clearly indicative of a substantial population of PPII at room temperature. Similar spectra were earlier obtained by

(39) Bochiccio, B.; Tamburro, A. M. *Chirality* **2002**, *14*, *782*.

Lisowski et al.[40] The observed PPII signal is significantly (by a factor of 3) more pronounced than that observed for A_3,[24] indicating a stabilization of PPII in A_4 in agreement with the result obtained from the vibrational spectroscopies. We performed a thermodynamic analysis by plotting $\Delta\epsilon_{216}(80°) - \Delta\epsilon_{216}(0°)$ as a function of temperature (Figure 5b) and by fitting the equation:[24]

$$\Delta(\Delta\epsilon)(T) = \frac{\Delta\epsilon_{\beta 0}\exp[(-\Delta H^{\varnothing}/RT_0) + (\Delta S^{\varnothing}/R)] + \Delta\epsilon_{\mathrm{PPII}}}{Z(T_0)} - \frac{\Delta\epsilon_{\beta 0}\exp[(-\Delta H^{\varnothing}/RT) + (\Delta S^{\varnothing}/R)] + \Delta\epsilon_{\mathrm{PPII}}}{Z(T)} \quad (17)$$

to these data. $\Delta\epsilon_\beta$ and $\Delta\epsilon_{\mathrm{PPII}}$ are the intrinsic $\Delta\epsilon$ values of β-strand and PPII conformer at the respective wavelength, $\Delta H^{\varnothing} = H_\beta - H_{\mathrm{PPII}}$ and $\Delta S^{\varnothing} = S_\beta - S_{\mathrm{PPII}}$ are the standard enthalpy and entropy differences between the two conformers, R is the gas constant, and T is the temperature in Kelvin. T_0 is the reference temperature (353.15 K in our case) and $Z(T)$ and $Z(T_0)$ are the partition sums at T and T_0, respectively. The fit visualized by the solid line in Figure 5 yielded $\Delta H^{\varnothing} = 19$ kJ/mol, $\Delta S^{\varnothing} = 53.1$ JK^{-1}, $\Delta\epsilon_{\mathrm{PPII}} = 13$ cm^{-1} mM^{-1} and $\Delta\epsilon_\beta = -10$ cm^{-1} mM^{-1}. The corresponding ΔG value at 300 K is 3.1 kJ/mol, which indicates a molar fraction of 0.78 for PPII. However, a correlation analysis of the fitting parameters suggests that this must be considered as the lower limit for the PPII conformation, since a correlation between $\Delta H^{\varnothing}$ and $\Delta\epsilon_{\mathrm{PPII}}$ allows equally good fits for higher $\Delta H^{\varnothing}$ values, which would lead to higher ΔG values and thus to an even higher molar fraction of PPII. Hence, the ECD spectra strongly corroborate our structure analysis in that A_4 is predominantly PPII.

The obtained thermodynamic parameters shed some light on the reason for the higher PPII preference of A_4 compared with A_3. Both the enthalpy and the entropy difference are larger for the former, but the enthalpy (favoring PPII) has increased by a factor of 1.7 whereas the entropy (favoring the extended conformation) increased only by a factor of 1.4. This led us to propose that the high PPII stability of longer alanine-containing peptides is because enthalpic stabilization of PPII exhibits a larger increase with the chain length than the entropic stabilization of the extended β-strand.

The applicability of eq 17 indicates that the PPII formation does not involve cooperativity. This notion is also supported by the temperature dependence of $\Delta\epsilon$(216 nm) depicted in the inset of Figure 5b. It reveals two phases and a transition temperature of ca. 55 °C.

Comparison with Literature and Conclusions. Besides demonstrating the capability of our theoretical approach to derive the dihedral angles of tetrapeptides from the amide I′ band profile of their IR, Raman, and VCD spectra, the most important result of this study is that the total propensity of A_4 for PPII is substantially larger than that observed for A_3. Since cooperativity can be ruled out, our result is still in agreement with Flory's isolated pair hypothesis,[41] as predicted for extended conformations.[42,43] This implies that the propensity of the individual residue depends on the number of residues in the peptide chain. We interpret this as indicating that (with respect to a tripeptide) the addition of an alanine residue changes the hydration shell of the peptide in a way that optimizes the contribution of peptide−solvent interactions to the stabilization of PPII. Earlier studies have shown that water is essential for the formation of this helix.[24,37,44,45] Avbelj and Baldwin provided strong evidence for the PPII stabilization by dipole (solvent)−dipole (peptide) interactions.[27]

The first theoretical study of the conformation of tetraalanine was carried out 30 years ago by Rossi et al.[46] These authors obtained the ground-state potential energy surface for tetraalanine by extended Hückel theory (EHT). Thus, they found three minima of comparable energy assignable to well-known secondary structure conformations, i.e., the left- and right-handed α helices, and the β-stranded chain conformation. The latter corresponded to a very broad and shallow minimum in the energy landscape. More recently, Pettit and associates performed molecular dynamic (MD) simulations of tetraalanine with a CHARMM24 force field and the explicit solvent model TIP3P for different protonation states of C- and N-terminus.[47] An analysis of the simulations identified four major conformational states in the sterically allowed region of the Ramachandran space. They found that the terminal charges have a limited influence on the conformation of ϕ_{12}, ψ_{12}, and ϕ_{23} (ϕ_2, ψ_2, and ϕ_3 in their notation), whereas ψ_{23} (ψ_3) was found to depend on the protonation state of the N-terminus. For the cationic species, the authors obtained the largest population for an α-helix-like conformation (ϕ_1, ψ_1) = (ϕ_2, ψ_3) = (−80°, −60°). A PPII-like conformation with (ϕ_1, ψ_1) = (ϕ_2, ψ_2) = (−80°, −165°) was found to be slightly less populated.[47] In a subsequent study from this laboratory, different treatments of the solvent were combined with CHARMM22 to study the free energy landscape of tetraalanine. The contour plots depict broad minima in the helical and β-strand region. Both studies have in common that they predict a substantial population of an α-helical conformation, in accordance with what was also obtained for the dialanine peptide.[48] Our experimental data are apparently at variance with this notion and, thus, suggest that the employed force fields overestimate the stability of helices.

The most recent MD simulations on alanine oligopeptides was done by Gnanakaran and Garcia.[25] Their article explicitly dealt with the overestimation of helical stability by CHARMM22 as well as by AMBER94. They used a modified AMBER force field termed A94/MOD in which the backbone dihedral angle energy terms for the rotation around ϕ and ψ was set to zero to study the conformational dynamics of peptides of different length in a water bath. For short peptides, they found that the overall PPII fraction substantially increases with the number of alanine peptides. For peptides with more than seven residues, the α-helical conformation becomes increasingly populated, in agreement with multiple spectroscopic experiments. PPII remains second in the conformational hierarchy at room temperature while it coexists with a more extended β-strand confor-

(40) Lisowski, M.; Siemion, I. Z.; Sobczyk, K. *Int. J. Pept. Protein Res.* **1983**, *21*, 301.
(41) Flory, J. P. *Statistical Mechanics of Chain Molecules*; Wiley: New York, 1969; p 252.
(42) Pappu, R. V.; Srinivasan, R.; Rose, G. D. *Proc. Natl. Acad. Sci U.S.A.* **2000**, *97*, 12565.
(43) Pappu, R. V.; Rose, G. D. *Protein Sci.* **2002**, *11*, 2437.
(44) Han, W.-G.; Jalkanen, K. J.; Elstner, M.; Suhai, S. *J. Phys. Chem. B* **1998**, *101*, 2587.
(45) Mu, Y.; Stock, G. *J. Phys. Chem. B* **2002**, *106*, 5294.
(46) Rossi, A. R.; David, C. R.; Schor, R. *J. Phys. Chem.* **1972**, *76*, 2793.
(47) Blatt, H. D.; Smith, P. E.; Pettitt, B. M. *J. Phys. Chem. B* **1997**, *101*, 7628.
(48) Rabhu, N. V.; Perkyns, J. S.; Blatt, H. D.; Smith, P. E.; Pettit, B. M. *Biophys. Chem.* **1999**, *78*, 113.

mation at high temperatures. This explains why ECD experiments indicate that thermal denaturation of helix forming alanine-based peptides yields a state with a substantial PPII contribution[16] (cf. also ref 5: in the manuscript the authors still interpret the CD signal as indicative of a random coil structure, which is not consistent with more recent findings). Even though the calculations by Gnanakaran and Garcia[25] overestimated the PPII population for trialanine (80 versus 50% at 300 K), the obtained results reproduce for the first time the experimental findings for trialanine[23,24] in that they predict a coexistence of PPII and an extended β-strand conformation without a significant α-helical contribution.[45] Moreover, their observation that the PPII fraction increases with the number of alanine residues is in line with the results of the present analysis. In this context, another study by Garcia[26] is noteworthy in which it is argued on the basis of MD simulations of blocked $AceA_{21}Nme$ that a peptide segment comprising four alanine residues is needed for the formation of a strongly hydrated groove around the peptide backbone. This explains why tetraalanine has a higher propensity for PPII than trialanine. It underscores the aforementioned relevance of the hydration shell for PPII stabilization. Hence, one is led to the prediction that the residue numbers 4, 8, 12, etc. might be considered magic numbers (borrowing a concept from nuclear physics) for the PPII stability of alanine-based peptides.

Our experimental data and the above-discussed MD simulations are mutually corroborative and lead us to challenge the classical understanding of the unfolded state as conformationally random. Our earlier studies[23,24,30] and multiple evidence from other experimental and theoretical investigations had already questioned the traditional view of the so-called random coil state as sampling the entire conformational allowed part of the Ramachandran space. Apparently, this view has to be modified in favor of a much more ordered conformation for the unfolded state of these peptides. The work of Garcia and associates[11,25,26] strongly suggests that water plays a major role in determining the conformational preference in the unfolded state. It is likely that this does not exclusively hold for alanine residues. The most recent investigations of K_3, D_3, and E_3 revealed a predominance of PPII already on the tripeptide level.[49] Hence, the frequently used Baldwin peptides, which contain mostly alanine with an admixture of ionizable residues to allow solubility in water, are likely to exhibit an even higher PPII propensity in their unfolded state. The individual propensities of other amino acids in an alanine context is currently under investigation in our laboratories.

Acknowledgment. Financial support was provided from the NIH-COBRE II grant for the Center for Research in Protein Structure, Function and Dynamics (P20 RR16439-01), from the NIH-SCORE grant (S06 GM008102-3052), and from the Fondos Institucionales para la Investigación of the University of Puerto Rico (20-02-2-78-514). R.S.-S. thanks Dr. Angel Garcia for a very useful discussion and providing us preprints of his articles prior to publication.

JA039452C

(49) Eker, F.; Griebenow, K.; Cao, X.; Nafie, L.; Schweitzer-Stenner, R. *Biochemistry* **2004**, *43*, 613.