

UV Resonance Raman Investigation of a 3₁₀-Helical Peptide Reveals a Rough Energy Landscape[†]

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ABSTRACT: We used UVRRS at 194 and 204 nm excitation to examine the backbone conformation of a 13-residue polypeptide (gp41_{659–671}) that has been shown by NMR to predominantly fold into a 3₁₀-helix. Examination of the conformation sensitive AmIII₃ region indicates the peptide has significant populations of β -turn, PPII, 3₁₀-helix, and π -helix-like conformations but little α -helix. We estimate that at 1 °C on average six of the 12 peptide bonds are in folded conformations (predominantly 3₁₀- and π -helix), while the other six are in unfolded (β -turn/PPII) conformations. The folded and unfolded populations do not change significantly as the temperature is increased from 1 to 60 °C, suggesting a unique energy landscape where the folded and unfolded conformations are essentially degenerate in energy and exhibit identical temperature dependences.

An understanding of the mechanisms of protein folding will enable the de novo design of proteins with profound commercial and medical applications (1–19). Over the past 50 years, significant effort to elucidate protein folding and unfolding mechanisms has been expended. Part of this effort has examined the thermodynamics and kinetics of small model systems such as β -hairpins (20–22) and alanine-based α -helices (23–33). These studies have provided detailed microscopic knowledge of the folding energy landscape of these isolated motifs. Theoretical simulations have suggested that 3₁₀-helices and/or type III β -turns may serve as intermediates in the α -helix folding pathway (34–39). The formation of kinetic intermediates such as β -turn or 3₁₀-helix lowers the entropic cost of nucleating the first helical residue, thus facilitating the helix folding transition (34, 40, 41).

The 3₁₀-helix is the fourth most common secondary structural motif in proteins (34–59). On average, in proteins, 3–4% of peptide residues occur in a 3₁₀-helix conformation (48). The 3₁₀-helix differs from an α -helix in that the tighter packing of the backbone forces the C=O \cdots H–N hydrogen bonds to point outward, away from the helical axis, resulting in decreased stability of the 3₁₀-helix compared to that of the α -helix (25, 48).

Recent ESR (39, 42) and NMR (43) work by Millhauser et al. (41) suggests the presence of 3₁₀-helix at the terminus of short alanine-based helical peptides. The authors proposed that 3₁₀-helices are a relic of the folding process. They reasoned that nascent helices contain mixtures of unfolded and turn conformations, specifically type III turns. As folding conditions begin to favor helical structures, the type III turn conformation (a single 3₁₀-helix unit) propagates; i.e., the chain adopts a 3₁₀-helix conformation. As the helical domain lengthens, the α -helix conformation competes with the 3₁₀-

helix conformation. Finally, at longer helical lengths, the α -helix conformation dominates. Some 3₁₀-helix/type III turn-like conformations may survive at the termini (41).

It should be noted that Millhauser's results have been disputed by Smythe et al. (44), who argued that the rotational diffusion of the flexible labels used in the ESR study of Milhauser et al. (41) confounded spectral interpretation. Using Toac residues as ESR labels, Smythe et al. found no evidence for a significant 3₁₀-helix population in a 17-residue, alanine-based helical peptide (44).

The inability to characterize simple monomeric 3₁₀-helices is a major stumbling block in advancing our understanding of the α -helix (un)folding process; furthermore, the lack of such a model system has made unambiguous identification of 3₁₀-helix spectroscopic markers difficult. Schievano et al. (45) recently reported the first water soluble 3₁₀-helix; however, this particular peptide consisted of unnatural amino acids. Unfortunately, the conformation dynamics of peptides with unnatural amino acids may be irrelevant for the study of biological problems. In this context, the recent discovery of Biron et al. of an all-natural amino acid tridecapeptide, gp41_{659–671} (48), that folds into a 3₁₀-helix is greatly significant since it enables the study of 3₁₀-helices.

gp41_{659–671} is a 13-residue peptide with the sequence E₆₅₉-LLELDKWAS₆₆₉LWN extracted from glycoprotein gp41 of HIV-1, corresponding to residues 659–671. NOE constraints indicate that in water at 277 K, the monomeric form of the peptide folds into a nine-residue 3₁₀-helix (48). Biron et al. proposed that the 3₁₀-helix is stabilized by favorable hydrophobic stacking interactions between L663, W666, L669, and W670 (48). Residues E662, K665, S668, and N671 form the polar face where E662 and K665 may engage in weak electrostatic interactions. Burial of considerable hydrophobic surface area is proposed to favor the 3₁₀-helix over the α -helix conformation (48).

In this work, we used UV¹ resonance Raman spectroscopy to examine gp41_{659–671}'s conformation. We studied the

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peptide secondary structure by exciting within the amide $\pi \rightarrow \pi^*$ transition with 204 and 194 nm excitation. Our results indicate that the 3_{10} -helix is in equilibrium with multiple secondary structures, namely, the π -helix, β -turns, and PPII conformation, creating an energy landscape where the folded and unfolded conformations are essentially degenerate in energy and exhibit identical temperature dependences.

EXPERIMENTAL PROCEDURES

The UV resonance Raman spectrometer has been described in detail elsewhere (60). Briefly, 204 nm UV light was generated by generation of the fifth anti-Stokes Raman harmonic of the third harmonic of a Nd:YAG laser (Coherent, Infinity). The 194 nm deep UV light was generated as the $(3\omega + \omega)$ fourth harmonic of a diode-pumped Ti:sapphire laser (Positive Light, Indigo-S). The sample was circulated in a free surface, temperature-controlled stream. A 135° backscattering geometry was used for sampling. The collected light was dispersed by a subtractive double monochromator onto a back-thinned CCD camera (Princeton Instruments, Spec 10).

The gp41_{659–671} peptide was obtained from the Pittsburgh Peptide Synthesis Facility (>95% pure) and used at a concentration of 1 mg/mL at pH 7. UVRR spectra measured at 20 °C during and after the Raman measurements were essentially identical, indicating the lack of significant photo degradation and/or thermal degradation. Raman spectra were normalized relative to the peak height of the 932 cm^{-1} ClO_4^- band. Concentrations of ClO_4^- of 0.05 and 0.1 M were used for the 204 and 194 nm excited UVRR measurements. Helix promoter TFE was acquired from Aldrich. The solution pH was adjusted by adding small aliquots of dilute HCl or NaOH. CD measurements were carried out on a JASCO 710 spectrometer using a peptide concentration of 0.3 mg/mL.

RESULTS AND DISCUSSION

Excitation (204 nm) Temperature Dependence. The 204 nm excitation Raman spectra (Figure 1) show bands arising from amide and to a lesser extent Trp vibrations. To facilitate spectral interpretation, we subtracted the Trp contribution from the temperature-dependent 204 nm excited gp41_{659–671} spectra (Figure 2). The band intensities of the resulting spectra were normalized with respect to the AmI band intensity. The spectra show an AmI vibration at 1668 cm^{-1} which is predominantly a C=O stretching vibration (61, 62). The 1556 cm^{-1} molecular oxygen stretching band (32, 63) overlaps with the 1558 cm^{-1} AmII band. The AmII vibration involves C–N stretching with some N–H bending (61, 62). The trough in the $1420\text{--}1500\text{ cm}^{-1}$ region is due to the 1483 cm^{-1} COO^- stretching band from the Trp monomer which was subtracted from the original peptide spectra (64, 65).

A weak 1355 cm^{-1} band (AmIII₁/residual W7) appears as a shoulder on the 1390 cm^{-1} $\text{C}_\alpha\text{--H}$ bending band. The $\text{C}_\alpha\text{--H}$ bending band intensity is derived from non- α -helical peptide bond conformations (61, 66). A weak AmIII₂ band is located at $\sim 1320\text{ cm}^{-1}$. A broad AmIII₃ band [predominantly C–N stretching and N–H bending mode (61, 66–

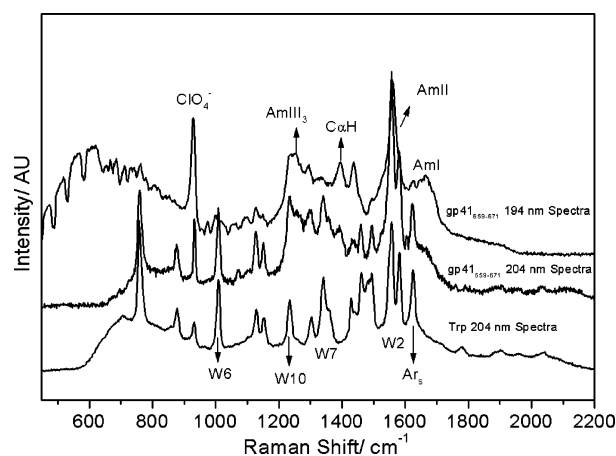


FIGURE 1: UV resonance Raman spectra (204 and 194 nm) of gp41_{659–671} measured at 1 °C. Also displayed are the 204 nm excitation UVRR spectra of the Trp monomer, where the 1483 cm^{-1} carboxylate stretching band overlaps with the W4 and W5 bands of Trp. As compared to the 204 nm spectra, the 194 nm excited gp41_{659–671} spectra show considerably less Trp spectral contribution. The smaller Trp contribution allows us to resolve amide bands from overlapping Trp bands.

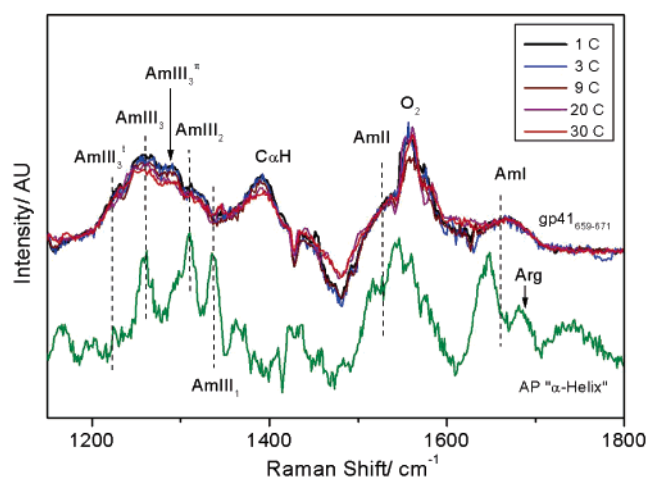


FIGURE 2: Top traces are 204 nm UV resonance Raman spectra of gp41_{659–671} measured between 1 and 30 °C. To facilitate spectral interpretation, the Trp contribution was subtracted. The resulting spectra exhibit resonance-enhanced amide vibrations. Below, the green trace shows the 204 nm excited pure α -helix spectra of AP peptide (adapted from ref 70) which shows its characteristic narrow AmIII₃ band at 1260 cm^{-1} .

69)] at 1257 cm^{-1} shows additional features at 1293 and 1226 cm^{-1} . Mikhonin et al. (70) recently demonstrated that the “pure” α -helix UVRR spectra show a narrow AmIII₃ band centered at 1260 cm^{-1} (Figure 2). The presence of a broad AmIII₃ band here indicates the existence of multiple conformations in gp41_{659–671}.

As the solution temperature is increased from 1 to 30 °C, the 1390 cm^{-1} $\text{C}_\alpha\text{--H}_b$ and AmIII₃ bands show a slight decrease in intensity (Figure 2). A similar temperature-induced intensity decrease was previously observed for a water-exposed, PPII peptide whose conformation was temperature-independent (24, 67). The lack of conformation-induced changes in the AmIII₃ and $\text{C}_\alpha\text{--H}_b$ region suggests that gp41_{659–671} does not undergo a thermally induced helix melting transition between 1 and 30 °C.

We can quantitate the number of helical amide bonds by using the Raman cross section of the $\text{C}_\alpha\text{--H}$ bending band

¹ Abbreviations: UV, ultraviolet; UVRRS, resonance Raman spectroscopy; CD, circular dichroism; Am, amide; NOE, nuclear Overhauser effect; TFE, 2,2,2-trifluoroethanol.

from a 21-residue, alanine-based, mostly α -helical peptide known as AP (24); we are utilizing the assumption that the gp41_{659–671}'s unfolded segment C_{α} –H bending bands have Raman cross sections similar to those of the unfolded/PPII conformation of AP. This is a reasonable estimate since C_{α} –H_b bands have similar cross sections in the PPII and extended β -strand conformations (61); we expect similar coupling between C_{α} –H_b and N–H_b bands for peptide bonds with similar Ψ angles (69). Since the α -helix and the 3_{10} -helix conformations have similar Ψ angles (45), we assume that as in the α -helix, the putative 3_{10} -helix will exhibit a negligible C_{α} –H band intensity. Thus, we use the C_{α} –H band intensity to estimate the number of bonds in the PPII conformation. On the basis of the C_{α} –H band cross sections of the alanine-based, mainly α -helical peptide AP (see ref 19), we calculate the number of nonhelical amide bonds in gp41_{659–671} as

$$n_A = (I_{C_{\alpha}H}N_{ClO_4^-} - \sigma_{ClO_4^-})/(\sigma_{C_{\alpha}H}N_P I_{ClO_4^-}) \quad (1)$$

where $\sigma_{C_{\alpha}H}$ and $\sigma_{ClO_4^-}$ are the Raman cross sections of the C_{α} –H band and the 932 cm^{-1} perchlorate band, respectively. $N_{ClO_4^-}$ and N_P are the numbers of perchlorate and gp41_{659–671} molecules in the scattering volume, respectively. $I_{ClO_4^-}$ is the perchlorate band (932 cm^{-1}) intensity, while $I_{C_{\alpha}H}$ is the intensity of the C_{α} –H band. n_A is the number of amide peptide bonds in gp41_{659–671} which contribute to intensity $I_{C_{\alpha}H}$. We calculate that at 1 °C six of 12 peptide bonds are in a PPII-like conformation while six peptide bonds adopt α -helix-like conformations.

As noted above, gp41_{659–671} does not exhibit a temperature-dependent decrease in helicity. The observed behavior can be rationalized by requiring the folded and unfolded state ensembles to be nearly degenerate in energy and equally disordered; i.e., the number of conformations comprising the folded and unfolded state are nearly equal. Under such circumstances, the peptide has an equal probability of populating either a helix or unfolded state, and as such, their relative population distribution would not be impacted by an increase in temperature. These results are consistent with the study of Biron et al., who reported a lack of 3_{10} -helix melting with an increase in temperature (48).

The gp41_{659–671} peptide, however, does exhibit a conformation change in TFE/water solutions (Figure 3). Manning and Woody (71) proposed that the CD spectra of 3_{10} - and α -helix should show different values of R ($\Theta_{222}/\Theta_{207}$). The 3_{10} -helix is expected to exhibit a lower R value (~ 0.3) than the α -helix (~ 1). Additionally, the 3_{10} -helix is expected to exhibit a weak positive band at ~ 195 nm, while the α -helix shows a relatively strong positive band at ~ 195 nm (59, 71). As shown in Figure 3 (inset), the CD spectra show an R value of ~ 0.3 in water and water/TFE solutions indicating the presence of 3_{10} -helices. In water, we observe a negative maximum at 200 nm without a trough at 222 nm. This suggests a significant population of unfolded conformations; UVRR indicates only 50% helicity in water. Our CD spectra of gp41_{659–671} differ from those of Biron et al. in that their peptide spectra in pure water exhibit greater 3_{10} -helix content. We do not know the reason for differences in the two measurements. Addition of 10% TFE does not impact the position of the 200 nm negative band, but 30% TFE red shifts the band maxima to 205 nm, indicating increased 3_{10} -helix

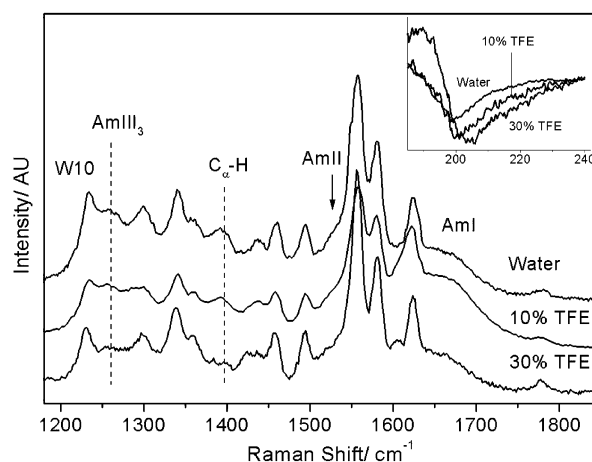


FIGURE 3: UV resonance Raman spectra (204 nm) of gp41_{659–671} in pure water or 10 or 30% (v/v) TFE at 1 °C. The TFE contribution has been subtracted from the spectra. The resulting spectra show bands arising from amide and Trp vibrations. In 10% TFE, the presence of a C_{α} –H band indicates $\sim 55\%$ helicity, whereas in 30% TFE, the C_{α} –H band shows negligible band intensity, indicating a 92% helical content. The inset shows CD spectra of gp41_{659–671} in water and 10 and 30% (v/v) TFE solutions. As discussed in the text, these CD spectra show the peptide's 3_{10} -helix content increases with TFE concentration.

content. These results are confirmed by our UVRR measurements where the peptide spectra in 10% TFE exhibit $\sim 55\%$ helicity, whereas in 30% TFE, the peptide exhibits negligible C_{α} –H band intensity, indicating $\sim 92\%$ helicity (Figure 3). The TFE-stabilized helix does not exhibit any appreciable melting with an increase in temperature (not shown).

It should be noted that our UVRR results do not necessarily suggest that a single, continuous 3_{10} -helix of six peptide bonds exists in water. Recently, Mikhonin and Asher (72) demonstrated that the resonance-enhanced AmIII and C_{α} –H bands scatter independently; i.e., there is negligible coupling between adjacent peptide bonds. Hence, the UVRR spectra in the AmIII and C_{α} –H region can be regarded as a linear sum of individual peptide bonds. We therefore envision a scenario in which, in water, the peptide is flickering between folded and unfolded conformations; the observed C_{α} –H band intensity is, therefore, a measure of the peptide's residence time in extended PPII-like conformations. Hence, the observed 50% helical content could indicate that during the measurement period a peptide bond has an equal probability of being in the folded or unfolded/extended conformation regions of the Ramachandran space.

CONFORMATION ANALYSIS

The conformational space sampled by the gp41_{659–671} peptide in water can be quantitatively analyzed by an examination of the AmIII₃ region. The subtraction of the Trp contribution, however, results in a noisy spectrum which complicates assignment of the AmIII₃ bands. Previously, Fodor et al. (73) reported that Trp cross sections weaken at shorter excitation wavelengths. Thus, by exciting the samples at 194 nm, we are able to selectively enhance the amide vibrations relative to Trp vibrations and acquire spectra dominated by amide vibrations.

We deconvoluted the measured 194 nm excitation spectrum into a sum of a minimum number of Voigt bands by using the peak fitting routine in Grams version 5 (Galactic

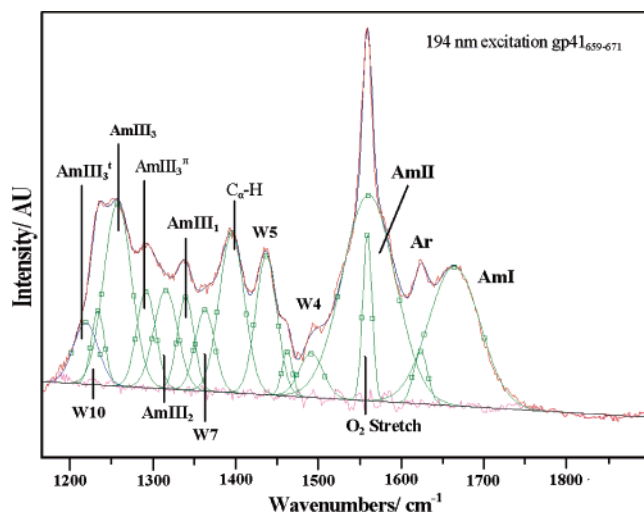


FIGURE 4: Spectral deconvolution of 1 °C, 194 nm UVRR gp41_{659–671} spectra with Voigt bands. The excellence of the fit is evident from the flat residual displayed underneath.

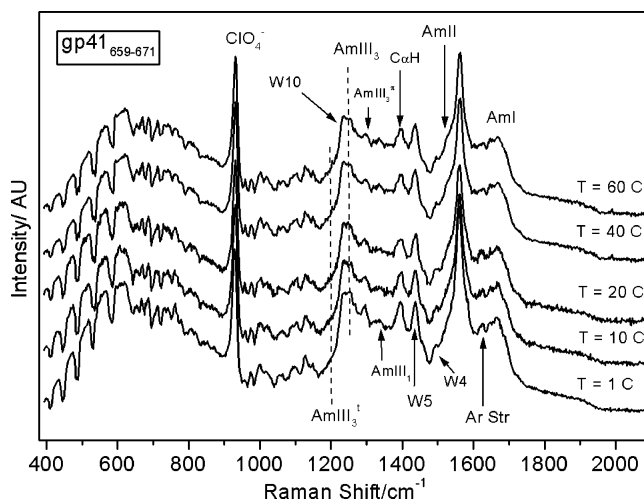


FIGURE 5: UV resonance Raman spectra (194 nm) of gp41_{659–671} measured between 1 and 60 °C. Three 10 min spectra were summed and scaled to the intensity of the 932 cm^{−1} perchlorate band. The bands arise mainly from amide vibrations except for those that derive from Trp ring vibrations.

Industries Corp., Figure 4). The 194 nm excitation UVRR spectra show an AmI band at 1664 cm^{−1} with a weak aromatic stretching band at 1665 cm^{−1}. The decreased aromatic stretching band intensity allows us to resolve the AmI band. An increased contribution from diatomic oxygen interferes with the AmII band located at ~1556 cm^{−1}. The W4 and W5 Trp bands are located at 1491 and 1437 cm^{−1}. An intense C α -H bending band located at 1394 cm^{−1} shows an only negligible temperature-induced decrease in intensity (Figure 5). A small AmIII₁ band at 1340 cm^{−1} overlaps the W7 band of Trp, while a weak AmIII₂ band is observed at ~1300 cm^{−1}. As with 204 nm excitation, we observe AmIII₃ bands at 1293, 1254, and 1224 cm^{−1}. As the temperature is increased from 1 to 60 °C, the AmII band intensity decreases while the band position downshifts from 1560 to 1557 cm^{−1} (Figure 5). The AmIII₁, AmIII₂, and 1293 cm^{−1} AmIII₃ band position remain more or less invariant with an increase in temperature. The broad 1254 cm^{−1} AmIII₃ band downshifts, while the 1224 cm^{−1} AmIII₃ band exhibits an increased band intensity with an increase in temperature.

Recently, Mikhonin et al. (74) quantitatively demonstrated that the AmIII₃ band frequency depends on the ψ dihedral angle and the hydrogen bonding state of amide bond ψ (67, 74). Using their methodology for correlating the ψ dihedral angle with the AmIII₃ band position, we determine that the 1224 cm^{−1} band could originate from either the distorted β -sheet ($\psi = 40^\circ$) or β -turn-like ($\psi = -3^\circ$) conformations.

In the 277–302 K temperature interval, Biron et al. (48) using analytical ultracentrifugation did not observe any significant aggregation or dimerization products of gp41_{659–671}. We therefore conclude that the 1224 cm^{−1} band originates from β -turn conformations and assign the 1224 cm^{−1} band as the β -turn amide III₃ (AmIII₃^t). The ψ dihedral angle of -3° indicated by the AmIII₃^t band position suggests the presence of type I turns.

The 1293 cm^{−1} AmIII₃ band does not exhibit any significant temperature-dependent shift in band position, indicating a conformation dominated by intrapeptide hydrogen bonds. According to the data of Mikhonin et al. (74), the band position likely correlates to a ψ of -75° , suggesting the presence of a π -helix-like conformation. Previous studies of peptides with significant 3_{10} -helix populations found appreciable π -helix populations in equilibrium with the native helix (38, 46). MD simulations suggest that the π -helix may serve as an intermediate in the α -helix folding pathway (38). Alternatively, Cartailier and Luecke suggest π -helices exist as defects in α -helices where the presence of a π -helix-like 1 \rightarrow 5 hydrogen bond (π -bulge) introduces a kink into the α -helix (75).

The broad 1254 cm^{−1} AmIII₃ band is composed of overlapping helical and PPII conformations. The 1254 cm^{−1} band maximum indicates a dominant 3_{10} -helix conformation, though it should be noted that the type III turn conformation (a single 3_{10} -helix unit) is expected to contribute in this frequency region as it populates similar dihedral angles (41). Typical α -helical peptides show an AmIII₃ band maximum at 1263 cm^{−1}. The 9 cm^{−1} shift in the AmIII₃ band position of gp41_{659–671} as compared to typical α -helical peptides such as the AP peptide (24) indicates the average ψ angle of gp41_{659–671} deviates from the typical α -helix conformation by 10°. This result demonstrates that UVRR can discriminate between conformationally similar α - and 3_{10} -helices.

Recent MD simulations have suggested that distorted α -helices exist in a rapid equilibrium with the 3_{10} -helix conformations (76). While we do not find any definitive evidence of significant concentration of α -helix conformation, the broad width of the AmIII₃ band suggests that a small α -helix population may exist (see Figure 2).

CONCLUSIONS

We examined the secondary structure of gp41_{659–671} by exciting the backbone at 204 and 194 nm. Our results indicate that six of the 12 peptide bonds adopt helical conformations (predominantly 3_{10} - or π -helix). An examination of the conformation sensitive AmIII₃ region indicates gp41_{659–671} exhibits a broad distribution of conformations that includes PPII, β -turn, 3_{10} -helix, and π -helix conformations but little α -helix; furthermore, we demonstrate that the AmIII₃ band position can be used to distinguish a 3_{10} -helix from an α -helix conformation. A lack of temperature-induced change in the AmIII₃ band intensities indicates an energy landscape where

the helical and unfolded conformations occupy degenerate energy levels with similar entropies. This allows for nearly equal populations of both the helical and unfolded conformations, as evidenced by the C α -H band intensity which indicates a 50% helical content, independent of temperature.

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