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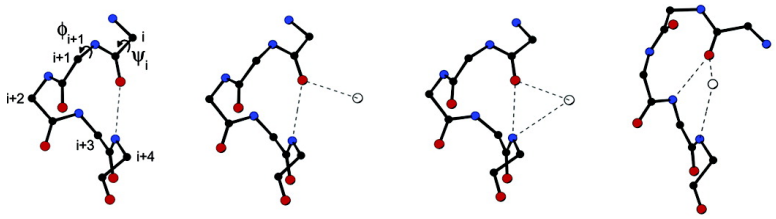

unhydrated (canonical, α_c) | external (open, α_o) | three centered | water inserted

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A Study of α-Helix Hydration in Polypeptides, Proteins, and Viruses Using Vibrational Raman Optical Activity

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Abstract: A vibrational Raman optical activity (ROA) study, supplemented by protein X-ray crystal structure data, of α-helices in polypeptides, proteins, and viruses has suggested that ROA bands in the extended amide III spectral region may be used to distinguish between two types of right-handed α-helix. One type, associated with a positive ROA band at ~1300 cm^{-1}, dominates in hydrophobic environments and appears to be unhydrated; the other, associated with a positive ROA band at ~1340 cm^{-1}, dominates in hydrophilic environments and appears to be hydrated. Evidence is presented to support the hypothesis that unhydrated α-helix corresponds to the canonical conformation α_c and hydrated α-helix to a more open conformation α_o stabilized by hydrogen bonding of a water molecule or a hydrophilic side chain to the peptide carbonyl. α-Helical poly(L-lysine) and poly(L-ornithine) in aqueous solution and poly(L-alanine) in dichloracetic acid display both bands, but α-helical poly(L-glutamic acid) in aqueous solution and poly(γ-benzyl L-glutamate) in $CHCl_3$ display only the ~1340 cm^{-1} band and so may exist purely as α_o due to enhanced stabilization of this conformation by particular side chain characteristics. The ROA spectrum of poly(β-benzyl L-aspartate) in $CHCl_3$ reveals that it exists in a single *left*-handed α-helical state more analogous to α_o than to α_c.

Introduction

The central role of water in peptide and protein science has long been appreciated.[1−5] However, the molecular details of hydration and how it influences structure and behavior are difficult to study in aqueous solution. It has been suggested that α-helix solvation in polypeptides and proteins in D_2O may be detected by characteristic FTIR bands, but the assignments are still the subject of debate[6−8] and may be due to structural elements other than the α-helix. Although NMR methods are used to study protein hydration, they suffer from several limitations. For example, high resolution ^1H NMR nuclear Overhauser effect spectroscopy is hampered by the fast chemical exchange between hydration water and bulk water,[9] and ^{17}O magnetic relaxation dispersion lacks the intrinsic spatial resolution of the nuclear Overhauser effect method.[10]

Insight into hydration has, however, been obtained from the study of water molecules in high resolution protein X-ray crystal structures, especially their hydrogen-bonding interactions with α-helices.[11−16] This insight follows from the observation that the α-helices in X-ray crystal structures of globular proteins usually deviate from the classic structure deduced by Pauling et al.[17] on the basis of model building and X-ray fiber diffraction data and which has $n = 3.65$ residues per turn achieved with planar peptides, Ramachandran ϕ, ψ angles of −48°, −57° and hydrogen bonds close to linear. In a pioneering study of water-induced distortions and the curvature of α-helices in three highly refined protein structures, Blundell et al.[11] found that, although a direct Ramachandran plot of ϕ_i versus ψ_i shows no systematic groupings, a plot of ϕ_{i+1} versus ψ_i shows groupings corresponding to residues in hydrophilic and hydrophobic environments. These angles are the torsion angles on each side of the $-(N-H)_{i+1}-(C=O)_i-$ peptide linkage between residues $i+1$ and i (Figure 1) and reflect a variation in the tilt of the peptide carbonyls in the two environments.[11−13] For all residues i that

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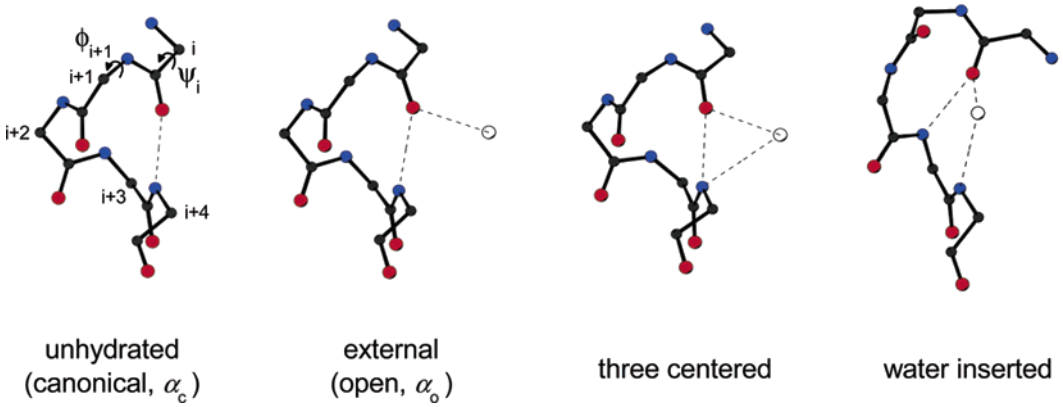

Figure 1. MOLSCRIPT diagrams of a segment of unhydrated (canonical, α_c) α-helix, together with segments displaying the external (open, α_o), three centered and water inserted types of hydration observed in the protein X-ray crystal structures analyzed in refs 11 and 14. All are drawn from X-ray crystal data as follows. Unhydrated: residues 20 to 24 in avian pancreatic polypeptide,[11] PDB code 1ppt. External: residues 102 to 106 in haemoglobin,[14] PDB code 1ecd. Three centered: residues 86 to 90 in troponin c,[14] PDB code 4tnc. Water inserted: residues 173 to 177 in tonin,[14] PDB code 1ton.

have hydrogen bonds to water or hydrophilic side chains, the mean values of ϕ_{i+1}, ψ_i are −66 (±5)°, −41 (±6)°, with n = 3.62 for the corresponding α-helix; whereas for residues without such hydrogen bonds the values are −59 (±6)°, −44 (±6)°, with n = 3.55.

In a detailed analysis of the hydration of α-helices in a set of 35 high-resolution protein X-ray crystal structures, Sundaralingam and Sekharudu[14−16] identified the three types of hydrogen bonding interactions between water molecules and the peptide backbone illustrated in Figure 1. In *external* hydration, the $(C{=}O)_i$ group already engaged in intrachain helix hydrogen bonding to $(NH)_{i+4}$ forms a hydrogen bond with an external water molecule. In *three-centered* hydration, besides the carbonyl group, the amide group as well forms a hydrogen bond with water molecules and hence participates in a bifurcated or three centered hydrogen bond. In *water inserted* hydration, the water molecule inserts itself between the $(C{=}O)_i$ and $(NH)_{i+4}$ groups, replacing the $i \rightarrow i{+}4$ α-helix hydrogen bond with a hydrogen bonded bridge. Water inserted hydration of α-helices in protein crystal structures generates a range of conformations including 3_{10}-helical elements (equivalent to type III turns) and type I turns containing $i \rightarrow i{+}3$ hydrogen bonds stabilized by the inserted water molecule, and extended β-strands.[14−16] Since these hydrated structures occupy contiguous regions of the Ramachandran surface, Sundaralingam and Sekharudu proposed that they may represent intermediates in the helix-coil transition pathway and hence provide insight into the role of the aqueous medium in promoting helix folding-unfolding.[14−16]

The external type of backbone hydration is much more common than the other two types and could be especially relevant to aqueous solution studies. It is present on the hydrophilic side of amphipathic α-helix, which has a distinct hydrophobic side protected from water and a distinct exposed hydrophilic side, and leads to helix bending due to slightly different Ramachandran ϕ,ψ angles on the two sides.[11−13] Other factors may contribute to helix bending: for example, hydrophilic side chains may also form hydrogen bonds with the peptide backbone in order to satisfy their hydrogen bonding potential.[3,18,19] However, as emphasized by Parthasarathy et al.,[13] it should not be assumed that there always will be a difference in the conformational parameters between the hydrophilic and hydrophobic sides of the helix since solvation can occur for hydrophobic as well as hydrophilic residues (the X-ray crystal studies revealed approximately equal numbers of hydrophobic and hydrophilic amino acid residues solvated by water molecules[14]).

That these X-ray studies of hydration in protein crystals have relevance to aqueous solution structure is indicated by recent ESR studies of double spin-labeled alanine rich peptides in aqueous solution that identified a new more open conformation of the α-helix, the experimental data being consistent with a model having ϕ,ψ angles of −70°, −45° and n = 3.8−3.9.[20,21] Computer modeling indicates that this more open geometry leaves the intramolecular hydrogen bonding network intact but changes the O=C−N angle resulting in a splaying of the backbone amide carbonyls away from the helix axis and into the solution. This more open structure may be the more preferred conformation in aqueous solution as it allows hydrogen bonding of the amide carbonyls with water molecules similar to the external hydration observed in X-ray crystal structures.

It therefore appears that we should allow for two distinct types of α-helix: a canonical form α_c that is favored in a hydrophobic or nonpolar environment and a more open form α_o favored in a hydrophilic or polar environment (Figure 1). Because of the small geometrical differences, barely perceptible in Figure 1, it is difficult to distinguish between the two conformations experimentally. However, the chiroptical technique of vibrational Raman optical activity (ROA), one manifestation of which is a small difference in the intensity of Raman scattering from chiral molecules in right- and left-circularly polarized incident light,[22−27] may have the necessary sensitivity. ROA can be applied to most biomolecules in aqueous solution and is an incisive probe of their structure and behavior.[28,29] In this paper we report an ROA

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study of selected polypeptides, proteins and viruses, most in aqueous but some in nonaqueous solution, supplemented by protein X-ray crystal structure data, to investigate α-helix hydration and to seek evidence for the putative α-helix conformations α_c and α_o.

Experimental Section

The polypeptide samples were purchased from Sigma and used without further purification. Dichloracetic acid (>99%) and $CHCl_3$ (Analar grade) were purchased from Riedel-de Haën and Hopkin & Williams, respectively. Poly(L-lysine) and poly(L-glutamic acid) support α-helical states at alkaline pH and low temperature and acid pH and ambient temperature, respectively.[30] The behavior of poly(L-ornithine) is similar in this respect to that of poly(L-lysine).[31] The α-helical states of poly(L-lysine), M_W 56 000, and poly(L-ornithine), M_W 33 000, were prepared by dissolving the dry material in distilled deionized H_2O and adjusting the pH to 11.0 with NaOH while maintaining a temperature of ~3 °C. The α-helical state of poly(L-glutamic acid), M_W 33 000, was prepared by dissolving the dry material in distilled deionized H_2O and adjusting the pH to 4.6 with HCl at room temperature. Solutions of α-helical poly(γ-benzyl L-glutamate),[32] M_W 26 000, and poly(β-benzyl L-aspartate),[32,33] M_W 26 000, were prepared by dissolving the dry material in $CHCl_3$; and for α-helical poly(L-alanine),[34] M_W 21 400, by dissolving in both neat dichloracetic acid and 30% dichloracetic acid/70% $CHCl_3$ (v/v). The protein and virus ROA spectra discussed in this paper were recorded in earlier studies and we refer to the associated publications, referenced later, for details of sample preparation.

The aqueous polypeptide solutions were mixed with a little activated charcoal to remove traces of fluorescing impurities, and centrifuged. The aqueous and nonaqueous solutions, with concentrations typically ~75 mg/mL, were subsequently filtered through 0.22 and 0.5 μm Millipore filters, respectively, directly into rectangular quartz microfluorescence cells which were centrifuged gently before mounting in the ROA instrument. All the polypeptide ROA spectra were measured at room temperature (~20 °C) except for poly(L-lysine) and poly(L-ornithine) which were studied at ~3 °C.

The instrument used for the Raman and ROA measurements has a backscattering configuration, which is essential for aqueous solutions of biomolecules, and employs a single-grating spectrograph fitted with a backthinned CCD camera as detector and a holographic notch filter to block the Rayleigh line.[35] ROA is measured by synchronizing the Raman spectral acquisition with an electrooptic modulator which switches the polarization of the incident argon-ion laser beam between right- and left-circular at a suitable rate. The spectra are displayed in analog-to-digital counter units as a function of the Stokes Raman wavenumber shift with respect to the exciting laser wavenumber. The ROA spectra are presented as circular intensity differences $I^R - I^L$ and the parent Raman spectra as circular intensity sums $I^R + I^L$, where I^R and I^L are the Raman-scattered intensities in right- and left-circularly polarized incident light, respectively. The experimental conditions were as follows: laser wavelength 514.5 nm; laser power at the sample ~700 mW; spectral resolution ~10 cm^{-1}; acquisition time ~10 h for the new polypeptide ROA spectra. For ROA measurements other than at room temperature, dry temperature controlled air was blown over the sample cell using an FTS Systems Model TC-84 Air Jet Crystal Cooler.

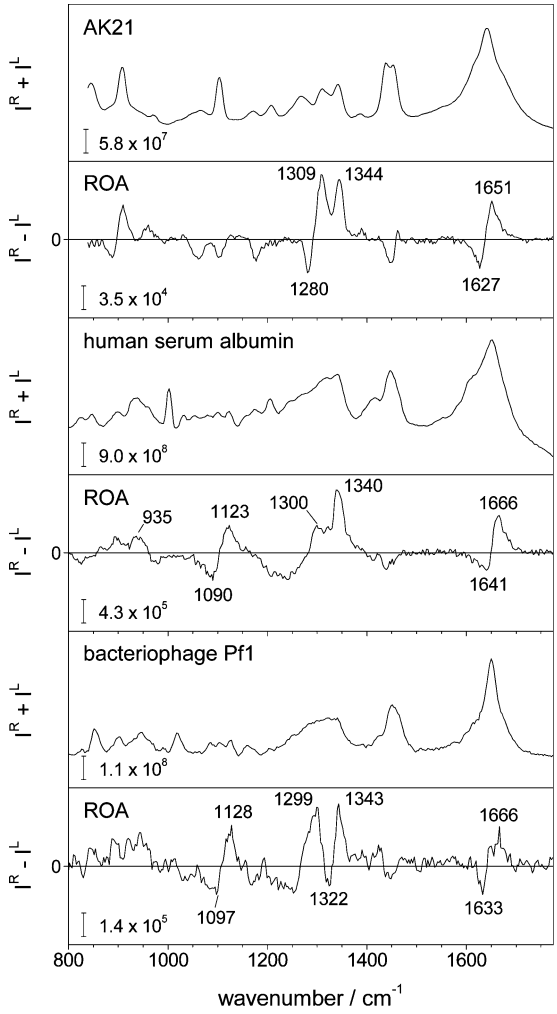

Figure 2. Backscattered Raman ($I^R + I^L$) and ROA ($I^R - I^L$) spectra of the alanine-rich peptide AK21, human serum albumin and filamentous bacteriophage Pf1 in aqueous solution.

Results and Discussion

Setting the Scene: Polypeptide, Protein and Virus ROA Spectra Displaying Bands Assigned to α-Helix in Different Environments. Polypeptides in model α-helical conformations, and proteins rich in α-helix, show a highly characteristic ROA band pattern,[28,29] quite different from those generated by β-sheet[36] and disordered structure,[37] that enables α-helix to be easily recognized. Three previously published examples, namely the alanine-rich peptide Ac−AAKAAAAKAAAAKAAAAK-AGY−NH_2 (AK21),[38] human serum albumin (HSA),[28] and the filamentous bacterial virus Pf1,[39] all in aqueous (H_2O) solution, are displayed in Figure 2. Focusing initially on HSA, the backscattered Raman and ROA spectra of which are shown as the middle pair of Figure 2, individual ROA bands characteristic

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of α-helix include the broad positive band with a peak at ~935 cm^{-1} and the couplet centered at ~1103 cm^{-1}, negative at low wavenumber and positive at high, both features being assigned to mainly $C_\alpha-C$, $C_\alpha-C_\beta$, and $C_\alpha-N$ stretching of the peptide backbone; the positive bands at ~1300 and 1340 cm^{-1} assigned to extended amide III type modes[40,41] involving the in-phase combination of the in-plane N−H deformation with the C−N stretch plus $C_\alpha-H$ deformations; and the couplet centered at ~1650 cm^{-1}, negative at low wavenumber and positive at high, arising from amide I modes involving the C═O stretch. Similar bands are observed in the ROA spectra of other α-helical proteins. Likewise for viruses with α-helical protein folds such as Pf1 and other filamentous bacterial viruses[39,42,43] the major coat proteins of which have extended α-helix folds, as well as tobacco mosaic virus and similar viruses[43−45] the coat proteins of which have helix bundle folds.

We generally observe the HSA type of overall ROA band pattern, albeit with small wavenumber shifts in the constituent bands, in heteropolypeptide α-helical sequences as in proteins; whereas in α-helical homopolypeptides, the backbone skeletal stretch region is sometimes rather different presumably due to contributions from the particular side chains (in heteropolypeptides the ROA contributions from side chains in this region appear to largely cancel each other out). Examples of this may be seen by comparing the ROA spectrum of AK21 with those of HSA and Pf1 in Figure 2. But in all cases, the general patterns in the extended amide III and the amide I regions are similar, consistent with the dominance of peptide backbone, rather than side chain, modes in the generation of most of the ROA bands in these regions.

The strong sharp positive ROA band at ~1340 cm^{-1} was tentatively assigned in early work to an extended amide III type mode in 3_{10}-helical elements:[46] however, this band was subsequently reassigned to a hydrated form of α-helix.[28] The positive ~1340 cm^{-1} band disappears immediately when the peptide, protein or virus is dissolved in D_2O. This indicates first that N−H deformations of the peptide backbone make a significant contribution to the generation of the ~1340 cm^{-1} ROA band because the corresponding N−D deformations contribute to normal modes in a spectral region several hundred wavenumbers lower; and second that, in proteins and viruses, the corresponding sequences are exposed to solvent, rather than being buried in hydrophobic regions where amide protons can take months or even years to exchange. Although the positive ~1300 cm^{-1} α-helix ROA band also changes in D_2O, again suggesting some involvement of N−H deformations, in systems containing a significant amount of α-helix in a protected hydrophobic environment quite a lot of intensity is often retained. Mainly for these reasons, the positive ~1300 and 1340 cm^{-1} ROA bands have been assigned to unhydrated and hydrated forms of α-helix, respectively,[28] and may correspond to the canonical and more open variants α_c and α_o, respectively, as described in the Introduction.

Filamentous bacterial viruses provide especially good examples of the distinct unhydrated and hydrated α-helix ROA bands,[39] and were instrumental in providing the initial insight. These viruses are long slender flexible rods consisting of a loop of single stranded DNA surrounded by a sheath made up of several thousand copies of the same major coat proteins each containing ~50 amino acid residues. X-ray fiber diffraction has shown that the major coat proteins have the fold of an extended helix and that within the sheath these helical coat proteins overlap each other like fish scales so that one-half of each major coat protein is exposed to water and the other half is protected.[47] The water-exposed half of the major coat proteins contains mixed hydrophilic and hydrophobic residues, whereas the buried half contains almost entirely hydrophobic residues. It is therefore compelling to assign the strong positive ~1300 and ~1340 cm^{-1} ROA bands in these viruses, illustrated in Figure 2 for Pf1, to these buried and exposed sequences, respectively.

Comparison of tobacco mosaic virus (TMV) with potato virus X (PVX) provides another good example from the virus world. Both show ROA spectra characteristic of helix bundle coat proteins.[45] However, the positive ~1340 cm^{-1} ROA band in PVX is significantly more intense than that in TMV. The implied increased hydration of the α-helices in the coat proteins of PVX is consistent with the characteristics of the PVX virus particles, which are flexuous filaments, compared with those of TMV, which are rigid rods, and with the greater solvent exposure of the PVX coat proteins implied by the deep grooves observed in the viral surface by X-ray fiber diffraction[48] and which have no counterpart in TMV.

Examples of Homopolypeptides in Aqueous Solution: Poly(L-lysine), Poly(L-ornithine) and Poly(L-glutamic Acid). Poly(L-lysine) (PLL) at alkaline pH, poly(L-ornithine) (PLO) at alkaline pH and poly(L-glutamic acid) (PLG) at acid pH have neutral side chains R = $(CH_2)_4NH_2$, $(CH_2)_3NH_2$ and $(CH_2)_2$-CO_2H, respectively, and so can support helical structures.[49] Figure 3 shows the backscattered Raman and ROA spectra of these three polypeptides in α-helical conformations. The new PLL and PLG ROA spectra are very similar to those published previously;[28] but no ROA spectra of PLO have been published before.

Remarkably, the ROA spectrum of α-helical PLG does not contain a counterpart of the positive α-helix band at ~1297 cm^{-1} present in the ROA spectrum of α-helical PLL. Instead, the spectrum in the extended amide III region contains just a single strong positive band at ~1341 cm^{-1}. This suggests that the positive ~1297 and 1341 cm^{-1} ROA bands in α-helical PLL originate in different variants of the α-helix and that only one variant is present in α-helical PLG. Although the linear side chains in both lysine and glutamic acid residues permit entry of a water molecule,[15] the peptide backbone is expected to be more accessible to solvent water in PLG than in PLL because the nonpolar parts of the side chains are shorter. This reinforces the proposition that the positive ~1341 cm^{-1} ROA band originates in a hydrated form of α-helix (α_o) and that at

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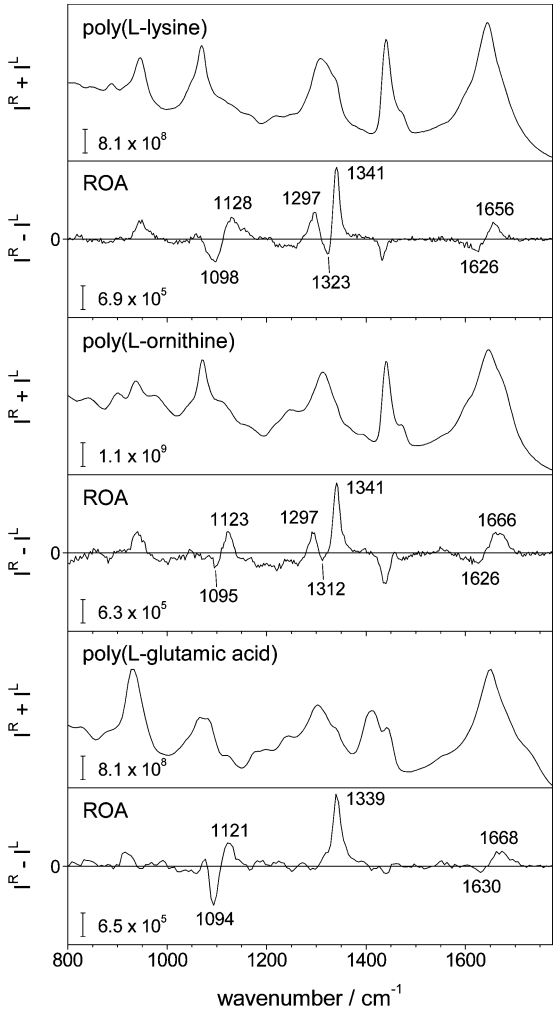

Figure 3. Backscattered Raman and ROA spectra of α-helical poly(L-lysine), poly(L-ornithine) and poly(L-glutamic acid) in aqueous solution.

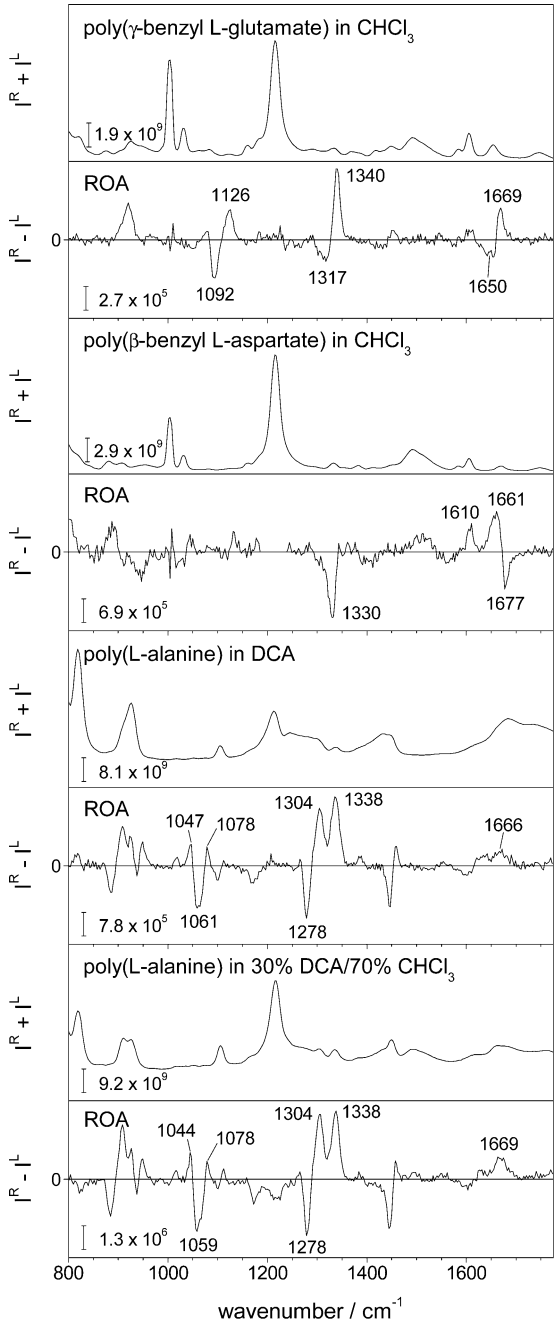

Figure 4. Backscattered Raman and ROA spectra of α-helical poly(γ-benzyl L-glutamate) in $CHCl_3$, poly(β-benzyl L-aspartate) in $CHCl_3$, poly(L-alanine) in dichloracetic acid, and poly(L-alanine) in 30% dichloracetic acid/70% $CHCl_3$. The poly(β-benzyl L-aspartate) α-helix is left-handed! A polarization artifact associated with the intense solvent Raman band at ∼1215 cm^{-1} has been deleted from the ROA spectrum of poly(β-benzyl L-aspartate).

∼1297 cm^{-1} originates in an unhydrated form (α_c). This proposition is further supported by the ROA spectrum of PLO in which the intensity of the positive ∼1297 cm^{-1} ROA band is ∼23% less than that in PLL, which is consistent with one less CH_2 group in the side chain since this would permit greater penetration of water to the backbone. The polar ends of the side chains could also interact with the backbone, thereby substituting for solvent water: in this respect, hydrogen bonding between peptide backbone amide carbonyl groups and protonated glutamic acid side chains is especially favorable.[3] The samples of PLL and PLO may therefore contain different equilibrium mixtures of residues with ϕ, ψ angles in the α_c and α_o conformations, whereas the sample of PLG may contain only α_o residues. It seems more likely that each homopolymer molecule contains a mixture of α_c and α_o residues rather than the sample being a mixture of molecules each made up purely of α_c or α_o residues.

Recent simulations of backbone hydration in an alanine-rich α-helical peptide containing both lysine and glutamic acid residues found that lysine shielded backbone hydrogen bonds from water much more than did glutamic acid.[50] As well as supporting the proposition put forward in the previous paragraph, this could also help to explain the rather large positive ∼1300 cm^{-1} band in the ROA spectrum of AK21 (Figure 2).

Examples of Homopolypeptides in Nonaqueous Solvents: Poly(γ-benzyl L-glutamate), Poly(β-benzyl L-aspartate) and Poly(L-alanine). Poly(γ-benzyl L-glutamate) (PBLG) is known to take up an α-helical conformation in organic solvents such as $CHCl_3$.[32] The backscattered ROA spectrum of PBLG in $CHCl_3$ shown in Figure 4 is very similar to that of PLG in aqueous solution in Figure 3, consistent with an α-helical conformation. However, despite no water being present, like in the PLG spectrum there is no positive ROA band at ∼1300

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cm^{-1} assigned to unhydrated α-helix. This may be due to the additional complexity introduced by the aromatic rings, which could interact with each other and with the backbone; but we have no definitive explanation for this observation.

Poly(*β*-benzyl L-aspartate) (PBLA) is thought to take up a left-handed α-helical conformation in $CHCl_3$.[32,33] The backscattered Raman and ROA spectra of this system are also shown in Figure 4, and it is reassuring that the ROA spectrum is similar to that of PBLG in the extended amide III and amide I regions, albeit with small wavenumber shifts, but with all the signs reversed! This suggests that the magnitudes of the ϕ, ψ angles of the associated left-handed α-helix correspond more closely to those of right-handed α_o than those of right-handed α_c. Whatever mechanism is responsible for the absence of a positive ∼1300 cm^{-1} ROA band in PBLG may also be operative in PBLA since it shows no negative ROA band at this wavenumber.

The vibrational modes of the α-helical structure that poly(L-alanine) (PLA), the simplest L-type polypeptide, is known to take up in strongly polar organic solvents such as dichloracetic acid (DCA)[32] have been extensively studied.[51−53] The α-helical state of PLA is therefore an important model system for ROA studies, but its ROA spectrum has not been published previously. The backscattered Raman and ROA spectra of α-helical PLA in pure DCA and in a mixture of 30% DCA and 70% $CHCl_3$ are shown in Figure 4. The ROA band structure in the extended amide III region is very similar to that of AK21 in aqueous solution (Figure 2) with strong positive bands at ∼1304 and 1338 cm^{-1}. In the DCA/$CHCl_3$ mixture, these two bands are of approximately equal intensity; but in pure DCA, the intensity of the lower wavenumber band is ∼17% less. Again, this observation is consistent with the presence of an equilibrium between residues in the α_c and α_o conformations characterized by the positive ∼1304 and 1338 cm^{-1} ROA bands, respectively, since in pure DCA, which is much more polar than $CHCl_3$, the equilibrium would be expected to shift toward α_o.

Examples of Proteins in Aqueous Solution: Lysozymes and α-Lactalbumins. Hen lysozyme was the first protein on which ROA measurements were performed,[54] with the original spectra being dominated by the positive ∼1300 and 1340 cm^{-1} α-helix bands under discussion. Subsequently, the lysozymes, together with the α-lactalbumins which have a very similar fold, have been studied extensively by ROA. Because they have also been studied extensively by other techniques, including X-ray crystallography and NMR, these proteins provide a good opportunity for attempting to obtain insight at atomic resolution into the structural differences between α-helices generating the positive ∼1300 and 1340 cm^{-1} ROA bands. Accordingly, we display in Figure 5 the backscattered aqueous solution Raman and ROA spectra of hen, human and equine lysozyme, together with that of bovine α-lactalbumin, all published previously.[38,55] It is intriguing that all four contain two strong positive ROA bands in the extended amide III region at wavenumbers close to those in the polypeptides above, but with large variations in the relative intensities despite all having similar amounts of α-helix (∼30%) and other types of structural element within the same basic fold as determined by X-ray crystallography. The X-ray crystal structure of hen lysozyme is displayed as a MOLSCRIPT diagram[56] in Figure 6 to illustrate the lysozyme/α-lactalbumin fold.

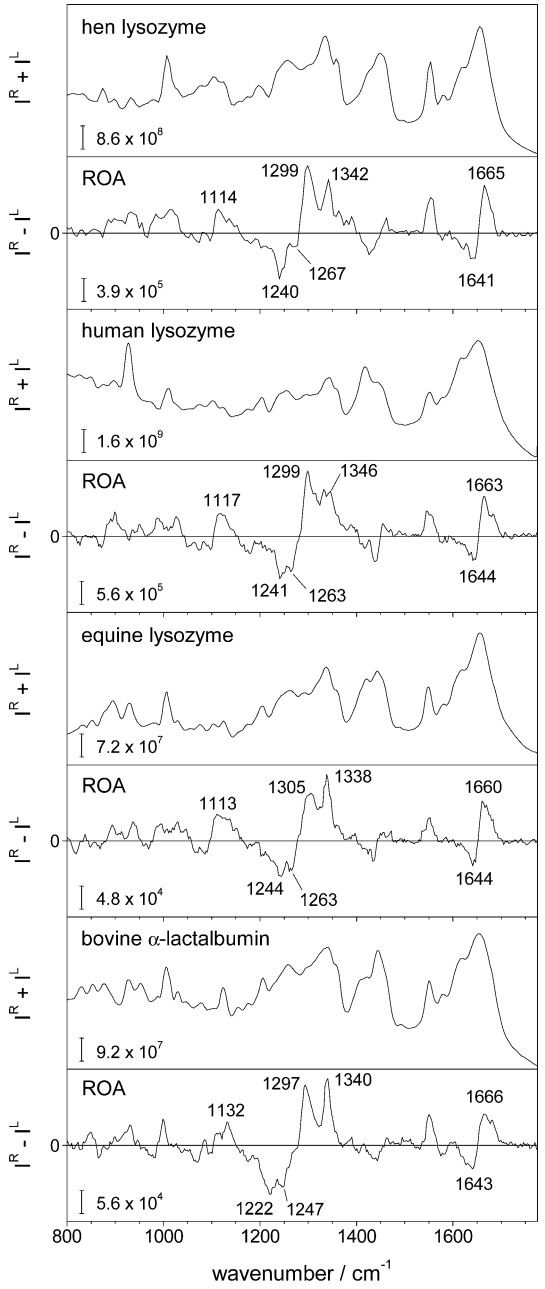

Figure 5. Backscattered Raman and ROA spectra of hen, human, and equine lysozyme, and of bovine α-lactalbumin, in aqueous solution.

The positive ∼1340 cm^{-1} ROA band is significantly more intense in bovine α-lactalbumin than in hen and human lysozyme. That this is not due to a subtle difference between the lysozyme fold and the α-lactalbumin fold is evidenced by the fact that the positive ∼1340 cm^{-1} band in equine lysozyme is similarly enhanced. The α-lactalbumins generally have greater flexibility relative to hen and human lysozyme, as judged by their average amide hydrogen−deuterium exchange protection

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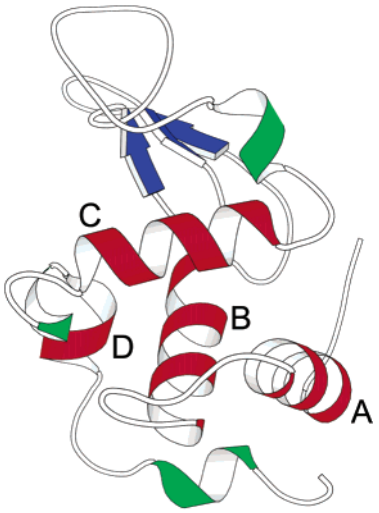

Figure 6. MOLSCRIPT diagram of the X-ray crystal structure of hen lysozyme (PDB code 1iee).

factors, measured by NMR, being 2 orders of magnitude smaller[57] and from large temperature factors in certain regions of their X-ray crystal structures.[58] The behavior of equine lysozyme is similar to that of the α-lactalbumins in this respect.[59] This would be consistent with the presence of more hydrated/exposed α-helix, as monitored by the enhanced positive ∼1340 cm^{-1} ROA bands in bovine α-lactalbumin and equine lysozyme.

Unfortunately, it was not possible to analyze directly the positions of water molecules bound to α-helices in the X-ray crystal structures of these proteins because of insufficient data quality of the best PDB files available. However, we obtained the mean of the angles ϕ_{i+1}, ψ_i for the residues within the α-helices (A, B, C, and D in Figure 6) which, as outlined in the Introduction, reflect differences in the tilt of the peptide backbone carbonyls in hydrophilic and hydrophobic environments. These are −66.9°, −39.6° for hen lysozyme (PDB code 1iee); −66.4°, −39.3° for human lysozyme (PDB code 1rex); −71.6°, −36.3° for bovine α-lactalbumin (PDB code 1hfz); and −70.7°, −38.6° for equine lysozyme (PDB code 2eql). According to the general trends mentioned in the Introduction (and bearing in mind the uncertainties of ∼±6° when considering the actual magnitudes), the larger mean negative ϕ_{i+1} and the smaller mean negative ψ_i angles for the α-helical residues in bovine α-lactalbumin and equine lysozyme compared with hen and human lysozyme correspond, on average, to a more hydrophilic environment in the first two proteins. This distinct shift of mean ϕ_{i+1}, ψ_i values for α-helical residues in bovine α-lactalbumin and equine lysozyme toward mean 'hydrophilic' values compared with hen and human lysozyme again correlates with the presence of more hydrated/exposed α-helix, as monitored by the enhanced positive ∼1340 cm^{-1} ROA bands in bovine α-lactalbumin and equine lysozyme. Because these protein X-ray crystal data reflect a variation in the tilt of the peptide carbonyls in the two environments, they are consistent with the assignment of the positive ∼1300 and 1340 cm^{-1} ROA bands in the corresponding protein ROA spectra in Figure 5 to residues that adopt conformations associated with α_c and α_o, respectively.

All four proteins show a very similar amide I ROA couplet centered at ∼1650 cm^{-1}, negative at low wavenumber and positive at high, which suggests that unhydrated and hydrated α-helix are not immediately distinguished by amide I ROA bands.

Vibrational Analysis of the α-Helix. Experimental[52] and theoretical[53] infrared and Raman studies of α-helical PLA performed on oriented films prepared from solutions in DCA have provided assignments of a number of the normal modes of vibration in the extended amide III region over the range in which we have identified α-helix ROA bands. These normal modes variously transform as the *A*, E_1 and E_2 symmetry species of the point group $C_{k/m}$ (for a helix with *k* residues in *m* turns) of a model infinite regular helix, the *A* and E_1 species being infrared active and *A*, E_1 and E_2 being Raman active. Of possible relevance here are a fundamental observed at 1306 cm^{-1} assigned to an *A* mode based mainly on $C_\alpha-H$ bending coordinates, and one observed at 1337 cm^{-1} assigned to an E_2 mode based mainly on $C_\alpha-H$ bending and $C_\alpha-C$ stretching coordinates.[52,53] In addition, a Raman band observed at ∼1340−1345 cm^{-1} in oriented α-helical polypeptides, proteins and filamentous viruses has been recognized as an α-helix marker and assigned to a coupled $C_\alpha-H$ bending and $C_\alpha-C$ stretching mode.[60] A more detailed analysis[61] on PLA and PBLG assigned it to an E_2 type mode localized in the $(O{=}C)-C_\alpha-H$ main chain network. On account of the large N-deuteration effect observed in the associated ROA band,[28,39] the possibility of a contribution from an N−H bending coordinate was acknowledged.[61]

However, the possible presence of two conformations α_c and α_o complicates the analysis. In fact, multiple bands have been observed in the infrared spectra of oriented films of PLA with relative intensities that depend on the residual solvent DCA content and which have been attributed to the presence of a structure in addition to the standard α-helical PLA structure assumed for the initial vibrational analysis.[62] Moreover, the normal mode description for long uniform helical *homo*polypeptides may not be valid for the more irregular helical *hetero*polypeptide sequences found in proteins. A further complication is that perturbations from the solvent water or DCA molecules responsible for stabilizing the α_o conformation will reduce the effective symmetry of the α-helix. It should also be appreciated that, due to the different selection rules for Raman scattering via the polarizability and optical activity tensors,[23−27] an intense ROA band may be associated with a weak or even unobserved Raman band, and vice-versa. In view of all these factors, no attempt is made here to provide definitive assignments of the positive ∼1300 and 1340 cm^{-1} ROA bands or to suggest mechanisms for how the ROA intensities are generated.

Conclusions

On the basis of ROA data from polypeptides, proteins, and viruses, together with data from other studies including X-ray, ESR, Raman, and infrared, it appears that the ROA band structure in the extended amide III region can distinguish

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α-helical residues in a hydrophobic or nonpolar environment from α-helical residues in a hydrophilic or polar environment. The first are identified by a positive ROA band at ~1300 cm^{-1} and may correspond to the canonical structure α_c; the second are identified by a positive ROA band at ~1340 cm^{-1} and may correspond to a more open conformation α_o that is stabilized by interactions with water molecules, polar organic solvent molecules or polar side chains. Small variations of geometry within α_c and α_o may be responsible for wavenumber shifts of up to ± 5 cm^{-1} around these nominal band positions. Although the external type of hydration illustrated in Figure 1 is most likely to be associated with α_o, other types of hydration such as three centered may also be present and may be responsible for wavenumber shifts, band broadenings and splittings.

Although it appears to be possible to study the α_o conformation in isolation via fully hydrated polypeptides such as PLG in aqueous solution, the α_c conformation is harder to study in isolation and this was not achieved in the present work. However, samples such as alanine-rich polypeptides and PLA itself, together with lysozymes and α-lactalbumins, and especially filamentous bacterial viruses, appear to contain large amounts of α_c that are easily distinguished from α_o by means of ROA.

The ability of ROA to distinguish hydrated from unhydrated α-helix will be valuable in studies of the aqueous solution structure and behavior of proteins. An especially interesting aspect of hydrated α-helix is its enhanced susceptibility to unfolding,[14−16] which ROA studies have already suggested may be primarily to the poly(L-proline) II type helix rather than the random coil, and which may have implications for amyloid fibril formation.[38,63]

Acknowledgment. We thank the EPSRC and BBSRC for research grants.

JA048991U

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