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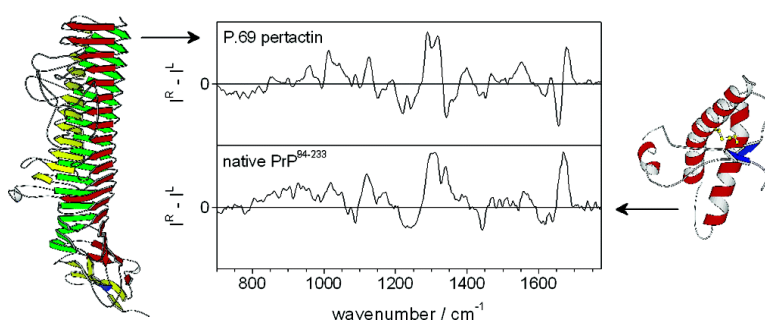# A New Perspective on β-Sheet Structures Using Vibrational Raman Optical Activity: From Poly(l-lysine) to the Prion Protein

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A New Perspective on β-Sheet Structures Using Vibrational Raman Optical Activity: From Poly(L-lysine) to the Prion Protein

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Abstract: The vibrational Raman optical activity (ROA) spectrum of a polypeptide in a model β-sheet conformation, that of poly(L-lysine), was measured for the first time, and the α-helix → β-sheet transition monitored as a function of temperature in H_2O and D_2O. Although no significant population of a disordered backbone state was detected at intermediate temperatures, some side chain bands not present in either the α-helix or β-sheet state were observed. The observation of ROA bands in the extended amide III region assigned to β-turns suggests that, under our experimental conditions, β-sheet poly(L-lysine) contains up-and-down antiparallel β-sheets based on the hairpin motif. The ROA spectrum of β-sheet poly(L-lysine) was compared with ROA data on a number of native proteins containing different types of β-sheet. Amide I and amide II ROA band patterns observed in β-sheet poly(L-lysine) are different from those observed in typical β-sheet proteins and may be characteristic of an extended flat multistranded β-sheet, which is unlike the more irregular and twisted β-sheet found in most proteins. However, a reduced isoform of the truncated ovine prion protein PrP^{94-233} that is rich in β-sheet shows amide I and amide II ROA bands similar to those of β-sheet poly(L-lysine), which suggests that the C-terminal domain of the prion protein is able to support unusually flat β-sheets. A principal component analysis (PCA) that identifies protein structural types from ROA band patterns provides a useful representation of the structural relationships among the polypeptide and protein states considered in the study.

Introduction

The two most common secondary structures found in globular proteins are the α-helix and the β-sheet. The α-helix comprises a single strand of a polypeptide chain and is stabilized mainly by hydrogen bonds between $C{=}O_i$ and NH_{i+4} groups within the same strand. The β-sheet, on the other hand, is built up from two or more different adjacent strands and is stabilized mainly by cross-strand $C{=}O$ to NH hydrogen bonds. The constituent β-strands may be either from the same polypeptide chain, and interspersed by loops and turns, or from different chains, with distinct patterns associated with parallel or antiparallel alignments of the individual strands. Consequently, considerably more structural diversity is found in β-sheet than in α-helix structures, with a variety of twisted and curved surfaces that depend on the number and length of the constituent strands and on the environment of the sheet.[1,2] All such details are revealed by protein structures determined at atomic resolution using X-ray crystallography or, to a lesser extent, by structures determined using multidimensional solution NMR.

Due to their wide general applicability, spectroscopic techniques are used extensively for studies of polypeptide and protein samples, especially in aqueous solution. Although there are assignments for β-sheet bands in infrared absorption (IR),[3] transparent[4] and ultraviolet resonance[5] Raman scattering, ultraviolet circular dichroism (UVCD),[6] and infrared vibrational circular dichroism (VCD),[7] these techniques have difficulty in discriminating between the different structural types of β-sheet

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found in proteins. These difficulties are due in part to overlap of the characteristic β-sheet bands with bands from other types of structures in protein spectra. However, it does appear to be possible to discriminate between some types of β-sheet in model polypeptides using IR and VCD.[8,9]

Spectroscopic methods have been particularly important in the identification of β-sheet in the context of protein misfolding and disease.[10] For example, both UVCD and IR were central to the discovery that the spongiform encephalopathies are associated with the change of the prion protein from its ubiquitous cellular form, PrP^C, which is rich in α-helix, to its amyloid scrapie form, PrP^{Sc}, which possesses elevated levels of β-sheet.[11] As well as being unusually aggressive, prion disease exhibits an infectious form along with genetic and sporadic forms, something not demonstrated in any other class of protein misfolding disease.[11] Although high-resolution structures are now available for refolded, recombinant PrP^C from solution NMR,[12,13] further understanding of the mechanism of prion disease is hampered by the lack of any high-resolution structure of PrP^{Sc} and the inability of spectroscopic techniques to discriminate between the various types of β-sheets that may be present. According to Cohen and Prusiner,[14] if we are to truly understand this disease process at a molecular level, we must endeavor to find routes to solubilize the PrP^{Sc} isoform or to identify polypeptides with PrP^{Sc}-like structures.

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,[15-17] can be applied to most biomolecules in aqueous solution and is an incisive probe of their structure and behavior.[18] However, the detailed characterization of β-sheet using ROA has been held back due to experimental difficulties in measuring the ROA spectra of polypeptides in model β-sheet conformations arising from the linear birefringence properties of the gel-like samples forming at the rather high concentrations currently required for ROA measurements. Thanks to improvements in instrumentation and careful choice of molecular weight, these experimental difficulties have now been overcome, and we accordingly report here the first ROA measurements on a model β-sheet polypeptide, poly(L-lysine), in both H$_2$O and D$_2$O and compare the results with ROA data on a number of proteins containing different types of β-sheet, including a β-sheet-rich isoform of the prion protein.

Methods

Poly(L-lysine) supports a disordered state at low pH and ambient temperature, an α-helix state at high pH and low temperature, and a

β-sheet state at high pH and high temperature.[19] Our sample of poly-(L-lysine), M_w 56 000, was purchased from Sigma. The α-helical state was prepared by dissolving the dry material in distilled deionized H$_2$O at a concentration of ~100 mg/mL and gently adjusting the pH to 11.0 with NaOH while maintaining a temperature of ~3 °C. Deuterated poly-(L-lysine) was prepared by dissolving the dry material in D$_2$O, allowing 24 h for exchange to occur, removing the liberated H$_2$O by lyophilization, and dissolving in D$_2$O. Poly(L-lysine) was first prepared in the α-helical state, and the corresponding Raman and ROA spectra were measured at 3 °C. Spectra over a range of gradually increasing temperatures up to 50 °C were measured subsequently in order to monitor the helix-to-sheet transition.

Commercial samples of jack bean concanavalin A and subtilisin Carlsberg from *Bacillus licheniformis* were supplied by Fluka. Virulence factor P.69 pertactin from *Bordetella pertussis* was expressed in inclusion bodies and purified as described previously.[20] To prepare the N-terminally truncated ovine PrP$^{94-233}$, inclusion bodies from recombinant bacteria expressing ovine PrP$^{94-233}$ were isolated and the protein purified by metal ion affinity followed by cation exchange chromatography. The normal α-isoform was generated by oxidizing the single disulfide bond by addition of an excess of copper ions before extensive dialysis into 50 mM sodium acetate at pH 5.5. To generate the β isoform, the protein was maintained under reducing conditions to prevent disulfide formation and dialyzed against 50 mM sodium acetate plus 5 mM dithiotheitol at pH 4.0 following the method of Jackson et al.[21] The presence or absence of the disulfide bond in the prion proteins was confirmed by mass spectrometry, and UVCD confirmed their tertiary structures. Further details of the preparation of the prion proteins will be given elsewhere.[22] All the proteins were studied at concentrations of ~50 mg/mL in various buffers as specified in the corresponding figure captions.

The instrument used for the Raman and ROA measurements features a backscattering configuration, which is essential for aqueous solutions of biopolymers, and employs a single-grating spectrograph fitted with a backthinned charge-coupled device (CCD) detector and a holographic edge filter to block the Rayleigh line.[23] ROA is measured by synchronizing the Raman spectral acquisition with an electro-optic modulator, which switches the polarization of the incident argon-ion laser beam between right- and left-circular at a suitable rate. One improvement that facilitated the acquisition of reliable ROA data on the gel-like state of β-sheet poly(L-lysine) was the insertion of a rotating half-wave plate into the incident beam after the electro-optic modulator: this averages the azimuths of any residual linear contaminants in the right- and left-circular polarization states of the laser beam and thereby helps to suppress artifacts from linear birefringence.[24] The spectra are displayed in analog-to-digital converter (ADC) units as a function of Stokes wavenumber shift with respect to the exciting laser wavenumber. The ROA spectra are presented as raw circular intensity differences $I^R - I^L$ and the parent Raman spectra as raw 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 times ~10-20 h. All the protein samples remained stable during the measurements despite the high concentrations, apart from concanavalin A, which showed slowly increasing light

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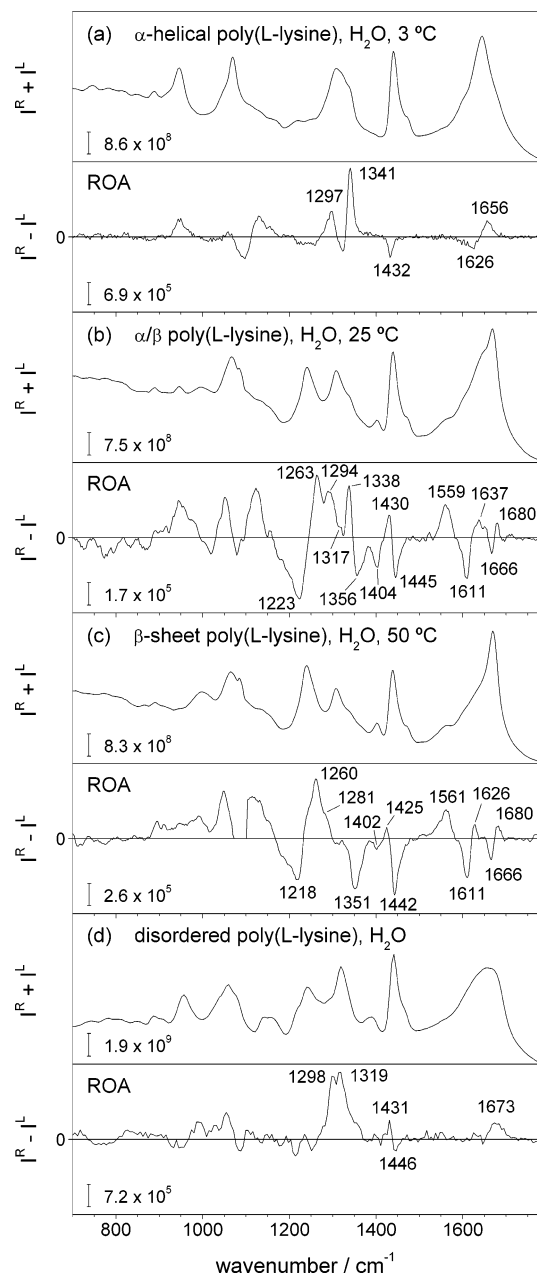(24) Syme, C. D. Doctoral Thesis, University of Glasgow, 2002.

Figure 1. Backscattered Raman ($I^R + I^L$) and ROA ($I^R - I^L$) spectra of poly(L-lysine) in H_2O at pH 11.0 and ~3 °C (a), 25 °C (b), and 50 °C (c) corresponding to α-helical, intermediate, and β-sheet states, respectively, and at pH 1.8 and 20 °C (d) corresponding to the disordered state. A large artifact in the ROA spectrum in (c) associated with a Raman band at ~1089 cm⁻¹ from a calcite component in the collection optics has been removed electronically (similarly in Figure 2a−c).

scattering due to slight aggregation, but no changes were observed in the parent Raman spectrum.

For ROA measurements at other than room temperature (~20 °C), air was blown over the sample cell using an FTS Systems Model TC-84 Air Jet Crystal Cooler, which can supply a stream of dry air over the range −85 to +100 °C.

Results and Discussion

Poly(L-lysine): The α-Helix → β-Sheet Transition. The backscattered Raman and ROA spectra of poly(L-lysine) at pH ~11.0 were measured over a range of temperatures at ~5 °C intervals between 3 and 50 °C; those recorded at 3, 25, and 50 °C are displayed in Figure 1a, b, and c, respectively. The α-helix

state is the most stable at 3 °C and the β-sheet state the most stable at 50 °C.[19] The 3 °C ROA spectrum has the appearance of that of the α-helix state reported previously.[18,25] The 50 °C ROA spectrum is quite different from the 3 °C α-helix spectrum and also from that of the disordered state, reported previously[18,25] and shown in Figure 1d, so we assume that it is characteristic of β-sheet poly(L-lysine). The parent conventional Raman spectra of the α-helix, β-sheet, and disordered states of poly-(L-lysine) displayed in Figure 1a, c, and d, respectively, are very similar to those in the literature.[26] At the high concentration used, the sample was transformed irreversibly into a gel at 50 °C and the Raman and ROA spectra remained unchanged on cooling the sample back down to 3 °C. Many differences between the ROA spectra of the α-helical, β-sheet, and disordered states of poly(L-lysine) are evident, so that ROA may be used to easily distinguish between them. The ROA spectra of poly(L-lysine) in D_2O solution at 3, 25, and 50 °C corresponding to α-helical, intermediate, and β-sheet states, together with the disordered state in D_2O, are presented in Figure 2. Some of the details of these β-sheet poly(L-lysine) spectra are discussed in the next section. Changes in the ROA spectra of the α-helical and disordered states in D_2O compared with H_2O have been discussed previously.[25]

Previous studies have reported a rather sharp α → β transition in poly(L-lysine) on heating, with no detectable disordered intermediate state.[26−28] Our results are in accord with these earlier reports with regard to backbone structure since the main bands from backbone vibrations in the Raman and ROA spectra measured in H_2O over the temperature range 3−50 °C may be reproduced approximately by taking appropriately weighted combinations of the 3 °C α-helix and 50 °C β-sheet spectra. However, this is not the case for some side chain bands, particularly those in the ~1400−1450 cm⁻¹ range arising from CH_2 deformations. This is especially noticeable for the ROA spectra measured in D_2O (Figure 2a−c) in the range ~1400−1480 cm⁻¹, where large ROA signals are generated from mixing of these side chain modes with the amide II′ vibration.

Comparison of β-Sheet Poly(L-lysine) ROA with that of β-Sheet Proteins. The backscattered Raman and ROA spectra of jack bean concanavalin A, subtilisin Carlsberg, P.69 pertactin, and the empty protein capsid of bacteriophage MS2 (published previously[29]) are shown in Figure 3 as examples of the spectral band patterns observed for proteins containing distinct types of β-sheet. According to the Protein Data Bank (PDB) X-ray crystal structure 1nls, jack bean concanavalin A contains 44.7% β-strand in antiparallel sheets, 0.0% α-helix, and 5.1% 3_{10}-helix and belongs to the mainly β-class with a jelly roll β-sandwich fold.[30] The PDB crystal structure 1sca of subtilisin Carlsberg contains 17.2% β-strand mostly in the form of parallel sheets, 29.6% α-helix, and 0.0% 3_{10}-helix and belongs to the αβ-class. The PDB crystal structure 1dab of P.69 pertactin contains 52.9% β-strand in parallel sheets, 0.0% α-helix, and 1.5% 3_{10}-helix

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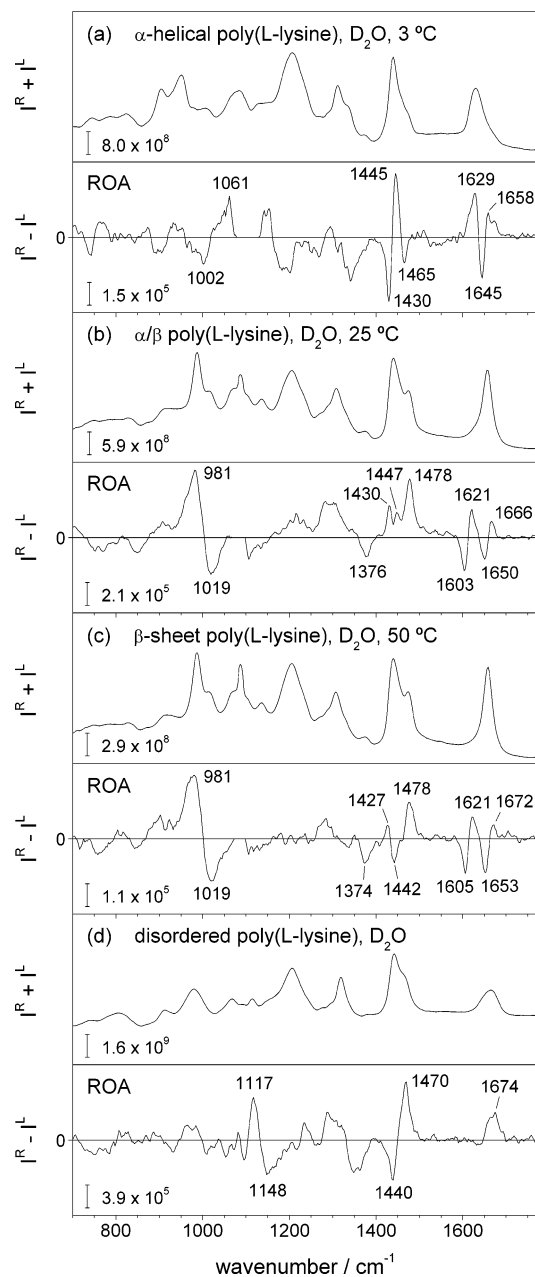
Figure 2. Backscattered Raman and ROA spectra of poly(L-lysine) in D$_2$O at pD 11.4 and ∼3 °C (a), 25 °C (b), and 50 °C (c) corresponding to α-helical, intermediate, and β-sheet states, respectively, and at pD 3.0 and 20 °C (d) corresponding to the disordered state.
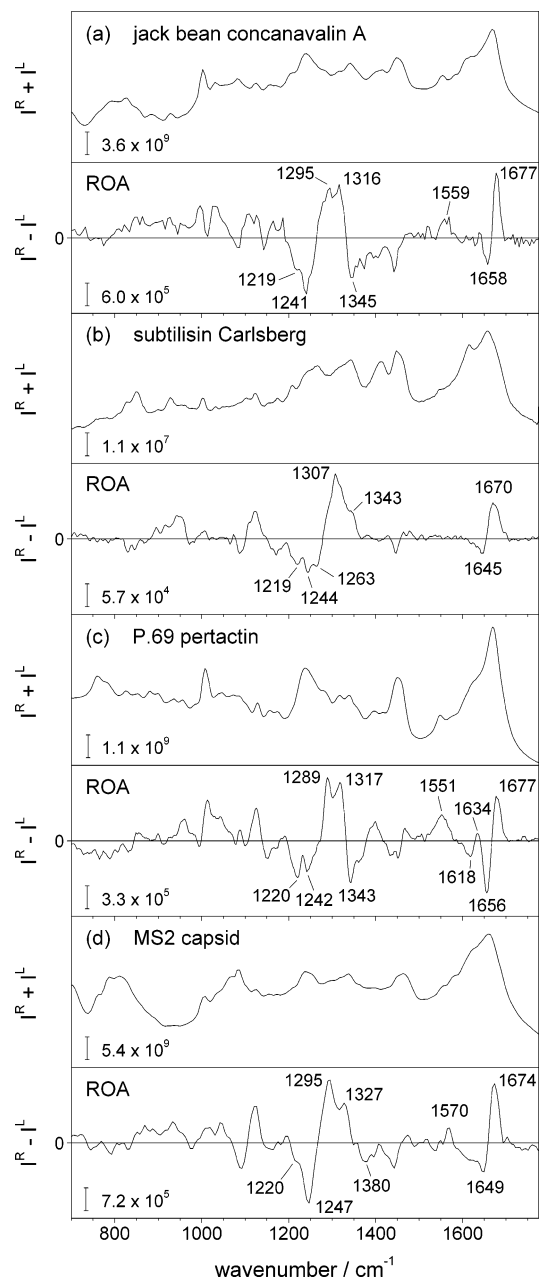

Figure 3. Backscattered Raman and ROA spectra of (a) jack bean concanavalin A in 0.1 M acetate buffer at pH 5.4, (b) subtilisin Carlsberg in 0.1 M acetate buffer at pH 5.4, (c) P.69 pertactin in 50 mM Tris/HCl at pH 8.8, and (d) empty bacteriophage MS2 protein capsid in 50 mM Tris/HCl at pH 7.2.

and belongs to the mainly β-class with a β-helix fold.[30] The PDB crystal structure 2ms2 of the MS2 capsid protein subunit contains 46.5% β-strand in up-and-down antiparallel sheets, 16.3% α-helix, and 2.3% 3$_{10}$-helix and belongs to the αβ-class with an unusual fold seen in the peptide binding domain of a class 1 major histocompatibility antigen.[30] MOLSCRIPT diagrams[31] of these structures are provided in Figure 4.

Vibrations of the backbone in polypeptides and proteins are usually associated with three main regions of the Raman spectrum.[4,32,33] These are the backbone skeletal stretch region ∼870−1150 cm^{-1}, originating mostly in the C$_\alpha$−C, C$_\alpha$−C$_\beta$,

and C$_\alpha$−N stretches; the amide III region ∼1230−1310 cm^{-1}, often assigned mostly to the in-phase combination of the in-plane N−H deformation with the C−N stretch; and the amide I region ∼1630−1700 cm^{-1}, which arises mostly from the C=O stretch. However, it is now recognized that the amide III region involves more mixing between N−H and C$_\alpha$−H deformations than previously thought and should be extended to at least 1340 cm^{-1}.[32−34] Amide II vibrations, which occur in the region ∼1510−1570 cm^{-1} and are assigned to the out-of-phase combination of the N−H in-plane deformation and the C−N stretch, either are very weak or are not observed at all in the conventional (nonresonance) Raman spectra of proteins,[4] but can be prominent in the UV resonance Raman spectra[5] and, as described below, in the ROA spectra.

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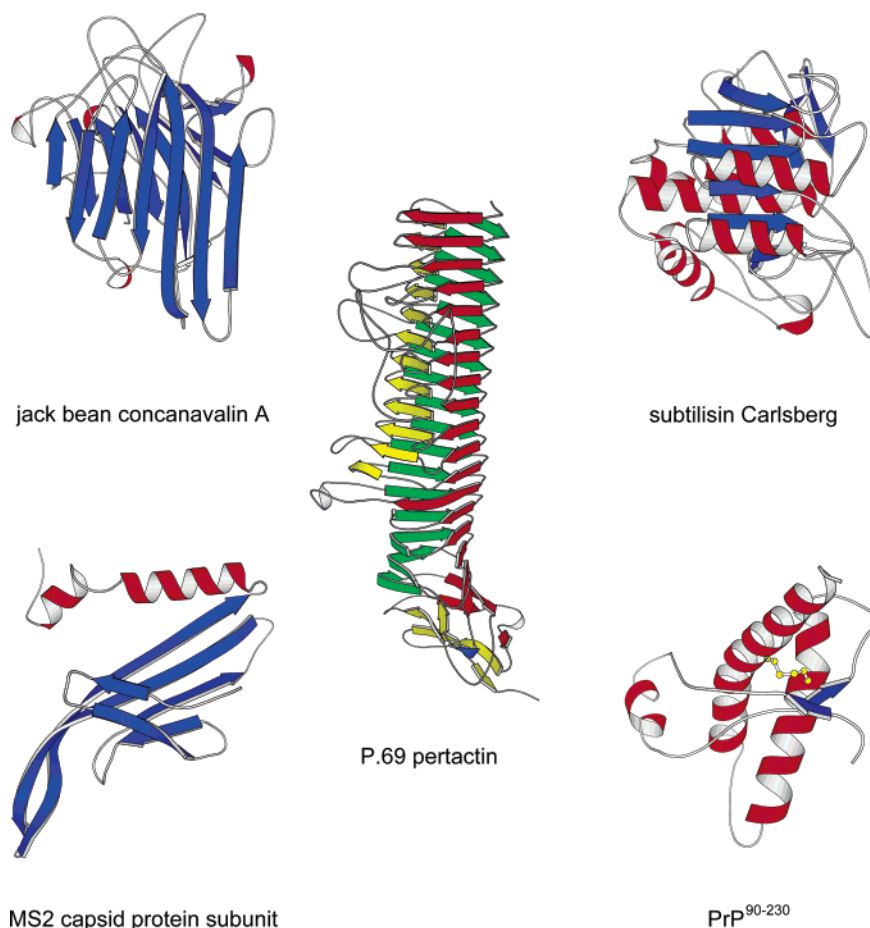
Figure 4. MOLSCRIPT diagrams of jack bean concanavalin A (1nls), subtilisin Carlsberg (1sca), P.69 pertactin (1dab), bacteriophage MS2 capsid protein subunit (2ms2), and human PrP[90-230] (1qm1) in which the single disulfide bond is shown in yellow.

The extended amide III region is particularly important for ROA studies because the coupling between N−H and C_α−H deformations is very sensitive to geometry[34] and generates rich and informative ROA band structure,[18] so this region will be considered first. The negative ROA bands at ∼1219−1247 cm^{-1} in the four proteins in Figure 3 are assigned to β-structure.[18] Since β-turn vibrations have been assigned to the ∼1260−1295 cm^{-1} region in the conventional Raman spectra of proteins,[35] we assign the positive band at ∼1295 cm^{-1} in the ROA spectra of concanavalin A and the MS2 capsid to the β-turns connecting some of the strands within the multistranded antiparallel β-sheet that each contains, and the positive ∼1289 cm^{-1} ROA band in pertactin to the β-turns connecting the strands in the parallel β-sheet making up the β-helix. In combination with the lower-wavenumber negative β-structure bands just described, this serves to generate a large ROA couplet that is probably characteristic of the β-hairpin motif[30] (or, more rarely, the β-helix): an especially clear example is seen in the MS2 capsid with negative and positive peaks at ∼1247 and 1295 cm^{-1}, respectively. Negative ROA bands in the range ∼1340−1380 cm^{-1} also appear to originate in similar β-turns, examples being seen in the ROA spectra of concanavalin A, pertactin, and the MS2 capsid in Figure 3. ROA band patterns similar to those seen in concanavalin A and the MS2 capsid in the region

∼1220−1380 cm^{-1} are observed in all the proteins containing multistranded up-and-down antiparallel β-sheet that we have studied, including immunoglobulin,[18] β-lactoglobulin,[18] serum amyloid P-component (unpublished), and avidin (unpublished). These β-turn assignments would explain the absence of similar positive bands in the range ∼1260−1295 cm^{-1} and negative bands in the range ∼1340−1380 cm^{-1} in the ROA spectrum of subtilisin Carlsberg since the parallel sheet in this protein is based on the β−α−β motif[30] in which the parallel strands are connected by α-helix loops. These bands are absent in the ROA spectra of other proteins we have studied having parallel β-sheet based on the β−α−β motif, including aldolase[18] and the receiver domain from MicA (unpublished), a response regulator from *Streptococcus pneumoniae*.[36]

The extended amide III region of the ROA spectrum of β-sheet poly(L-lysine) displayed in Figure 1c is dominated by a large couplet, negative at ∼1218 and positive at ∼1260 cm^{-1} and similar to the one observed in the β-sheet proteins but shifted by ∼30 cm^{-1} to lower wavenumber. Amide III bands from β-sheet in conventional Raman spectroscopy are assigned to the region ∼1230−1245 cm^{-1},[4,37] an example being the strong band at ∼1239 cm^{-1} in the parent Raman spectrum of β-sheet poly(L-lysine), which coincides with the center of the large poly-(L-lysine) ROA couplet discussed above. In the ROA spectrum of β-sheet poly(L-lysine) in D_2O, displayed in Figure 2c, both

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this ROA couplet and its associated parent Raman band have disappeared, with a new large couplet positive at ~981 and negative at ~1019 cm⁻¹ appearing that is associated with two new Raman bands at similar wavenumbers. This observation confirms that N−H deformations make significant contributions to the generation of this ROA couplet, with corresponding N−D deformations contributing to the new ROA couplet shifted some 240 cm⁻¹ to lower wavenumber. The ROA spectrum of β-sheet poly(L-lysine) also displays a large negative band at ~1351 cm⁻¹ assigned to β-turns, but it is reassuring that there is no positive ROA signal in the range ~1314−1325 cm⁻¹ as observed in disordered poly(L-lysine) (Figure 1d) and assigned to polyproline II (PPII) helix structure.[18,38] We therefore envisage our sample of β-sheet poly(L-lysine) to contain up-and-down β-sheet based on the hairpin motif. This supports the idea that, at least for poly(L-lysine) samples of molecular weight greater than ~50 000, there is significant *intra*molecular, in addition to *inter*molecular, β-sheet formation as first suggested by Wooley and Holzwarth[28] from UVCD studies as a function of concentration.

In the amide I region a large couplet, negative at ~1658 cm⁻¹ and positive at ~1677 cm⁻¹, in the ROA spectrum of con-canavalin A in Figure 3a is another signature of β-sheet and can be distinguished easily from a similar amide I couplet produced by α-helix, which typically occurs ~10 cm⁻¹ lower.[18] A similar large couplet is observed in the ROA spectrum of the MS2 capsid. This correlates with β-sheet amide I bands in conventional Raman spectroscopy which occur in the range ~1665−1680 cm⁻¹.[4,37] However, the corresponding amide I couplet in pertactin is most unusual in that it displays a much larger negative component that has not been observed in the ROA spectrum of any other β-sheet protein to date and may provide a unique signature of the β-helix fold.

The negative−positive−negative−positive band pattern observed in the range ~1600−1690 cm⁻¹ in the ROA spectrum of β-sheet poly(L-lysine) displayed in Figure 1c is rather different from the band patterns observed in this region in the ROA spectra of the β-sheet proteins displayed in Figure 3 and may be characteristic of extended flat multistranded β-sheet, the wavenumber range being similar to that observed in IR spectra[3] and computed[9] for the amide I vibrations of such structures. The suppressed intensity of the amide I ROA couplet centered at ~1672 cm⁻¹ in poly(L-lysine) relative to the large couplet centered at ~1665 cm⁻¹ in concanavalin A, for example, parallels the weak amide I VCD observed for such model β-sheet structures compared with the large VCD signals seen in typical β-sheet proteins.[9] This may result from the "planar" nature of the constituent strands within the flat multistranded β-sheet supported by the polypeptide for which the intrinsic skeletal chirality, and hence ROA and VCD intensities, are smaller than for the twisted strands present in the more irregular β-sheet structures found in typical native proteins.[9] Since intense IR bands are observed in the range ~1610−1620 cm⁻¹ in β-sheet polypeptides and some denatured proteins,[8] the absence of clear parent Raman bands associated with the negative ~1611 cm⁻¹ and positive ~1626 cm⁻¹ ROA bands in Figure 1c, which is even more apparent in the spectrum in D₂O in Figure 2c, is
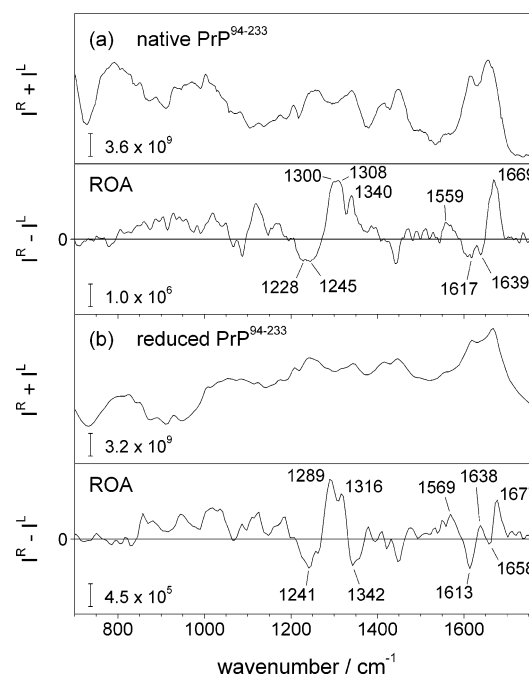

Figure 5. Backscattered Raman and ROA spectra of (a) native ovine PrP⁹⁴⁻²³³ in H₂O at pH 6.0 and (b) reduced ovine PrP⁹⁴⁻²³³ in H₂O at pH 4.0.

probably due to the corresponding modes being forbidden in conventional Raman but allowed in ROA (the transition optical activity tensors responsible for ROA transform differently to the transition polarizability responsible for conventional Ra-man[16]).

The positive ROA band at ~1561 cm⁻¹ in β-sheet poly(L-lysine) is assigned to the amide II vibration. It is evident from Figure 2c that this ROA band disappears when β-sheet poly-(L-lysine) is prepared in D₂O solution, with a new positive ROA band assigned to the amide II′ appearing at ~1478 cm⁻¹ together with other changes, confirming that N−H deformations are heavily involved. In contrast, the ROA band pattern in the range ~1600−1690 cm⁻¹ remains virtually unchanged, with small shifts to lower wavenumber, which is consistent with the assignment to amide I vibrations. Some β-sheet proteins show weak positive ROA bands at ~1560−1570 cm⁻¹ that may be assigned to the amide II vibration: examples are seen in the ROA spectra of concanavalin A and the MS2 protein capsid in Figure 3. This ROA band may sometimes be confused with that from the W3-type vibration of the indole ring in tryptophan side chains which occurs in the region around 1550 cm⁻¹.[37,39] It is possible that an unusually large positive amide II ROA band is characteristic specifically of the extended multistranded flat type of β-sheet found in β-sheet polypeptides.

The Prion Protein. We studied the N-terminal truncated ovine prion protein PrP⁹⁴⁻²³³ in native and reduced states. The first solution NMR structures of a full-length recombinant PrP revealed a structured C-terminal domain containing mainly α-helical sequences in the form of a compact three-helix bundle stabilized by a single disulfide bond and a long flexibly disordered N-terminal domain of 80−100 amino acids.[12,13]

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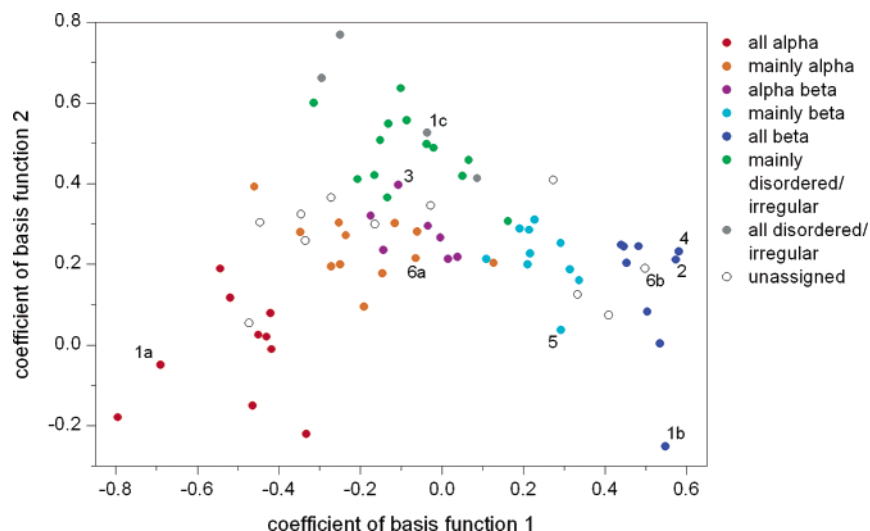
Figure 6. Plot of the PCA coefficients for the two most important basis functions for a set of 75 polypeptide, protein, and virus ROA spectra. The examples discussed in this article are labeled as follows: α-helical (1a), β-sheet (1b), and disordered (1c) poly(L-lysine); jack bean concanavalin A (2); subtilisin Carlsberg (3); P.69 pertactin (4); empty MS2 protein capsid (5); native ovine PrP$^{94-233}$ (6a); reduced ovine PrP$^{94-233}$ (6b). More complete definitions of the structural types are as follows: all-α, > ~60% α-helix with little or no other secondary structure; mainly α, > ~35% α-helix and a small amount of β-sheet (~5–15%); α–β, similar significant amounts of α-helix and β-sheet; mainly β, > ~35% β-sheet and a small amount of α-helix (~5–15%); all-β, > ~45% β-sheet with little or no other secondary structure; mainly disordered/irregular, little secondary structure; all disordered/irregular, no secondary structure.

The solution structure[40] of recombinant human prion protein PrP$^{90-230}$ (PDB structure 1qm1) is presented as a MOLSCRIPT diagram in Figure 4. Since the structure of the ovine prion protein has yet to be determined, and the structures of the prion proteins from all other mammalian species determined to date are very similar, we assume here for the purposes of discussion that the structure of ovine PrP$^{94-233}$ is very similar to that of human PrP$^{90-230}$ shown in Figure 4. UVCD confirmed that our sample of reduced PrP$^{94-233}$ at pH 4.0 has a structure rich in β-sheet, as found previously for reduced forms of other truncated recombinant mammalian prion proteins under similar conditions.[21,41,42] Although remaining compact with some tertiary structure, these reduced prion proteins exhibit certain molten globule-like properties[21,42,43] and are fibrillogenic at high ionic strength.[21,42]

The ROA spectrum of native ovine PrP$^{94-233}$, displayed in Figure 6a, shows features characteristic of significant amounts of both α-helix and β-sheet and is quite similar to that of subtilisin Carlsberg in Figure 3b, whereas that of the reduced protein in Figure 6b shows no α-helix bands, being dominated instead by band patterns characteristic of β-sheet. The ROA band pattern of the reduced protein in the range ~1220–1350 cm^{-1}, which includes the extended amide III region, is similar to that observed in the ROA spectrum of concanavalin A displayed in Figure 3a and is even more similar to that of immunoglobulin.[18] This indicates a structure based on multi-stranded β-sheet, together with β-turns and longer loops associated with the residual tertiary structure in the molten globule-like state. However, the general band pattern covering the amide I and amide II regions (~1550–1690 cm^{-1}) of the ROA spectrum of reduced ovine PrP$^{94-233}$ is not observed in

typical native proteins rich in β-sheet but is similar to that observed in this region in β-sheet poly(L-lysine) (Figure 1c) and therefore suggests that the β-sheet is unusually flat and regular.

It has been suggested[43] that the reduced human prion protein studied in the earlier work[21,41,42] must have been oligomeric, rather than monomeric as claimed.[21] Whether monomeric or oligomeric, it is significant that, as revealed by this ROA study, the C-terminal domain of a prion protein in a reduced state, which it has been suggested[21,42] may be present in intracellular compartments and involved in the conversion of PrP^C to PrP^{Sc}, is able to support a multistranded β-sheet in a relatively flat and soluble form. This is different from the more irregular twisted types of β-sheet found in typical native proteins, and we have not observed it in any other fibrillogenic protein state, including destabilized human lysozyme[44] and the natively unfolded brain proteins α-synuclein and tau,[45] all of which contain large amounts of PPII helix.

In a recent electron crystallographic study, Wille et al.[46] suggested that the structure of PrP^{Sc} is based on *parallel* β-sheets, possibly with a β-helix fold like pertactin. Since the ROA spectrum of reduced PrP$^{94-233}$ is more like that of β-sheet poly(L-lysine) in the amide I and amide II regions than that of pertactin, it probably does not adopt a β-helix structure, which in any event is unlikely to be supported in a molten globule-like state. If the structure of PrP^{Sc} is indeed based on a β-helix fold, which is still open to question due to the low-resolution of electron crystallographic data and the extensive modeling and assumptions used in reaching this conclusion,[46] then reduced prion proteins are not realistic models for PrP^{Sc}.

Principal Component Analysis. Pattern recognition methods are becoming important for protein ROA studies due to the large

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number of structure-sensitive ROA bands and the realization that, although some band assignments may be uncertain or not even valid due to extensive vibrational coupling, overall band patterns can be characteristic of certain structural elements, motifs, and perhaps even folds.[29] Here we apply the standard method of principal component analysis (PCA)[47] to obtain a representation of the structural relationships among the polypeptide and protein states discussed in this paper.

From the experimental ROA spectral data, PCA calculates a set of subspectra that serve as basis functions, the algebraic combination of which with appropriate expansion coefficients can be used to reconstruct any member of the original set of ROA spectra. The ROA spectra are specified by intensities for the same set of wavenumbers and are normalized by scaling a spectrum such that the sum of the squared intensities is unity. Most of the polypeptides and proteins in our set of ROA spectra may be divided into two sets, one containing α-helices and β-sheets in various amounts, the other containing mainly disordered or irregular structures. This is reflected in the coefficients of the two most important basis functions. For the first and most important basis function, large coefficients are associated with polypeptides and proteins containing large amounts of α-helix or β-sheet. Since the α-helix and β-sheet contents are inversely correlated (the larger the amount of one, the smaller the amount of the other), the coefficients associated with α-helix and β-sheet have opposite signs, here being negative for α-helix and positive for β-sheet. The coefficients for the second basis function reflect the amount of disordered structure, large positive coefficients being associated with mainly disordered polypeptides and proteins. Proteins with small coefficients, positive or negative, for the first basis function, together with small coefficients for the second basis function, have similar significant amounts of α-helix and β-sheet. The raw basis functions do not have any direct physical interpretation beyond this.

Figure 6 shows a plot of the coefficients for our current set of 75 polypeptide, protein, and virus ROA spectra for the two most important basis functions. The protein positions are color-coded with respect to the seven different structural types listed in the figure, which provide a useful initial classification. The spectra separate into clusters corresponding to different types of structure, with increasing α-helix content to the left, increasing β-sheet content to the right, and increasing disordered or irregular structure from bottom to top. Poly(L-lysine) in α-helical, β-sheet, and disordered states lie in the lower left, lower right, and upper center of the plot, as expected. Jack bean concanavalin A and P.69 pertactin lie within the all-β-region well to the right, reflecting their high β-sheet content with no α-helix, but somewhat higher than β-sheet poly(L-lysine) due to the significant amount of disordered or irregular structure in the form of loops and turns present in the native folds. Concanavalin A and pertactin lie close together because basis functions 1 and 2 are not able to discriminate between their distinct jelly roll and β-helix folds: higher-order basis functions are required to distinguish them. The MS2 protein capsid lies

within the mainly β-region, consistent with its unusual fold containing mostly β-sheet plus some α-helix. Subtilisin Carlsberg lies within the α−β-region, but quite far from the bottom, reflecting quite a lot of disordered or irregular structure in its many long loops. Ovine PrP[94-233] also lies in the middle of the α−β-region, which reinforces the close similarity of its ROA spectrum to that of subtilisin Carlsberg and the impression from its ROA spectrum of a significant amount of β-structure in addition to the expected α-helix. Reduced PrP[94-233] lies well to the right of native PrP[94-233], within the all-β-region and close to concanavalin A, confirming that it contains a large amount of β-sheet with little or no α-helix.

Conclusions

Our results have shown that the ROA spectrum of poly(L-lysine) in the β-sheet state contains many bands quite distinct from those observed in the α-helix and disordered states. This facilitated a study of the α-helix → β-sheet transition, which revealed no significant population of a disordered intermediate backbone state but did reveal some intermediate side chain structure not present in the initial or final states. On account of the large number of structure-sensitive bands in polypeptide and protein ROA spectra, ROA has the potential to discriminate between different types of β-sheet. One example is the presence of ROA bands assigned to β-turns in the extended amide III region, which enables up-and-down antiparallel β-sheet based on the hairpin motif to be identified in some cases. Although the absence of β-turn ROA bands in proteins containing β-sheet indicates the sheet is *parallel* and based on the β−α−β motif, the presence of β-turn ROA bands does not always mean the sheet is *antiparallel* since such turns are present in the β-helix fold which is based on a parallel sheet. However, ROA may still be able to recognize the β-helix fold from other characteristic bands. The striking amide I and amide II ROA bands that are observed in β-sheet poly(L-lysine) and which appear to be characteristic of flat multistranded β-sheet are not observed in most native proteins rich in β-sheet, but are observed in a reduced β-rich isoform of a truncated prion protein, which suggests that it is able to support an unusually flat β-sheet. Although some individual band assignments may still be uncertain, or not even valid due to the extensive vibrational coupling often involved in the generation of large ROA signals, overall band patterns can be characteristic of certain structural elements, motifs, and perhaps even folds. Consequently pattern recognition methods such as PCA, a simple version of which was used to provide an initial representation of the structural relationships among the polypeptide and protein states considered in the study, will become increasingly important in the analysis of protein ROA spectra.

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