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UV Resonance Raman Investigation of the Conformations and Lowest Energy Allowed Electronic Excited States of Tri- and Tetraalanine: Charge Transfer Transitions

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Abstract

UV resonance Raman excitation profiles and Raman depolarization ratios were measured for trialanine and tetraalanine between 198 and 210 nm. Excitation within the $\pi \rightarrow \pi^*$ electronic transitions of the peptide bond results in UVRR spectra dominated by amide peptide bond vibrations. In addition to the resonance enhancement of the normal amide vibrations, we find enhancement of the symmetric terminal COO^- vibration. The Ala_3 UVRR AmIII_3 band frequencies indicate that poly-proline II and 2.5_1 helix conformations and Type II turns are present in solution. We also find that the conformation of the interior peptide bond of Ala_4 is predominantly poly-proline II-like. The Raman excitation profiles of both Ala_3 and Ala_4 reveal a charge transfer electronic transition at 202 nm, where electron transfer occurs from the terminal nonbonding carboxylate orbital to the adjacent peptide bond π^* orbital. Raman depolarization ratio measurements support this assignment. An additional electronic transition is found in Ala_4 at 206 nm.

INTRODUCTION

UV resonance Raman (UVRR) spectroscopy is recognized as a powerful technique for probing peptide and protein secondary structure.^{1–14} UVRR spectroscopy also provides insight into the geometry of the excited states and electronic transitions through Raman excitation profiles and Raman depolarization ratios.^{11–13} Excitation between 180 and 215 nm is in resonance with the amide $\pi \rightarrow \pi^*$ electronic transition of the peptide backbone, resulting in enhancement of amide vibrations.^{1–13} In the work here, we use UVRR spectroscopy to examine the secondary structure conformations and underlying electronic transitions of both Ala_3 and Ala_4 .

Alanine-based peptides are often used as theoretical and experimental models to study protein conformation and folding.^{1, 2, 15–25} The folded state of longer alanine peptides is predominantly α -helix-like^{1–3, 19, 24, 25}, while the conformation of the unfolded state has been established to be a polyproline II (PPII) helix-like structure,^{9, 20–23, 26} (although there remains some controversy with this assignment.^{27, 28}) Short alanine peptides, such as trialanine (Ala_3) and tetraalanine (Ala_4), are often used as models for the unfolded state of peptides and proteins. Although there have been numerous studies of Ala_3 and Ala_4 which have identified a number of equilibrium conformations, the conformational distributions are still not clear.^{15, 29–43}

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Most studies agree that the unfolded state for both peptides is populated mainly by the polyproline II helix (PPII) conformation.^{15, 29–36, 38, 41–43} There is, however, little agreement on the other conformations present in equilibrium. The reported conformational distributions include: only PPII^{31, 39, 41, 42}; PPII, with either an additional β structure^{29, 30, 33} or a right-handed α -helix (α_R)⁴³; and a β structure with α_R -helix.^{15, 32, 35, 36, 40} Another study reported that the conformations in solution include PPII, a β -like structure, an α_R -helix, and a γ -turn.³⁸ A few studies that found only one conformation present assigned it to either a left-handed helix³⁴ or an extended β -helix-like structure,³⁷ both of which are similar to a PPII-like conformation.

In the study here we measured the UVRR excitation profiles and Raman depolarization ratios of aqueous solutions of Ala₃ and Ala₄ between 198 to 210 nm at 25 °C to determine the solution conformation(s) and electronic transitions of Ala₃ and Ala₄. We find that both Ala₃ and Ala₄ adopt three primary conformations: PPII-like, 2.5₁ helix-like, and Type II turns; and that Ala₄ may also adopt an additional unknown conformation. We observe the previously described charge transfer transition¹² at 202 nm in both peptides. We also observe a transition in Ala₄ at 206 nm, whose origin remains murky.

MATERIALS AND METHODS

Sample Preparation

Dialanine (Ala₂), trialanine (Ala₃) and tetra-alanine (Ala₄) were purchased from Bachem (Torrance, CA) and used as received. We used 6.6 mM, pH 7 solutions of Ala₃ and Ala₄ for the UVRRS measurements. Each sample contained sodium perchlorate (0.2 M) as an internal standard. The UVRR spectral measurements were taken at 25 °C $\pm$ 0.5 °C.

Raman Instrumentation

The UVRR instrumentation was previously described.⁴⁴ The laser source used was a Positive Light Co. Indigo-DUV Ti:Sapphire laser system (Coherent, Santa Clara, CA). The Indigo-DUV system utilizes an intra-cavity frequency doubled, Q-switch pulsed Nd:YLF Evolution 15 laser (527 nm, 30 nsec pulse width, 5 kHz repetition rate, 10 W average power) to pump a Ti:Sapphire oscillator, which generates tunable radiation from 772 to 840 nm. Raman excitation in the deep UV is obtained by mixing the third harmonic with the fundamental, producing tunable radiation between 193 and 210 nm. The average powers in the deep UV vary between 2–5 mW.

The laser beam was focused into a temperature-controlled, circulating flow stream. The flow stream was purged with N₂ to eliminate Raman scattering from the O₂ band at 1555 cm⁻¹. Each 20-mL sample was irradiated for a maximum of 15 min. The scattered light was directed into a subtractive double monochromator⁴⁴ and the Raman scattered light was detected by a liquid nitrogen cooled CCD (Princeton Instruments, Spec-10:400B). The Raman intensities were normalized to that of the 932 cm⁻¹ perchlorate (ClO₄⁻) symmetric stretch vibration. The spectra were analyzed and deconvoluted using Grams/32 AI 8.0 software (Thermo Electron Corporation, Waltham, MA).

UV Raman depolarization ratios (ρ) were measured using a 180° back scattering geometry. The light collected from the sample was directed through a UV linear dichroic polarizer (Oriol Instruments, Stratford, CT) followed by a crystalline quartz polarization scrambler (Spex Industries, Edison, NJ) before the monochromator entrance slit. The depolarization ratio ρ was calculated as a ratio of the perpendicularly polarized light ($I_{\perp}$) to the parallel polarized light ($I_{\parallel}$):

$$\rho = \frac{I_{\perp}}{I_{\parallel}} \quad (1)$$

The depolarization ratios of ClO_4^- and cyclohexane were used as standards¹⁴ to verify the accuracy of the measured depolarization ratios.

Spectrometer Efficiency

The spectrometer used was a modified Spex 1401 double monochromator that operates over the 193–270 nm range. We corrected for the wavelength dependence of throughput efficiency by using previously determined spectrometer efficiencies.⁴⁴

Absorption Measurements

The UV absorption spectra of Ala₂, Ala₃ and Ala₄ between 190 and 250 nm were measured using a Cary 5000 Varian UV-Vis-NIR spectrophotometer. The absorption measurements were taken with solution concentrations of 0.5 mM at pH 7 and pH 2 at room temperature (25 °C ± 0.5 °C).

RESULTS & DISCUSSION

Absorption Spectra

The absorption spectra of Ala₂, Ala₃ and Ala₄ are shown in Figure 1. The molar absorptivities increase for all three peptides at both pH 7 and pH 2 as the wavelength decreases from 250 nm to 190 nm.

For Ala₂, the broad absorption band centered below ~190 nm derives from the amide $\pi \rightarrow \pi^*$ NV₁ electronic transition for both pH 7 and pH 2 (Fig. 1a). The absorption maximum of the pH 2 spectrum, where the carboxylate group is protonated, appears to be blue-shifted compared with the pH 7 spectrum. The pH difference spectrum for Ala₂ indicates the presence of an underlying transition in the pH 7 absorption spectrum at ~202 nm, which disappears at pH 2. The absorption difference spectra for each peptide can be well fit with two Gaussian bands (Fig. 2a–c). For Ala₂, the Gaussian bands are centered at 180 nm (not shown) and 203 nm (Fig. 2a). The 180 nm band is assigned to the amide NV₁ $\pi \rightarrow \pi^*$ electronic transition, and we assign the ~203 nm band to a charge transfer transition, similar to those previously found in Gly dipeptides.¹² Chen et al.¹² previously demonstrated that a charge transfer transition occurs at ~200 nm in short Gly peptides. The transition involves transfer of charge from the nonbonding carboxylate orbital to the amide π^* orbital (Fig. 3). This charge transfer band disappears in the low pH protonated species.

For Ala₃ (Fig. 1b) and Ala₄ (Fig. 1c), the broad absorption band centered below ~190 nm derives from the amide $\pi \rightarrow \pi^*$ NV₁ electronic transitions at both pH 7 and pH 2. The Ala₃ and Ala₄ pH 7 spectra are broader and absorb more than the pH 2 spectra.

UVRR Spectra

The UVRR spectra for Ala₃ and Ala₄ at 25 °C excited between 198 and 210 nm are shown in Figs. 4 and 5, respectively. All spectra were normalized to the symmetric perchlorate stretch (932 cm⁻¹).

Vibrations enhanced in both Ala₃ and Ala₄ include the Amide I (AmI) vibration at ~1655 cm⁻¹, which is primarily a C=O stretching vibration (st). The enhanced Amide II (AmII) vibration involve C–N stretching (st), coupled with N–H bending (b) at ~1550 cm⁻¹. The

symmetric stretching of the carboxylate appears at $\sim 1400\text{ cm}^{-1}$ but is overlapped by other bands. The symmetric b vibrations of the CH_3 and $\text{C}_\alpha\text{-H}$ groups occur at 1371 cm^{-1} and 1332 cm^{-1} , respectively. There is an additional vibration present at 1391 cm^{-1} assigned as $\text{CH}_3 + \text{C}_\alpha\text{-H}$ bending, which most strongly overlaps the symmetric carboxylate stretch. The AmIII (AmIII) region is broad and spans the $1200 - 1300\text{ cm}^{-1}$ range. The bands in the AmIII region result primarily from vibrations composed of C-N st coupled to N-H b, but can also include $\text{C}_\alpha\text{-C}$ st, N-C st, C-N st, and some $\text{C}_\alpha\text{-H}$ b.⁹ Both peptides show the highest relative band intensities for 202 nm excitation. The relative band intensities within the spectra are similar across the range of excitation wavelengths, indicating that similar or identical electronic transitions are involved in the enhancement.

Our group has previously shown that for a 21-residue, primarily Ala peptide in mixtures of $\text{H}_2\text{O}/\text{D}_2\text{O}$, the spectra of the partially deuterated chains can be modeled as a statistically weighted linear sum of the deuterated and protonated segments of the peptide.^{10, 45} This result indicates that the peptide bond vibrations are localized within the individual peptide bonds. More recently, we also showed that for short Gly peptides, there is also a lack of coupling between adjacent peptide bond vibrations.⁴⁶ These results allow for accurate modeling of the Raman spectra of peptides in solution because the spectra are simply a linear sum of the terminal and internal peptide bonds. The spectra for the terminal and internal peptide bonds significantly differ.⁴⁶ We showed that the spectrum of a three residue peptide approximates the spectrum of the two terminal peptide bonds of an oligopeptide, while the difference spectrum between a six residue peptide and a five residue peptide accurately models the spectra of an internal peptide bond. We use this methodology to resolve the spectra of the COO^- vibration for both Ala₃ and Ala₄.

To resolve the COO^- vibration in Ala₃ we assume that UVRR spectrum of the Ala₃ NH_3^+ end peptide bond is similar to that of the interior peptide bond of Ala₄, which we model as that resulting from subtraction of the Ala₄ – Ala₃ UVRR (Fig. 12). The underlying reasoning is that this peptide bond has the standard peptide bond $\pi \rightarrow \pi^*$ transition as the internal peptide bonds, without the charge transfer band of the carboxylate terminal peptide bonds.

We then model the Ala₃ UVRR spectra of the carboxylate end peptide bond as resulting from the UVRR spectral subtraction $\text{Ala}_3 - (\text{Ala}_4 - \text{Ala}_3) = 2 * \text{Ala}_3 - \text{Ala}_4$, where the NH_3^+ end peptide bond UVRR (interior Ala₄ peptide bond spectrum) is subtracted from the Ala₃ spectrum (Fig. 6a). This spectral subtraction is obtained using the perchlorate internal standard intensity. This spectrum clearly shows the strong enhancement of the 1405 cm^{-1} carboxylate stretching vibration due to excitation within the charge transfer absorption spectrum.

Identically we can resolve the Ala₄ COO^- band by the UVRR subtraction $\text{Ala}_4 - 2 * (\text{Ala}_4 - \text{Ala}_3) = 2 * \text{Ala}_3 - \text{Ala}_4$, where we assume that the UVRR spectrum of the Ala₃ NH_3^+ end peptide bond is similar to that of the interior Ala₄ peptide bond, which we model as that resulting from subtraction of the Ala₄ – Ala₃ UVRR (Fig. 6b). The resulting spectrum identically shows the strong Ala₄ enhancement of the 1405 cm^{-1} carboxylate stretching vibration due to excitation within the charge transfer absorption spectrum.

Absolute Raman Cross Sections

To quantitatively compare the spectra across the range of excitation wavelengths, we calculated the Raman cross sections for each amide band at each wavelength, using ClO_4^- as the internal standard. It has been shown that the ClO_4^- symmetric stretching band has an Albrecht A-term frequency dependence for excitation wavelengths from the visible to the UV (to 220 nm).⁴⁷ We extrapolated the ClO_4^- cross sections to 198 nm.

The absolute Raman cross sections (with a correction factor for self absorption) were calculated from:

$$\sigma_{\text{peptide}} = \frac{I_{\text{band}} \cdot k(\lambda_{\text{ClO}_4}) \cdot C_{\text{ClO}_4} \cdot \sigma_{\text{ClO}_4}}{I_{\text{ClO}_4} \cdot k(\lambda_{\text{band}}) \cdot C_{\text{peptide}} \cdot n_A} \cdot \left[\frac{\epsilon_s + \epsilon_o}{\epsilon_r + \epsilon_o} \right] \quad (3)$$

where I_{band} and I_{ClO_4} are the relative intensities of the amide and ClO_4^- bands, respectively; $k(\lambda_{\text{band}})$ and $k(\lambda_{\text{ClO}_4})$ are the spectrometer efficiencies at the wavelengths of the Raman bands; C_{peptide} and C_{ClO_4} are the concentrations (M) of the individual Ala peptides and perchlorate; σ_{ClO_4} is the calculated ClO_4^- cross section at the band wavelength; n_A is the number of amide bonds in the peptide; ϵ_o is the molar absorptivity of the peptide at the laser excitation frequency; ϵ_s is the molar absorptivity of the peptide at the Raman band wavelength; and ϵ_r is the molar absorptivity at the ClO_4^- band wavelength. The expression in the brackets corrects the measured Raman intensities for self absorption.^{48, 49}

These Raman cross sections of the AmI, AmII, AmIII, the $\text{C}_\alpha\text{-H}$ and CH_3 bends are actually the summed Raman cross sections of each of these Raman bands over all of the peptide bonds, which as we have shown previously scatter independently⁴⁶ (except for the AmI band of the α -helix conformation). In contrast, only the terminal COO^- peptide bond gives rise to enhancement of the symmetric COO^- stretch. Thus, we evaluated its cross section from the difference spectra obtained as shown in Fig. 6.

Excitation Profiles

The excitation profiles for Ala₃ shown in Fig. 7 indicate that the Raman cross sections increase as the excitation wavelength decreases from 210 nm to maxima at 202 nm. It is well-established that the $\text{NV}_1 \pi \rightarrow \pi^*$ electronic transitions in short peptides occur at or below 190 nm (Fig. 1).^{50–52} The excitation profile maxima at 202 nm occur close to the maximum wavelength of the charge transfer transition found in the pH difference absorption spectrum of Ala₃.

Examination of the Fig. 7 Ala₃ excitation profiles indicates that the largest cross sections occur for the AmII vibration, which is mainly a C–N st coupled to an N–H b. A similar maximal AmII band enhancement is also seen in the longer, 21 – residue alanine peptide (AP).⁴ The next largest cross section occurs for the AmIII vibration, which is also composed of a C–N st and N–H b. The AmIII cross section in AP was also found to be less than that of the AmII.⁴ Normal mode calculations of polyalanine indicate that C–N motion contributes twice as much to the AmII vibration than to the AmIII vibration.⁵³ The COO^- symmetric stretch shows the next largest excitation profile cross section.¹² The COO^- symmetric st is selectively enhanced by the ~202 nm charge transfer transition.¹²

All of the excitation profiles are much narrower than the charge transfer absorption difference spectra. The resonance Raman cross sections of CH_3 , C_αH , and the AmI vibrations are significantly smaller with only a modest charge transfer enhancement.

The Raman excitation profiles for Ala₄ shown in Fig. 8 differ significantly from those of Ala₃ in that they show two distinct maxima. There are two peptide bonds in Ala₃, whereas Ala₄ contains three. The AmII and AmIII vibrations once again have the largest cross sections, with the AmIII vibration having a significantly larger relative cross section than in Ala₃. The AmI band cross section per peptide bond at 202 nm is approximately twice that in Ala₃.

As the excitation wavelength decreases from 210 to 198 nm, a small excitation profile maximum is observed at 206 nm, followed by a larger maximum at 202 nm. The excitation profile maximum at 202 nm for Ala₄ is assumed to be due to the charge transfer transition in

the pH difference absorption spectrum (Fig. 1c and 2c), while understanding the origin of the 206 nm maximum will require additional information. It is important to note that the cross sections per peptide bond are roughly similar between Ala₃ and Ala₄ (except for the AmI) but the peak values are smaller in Ala₄ while the excitation profiles appear broader than those of Ala₃.

The lowest order theory⁵⁴ indicates that the resonance Raman excitation profiles will scale as the square of the absorbance band molar absorptivity. Figs. 7 and 8 also compare the excitation profiles of Ala₃ and Ala₄ to the squares of their absorption difference spectra. This comparison shows more similar bandwidths.

The COO⁻ symmetric st is clearly visible for short peptides such as Ala₃ and Ala₄. However, the COO⁻ symmetric st will be less evident in longer peptides such as AP, where the peptide bond NV₁ $\pi \rightarrow \pi^*$ transitions dominate the excitation profile resulting in a small relative enhancement of the COO⁻ symmetric st.

Raman Depolarization Ratios, Excitation Profiles, and Absorption Spectra

In order to gain insight into the origin of the excitation profiles, we measured the dispersions of the resonance Raman depolarization ratios. Fig. 9 compares the Ala₃ absorption difference spectrum, the Raman excitation profile averaged over the enhanced vibrations and the Raman depolarization ratios. The excitation profile is slightly blue shifted and much narrower than the absorption difference spectrum.

The Ala₃ depolarization ratio shows a minimum of $\rho \sim 0.33$ at the excitation profile maximum, indicating that a single nondegenerate electronic transition, presumably the charge transfer transition, dominates the resonance Raman enhancement at 202 nm.^{4, 12, 13, 55} At excitation wavelengths above and below the excitation profile maximum $\rho > 0.33$, indicating contributions of additional electronic transitions.^{4, 12, 13, 55} This excitation profile behavior, where $\rho > 0.33$ for preresonance excitation and also $\rho > 0.33$ values from 201 to 198 nm, between the charge transfer transition and the NV₁ transition, are not possible if the 202 nm transition and the NV₁ are the only transitions involved in enhancement. There is a hint of another transition at 206 nm evidenced by the skewed Raman excitation profile.

Fig. 10 shows the Ala₄ pH absorption difference spectrum, the average resonance Raman excitation profile and the depolarization ratio dispersion measurements. The Ala₄ excitation profile shows a second maximum at 206 nm, $\sim 1000 \text{ cm}^{-1}$ lower in frequency than that of the $\sim 202 \text{ nm}$ charge transfer transition. As observed for Ala₃, a global depolarization minimum occurs with $\rho \sim 0.33$ at the charge transfer excitation profile maximum at 202 nm. Also, as for Ala₃, $\rho > 0.33$ from 201 to 198 nm, between the charge transfer transition and the NV₁ transition at $\sim 190 \text{ nm}$. In contrast to Ala₃, a local depolarization ratio maximum occurs at the 206 nm second excitation profile maximum ($\rho \sim 0.5$). $\rho > 0.33$ in preresonance with all of these transitions for the carboxylate stretch and the AmII and III bands (although less so than for Ala₃).

We do not, as yet, have a simple explanation for these results. One possibility would require two charge transfer transitions close in energy that give rise to the two excitation profile peaks in Ala₄ and the very weak 206 nm shoulder in Ala₃. These charge transfer transitions would involve two different sets of carboxylate non-bonding electrons undergoing a transition to the adjacent peptide bond π^* orbital. The weakness of the 206 nm excitation profile maximum in Ala₃ could result from destructive interference between the contributions from these two charge transfer transitions and the NV₁ transition. Although, the observed 206 nm Raman intensities could be dramatically attenuated by the destructive interference, the 206 nm transition could still significantly increase ρ in the wings of the excitation profile maxima.

The dispersion of ρ can be understood if the contribution to the polarizability of the NV_1 transition and the 202 nm charge transfer transition were of the same sign and the 206 nm transition contribution were of opposite sign. This would yield the dispersion observed with $\rho > 0.33$ between 201 to 198 nm, if the NV_1 transition and the 202 nm charge transfer transition dominated in this spectral region and the NV_1 transition and the 202 nm charge transfer transition contributed similarly to that of the 206 nm transition.

The alternative explanation would assign the 206 and 202 nm transitions to the 0-0 and 0-1 vibronic components of the charge transfer transition. The $\sim 1000\text{ cm}^{-1}$ difference in the peak maxima would indicate a decreased excited state carboxylate frequency. A detailed theoretical calculation would be required to understand the dispersion of the depolarization ratios around the 206 nm transition.⁵⁶

Another possible origin of the 206 nm band is that second conformation of the Ala_4 peptide occurs in solution, which has its charge transfer transition shifted to 206 nm. To search for evidence for this additional conformation, we examined the $AmIII$ region, which is sensitive to the peptide bond conformations⁷.

Deconvolution of the $AmIII$ Region

We deconvoluted the $AmIII_3$ region in both Ala_3 and Ala_4 (Fig. 11) and found that the $AmIII_3$ region could be well fit with three Gaussian bands, at $\sim 1226\text{ cm}^{-1}$, 1249 cm^{-1} , and 1268 cm^{-1} . We utilized the methodology of Mikhonin et al.⁷ which correlates Raman $AmIII_3$ frequency to Ψ angle, specifically using their equation 6A which is applicable to peptide bonds fully exposed to water, such as in poly-proline II (PPII) helix, 2.5_1 helix, extended β -strand conformations, and certain turn structures. The deconvoluted peak positions correlate to the following Ψ angles: $\sim +120^\circ$, $\sim +150^\circ$, $\sim +170^\circ$, which most likely derive from Type II turns, PPII helix-like, and 2.5_1 helix-like conformations, respectively. The Ψ angle of 120° is also found for Type VIa and Type VIII turns, however these are less likely structures to occur. Also, note that the Raman frequencies correspond only to Ψ angle values, with no information about the Φ angle. Thus, there are structures with Type II turn Ψ angles, but it is possible that a significant Φ angle variation could be present. Comparing Fig. 11a and 11b, assuming identical Raman cross sections for these species, we find that the relative contribution of the 2.5_1 helix-like structure in Ala_4 is less than in Ala_3 , and conversely, the PPII-helix-like contribution is larger in Ala_4 than in Ala_3 .

Assuming identical Raman cross sections per peptide bond for each conformation, we can roughly estimate the population distributions from the $AmIII_3$ bands of both peptides. We find that the relative population distributions of both peptides are independent of wavelength. For Ala_3 , the Type II turn has the lowest relative contribution ($\sim 8\%$), the PPII-like conformation contribution is $\sim 29\%$, and the 2.5_1 helix contribution is $\sim 63\%$.

Although there have been numerous theoretical and experimental studies performed on Ala_3 using a variety of techniques, there is no existing consensus on the conformational distribution for Ala_3 in solution. Most studies agree that Ala_3 occurs in a PPII-like conformation.^{15, 29–36, 38, 41–43} Other possible conformations include β -sheet-like,²⁹ β -strand,^{15, 30} β structures,^{32, 33, 35, 36, 38, 43} right-handed α -helix,^{15, 33, 35, 36, 38, 43} and γ -turn.³⁸ No previous study identified the 2.5_1 helix conformation, although there is a suggestion of an extended β -strand-like conformation with a similar Ψ angle ($\sim 170^\circ$).^{15, 37}

For Ala_4 , the conformations found are similar to those in Ala_3 , although the distribution is different. There is a larger contribution from the PPII-helix-like structure (39%). The type II turn contribution is similar (8.5%), while the 2.5_1 helix contribution is less than that in Ala_3 (52%). Garcia⁵⁷ determined that four consecutive amino acid residues are required to form a

water stabilized PPII structure, lending support to the result that Ala₄ should show a larger contribution from PPII-like structures than Ala₃. Again, there is little consensus on the conformational distribution in Ala₄, although there is agreement that the peptide adopts both PPII-like and β -like structure,^{39, 40} with a small amount of a right-handed α -helix-like conformation.⁴⁰ We assign the 202 nm excitation maximum as the charge transfer transition for all three conformations of Ala₄. None of these conformations should obviously give rise to a shifted 206 nm charge transfer band.

Ala₄ and Ala₃ Difference Spectra

To differentiate the conformational distribution of the internal versus terminal peptide bonds of Ala₄ we examined the Ala₄ – Ala₃ difference spectrum (Fig. 12a), which models the internal Ala₄ peptide bond UVRR by removing the contribution of the terminal carboxylate and amine groups. We note that the COO[−] symmetric stretching vibration is absent in the difference spectra. The contributions of the carboxyl end group of both peptides were removed by this subtraction. The relative intensities and frequencies of the bands within each spectrum are similar across the range of excitation wavelengths, once again indicating similar enhancement of the bands.

We calculated the Raman cross sections of the AmIII₃ bands (Fig. 12b). We find that the internal residue of Ala₄ is predominantly PPII-like (~72%), with smaller contributions from 2.5₁ helix (19%) and Type II turn structures (~9%). A previous MD simulation study on the middle residue of Ala₃ found that it was ~85% PPII-like,³¹ which closely agrees with our findings here. We can also infer that the 2.5₁ helix-like conformation is largely found at the peptide ends, which could be representative of a less compact structure at the peptide terminal ends.

An interesting feature in the excitation profiles of Ala₄ – Ala₃ is that the 202 nm and 206 nm excitation profile maxima are present for both the PPII-like and 2.5₁ helix conformation profiles, but less evident in the Type II turn profiles. Thus, as expected, we see less contribution from the charge transfer enhancement in the estimated excitation profile of the internal peptide bond. This residual enhancement may result because our assumption that the difference spectrum results completely from the Ala₄ internal peptide bond is not completely accurate.

Conclusions

We measured the Raman excitation profiles and depolarization ratios for both Ala₃ and Ala₄. Both peptides show excitation profile maxima at 202 nm, which we assign to charge transfer transitions of the carboxylate terminated peptide bonds clearly observed in the pH absorption difference spectra. Ala₄ shows an additional smaller excitation profile maximum at 206 nm whose origin remains unclear.

We correlated the AmIII₃ band Raman frequencies to the Ψ angle of both Ala₃ and Ala₄ to determine the conformational distribution of these peptides. We found Type II turns, as well as PPII-like and 2.5₁ helix-like conformations in solution. The Ala₄ – Ala₃ Raman difference spectra, allowed us to separately study the interior peptide bond of Ala₄, which is found to be predominantly PPII-like.

We assign the charge transfer transition at 202 nm to the three β -type structures deconvoluted from the Raman spectra. We are also able to resolve the COO[−] vibration for both Ala₃ and Ala₄. We find a shoulder in the Ala₄ excitation profile at 206 nm, but are unable at this time to definitively assign its origin.

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Figure 1.

UV absorption spectra at pH 7 (black), pH 2 (red), and the pH absorption difference spectra (blue) for (a) Ala₂, (b) Ala₃, and (c) Ala₄. All absorption measurements were taken of 0.5 mM peptide solutions at 25 °C.

Figure 2. Deconvolution of absorption spectra. The pH7-pH2 difference spectra (black) are fit with two Gaussian bands (blue), the resulting fit curve (red) matches the experimental curve remarkably well. (a) Ala₂, (b) Ala₃, (c) Ala₄.

Figure 3. Ala₃ zwitterion in an extended β conformation. The electron density charge transfer from carboxylate to amide is indicated by the blue arrow. (Picture courtesy of Nataliya Myshakina)

Figure 4.

UVRR spectra of Ala_3 ($6.6 \times 10^{-3} \text{ M}$) at 25°C excited between 198 and 210 nm. Spectra were collected for 15 min each. The spectral resolution is $\sim 5 \text{ cm}^{-1}$. Spectra were not corrected for self-absorption or spectrometer throughput efficiency.

Figure 5. UVRR spectra of Ala_4 (6.6×10^{-3} M) at 25°C excited between 198 and 210 nm. Spectra were collected for 15 min each. The spectral resolution is $\sim 5\text{ cm}^{-1}$. Spectra were not corrected for self-absorption or spectrometer throughput efficiency.

Figure 6.

Resolving the carboxylate stretching vibration in 200 nm excited (a) Ala₃: measured spectrum (red) and Ala₃ - (Ala₄ - Ala₃) difference spectra (blue) at showing COO⁻ vibration enhanced in COO⁻ end peptide bond. (b) Ala₄: measured spectrum (red) and Ala₄ - 2*(Ala₄ - Ala₃) difference spectra (blue) showing COO⁻ vibration enhanced in COO⁻ end peptide bond.

Figure 7.

Absolute Raman cross section excitation profiles of Ala₃ (mbarn/sr) between 198 and 210 nm. See text for details. Also shown is the squared pH absorption difference spectrum from Fig. 2. The isolated COO⁻-profile was calculated using the Ala₃ – (Ala₄ – Ala₃) difference spectra shown in Fig. 6.

Figure 8.

Absolute Raman cross section excitation profiles of Ala_4 (mbarn/sr) between 198 and 210 nm. See text for details. Also shown is the squared pH absorption difference spectrum from Fig. 2. The isolated COO^- profile was calculated using the $\text{Ala}_4 - 2 \cdot (\text{Ala}_4 - \text{Ala}_3)$ difference spectra shown in Fig. 6.

Figure 9.

(a) The average Raman excitation profile for the Ala₃ amide bands at 25 °C between 198 to 210 nm and the pH 7-pH 2 absorption difference spectrum of Ala₃ at 25 °C between 190 to 250 nm. (b) Raman depolarization ratios of Ala₃ at 25 °C. The dashed line indicates the coincidence between the excitation maximum of the excitation profile, the pH absorption difference spectrum, and the depolarization ratio minimum with $\rho \approx 0.33$ at 202 nm.

Figure 10.

(a) The averaged Raman excitation profile for Ala₄ at 25 °C between 198 to 210 nm and the pH7 – pH2 absorption difference spectrum of Ala₄ at 25 °C from 190 to 250 nm. (b) Raman depolarization ratios of Ala₄ at 25 °C. The dashed line indicates the coincidence between the maximum of the excitation profile, the pH absorption difference spectrum, and the depolarization ratio minimum with $\rho \approx 0.33$ at 202 nm.

Figure 11.

(a) Deconvolution of Ala₃ AmIII₃ region excited at 204 nm with three Gaussian bands. (b) Deconvolution of Ala₄ AmIII₃ region excited at 204 nm, also with three Gaussian bands. Different conformational distributions are present. There is excellent agreement between the modeled peaks and the observed data ($R^2 > 99\%$).

Figure 12.

(a) Ala₄ – Ala₃ difference spectra, from 198 to 210 nm. (b) The AmIII₃ band Raman cross sections of the three conformations (Type II turn, PPII-helix-like, 2.5₁ helix) in the Ala₄ – Ala₃ difference spectra.