UVRR Spectroscopy of the Peptide Bond. 1. Amide S, a Nonhelical Structure Marker, Is a $C_\alpha H$ Bending Mode

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Abstract: Raman spectra with deep UV excitation are reported for *N*-methylacetamide. In addition to the well-known amide I, II, and III bands, an enhanced band at 1385 cm^{-1}, labeled amide S, is identified as a C-H bending mode of the acetyl group via its disappearance upon $C-CD_3$ substitution. At the same time, the nearby amide III shifts up from 1316 to 1347 cm^{-1}, showing it to be coupled with amide S and thereby accounting for the enhancement of the latter. This assignment is, in addition, supported by the UV resonance Raman spectra of a series of *N*-methylacetamide derivatives that have different numbers of C_α-H's. The ca. 1395-cm^{-1} UVRR band of peptides is likewise shown to be a C_α-H bending mode via C_α deuteration of the oligopeptides triglycine and *N*-acetyltrialanine methyl ester. This band disappears when polylysine is converted to the α-helical form at high pH, and likewise when polyglutamate is converted to the α-helical form at low pH. Its disappearance is proposed to result from uncoupling of the amide S and amide III modes when the C_α-H bond is rotated into an orientation which is cis to the nearby peptide carbonyl group in α-helices, from the trans orientation found in β-sheet or loop structures. The disappearance of amide S upon NH/D exchange in D_2O is likewise attributable to uncoupling, due to the large shift in amide III. These amide uncoupling effects as well as the uncoupling of amide II from amide III and amide S in *cis*-amides are mainly due to kinematic changes in the various conformers, as revealed by normal mode analysis of NMA. Quantitative analysis of UVRR spectra for a range of proteins shows the amide S intensity to be linearly related to the nonhelical content and to extrapolate to zero for 100% α-helix, consistent with its behavior in polylysine and polyglutamate. Intensity values are given for amide S and for amide II, which can be used to determine helix content in polypeptides and proteins.

Introduction

Ultraviolet resonance Raman (UVRR) spectroscopy has recently emerged as a promising structure probe for proteins.[1-14] The availability of laser excitation sources with wavelengths deep in the ultraviolet has made it possible to enhance selectively the vibrational modes of aromatic side chains and of the amide bonds. Especially strong enhancement is seen for the amide II vibration,[15,16] and its intensity has been found to depend strongly on the α-helical content,[11,12] an effect attributable to the hypochromic alignment of the amide electronic transition dipoles in the α-helix. Amide II cross sections have been measured at different wavelengths,[12,17] which provide a basis for secondary structure determination via UVRR spectroscopy.

Another UVRR band, at 1395 cm^{-1}, has recently been noted to be sensitive to structure.[17-20] Because only three modes above 1200 cm^{-1} have been thought to involve the amide group directly, amide I, II, and III (consisting primarily of C=O stretching and coupled C-N stretching and N-H bending), the additional 1395-cm^{-1} band was judged not to be a fundamental mode. It was assigned by Krimm and Asher and co-workers[18,19] to the overtone of amide V, composed mainly of N-H out-of-plane bending. Since the amide V coordinate produces a twist about the C-N bond, enhancement of its overtone would be a reasonable consequence of a twisted excited state, by analogy with ethylene, for which enhancement of the twisting mode overtone has been demonstrated in resonance with its first allowed excited state.[21,22] In this study we show, however, that the 1395-cm^{-1} band, called amide S[20] (for secondary structure-sensitive), actually arises from bending of the C-H bond associated with the C_α atom, since it is specifically eliminated by C_α-D substitution. Ordinarily, C-H bending modes are not resonance enhanced unless they are coupled to a displacement mode of the chromophore. The amide S mode is enhanced via vibrational coupling with the nearby amide III mode, as evidenced by a significant upshift in the frequency of the latter upon C_α-D substitution. Uncoupling also explains the disappearance of amide S upon amide N-H/D exchange, when the amide III mode is shifted out of the region. *N*-methylacetamide (NMA), a much studied model of the peptide bond, also shows a 1385-cm^{-1} UVRR band which is assignable to a C-H bending mode of the carbon-bound methyl group, since it disappears on CD_3 substitution, while the nearby amide III band again shifts up.

Krimm and Asher suggested that the 1395-cm^{-1} band shifts to ca. 1335 cm^{-1} for α-helical peptides.[18] We present evidence, however, that the amide S band disappears in α-helices. Its intensity extrapolates smoothly to zero when plotted against α-helical content for synthetic polypeptides or native proteins, and there is no compensating intensity increase for any other band. Part of the intensity loss is attributable to hypochromism of the resonant $\pi-\pi^*$ electronic transition, as documented for the amide II band.[11] But the amide II intensity does not vanish for α-helices,

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and an additional loss mechanism is required to explain the absence of α-helical amide S intensity. This additional mechanism is suggested to be uncoupling of the C_α–H bending motion from the amide III mode, due to an unfavorable geometry. The amide ϕ,ψ angles enforced by the α-helix require that the C_α–H bond be oriented cis to the C=O bond, whereas the orientation is trans in β-sheet and loop structures. Normal mode calculations on NMA conformers confirm that this cis orientation diminishes the kinematic coupling of the C_α–H bend with amide III, and also that in *cis*-amides, amide II is uncoupled from both amide III and amide S, consistent with the *cis*-amide UVRR spectra.[19,20,23] The amide S intensity shows an accurate negative linear dependence on α-helical content and can therefore be used to estimate non-helical content in polypeptides and proteins.

Experimental Section

Materials. *N*-methylacetamide (NMA) and *N*-methylpropionamide (NMP) were purchased from Aldrich. Acetamide (Aldrich) was used to synthesize $(CD_3)NHCOCH_3$ via acetylation of $(CD_3)NH_2$;[24] (CD_3)-$NHCO(CD_3)$ was prepared in the same way, starting with CD_3-acetamide. Likewise, *N*-methylisobutyramide and *N*-methylpivalamide were prepared by acyl transfer to CH_3NH_2 from isobutyramide and pivalamide (Aldrich). These products were purified by distillation. Samples of peptides, polypeptides, and proteins (see text) were purchased from Sigma. Bovine somatotropin (BST) was a gift of Doctors Gerald Stockton and Patrick Mowery, American Cyanamid Company.

H/D exchange at the peptide C_α atom was carried out by adjusting a concentrated D_2O solution to pD 11 and incubating at 50 °C for 24–48 h. The pD was then readjusted to neutrality, and the samples were diluted (30–40×) rapidly in aqueous buffer, allowing the peptide N–D to reexchange for H. The exchange was monitored for *N*-acetyltrialanine methyl ester via the intensity of the C_α–H ^{1}H NMR signal, which decreased by 78% in 24 h.

Spectroscopy. The UVRR spectrometer has been described elsewhere.[25] Excitation was provided by a 10-Hz H_2-Raman-shifted Nd:YAG laser, operating at 200 or 192 nm. It was focused onto the front surface of a flowing flat stream of the sample.[25] For protein spectra (Figure 12), a 300-Hz excimer/dye laser[26,27] (Lambda Physik LPX130/FL3002) was used. Excitation at 200 nm was obtained by mixing the fundamental (600 nm) and the second overtone (300 nm) of the rhodamine 6B (Exciton) dye laser output, in a β-BaB_2O_4 (BBO) crystal. We found it convenient and efficient to generate the sum frequency by placing a polarization rotating plate after the second harmonic generation crystal, in order to rotate the polarization of the 300-nm output but not the 600-nm fundamental. This procedure improves efficiency but limits tunability (2–3 nm). The scattered photons were dispersed with a SPEX 1269 single monochromator, equipped with UV-enhanced optics and a 2400 grooves/mm holographic grating. The spectrum was recorded in spectrograph mode with an intensified diode array detector (Princeton Instruments) or in scanning mode with a solar blind photomultiplier (Hamamatsu) and electronic integrator (Stanford Research). The diode array response function was recorded with a diffuse white light source and used to correct the spectra. A polarization scrambler was used to correct the intensity variation due to polarization.

The spectral data were stored on a computer disk and processed with Lab Calc software.[28] Intensities were determined via the deconvolution routine, which iteratively fit the data to within 0.1%. A minimal set of Lorentzian bands was used in the fitting.

Results and Discussion

A. The 1385-cm^{-1} UVRR Band of NMA Is a Fundamental Mode Due to Acetyl C–H Bending. Figure 1 compares 200-nm-excited RR spectra of NMA and its N–CD_3 and C–CD_3 isotopomers, and of NMA in D_2O, in which the N–H proton is exchanged for a deuteron. The NMA bands above 1200 cm^{-1} (spectrum a) are assigned to the amide I, II, III, and S modes. Amide I (the shoulder at about 1645 cm^{-1}; this band shifts up and becomes stronger in nonaqueous solvents[22,23]) is mainly carbonyl C=O stretching, while amide II at 1577 cm^{-1} and III at 1316 cm^{-1} are coupled C–N stretching and N–H bending.[29] The N–H/D substitution in D_2O (spectrum d) eliminates the coupling by shifting the N–H bending frequency out of the region, leaving an intense band, amide II′, at an intermediate frequency, 1517 cm^{-1}, which is essentially pure C–N stretching in character. This band has a doublet appearance, which is apparently connected to some involvement of the N–CH_3 group, since a single band is seen for the N–CD_3 isotopomer in D_2O[20] (spectrum e). The N–H/D exchange also eliminates the amide S band; this behavior was the basis for our earlier suggestion[20] that amide S might be the overtone of amide V, although the 100-cm^{-1} deficit from the expected overtone frequency[18] made the assignment problematic. It is ruled out altogether by the disappearance of amide S when the C–CH_3 protons are replaced by deuterons (spectrum c), a substitution which should not influence the amide V overtone. (This observation has also been made by Song et al.[19]) Amide S is assigned instead to a C–H bending mode; its frequency, 1385 cm^{-1}, is characteristic for the umbrella mode of methyl groups attached to C atoms, and this assignment has previously been made from nonresonance Raman spectra.[30] On the other hand, deuteration of the N–CH_3 group alone (spectrum b) has no effect beyond a slight shift in amide II.

Ordinarily, modes of methyl groups are not subject to resonance enhancement, even in the UV region, and indeed the N–CH_3 umbrella mode, assigned at 1414 cm^{-1} in nonresonance Raman spectra,[30] is not seen in the UVRR spectrum. It is not impossible that a methyl C–H bending mode could be displaced in the excited state of an adjacent chromophore, thereby producing RR intensity. Enhancement of ethyl modes has been observed in nickel octaethylporphyrin RR spectra excited in resonance with the porphyrin π–π^* transitions and was attributed to excited-state displacement due to hyperconjugation.[31] If hyperconjugation were an effective mechanism in NMA, however, it might be expected to apply equally to the N-bound methyl, which is also adjacent to the C–N bond. An alternative mechanism is intensity borrowing from another enhanced mode. This mechanism *does* apply to amide S, since the amide III band shifts up significantly when all three H atoms of the C–CH_3 group are replaced by D (spectrum c), indicating that amide III must be mixed with a C–H bending coordinate. Vibrational coupling can also explain the disappearance of amide S upon N–H/D replacement, since amide III, upon which it depends for intensity, is shifted out of the region. We infer that the intensities of amide II, III, and S are all dependent primarily on the C–N displacement in the excited state, amide II and III directly through mixing of the C–N stretching and N–H bending coordinates, and amide S indirectly through its subsidiary coupling with amide III. Consistent with this view is the very large intensity of the amide II′ band, in the N–D isotopomer, which is greater than the amide II and III intensities combined. Moreover, all three band intensities change in parallel when the excitation wavelength or the solvent polarity is varied, as demonstrated in the following paper.[23]

We note that a similar inference of amide III mixing with C–H bending coordinates has been made by Diem and co-workers[32a,b] on the basis of deuteration effects on the alanylalanine nonresonance Raman spectrum.

Further evidence concerning the C–H bending character of amide S is provided by the UVRR bands (Figure 2) of the NMA derivatives *N*-methylpropionamide, *N*-methylisobutyramide, and *N*-methylpivalamide. As the C–CH_3 H atoms of NMA are

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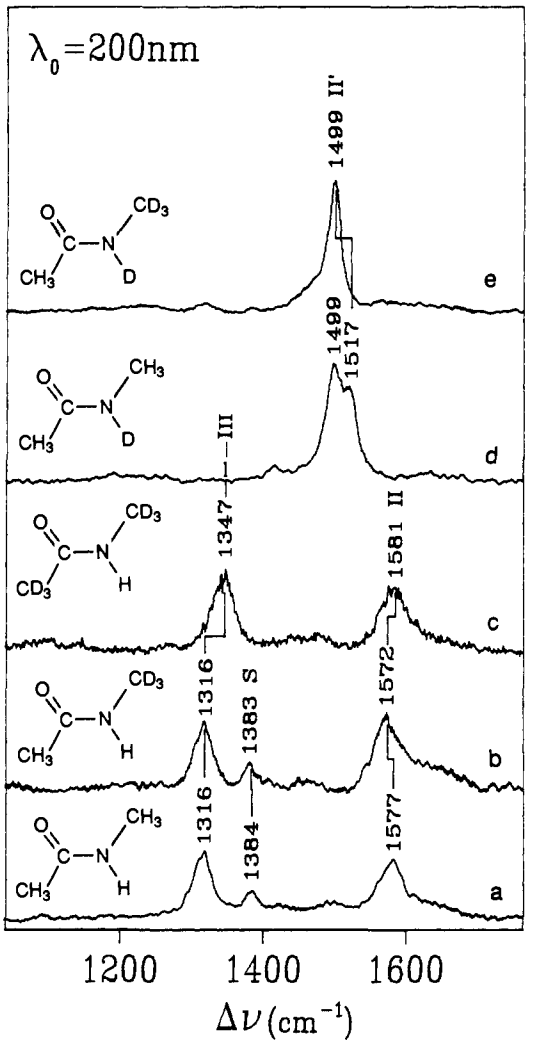

Figure 1. The 200-nm-excited UVRR spectra of NMA (4 mM) in H_2O (a) and D_2O (d) and of its N–CD_3 (b and e) and C,N–$(CD_3)_2$ (c) isotopomers: diode array detection, eight minutes collection time.

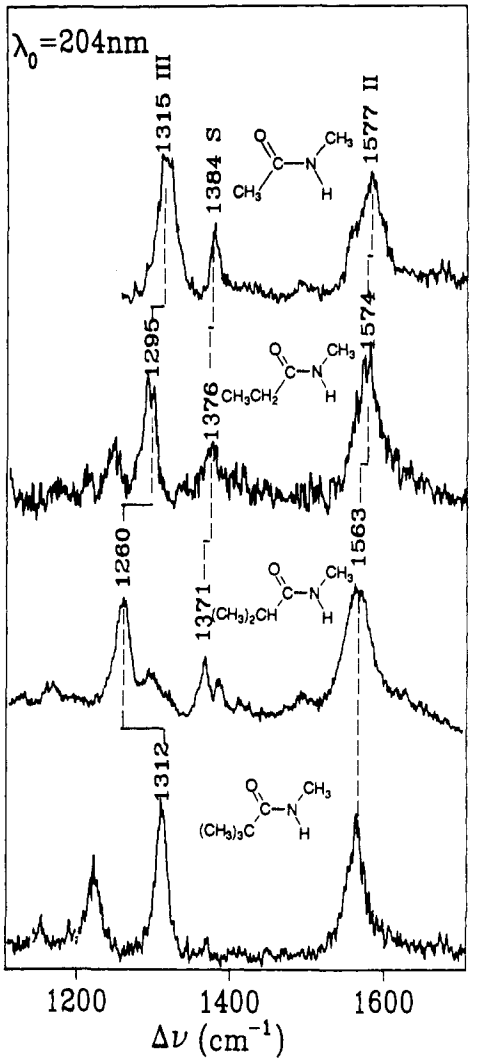

Figure 2. The 204-nm-excited UVRR spectra of ca. 5 mM NMA (a), *N*-methylpropionamide (b), *N*-methylisobutyramide (c), and *N*-methylpivalamide (d). The amide III shifts down from (a) to (c), and little change is observed for amide S. When there is no α proton as in (d), amide S disappears and amide III shifts up: scanning mode, 1 cm^{-1}/s, average of five scans.

successively replaced by one and two methyl groups, the amide S and III bands shift to lower frequency, but the third replacement eliminates amide S and shifts amide III back *up* to 1312 cm^{-1}. Thus, the appearance of amide S and the simultaneous depression of the amide III frequency are clearly associated with the presence of at least one H atom on the C atom attached to the carbonyl group.

We interpret this spectral pattern in the following way: Amide S is a C–H bending mode of the carbonyl-bound C atom, which gains its intensity by mixing with amide III. This mixing also depresses the frequency of amide III. When all three C–CH_3 H atoms on NMA are replaced by D atoms (spectrum c, Figure 1) or by methyl groups (*N*-methylpivalamide, Figure 2), this mixing is eliminated. In the latter case, however, the greater effective mass of the carbonyl substituent also depresses the amide III frequency, which is 35 cm^{-1} lower in $(CH_3)_3C(CO)NHCH_3$ (1312 cm^{-1}) than in $CD_3(CO)NHCD_3$ (1347 cm^{-1}). Increasing effective mass can also account for the drop in the amide III frequency from $CH_3(CO)NHCH_3$ (1316 cm^{-1}) to $(CH_3)CH_2(CO)NHCH_3$ (1295 cm^{-1}) to $(CH_3)_2CH(CO)NHCH_3$ (1260 cm^{-1}). It has been pointed out to us (Krimm, S., personal communication) that the amide S band of $(CH_3)CH_2(CO)NHCH_3$ and $(CH_3)_2CH(CO)NHCH_3$ might arise from the umbrella modes of their methyl groups rather than from C–H bending of the carbonyl-bound C atom. But these methyl modes lack a plausible RR enhancement mechanism, and, if they nevertheless did have significant intensity, it would then be inexplicable why the same modes of $(CH_3)_3C(CO)NHCH_3$ are not seen, or why its amide III frequency shifts up relative to $(CH_3)_2CH(CO)NHCH_3$ or $(CH_3)CH_2(CO)NHCH_3$. It should be pointed out that the amide S frequencies of the last two molecules do not correspond to the group frequencies for methylene or methine C–H bending in hydrocarbons, ~1460 and ~1300 cm^{-1}, but on the other hand, these group frequencies are known not to apply in carbonyl compounds. Thus, the CH_2 deformation is lowered to ~1425 cm^{-1} when CH_2 is next to a carbonyl group, while the in-plane CH bend of an aldehyde is at 1370–1410 cm^{-1}.[32c] Consequently, a ~1380-cm^{-1} frequency may not be unreasonable for CH_2 or CH bends next to the carbonyl group of an amide.

B. The 1395-cm^{-1} UVRR Band of Peptides Is Due to C_α–H Bending. Di- and polypeptides also show a UVRR band at 1395 cm^{-1}, which Krimm and Asher[18,19] have assigned to the amide V overtone, based on the frequency match with twice the fundamental frequency. Figure 3 shows, however, that this band also disappears upon H/D exchange at the C_α position of the oligopeptides triglycine and *N*-acetyltrialanine methyl ester, whereas the amide V overtone should be unaffected, since C_α–H contributes negligibly to the mode composition (N–H out-of-plane bending). This H/D exchange was carried out by incubation in D_2O at pD 11 at 50 °C for 24–48 h, followed by neutralization and rapid dilution in H_2O, a procedure that introduces D at the C_α atom but maintains H at the N atom.

The triglycine UVRR spectrum shows a weak amide I band, at ca. 1660 cm^{-1}, and strong amide II, S, and III bands, at 1558, 1394, and 1266 cm^{-1}, due to the two peptide bonds. Upon C_α–

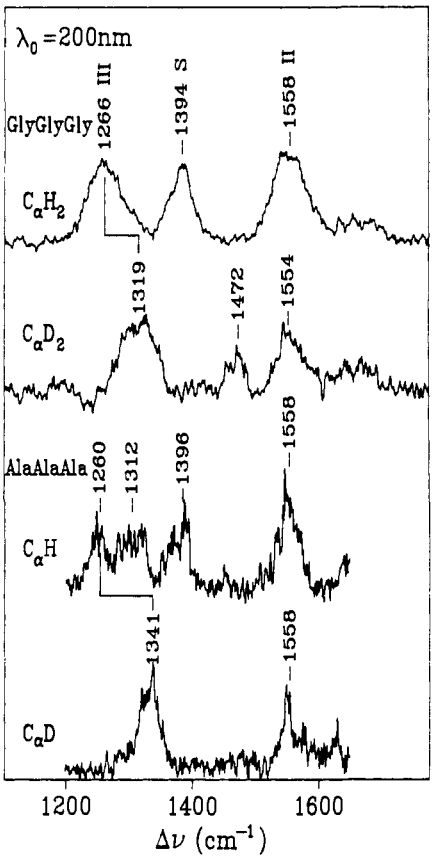

Figure 3. Amide III/S region of 200-nm-excited RR spectra of aqueous triglycine (GlyGlyGly) and of *N*-acetyltrialanine methyl ester (AlaAlaAla), before and after H/D exchange at the peptide C_α atoms.

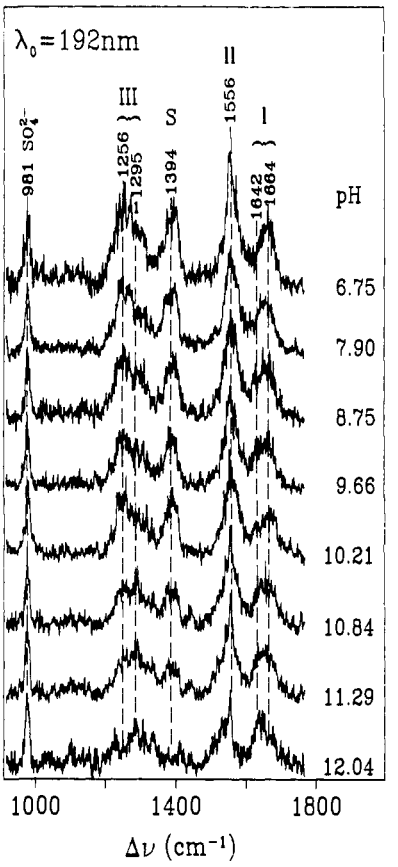

Figure 4. The 192-nm-excited UVRR spectra of aqueous polylysine (3 mM) at the indicated pH values: scanning mode, 1 cm^{-1}/s, average of five to eight scans.

H/D exchange, the amide S and III bands are replaced by a single band at 1319 cm^{-1}. The band at 1472 cm^{-1} is the amide II′ mode of residual N–D isotopomer remaining after the H_2O dilution (~10-fold in this case) of the D_2O reaction mixture. The amide III/S coupling is stronger in triglycine than in NMA, as judged by the nearly equal intensities and larger frequency separation of the two bands in the $C_\alpha H_2$ sample. Interestingly, the amide III frequency is nearly the same for $C_\alpha D_2$ triglycine as for NMA, implying that in the absence of δC–H coupling, the C–N bonding strength, and the amide mode couplings are virtually the same for NMA and for the peptide units.

The *N*-acetyltrialanine methyl ester spectra are very similar to those of triglycine. However, the C_α–H molecules show a broad extra band, at ~1312 cm^{-1}, between amide III and amide S. We note that a band near this frequency (1325 cm^{-1}) is seen in nonresonance Raman and infrared spectra of alanylalanine and that it also gives an infrared circular dichroism signal.[32a,b] It may arise from an out-of-plane C_α–H bending mode.[32b] A similar assignment may apply for the ~1312-cm^{-1} band of *N*-acetyltrialanine methyl ester. Along with amide III and amide S, the 1312-cm^{-1} band is replaced by a single 1341-cm^{-1} band upon C_α deuteration.

C. Amide S Disappears in α-Helical Polypeptides and Proteins. In Figure 4, UVRR spectra are shown for polylysine as a function of solution pH, with 192-nm excitation, in resonance with the amide π–π^* transition. It is known[33] that polylysine converts from an unordered to an α-helical structure when the ammonium side chains are neutralized at high pH, and this transition is readily seen in the UVRR spectra. The following changes accompany α-helix formation: (1) The amide I frequency shifts from 1664 to 1642 cm^{-1}, a classical α-helix value.[34] (2) The amide II intensity decreases greatly, an effect attributed to hypochromism of the resonant electronic transition.[11] The UV absorptivity is halved when a polypeptide is coiled into an α-helix,[33] due to the alignment of the transition dipoles, and the amide II RR intensity decreases by a factor of about four, as expected from the scaling of resonance enhancement with the square of the absorption strength.[11] (3) The amide III band also loses intensity, but in addition shifts up in frequency, from 1265 to 1295 cm^{-1}, maintaining a constant intensity ratio with the amide II band. Another band, slightly above 1300 cm^{-1}, can also be seen at neutral pH and may be an out-of-plane C–H bend like the ~1312-cm^{-1} band of *N*-acetyltrialanine methyl ester. (4) The amide S band at 1394 cm^{-1} disappears and is not replaced by a nearby band with significant intensity. In Figure 5 the band intensity changes are plotted against pH. All of the changes are concerted, and the transition pH is seen to be in the vicinity of 10.5, as expected for ammonium group neutralization, and consistent with previous studies.[33]

Figure 6 shows 200-nm-excited spectra for polyglutamate. This polypeptide is converted to an α-helical form at low pH (pK_a = 5), where the anionic side chains are neutralized. As in the case of polylysine, the coil → helix transition is accompanied by a downshift of amide I, an upshift of amide III, and intensity losses for amide II, III, and S relative to amide I. The amide S band does not disappear completely in the pH 4 spectrum, but this remnant intensity is attributed to incomplete conversion to α-helix, the pH being insufficiently below the transition pK_a. Lowering the pH further resulted in polypeptide precipitation. A band is also seen at 1320 cm^{-1}, on the side of amide III. This band does not lose intensity at low pH and therefore becomes more noticeable, in comparison with amide III. Krimm et al.[18b] assigned this band (listed as 1338 cm^{-1}) to the amide V overtone in the α-helical structure, shifting down from the 1395-cm^{-1} frequency assigned to this mode in the coil structure (here reassigned to

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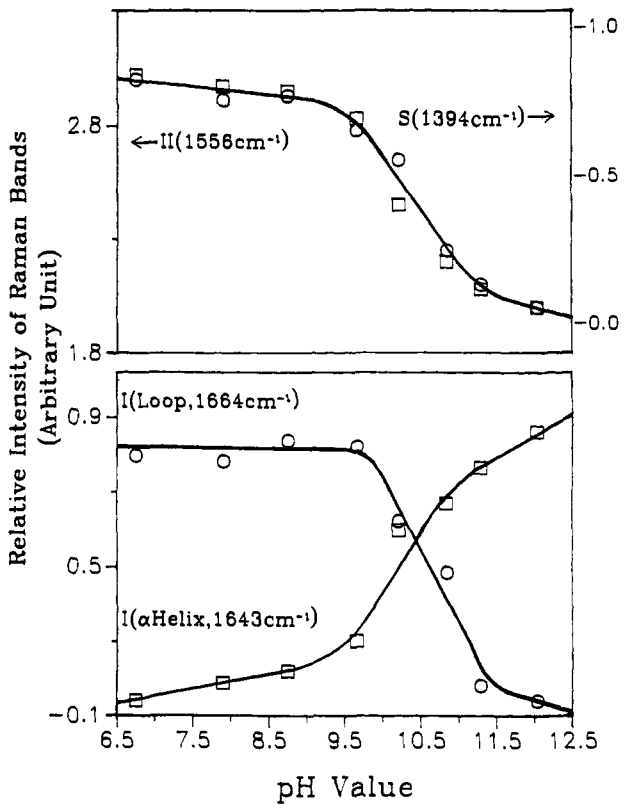

Figure 5. Relative peak area intensities (from the spectra in Figure 4) for polylysine amide II (◊) and S (○) bands and of the loop (□) and α-helical (△) amide I bands at 1664 and 1642 cm^{-1}. The intensity changes are concerted and show a pK_a of ~10.5.

amide S) due to the altered H-bonding. It is clear from the top spectrum in Figure 6, however, that the 1320-cm^{-1} band is also present in the high pH coil structure and has the same intensity relative to amide I as it does in the low pH α-helical form. The fact that it does not lose intensity in parallel with amide II and III, upon α-helix formation, indicates that it is not coupled with these modes and is not resonance enhanced. It is tentatively assigned to side-chain C–H bending, its intensity resulting from the large number of side-chain C–H bonds. When polyglutamate was subjected to H/D exchange at the C_α atoms, with the same procedure described above for the oligopeptides, the amide III and S bands were again observed to collapse to a single band at an intermediate frequency (bottom spectrum).

These UVRR characteristics of α-helical polypeptides can also be observed in proteins, as demonstrated by the comparison of the 200-nm-excited spectra of myoglobin (Mb) and concanavalin A (Con A) in Figure 7. The secondary structure is mostly (85%) α-helical in Mb[35] but mostly (65%) β-sheet in Con A which has less than 3% α-helical structure.[36] As indicated in the figure, these spectra contain bands arising from aromatic residues,[1] but the amide bands stand out clearly. The amide I and III frequencies are 1644 and 1287 cm^{-1} for Mb and 1668 and 1238 cm^{-1} for Con A, values that are characteristic for α-helical and β-sheet structures.[34] The amide II and III intensities are much lower for Mb than Con A, and the amide S band, which is prominent for Con A, essentially disappears for Mb.

The resonant $\pi-\pi^*$ transition involves displacement of the amide C–N bond stretch, primarily.[15] This displacement is divided between the amide II and III modes, and, as seen in the present study, is shared significantly by the amide S mode as well. Consequently, it is expected that all three bands should lose intensity upon α-helix formation, due to the loss of $\pi-\pi^*$ absorption strength.[11] Amide II and III, however, retain significant intensity

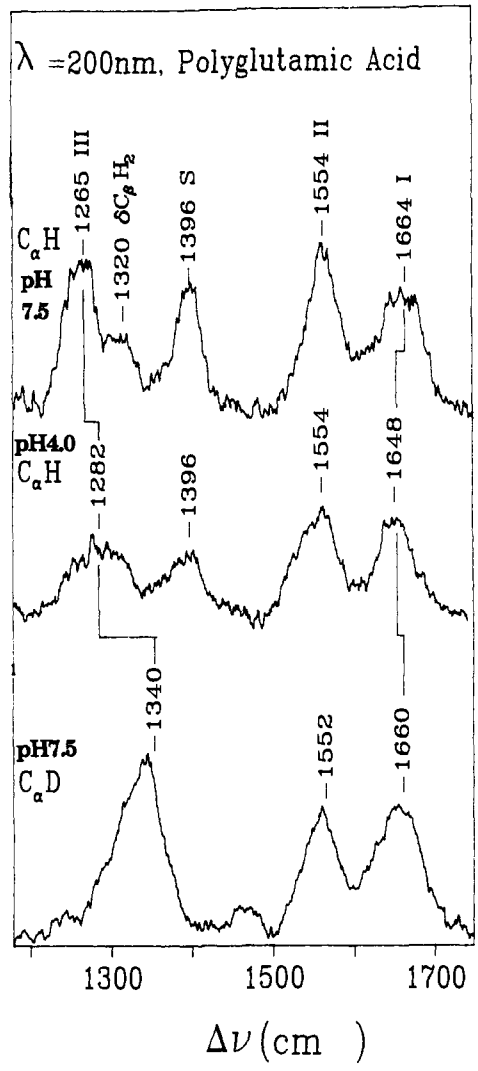

Figure 6. The 200-nm-excited UVRR spectra of polyglutamic acid at pH 7.5 (top) and 4 (middle), and at pH 7.5 after H/D exchange at the C_α atoms (bottom).

in the α-helix spectrum, whereas amide S disappears. Thus, an additional intensityless mechanism is implied. This mechanism is suggested to involve uncoupling of the amide III and S modes. Supporting this suggestion is the upshift in amide III upon α-helix formation; indeed, the disappearance of amide S and the upshift of amide III are reminiscent of the changes seen upon C_α–H/D exchange.

Why should amide S uncouple from amide III in α-helices? The answer is suggested to lie in the range of accessible ϕ and ψ angles. In the Ramachandran plot these angles cluster near −60°, −60° for α-helices and near −90°, +120° for both β-sheet and loop structures.[37] As shown in Figure 8, the 180° rotation of the ψ angle carries the C_α–H bond from an orientation trans to the C=O bond in the β-sheet or loop structure to an orientation cis to the C=O bond in the α-helix. This reorientation may alter the kinematics sufficiently to inhibit coupling of the C_α–H bending coordinate with the C–N stretch.

D. Modeling the Amide Mode Couplings with Normal Coordinate Calculations. In order to investigate the coupling effects suggested by the UVRR spectra, we carried out a normal coordinate analysis on NMA, paying particular attention to the orientation of the C–CH_3 group. For this purpose the empirical valence force field developed by Jakes and Krimm[30] was employed, with a slight adjustment of the parameters to fit the more recent NMA vibrational frequencies and geometry reported by Tsuboi.[38]

(35) Dickerson, R. E.; Geis, I. In *Hemoglobin*; Benjamin/Cummings: Menlo Park, CA, 1983; pp 159–197.
(36) Levitt, M.; Greer, J. *J. Mol. Biol.* **1977**, *114*, 181–293.
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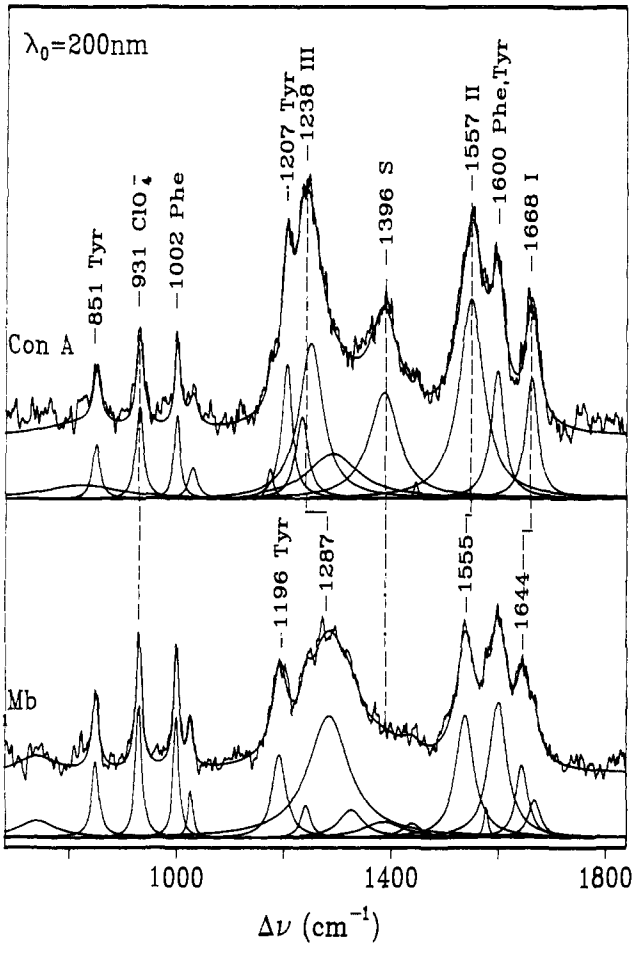
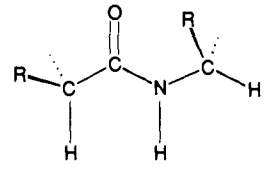
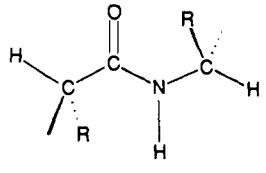


Figure 7. The 200-nm-excited UVRR spectra of concanavalin A (top) and myoglobin (bottom) at 1 mg/mL diode array detection, 10-min collection. Spectral deconvolution with Lab Calc software is shown.



Trans C=O/C-H (β-Sheet and Loops)



Cis C=O/C-H (α-Helix)

Figure 8. Structural diagrams of the peptide conformation in α-helices and in β-sheet or loop secondary structure, showing the trans vs cis orientation of the C_α–H bond relative to the C=O bond.

Table I shows that the experimental frequencies are well reproduced, as are the deuteration shifts. The underlined entries correspond to bands observed in the UVRR spectra. Their eigenvectors are drawn in Figure 9. As expected,[30] amide I has mainly C=O stretching character, while amides II and III are mixed C–N stretching and N–H bending. The C–CH_3 symmetric deformation mode, δ_s, is selected as amide S, because its eigenvector shows substantial N–H bending; at the same time the amide III eigenvector shows substantial C–CH_3 δ_s character. In the C–CD_3 isotopomer, amide III shifts *up* 25 cm^{-1}, as the coupling with C–CH_3 δ_s is relieved.

Table I. Experimental and Calculated Frequencies (cm^{-1}) for NMA Isotopomers

	na[a]		N–D		C–CD_3		N–CD_3	
mode	exptl[b]	calc[c]	exptl	calc	exptl	calc	exptl	calc
amide I[d]	1654	1653	1635	1640	1644	1648	1651	1650
amide II	1564	1566	1479	1483	1564	1563	1660	1554
N–CH_3 δ_{as}	1448	1450	1442	1444	1457	1452	1065	1041
C CH_3 δ_{as}	1448	1445	1442	1440	1036	1044	1442	1439
N CH_3 δ_{as}	1414	1417	1406	1408	1416	1420	1035	1031
C CH_3 δ_s[e]	1374	1376	1370	1368	1036	1032	1373	1374
amide III	1302	1296	965	970	1325	1320	1304	1297

[a] Natural abundance, na. [b] Experimental values from ref 38. [c] Calculated on the basis of a valence force field modified slightly from ref 30a. [d] Underlined amide bands are observed in UVRR spectra. [e] Assigned as amide S.

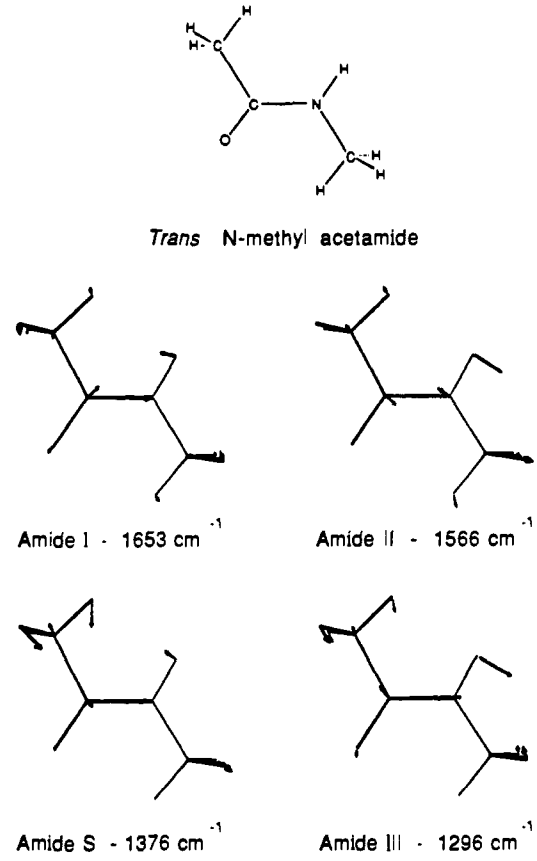


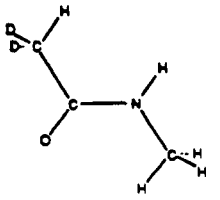
Figure 9. Eigenvectors of the amide I, II, III, and S modes of *trans*-NMA, calculated as described in the text.

The amide III/S coupling is due to the substantial interaction force constant between the C–CH_3 δ_s coordinate and the C–C stretching coordinate which is required to bring the symmetric deformation frequency below that of the asymmetric deformation. In the absence of this interaction constant the calculated frequency order is reversed, contrary to experiment. This requirement is a well-known feature of the force field of carbon-bound methyl derivatives.[39] The interaction constant permits the mixing of symmetric CH_3 deformation with N–H bending via the intervening C–C and C–N bonds. The behavior of amide III and S in the NMA UVRR spectrum constitutes striking confirmation of this interaction. No mixing is calculated for the C–CH_3 asymmetric deformation or for either of the N–CH_3 deformation modes, consistent with the experimental evidence.

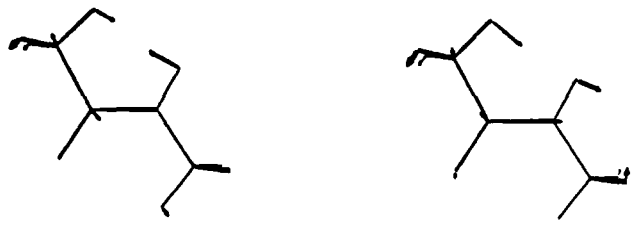
In order to model the behavior of the amide S mode of peptides, we calculated the frequencies of the C–CD_2H isotopomer of NMA

(38) Sugawara, Y.; Hirakawa, A. Y.; Tsuboi, M. *J. Mol. Spectrosc.* **1986**, *108*, 206–214.

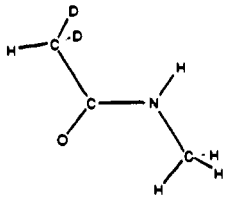
(39) Snyder, R. G.; Schachtschneider, J. H. *Spectrochim. Acta* **1965**, *21*, 169–195.



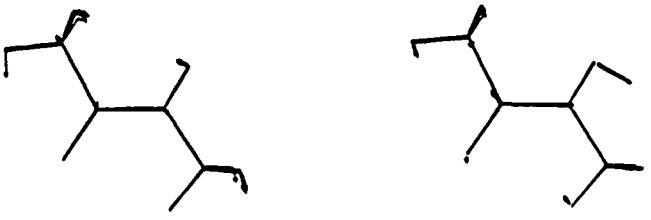
$CHD_2CONHCH_3$, *trans* C-H/C=O



Amide S - 1312 cm^{-1} Amide III - 1250 cm^{-1}



$CHD_2CONHCH_3$, cis C-H/C=0



CH bend - 1546 cm^{-1} Amide III - 1294 cm^{-1}

Figure 10. Eigenvector calculated for the $C-CD_2H$ isotopomer of NMA, with the C–H bond oriented trans and cis to the carbonyl group.

Table II. Calculated Amide Frequencies for $CHD_2CONHCH_3$ with the C–H Bond of the CHD_2 Group Oriented Trans and Cis to the C=O Bond

	trans-C–CHD_2	cis-C–CHD_2
amide I	1652	1658
amide II	1564	1566
amide S	1312	(1546)[a]
amide III	1250	1294

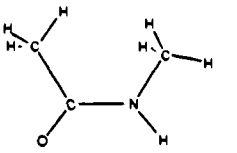
[a] Among the calculated modes this one shows the largest CD_2–H bending displacement (see eigenvector, Figure 10).

(Figure 10). This isotopomer has a single C_α–H bond, as do all peptide bonds except glycine and proline. The results are given in Table II; the $N-CH_3$ and $C-CD_2$ modes have been omitted for clarity. Two conformers of the CD_2H group were considered, with the C–H bond in the amide plane and oriented either cis or trans to the C=O bond, modeling the conformation in α-helices and loop or β-strand structures, respectively. In the trans orientation, the C–H deformation does indeed act like amide S. The eigenvectors of this mode and of amide III are very similar to the amide S and III eigenvectors of natural abundance NMA (Figure 9). In the cis orientation, however, there is no amide S. The mode with the largest $C-CD_2-H$ bend is one calculated at the rather high frequency of 1546 cm^{-1}. This mode has little N–H bending character, which is concentrated instead in amide III. Strikingly, the amide III frequency shifts *up* from 1250 cm^{-1} in the trans orientation to 1294 cm^{-1} in the cis orientation, as the coupling with amide S is relieved. This upshift is quite close to that observed

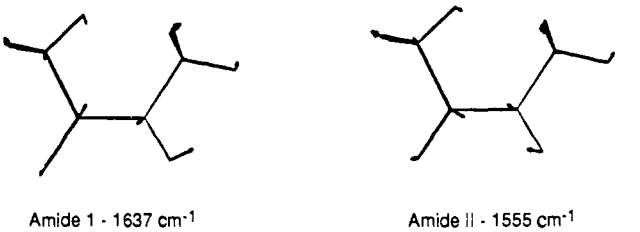
Table III. Calculated Amide Frequencies (cm^{-1}) for *trans*- and *cis*-Amide Isomers of NMA

	trans	cis
amide I	1653	1637
amide II	1566	1555
amide S	1376	(1372)[a]
amide III	1296	1379

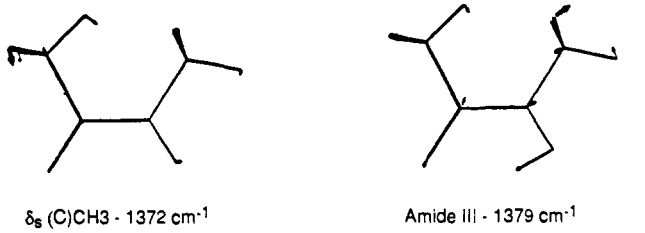
[a] Among the calculated modes this one shows the largest CD_2–H bending displacement (see eigenvector, Figure 10).



Cis N-methyl acetamide



Amide I - 1637 cm^{-1} Amide II - 1555 cm^{-1}



δ_s (C)CH3 - 1372 cm^{-1} Amide III - 1379 cm^{-1}

Figure 11. Eigenvectors calculated for the *cis*-amide isomer of NMA.

(1260 → 1295 cm^{-1}) in the transition from loop to α-helix polypeptides and provides strong support to the uncoupling explanation for the disappearance of amide S in α-helix UVRR spectra.

On the other hand, the amide S frequency calculated in the model with trans orientation, 1312 cm^{-1}, is substantially lower than that observed in peptides, 1395 cm^{-1}, and the frequency separation between amide S and III is correspondingly smaller. Thus, the C–H deformation force constant must be higher for peptides than for NMA, and the interaction with the C–C stretch may also need to be adjusted in order to get the coupling right. The work of Krimm and Bandekar[40a] indicates that other force constant adjustments, relative to NMA, are also required for peptides. More accurate modeling of the peptide UVRR spectra is left for future work.

One other effect was explored in the NMA calculations, namely the uncoupling of amide II and III in the *cis*-amide isomer of secondary amides. As shown elsewhere via the UVRR spectrum of caprolactam,[20,23] the UVRR spectrum of the cis isomer is strikingly different from the trans. In place of the amide II, III, and S bands, one sees a single intense band at an intermediate frequency, 1495 cm^{-1}. This frequency is nearly insensitive to N–H/D exchange, and it is very close to the amide II′ frequency of the trans isomer in D_2O, implying that the cis isomer amide II mode is no longer mixed with N–H bending. The frequencies of *cis*-NMA were calculated with the *trans*-NMA force field and are listed in Table III, while the eigenvectors are plotted in Figure 11. Amide III is seen to shift *up* by 77 cm^{-1}, due to uncoupling, and it is mostly N–H bending in character, although some coupling with amide I is now seen. At the same time, amide II is mainly C–N stretching in character, with little N–H bending. This composition is also consistent with recent ab initio calculations

Table IV. Molar Raman Scattering Ratios[a] of Several Native Proteins Measured with 200-nm Excitation

		molar Raman scattering ratio				
		peak height		peak area		
protein	% helix	amide S	amide II	amide S	amide II	ref[b]
Con A	2	17.7	41.0	46.3	136	36
sup dis	6	16.4	37.7	45.0	126	42
trypsin	8	19.7	39.4	49.0	144	43
Rnase A	23	14.1	34.4	45.5	134	36
cyt *c*	39	10.5	29.8	25.0	117	44
lysozyme	46	8.9	28.5	20.0	112	36
BST	55	7.2	23.3	19.4	93.8	45
Hb	78	5.6	21.0	13.6	48.9	35
Mb	89	3.0	13.5	8.9	41.1	35
tropomyosin	95	1.0	10.2	6.0	32.5	47

[a] Intensity relative to the perchlorate 931-cm^{-1} band, per mol of peptide bonds and of perchlorate; $R = (I_a/I_r) \times (C_r/C_a)$. [b] Reference for helix content.

by Mirkin and Krimm[40b] and with the larger $^{13}C^{15}N$ isotope shift seen by Song et al.[19] in *cis*- than in *trans*-diglycine. The symmetric CH_3 bend, at 1372 cm^{-1}, now shows no mixing with other coordinates. These unmixing effects explain the enhancement of a single dominant band in the UVRR spectrum of *cis*-amide, since C–N stretching is the main distortion coordinate in the resonant excited state. We note that uncoupling of C–N stretching and N–H bending has also been found by Cheam and Krimm[41] in diketopiperazine, another *cis*-amide model, although the C–N Raman band (amide II) is raised to 1520 cm^{-1} in this cyclic molecule with two peptide units.

The calculated *cis*-amide II frequency, 1555 cm^{-1}, is too high, suggesting that isomerization alters the force field somewhat, consistent with the preliminary results reported on the basis of ab initio calculations.[40b] For *N*-methylformamide, the reported ab initio N—C=O bending and C–N–C bending force constants are about 15% lower in the cis than the trans isomer.[42] Trial modeling suggests that similar adjustments could bring the *cis*-NMA frequencies into line with the experimental data on *cis*-amides.

E. Amide II and S Intensities Quantitate Nonhelical Content in Proteins. Figure 12 shows 200-nm-excited UVRR spectra for a number of proteins, chosen to represent a wide range of secondary structure compositions. The solutions contained 1 mg/mL of protein, corresponding to a peptide bond concentration of ca. 8 mM. At this concentration, contributions from the broad water band at 1640 cm^{-1}, or from most buffer ions, are negligible. The band intensities were determined via band deconvolution, as illustrated in Figure 7, and were referenced to the 931-cm^{-1} band of perchlorate ion, which was added to the solutions (0.15 M) as an internal standard. Both peak heights and peak areas were determined (Table IV) and are plotted against α-helical content in Figure 13. The values are given as the molar scattering ratio R with the equation

$$R = (I_a/I_r) \times (C_r/C_a) \quad (1)$$

where I_a is the intensity of the amide peak (S or II), I_r is the reference (ClO_4^-) peak intensity, C_r is the reference concentration, and C_a is the concentration of peptide bonds. Since this ratio is alternatively defined as $R = \sigma_a/\sigma_r$, where σ is the absolute scattering cross section (mbarn/molecule·steradian), the cross section values for the Raman peaks can be determined with the standard value for perchlorate.[43]

Table V. 200 nm UVRR Parameters for Determination of α-Helix Content

		molar Raman scattering ratio	
vibrational mode	intensity	0% helix (J_{NH})	100% helix (J_H)
amide S	peak height	18	0
	peak area	49	0
amide II	peak height	41	11
	peak area	150	32

The peak area and height values differ substantially, because the amide bands are much broader than the perchlorate band. Clearly, the peak area is the correct measure of cross section. Moreover, the peak area should be the more reliable intensity measure for protein bands, since the many contributing peptide bonds might have slightly different frequencies. On the other hand, the peak area is more subject than is the peak height to deconvolution errors associated with noise or with unrecognized peaks in the tails of the band under consideration.[27] The deconvolution program accommodates these contributions by widening the peak, thereby increasing the measured area; the peak height, however, is little affected. Thus, the peak height may be a more reliable measure of the intensity when the band is poorly resolved or has excessive noise or suspicious features in the tails. Indeed, the observed scatter in the amide II intensities is slightly worse for the peak area measurements, perhaps reflecting deconvolution errors from variable overlap with the phenylalanine and tyrosine bands.

The peak area and height intensities both follow negative linear correlations with α-helix content for both amide II and amide S and do so quite accurately over the entire range of composition. For a two-component system consisting of helical (H) and nonhelical (NH) contributions to the Raman intensity, the molar scattering ratio can be expressed as[11]

$$R = J_H f_H + J_{NH} f_{NH} \quad (2)$$

where f_H and f_{NH} are the mole fractions for the two structural types with associated molar scattering coefficients J_H and J_{NH}. Since $f_H + f_{NH} = 1$ we can relate R to f_H via the equation

$$R = (J_H - J_{NH})f_H + J_{NH} \quad (3)$$

which gives the linear plots shown in Figure 13 for amide S and amide II. The coefficients, listed in Table V, are the R-axis intercepts at 0% (J_{NH}) and 100% (J_H) helix content. It is evident that one can determine the fraction of a protein having helical structure from simple interpolation:

$$f_H = (J_{NH} - R)/(J_{NH} - J_H) \quad (4)$$

While both amide II and amide S can be employed to determine f_H, and the two together provide a useful consistency check, amide S provides the more sensitive measure because J_H is zero. Also, amide II is subject to interference from the tryptophan mode W_3, which is found at the same frequency, 1555 cm^{-1}.[1] Although the W_3 enhancement at 200 nm is considerably lower than its peak value at 220 nm,[27] it might nevertheless contribute nonnegligible intensity to the amide II band if tryptophan is abundant. The amide S band, however, is at a frequency that is relatively free of interferences. In order to accurately determine the helical contents of uncharacterized proteins it is suggested that several

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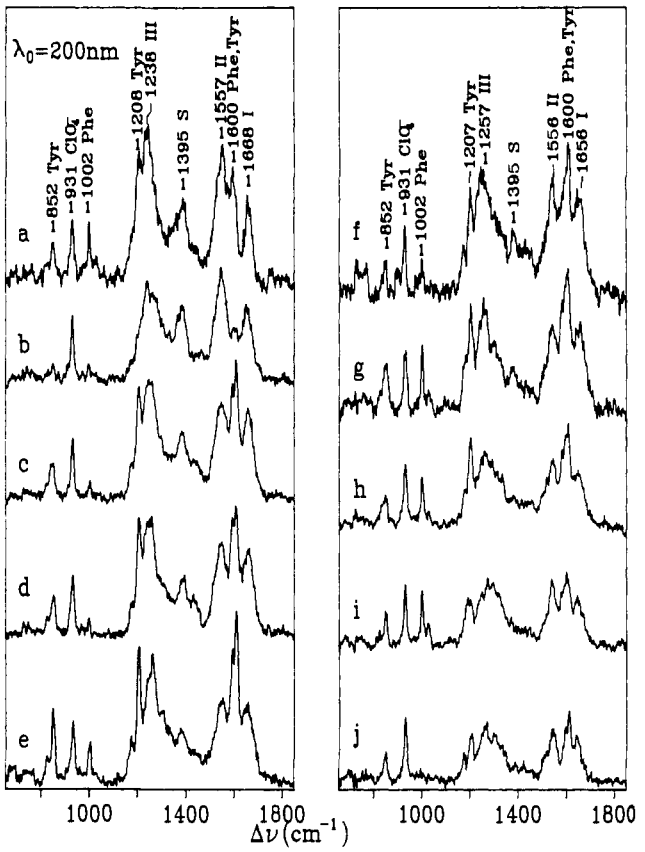

Figure 12. UVRR spectra of aqueous protein samples (1 mg/mL): (a) concanavalin A, (b) superoxide dismutase, (c) trypsin, (d) RNase A, (e) cytochrome c, (f) lysozyme, (g) BST (bovine somatotropin), (h) hemoglobin, (i) myoglobin, and (j) tropomyosin. pH = 9 for BST, pH = 7 for other proteins. The excitation source was a 300-Hz excimer/dye laser system, operating at 200 nm. The 0.15 M sodium perchlorate was used as an internal intensity standard. Conditions: 10–20-min accumulation time, diode array detection.

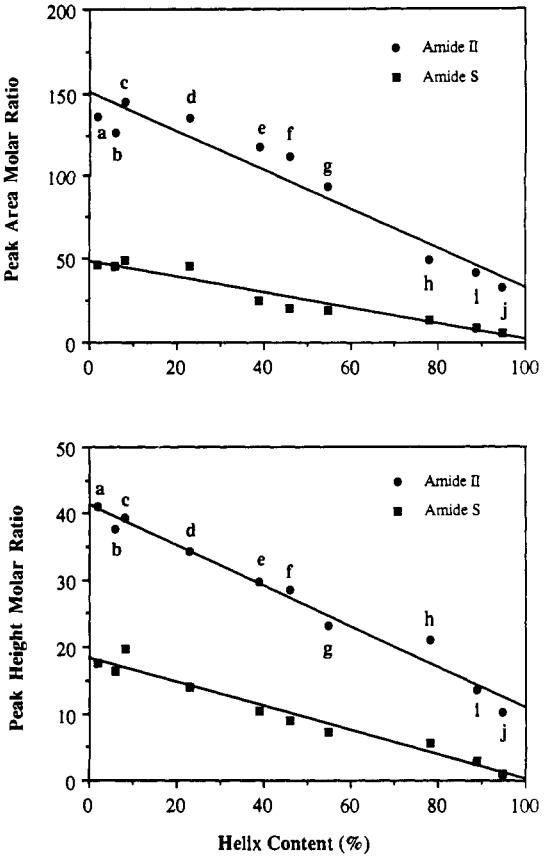

Figure 13. Plots of amide II and amide S molar scattering ratio R vs α-helical content for various proteins (labels as in Figure 12). Peak heights (bottom) and areas (top) were both determined relative to the 931-cm^{-1} ClO_4^- band, and R was calculated with the equation $R = (I_a/I_r) \times (C_r/C_a)$ (see text for details).

standard proteins be used to create a calibration curve similar to Figure 13, thereby reducing systematic error arising from system response.

Song and Asher[17] have reported amide scattering factors for 220-nm excitation. Because this wavelength is further from resonance than is 200 nm, the enhancement of the amide bands is much lower, and correspondingly higher protein concentrations are required for adequate signal/noise. In our experience, protein concentrations of 6–10 mg/mL are required to obtain 220-nm-excited amide bands at signal strengths comparable to those in Figure 12. An additional difficulty is that the relative contribution of aromatic residue bands is greater at 220 than at 200 nm; in particular, this wavelength is at the peak of the tryptophan excitation profile.

The spectra in Figure 12 were obtained with a 300-Hz excimer laser source, with an average of 10-min collection times. Aside from the rapid spectral accumulation, this high repetition rate laser minimizes saturation effects, relative to the 10-Hz YAG laser, because the same average power can be achieved with lower peak power.[26,27] Nevertheless, we have not observed significant saturation effects in YAG spectra excited at 200 nm (they can be pronounced at longer wavelengths,[26,27] however, where the accessible peak powers are much higher). Although longer accumulation times (20–30 min) are required, results similar to those in Figure 12 can be obtained with YAG excitation. We note, however, that the intensities plotted for amide II in Figure 13 deviate significantly from those reported earlier from this laboratory.[11] The discrepancies can be traced to the use of sulfate as the internal standard together with the earlier practice of measuring peak heights from the spectra without benefit of band deconvolution. As can be seen in Figure 7, the 1004-cm^{-1} band of phenylalanine is close enough to the sulfate 981-cm^{-1} band to influence its apparent peak height. This interference is believed to account for the different scattering factors reported in the earlier work as well as the observation of apparent deviations[11] from the α-helix correlation of two proteins having large fractions of β-sheet structure, concanavalin A and superoxide dismutase, which have little phenylalanine. As seen in Figure 13, these two proteins fall on the α-helix correlation for both amide II and amide S, when the perchlorate 931-cm^{-1} band is used for reference, and the bands are properly deconvoluted. We conclude that the scattering factor differs insignificantly for β-sheet and loop structures with 200-nm excitation.

Summary

A strongly enhanced UVRR band of nonhelical peptides at 1395 cm^{-1} is shown by isotopic and chemical substitution studies to be associated with bending of the C–H bond of the C_α atom, attached to the carbonyl group. It gains its intensity by coupling with the nearby amide III mode. The coupling is revealed by the effects of C_α–H/D substitution and is supported by normal coordinate calculations; it arises via the interaction force constant between C–H bending and C–C stretching.

This band disappears in α-helical polypeptides and is labeled amide S, for secondary structure-sensitive. Normal coordinate calculations support the view that its disappearance results from uncoupling with amide III when the C_α–H bond is rotated into an orientation cis to the carbonyl group, in α-helices, from the trans orientation found in loop or β-sheet structures. This uncoupling is a kinematic effect and results in the characteristic upshift of amide III in α-helical polypeptides.

The high sensitivity of amide S to α-helix formation, and the absence of interfering UVRR bands in its vicinity, makes it an excellent marker for determining the population of nonhelical structures. For a wide range of proteins its intensity is linearly related to α-helix content, with a zero intercept. Intensities are

given for peak-height or area measurement of amide S as well as of amide II with which α-helical content can be determined for any protein.

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UVRR Spectroscopy of the Peptide Bond. 2. Carbonyl H-Bond Effects on the Ground- and Excited-State Structures of *N*-Methylacetamide

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Abstract: Ultraviolet resonance Raman (UVRR) spectra of *N*-methylacetamide (NMA) are markedly solvent dependent. When non-H-bonding solvents are compared with water, the amide I band is seen to intensify and shift up in frequency, while the amide II, III, and S bands weaken and shift down in frequency. The amide I frequency is linearly correlated with the solvent acceptor number (H-bond donating tendency), while the N-H stretching infrared frequency is linearly correlated with the solvent donor number (H-bond accepting tendency), but there is no cross-correlation. Thus, the ground-state structure of the amide bond is sensitive to C=O but not to N-H H-bonding. Raman excitation profiles (EPs) in water show maxima at the first allowed absorption maximum, 188 nm, for amide II, III, and S but not for amide I, which shows only preresonance enhancement from a transition at ca. 165 nm. In acetonitrile, the intensity order is amide I > III > II > S, and the EPs all rise strongly toward the blue-shifted first absorption maximum. These dramatic changes are suggested to arise from stabilization of the C=O π^* fragment orbital by H-bonding, resulting in an altered orbital pattern and lowered energy for the first π-π^* excited state. In stationary samples, a 1495-cm^{-1} band grows in with increasing laser power. It is assigned to the amide II band of the *cis*-amide isomer by comparison with the UVRR spectrum of the cyclic *cis*-amide, caprolactam. At laser powers sufficient to convert 20% of the NMA to the cis isomer, the Stokes/anti-Stokes intensity ratio of an 831-cm^{-1} probe band of deuterated acetonitrile revealed a temperature rise within the scattering volume of 25 °C, sufficient to account for an increase of only 1% in the cis isomer fraction via heating. Most of the isomerization is therefore induced by photoexcitation. MINDO/3 calculations of the potential with respect to twisting about the C-N bond show a minimum energy for the S_1 state at 90°, suggesting a facile pathway for photoisomerization. The photoisomerization yield is much higher in water than in acetonitrile, an effect attributable to a deeper excited-state potential well, due to the same orbital changes that account for the altered EPs. The yield also decreases with steric bulk of substituents on the amide C and N atoms, as expected from steric clashes in the 90° twisted geometry. Photoisomerization is seen for glycylglycine but is barely detectable for glycyllysine and alanylalanine. For hexaglycine, the photoisomerization yield, per amide bond, is only about 40% of that seen for glycylglycine, suggesting that only the outer two of the five amide bonds are affected. Consequently, photoisomerization is expected to be unimportant for polypeptides and proteins except for terminal glycine residues. The overtone of the amide V out-of-plane N-H bend is not observed in NMA UVRR spectra, even directly on resonance at 192 nm. The low enhancement is suggested to reflect a relatively shallow slope for the S_1 twisting potential or a small degree of twist in the amide V normal coordinate.

Introduction

N-Methylacetamide (NMA) is the simplest realistic model compound for the peptide bond. Its ultraviolet resonance Raman (UVRR) spectrum has recently been examined in connection with the development of UVRR probes of protein structure.[1-7] These studies revealed enhancement of amide vibrational bands near resonance with the strong 190-nm absorption band assigned to the first π-π^* transition, as expected, but they also contained surprises, which have prompted the present investigation. Thus, Hudson and co-workers found strong enhancement of the carbonyl stretching mode, amide I, in the vapor phase or in acetonitrile solution, but could not detect the band in aqueous solution.[1,2] They suggested that a distribution of H-bonded structures in water might broaden the amide I band out of recognition. In the present study, we examine the UVRR spectra of NMA in various solvents and find that amide I is hidden by amide II in aqueous solution, because H-bonding solvents shift the frequencies of these modes in opposite directions and also reverse their intensities. Both of these effects can be understood on the basis of H-bond influences on the amide resonance structures or, equivalently, on the HOMO/LUMO orbital compositions. Interestingly, the H-bond effect on the amide electronic structure is due exclusively to interactions at the carbonyl group and not at the N-H group.

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