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FOR THE RECORD

New insight into the pH-dependent conformational changes in bovine β-lactoglobulin from Raman optical activity

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Abstract: We have studied the conformation of β-lactoglobulin in aqueous solution at room temperature over the pH range ~2.0–9.0 using vibrational Raman optical activity (ROA). The ROA spectra clearly show that the basic up and down β-barrel core is preserved over the entire pH range, in agreement with other studies. However, from the shift of a sharp positive ROA band at ~1268 to ~1294 cm^{-1} on going from pH values below that of the Tanford transition, which is centered at pH ~7.5, to values above, the Tanford transition appears to be associated with changes in the local conformations of residues in loop sequences possibly corresponding to a migration into the α-helical region of the Ramachandran surface from a nearby region. These changes may be related to those detected in X-ray crystal structures which revealed that the Tanford transition is associated with conformational changes in loops which form a doorway to the interior of the protein. The results illustrate how the ability of ROA to detect loop and turn structure separately from secondary structure is useful for studying conformational plasticity in proteins.

Keywords: β-lactoglobulin; conformational plasticity; Raman optical activity; Tanford transition

On account of its abundance and ease of purification, together with its importance in the dairy industry, the mammalian milk protein β-lactoglobulin has been the subject of many physicochemical studies (see Hambling et al., 1992, for a review). Its function is still not clear, but since its structure is very similar to that of plasma retinol binding protein and it is able to bind many hydrophobic molecules including retinol itself, β-lactoglobulin has been included in the broad class of hydrophobic ligand binding and transporting proteins sometimes called lipocalins (Sawyer, 1987; Banaszak et al., 1994). Six different genetic variants have been identified in the cow, the most common being variants A and B which differ at positions 64 (Asp/Gly) and 118 (Val/Ala).

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As well as changes in oligomerization, β-lactoglobulin also undergoes interesting conformational changes with changes in pH, temperature and concentration which have attracted attention due to their possible relevance to general problems in protein folding, function, and design (Molinari et al., 1996; Hoffmann et al., 1997; Ikeguchi et al., 1997; Qi et al., 1997; Ragona et al., 1997). The most important of the pH-induced conformational changes shown by β-lactoglobulin is a reversible transition, first detected by Tanford et al. (1959) using optical rotatory dispersion, which occurs near pH 7.5 at 25 °C. This transition is characterized by the release of a buried carboxyl group, an increase in the reactivity of a free sulfhydryl group, and a change in the environment of a tyrosine residue (Hambling et al., 1992) and may be of functional importance since it occurs close to physiological pH. Some molecular details of this conformational change have recently been revealed from X-ray structures using crystals grown at pH values on either side of the Tanford transition (Brownlow et al., 1997; Qin et al., 1998).

A promising technique for studying conformational changes in biopolymers in aqueous solution is Raman optical activity (ROA), which measures vibrational optical activity in chiral molecules by means of a small difference in the intensity of Raman scattering in right- and left-circularly polarized incident light (Barron et al., 1996, 1998; Hecht & Barron, 1994) or, equivalently, by means of a small circular component in the scattered light (Nafie, 1996). ROA is proving particularly useful in this area because it is able to cut through the complexity of the conventional vibrational spectra of biopolymers due to the fact that only those few local vibrational coordinates within a complicated normal mode which sample the skeletal chirality most directly make the largest contributions to the ROA intensity. Proteins are particularly favorable for such studies since their ROA spectra contain distinct bands arising from loops and turns, as well as from secondary structure and side groups, and so can provide information about the tertiary fold of the peptide backbone and its changes with pH, temperature, etc. (Wilson et al., 1995; Teraoka et al., 1998).

Here we present an ROA study of bovine β-lactoglobulin over the pH range ~2.0–9.0. The availability of X-ray crystal structures on either side of the Tanford transition (Brownlow et al., 1997; Qin

et al., 1998) provides a rare opportunity to compare ROA data on delicate conformational changes in a protein with the detailed changes seen at the atomic level in the crystal structures. Our results accord not only with a Fourier transform infrared spectroscopy study, which showed the total amount of β-structure to remain constant between pH 2 and pH 10 (Casal et al., 1988) but also with the more specific findings from the above X-ray studies that bovine β-lactoglobulin retains a well-defined β-sheet core through the Tanford transition and from NMR studies that it persists even at pH 2.0 (Molinari et al., 1996; Belloque & Smith, 1998; Fogolari et al., 1998; Kuwata et al., 1998). Our results also demonstrate that the subtle loop conformation changes that accompany the Tanford transition can be monitored clearly and directly using ROA. The upper limit of ~50 °C for temperature-dependent measurements with our current ROA instrument meant that we were unable to use ROA to monitor the interesting temperature induced conformational changes to partly unfolded molten globule like states since these occur at ~70 °C at neutral pH (Qi et al., 1997).

To facilitate discussion of the ROA spectra, we briefly review the main features of the structure of bovine β-lactoglobulin. The protein usually exists as a dimer at neutral pH, but dissociates into monomers below pH ~3.0. Each monomer has a molecular weight of 18,350 Da and comprises 162 amino acids with one free cysteine and two disulfide bridges. The subunit structure (Brownlow et al., 1997) is sketched in Figure 1 and shows it to be dominated by nine β-strands (labeled A to I). The core of the structure is an up and down β-barrel composed of the eight antiparallel β-strands A to H folded into a flattened cone (a calyx) with the front and back surface β-sheets comprising strands A to D and E to H orthogonal. The β-sheet E to H is flanked on its outer surface by a three-turn α-helix (residues 130–140), followed by the ninth β-strand, I, which forms an extension to β-sheet E to H. There are five short loops, namely BC, CD, DE, EF, and FG, which constitute β-turns linking the ends of the corresponding strands; and four long loops, namely AB (residues 27–41), GH (residues 109–117), and one in each of the N- and C-terminal regions, which all contain short helical segments. The hydrophobic pocket within the calyx is able to bind small hydrophobic molecules. The two monomers are linked in the dimer via a short stretch of double stranded β-sheet comprising strand I from each subunit.

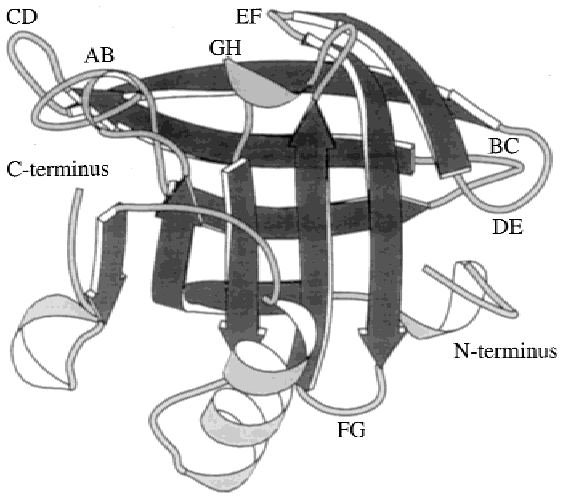

Fig. 1. Diagram of a single subunit of bovine β-lactoglobulin, within lattice X crystals grown at pH 6.5, drawn from data of Brownlow et al. (1997) in the PDB (code 1beb) using the program MOLSCRIPT (Kraulis, 1991). The labels of the loops correspond to the β-strands, which they link.

Comparison of the protein dimer structures of a mixture of variants A and B within triclinic lattice X crystals grown at pH 6.5 (resolution 1.8 Å) and triclinic lattice Z crystals grown at pH 7.8 (resolution 3.0 Å) revealed that the Tanford transition changes the relative orientation of the monomers in the dimer: raising the pH induces a rotation of ~5°, which breaks a number of hydrogen bonds between the two subunits (Brownlow et al., 1997). A later study which compared the structures of pure variant A within lattice Z at pH 6.2, 7.1, and 8.2 (resolution 2.56, 2.28, and 2.49Å, respectively) has, in addition, revealed a large conformation change in loop EF (residues 85–90) showing that this loop serves as a lid, which is closed at low pH, thereby blocking access to the interior of the calyx but which opens at high pH allowing access for substrates (Qin et al., 1998).

Like the first of the X-ray studies mentioned above, our ROA measurements were performed on a mixture of variants A and B.

Results: Figure 2 shows the backscattered Raman and ROA spectra of bovine β-lactoglobulin in H_2O solution at pH 2.0 (top pair), pH 6.8 (middle pair), and pH 9.0 (bottom pair). All were measured at room temperature (~21 °C). The ROA spectrum at pH 6.8 is very similar to an ROA spectrum of β-lactoglobulin published a few years ago (Wen et al., 1994). A general preliminary account of the assignments of protein ROA bands (which are being continuously refined as more protein ROA data accumulate) can be found in Barron et al. (1996), but a brief summary is given here for convenience. Vibrations of the peptide backbone in proteins are usually associated with three main regions of the Raman spectrum: the backbone skeletal stretch region comprising the C_α-C stretch region ~870–950 cm^{-1} and the C_α-N stretch region ~1,020–1,150 cm^{-1}, the extended amide III region ~1,230–1,350 cm^{-1} that arises from normal modes containing various combinations of the C_α-H and N-H deformations and the C_α-N stretch, and the amide I region ~1,630–1,700 cm^{-1} that arises predominantly from the C=O stretch. ROA signals in the backbone skeletal stretch region appear to arise mainly from secondary structure, those in the extended amide III region from loops and turns as well as secondary structure, and those in the amide I regions mainly from secondary structure with some contributions from loops and turns. Glycine and proline residues make quite different contributions to the ROA in the extended amide III region, and on either side, due to the backbone CH_2 deformations in the former and the C-H and CH_2 deformations but no N-H deformations in the latter, but to date the assignments of the associated ROA signals are not complete. Side group vibrations also generate characteristic Raman bands some of which give rise to useful ROA signals.

An important feature of all three ROA spectra is a strong positive band at ~1,315 cm^{-1} together with a weaker negative band at ~1,360 cm^{-1}. Previous studies suggested that this feature originates in antiparallel β-sheet together with associated turns (Wen et al., 1994). The negative bands at ~1,220 and 1,248 cm^{-1} are also associated with the β-barrel so that, whatever the actual band assignments (vide infra), the overall negative-negative-positive-negative ROA pattern generated by the bands at ~1,220, 1,248, 1,315, and 1,360 cm^{-1} can be taken as characteristic of a β-barrel with the specific details (relative band intensities and precise wavenumbers) corresponding to an up and down β-sheet motif since the

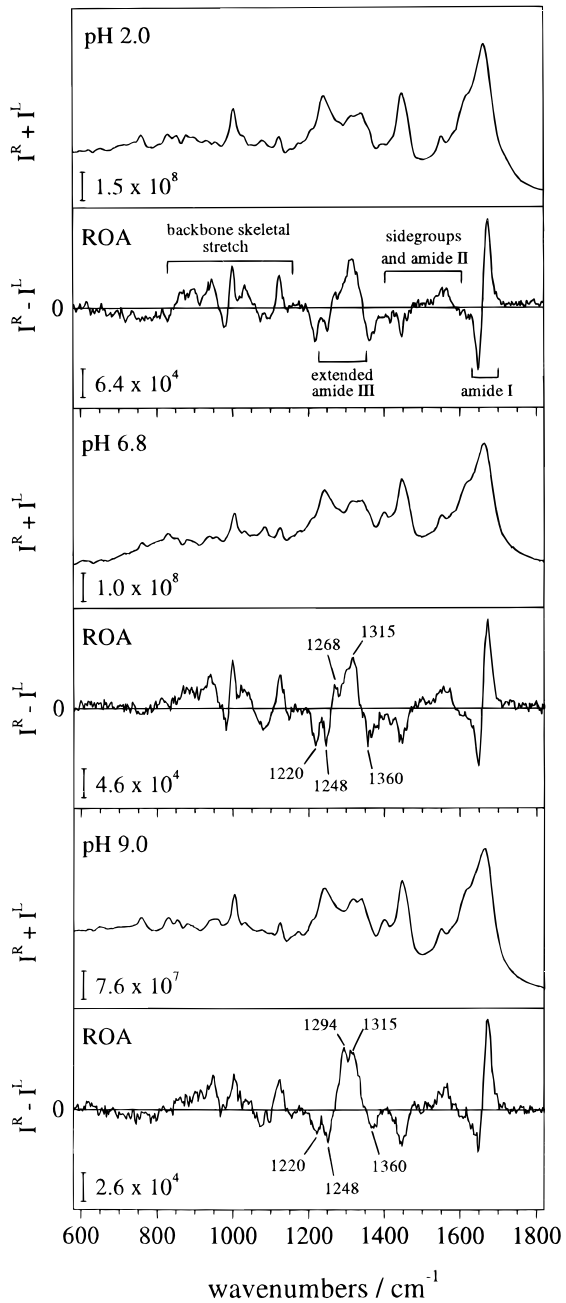

Fig. 2. The backscattered Raman and ROA spectra of bovine β-lactoglobulin in H_2O solution at pH 2.0 (top pair), 6.8 (middle pair), and 9.0 (bottom pair). All were measured at room temperature.

ROA spectra of proteins such as immunoglobulins containing β-barrels with the distinct Greek key β-sheet motif show rather different details (E. Smyth & L.D. Barron, unpubl. results). The constancy of this pattern in all three ROA spectra reveals that the basic β-barrel core is preserved over the entire pH range ~2.0–9.0, albeit with small changes of detail associated with small changes in the relative intensities of the four bands.

The negative ROA band at ~1,220 cm^{-1} may be characteristic of some of the short turns found in up-and-down β-barrels. It appears strongly in the ROA spectrum of the model β-turn peptide L-Pro-L-Leu-Gly-NH_2 (Wen et al., 1994) where, based on a detailed vibrational analysis (Naik & Krimm, 1984), it was suggested to originate in CH_2 deformations coupled with in-plane backbone NH deformations. The involvement of NH deformations in structure exposed to solvent is evidenced by the loss of much of the intensity of this ROA band in both β-lactoglobulin and the model peptide in D_2O solution (data not shown) due to exchange of the amide protons with deuterium which greatly changes the normal mode composition with the N-D deformations now contributing to normal modes at lower wavenumbers. The negative ROA band at ~1,248 cm^{-1} was previously suggested to originate in residues, within loop structure, having local order that of the poly-L-proline II (PPII) helix (Wilson et al., 1996). However, another possibility we are currently investigating is that it originates in β-strands and that the positive ROA band at ~1,315 cm^{-1} originates in PPII residues.

An unusual feature of the ROA spectra of β-lactoglobulin at pH values below that of the Tanford transition (in that it is not so prominent in any other protein ROA spectrum measured to date) is a sharp positive ROA band at ~1,268 cm^{-1}, which is of central interest in the present study because it undergoes a dramatic shift to ~1,294 cm^{-1} at pH values above that of the Tanford transition. This band can also be assigned to structure exposed to solvent because much of its intensity is lost in D_2O solution (data not shown).

The backbone skeletal stretch region is similar in all three ROA spectra. The features reflect the presence of α-helix (positive intensity in the range ~880–965 cm^{-1} plus the couplet centered at ~1,100 cm^{-1}, negative at low wavenumber and positive at high) and β-sheet (positive intensity in the range ~1,000–1,050 cm^{-1}). The minor changes in some of the ROA band details may be related to small changes in the β-sheet sequences and the connecting loops.

The large ROA couplet in the amide I region, negative at ~1,648 cm^{-1} and positive at ~1,672 cm^{-1}, has the characteristics usually found in β-sheet proteins (Wen et al., 1994). A similar ROA couplet is exhibited by α-helical proteins but shifted by ~10 cm^{-1} to lower wavenumber.

The negative ~1,248 and positive ~1,268 cm^{-1} ROA bands are a little weaker at pH 2.0 suggesting that breaking of the dimer interface has led to mild denaturation. This conclusion is in accord with the observation of disordered loop regions and terminal segments in an NMR study of β-lactoglobulin at pH 2.0 (Fogolari et al., 1998).

Discussion: *Molecular basis of the Tanford transition:* This ROA study of β-lactoglobulin has revealed that the basic β-barrel core is preserved over the pH range ~2.0–9.0 and is not significantly affected by the Tanford transition. However, the ROA spectra measured at pH values of ~6.8 and 9.0 (Fig. 2), which are on either side the of the value at which the Tanford transition occurs, clearly reveal that there is a change in loop structure monitored by a positive band at ~1,268 cm^{-1} shifting to ~1,294 cm^{-1} with increasing pH.

Brownlow et al. (1997) provided a preliminary discussion of the molecular basis of the Tanford transition from a comparison of the X and Z crystal structures, although a detailed comparison was precluded by the fact that the Z crystal structure was limited to 3.0 Å resolution. The two subunits of the dimer, which exists above pH ~3.0, were seen to be rotated by ~5.1° relative to each other in the two crystal forms but without affecting the linking

double stranded β-sheet at the dimer interface comprising strand I from each subunit. The adjacent AB loops are moved further apart in lattice Z so that most hydrogen bonds between the loops (involving Asp33, Ala34, and Arg40) are broken, becoming more exposed and mobile as judged by weaker electron density in lattice Z.

Since the ~1,268 cm^{-1} ROA loop band retains the same frequency (albeit with reduced intensity) when the protein is in the monomeric state at pH ~2.0, it seems that the conformational change associated with the frequency shift to ~1,296 cm^{-1} induced by the Tanford transition is not primarily due to the breaking of the hydrogen bonds between the AB loops. This conclusion is consistent with the more detailed structural basis for the Tanford transition provided by the later study of Qin et al. (1998), who identified a further two distinct intermolecular contacts in addition to the dimer interface described previously. One is a substantial interface created by insertion of Lys8 into a pocket of a neighboring molecule; the other involves mutual contacts between pairs of the loops CD (residues 61–67), EF (residues 85–90), and GH (residues 109–117) although there do not appear to be any specific interactions between these rather mobile regions. As can be seen from the subunit crystal structure at pH 6.5 shown in Figure 1, these three loops, especially EF, form the doorway to the interior of the calyx. As the pH increases through the Tanford transition region, there is an expansion in the volume with a concomitant loosening of intermolecular contacts in the first dimer interface, as described earlier, as well as in the second interface. More important, however, is that a major conformational change occurs for loop EF. At low pH, loop EF serves as a lid which blocks access to the interior of the calyx; at high pH loop EF flips away and the interior becomes accessible to substrates. In addition, this conformation change leads to Glu89 changing from a buried situation at low pH to exposed at high pH.

It has been shown that the ϕ,ψ angles of individual residues within loop sequences in proteins tend to cluster in well defined regions of the Ramachandran potential energy surface (Efimov, 1993a, 1993b; Swindells et al., 1995; Donate et al., 1996; Geetha & Munson, 1997). Some protein ROA signals in the extended amide III region appear to be sensitive to the local geometry of the individual residues irrespective of whether a particular residue is part of extended secondary structure or is part of a loop or turn lacking extended order (perhaps with small wavenumber changes for a given residue conformation in different contexts). By assuming that the shift of the positive ROA band at ~1,268 cm^{-1} to that at ~1,294 cm^{-1} on going through the Tanford transition is primarily associated with the large conformation change of loop EF, we had hoped to be able to use the X-ray structure data of Qin et al. (1998) to assign these ROA bands to residues clustering in two distinct and well-defined regions of the Ramachandran surface, but unfortunately this has not been possible because the relevant ϕ,ψ angles are not sufficiently well defined. While the overall path taken by loop EF at different pH values is clear, the actual values of ϕ and ψ are rather imprecise since this loop in lattice Z is quite mobile. Hence, it is not clear whether or not the shift of the positive ~1,268 cm^{-1} ROA band to ~1,294 cm^{-1} is directly monitoring the changes in the residue conformations within loop EF, or originates in changes in other loop sequences.

This highlights a general problem that has become apparent as protein ROA spectra have accumulated, namely that only a rather small repertoire of often quite sharp ROA bands within small wavenumber ranges appears; whereas from the often rather large spread of ϕ,ψ angles specified within a given type of secondary, turn or loop element in X-ray crystal structures much broader ROA bands would have been expected. One possible explanation is that X-ray diffraction measures the spatial distribution of atomic positions over many protein conformational substates (Frauenfelder et al., 1979; Rader & Agard, 1997), whereas the ROA spectrum is a superposition of well-resolved "snapshot" spectra from individual protein molecules in distinct conformational substates within each of which the ϕ,ψ angles may be tightly constrained around standard values.

Positive ROA intensity in the range ~1,295–1,305 cm^{-1} has been previously assigned to residues with ϕ,ψ angles lying within the α-helix region of the Ramachandran surface (Wilson et al., 1996; Teraoka et al., 1998), so the positive ~1,294 cm^{-1} ROA band may originate in loop residues that are clustering in the α-helix region of the Ramachandran surface. The assignment of the positive ~1,268 cm^{-1} ROA band is less clear. It may originate in residues lying within a distinct but nearby region such as the type-I β-turn region, or even the inverse γ-turn region since Krimm and Bandekar (1986) have calculated a frequency of 1,268 cm^{-1} for one of the normal modes of a model inverse γ-turn peptide. The positive ~1,268 cm^{-1} ROA band may even originate in residues lying within the 3_{10}-helix region. It was tentatively suggested that 3_{10}-helix may be responsible for a positive band at ~1,340 cm^{-1} in the ROA spectra of some proteins (Barron et al., 1996; Teraoka et al., 1998). However, there is no sign of such a band in the ROA spectrum of β-lactoglobulin despite 3_{10}-helix being present in some of the loop sequences in the X-ray crystal structures. Some of the 3_{10}-helix present in the lattice X crystal structure (Brownlow et al., 1997) is observed as α-helix in the lattice Z crystal structure (Qin et al., 1998), which suggests that 3_{10}- and α-helical elements may interconvert quite readily.

Hence, from the perspective of ROA, the Tanford transition is associated with changes in the local conformations of residues in loop sequences around a conserved β-barrel core. These residue conformation changes appear to correspond to a migration into the α-helical region of the Ramachandran surface from some nearby, but as yet unidentified, region of the surface. The parts of the protein which may be involved in the changes sensed by ROA are identified by the X-ray data as the doorway loops CD, EF, and GH, together with other loops such as AB that become less constrained at pH values above ~7.5.

Conformational plasticity: There has been much discussion recently of the dual nature of certain peptide sequences which can be made to switch between α-helical and β-sheet conformations within proteins by manipulating the balance of local and long-range interactions to overcome individual residue propensities (Minor & Kim, 1996; Dalal et al., 1997). In the case of β-lactoglobulin, Kuroda et al. (1996) have shown that isolated 17–18 residue peptides corresponding to the β-strands A, D, and F within the native structure adopt residual helicities of up to 17% in aqueous solution at pH ~2.0, which could be further stabilized by the addition of trifluoroethanol. This is consistent with the observation of a high helical preference for the complete amino acid sequence of β-lactoglobulin (Shiraki et al., 1995). Our ROA observations appear to fit within this general trend in that, once released from the constraints that stabilize whatever structure is responsible for the positive ~1,268 cm^{-1} ROA band at pH values below ~7.5, the associated residues appear to migrate to the α-helical region of

the Ramachandran surface thereby generating the positive ~1,294 cm^{-1} ROA band. Interestingly, other proteins containing highly conserved β-barrel cores together with long loops, such as immunoglobulins, often show a strong positive ROA band near 1,294 cm^{-1} (E. Smyth & L.D. Barron, unpubl. data), which may similarly originate in loop residues clustering in the α-helix region. Migration of residue conformations between different regions of the Ramachandran surface corresponds to what has been called "plasticity," which allows for fluctuations between discrete energy wells associated with distinct conformational substates (Rader & Agard, 1997).

This study has demonstrated that, on account of its ability to detect loop and turn structure separately from secondary structure, ROA is able to monitor delicate changes in details of the fold, something inaccessible to conventional solution phase techniques such as ultraviolet circular dichroism and NMR (Dalal et al., 1997). Hence, ROA may help to provide a better understanding of the conformational plasticity in proteins that is central to folding, function, and design.

Materials and methods: *Proteins, reagents, and sample handling:* Bovine β-lactoglobulin (three times crystallized and lyophilized) was purchased from Sigma-Aldrich Company Ltd., Poole, Dorset, United Kingdom and used without further purification. The protein was dissolved in dilute HCl, H_2O or dilute NaOH as required, and the final pH adjusted by addition of acid or base. The pHs of the solutions were measured using a pH meter with a glass electrode.

The protein solutions, with concentrations typically ~75 mg/mL, were made up in small glass sample tubes, mixed with a little charcoal to remove traces of fluorescing impurities, and centrifuged. The solutions were subsequently filtered through 0.22 μm Millipore filters directly into rectangular quartz microfluorescence cells which were again centrifuged gently prior to mounting in the ROA instrument. Residual visible fluorescence from traces of impurities, which can give large backgrounds in Raman spectra, was allowed to "burn down" by leaving the sample to equilibrate in the laser beam for several hours before acquiring ROA data.

ROA spectroscopy: The instrument used for the Raman and ROA measurements has a backscattering configuration, which is essential for aqueous solutions of biopolymers, and employs a single grating spectrograph fitted with a backthinned CCD camera as detector and a holographic notch filter to block the Rayleigh line (Hecht & Barron, 1994). 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. 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.

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