

COMMUNICATION

Raman Optical Activity of Filamentous Bacteriophages: Hydration of α -Helices**Ewan W. Blanch¹, Alasdair F. Bell¹, Lutz Hecht¹, Loren A. Day^{2*} and Laurence D. Barron^{1*}**¹*Chemistry Department
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We report the first observations of vibrational Raman optical activity (ROA) on intact viruses. Specifically, ROA spectra of the filamentous bacteriophages Pf1, M13 and Ike in aqueous solution were measured in the range ~ 600 – 1800 cm^{-1} . On account of its ability to probe directly the chiral elements of biomolecular structure, ROA has provided a new perspective on the solution structures of these well-studied systems. The ROA spectra of all three are dominated by signatures of helical elements in the major coat proteins, as expected from pre-existing data. The helical elements generate strong sharp positive ROA bands at ~ 1300 and 1342 cm^{-1} in H_2O solution, but in $^2\text{H}_2\text{O}$ solution the $\sim 1342\text{ cm}^{-1}$ bands disappear completely. The spectra are similar to those of polypeptides under conditions that produce α -helical conformations. Our present results, together with results from other studies, suggest that the positive $\sim 1342\text{ cm}^{-1}$ ROA bands are generated by a highly hydrated form of α -helix, and that the positive $\sim 1300\text{ cm}^{-1}$ bands originate in α -helix in a more hydrophobic environment. The presence of significant amounts of highly hydrated helical sequences accords with the known flexibility of these viruses. Differences of spectral detail for Pf1, M13 and Ike demonstrate that ROA is sensitive to subtle variations of conformation and hydration within the major coat proteins, with M13 and Ike possibly containing more non-helical structure than Pf1. The ROA spectra of Pf1 at temperatures above and below that at which a structural transition is known to occur ($\sim 10^\circ\text{C}$) reveal little difference in the protein conformation between the two forms, but there are indications of changes in DNA structure.

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The filamentous bacteriophages are flexible rods approximately 800 to 2000 nm long and 6 to 7 nm in diameter, comprised of single-stranded circular DNA molecules surrounded by cylindrical protein shells. Each protein shell is a helical array of several thousand identical copies of a major coat protein subunit, of about 50 amino acid residues; the shell is terminated at both ends by a few copies of minor coat proteins. The packaged DNA

genome constitutes from 6 to 14% of the total virion mass, depending on the viral strain. Observations made with a variety of techniques, including X-ray and neutron diffraction from oriented fibres as well as NMR, Raman, IR, fluorescence and UV CD, have established that there are two types of fundamental helical symmetry for the protein shells, that the conformations of the individual major coat proteins are predominantly α -helical, that the positively charged C-terminal domain of each subunit interacts with the DNA, whereas its negatively charged N-terminal domain is on the virion exterior, and that there are striking differences in DNA conformation (for reviews, see Day *et al.*, 1988a,b; Marvin, 1998). Since filamentous bacteriophages have not been crystallized, the

Abbreviations used: ROA, Raman optical activity; NMR, nuclear magnetic resonance spectroscopy; IR, infrared spectroscopy; UV CD, ultraviolet circular dichroism.

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details of the structures have to be deduced with the aid of modeling studies for which the entire set of data provides critical constraints. Several different models have been proposed, the relative merits of which are the subject of current debate (Marvin, 1990; Nambudripad *et al.*, 1991; Liu & Day, 1994; Gonzalez *et al.*, 1995; Matsuno *et al.*, 1998; Welsh *et al.*, 1998; Wen *et al.*, 1999).

Raman spectroscopy is a valuable technique for studies of viruses at the molecular level on account of its ability to provide structural information about both protein and nucleic acid constituents of intact virions and viral precursors over a broad range of sampling conditions (for a review, see Thomas, 1999). A novel form of Raman spectroscopy called Raman optical activity (ROA), which measures small differences in the Raman spectra of chiral molecules acquired using right and left circularly polarized incident light, has recently been applied to biomolecules and provides a new perspective on their solution structure and dynamics (Barron *et al.*, 1996, 1998; Vargek *et al.*, 1997; Hecht *et al.*, 1999). ROA bears the same relation to conventional Raman spectroscopy as does UV CD to conventional UV absorption spectroscopy. ROA is able to cut through the complexity of the conventional vibrational spectra of biopolymers because only those few local vibrational coordinates within a complicated normal mode which sample the chiral structural elements most directly contribute to the ROA intensity. As well as bands arising from secondary structure and side-chains, protein ROA spectra also contain distinct bands from loops and turns, and so can provide information about the tertiary fold of the peptide backbone and its changes with pH, temperature, and other variables (Wilson *et al.*, 1995; Teraoka *et al.*, 1998; Blanch *et al.*, 1999). Nucleic acid ROA spectra contain information on base stacking, the mutual orientations of sugar and base rings, and the sugar ring and sugar-phosphate backbone conformations (Bell *et al.*, 1997a, 1998). Here we report the first measurements of the ROA spectra of nucleoproteins, specifically three filamentous bacterial viruses.

Figure 1 displays the backscattered Raman and ROA spectra of bacteriophages Pf1 (top pair) and M13 (bottom pair) in H₂O solution at concentrations of ~10 and 18 mg/ml, respectively. We also performed preliminary measurements on IKE, which gave an ROA spectrum with an appearance more similar to that of M13 than Pf1 (data not shown). At higher concentrations anisotropic liquid crystal structures start to form (Tang & Fraden, 1995) in solutions of these slender rod-like structures and these produce large birefringence artefacts which prevent the acquisition of ROA spectra. We were, however, aided by the reduced Rayleigh scattering resulting from these particular shapes. Although the allowed concentrations are ~fivefold lower than those normally used in ROA studies of proteins and nucleic acids, the signal-to-noise ratio is similar. The reasonable data quality

at lower concentrations may result from restrictions on the mobility of components packed in virions because, as previously noted (Bell *et al.*, 1997b), the dependence of ROA on absolute chirality confers a special sensitivity to conformational mobility due to cancellation of contributions from conformational sub-states having approximately enantiomeric structures.

The extra incisiveness of ROA compared to conventional Raman spectroscopy is illustrated by the fact that, although the parent Raman spectra of the three bacteriophages are very different (due to the different protein side-chains and different DNA contents and base compositions), the ROA spectra of all three have the same general appearance. This appearance is very similar to that of the ROA spectrum of poly(L-lysine) in a model α -helical conformation (Wilson *et al.*, 1996) together with those of other polypeptides in α -helical states (unpublished results). As well as comparison with the ROA spectra of polypeptides in model conformations, assignments of protein ROA bands are based mainly on comparison of ROA spectra of proteins for which X-ray crystal structures and/or NMR structures are available (Barron *et al.*, 1996; Teraoka *et al.*, 1998; Blanch *et al.*, 1999) and are continually being refined as new data become available. The bacteriophage spectra are dominated by the following features assigned to α -helix: positive intensity in the range ~850-970 cm⁻¹ and the couplet centred at ~1100 cm⁻¹ negative at low wavenumber and positive at high, both assigned to mainly C ^{α} -C, C ^{α} -C ^{β} and C ^{α} -N stretching of the peptide backbone; positive intensity at ~1300 cm⁻¹ from an extended amide III mode involving N-H and C ^{α} -H deformations and the C ^{α} -N stretch; and a couplet centred at ~1644 cm⁻¹ that is negative at low and positive at high wavenumber from peptide amide I modes involving mostly C=O stretching. A similar amide I ROA couplet but shifted by ~10 cm⁻¹ to higher wavenumber is characteristic of β -sheet. All three virus spectra also show a strong sharp positive ROA band at ~1342 cm⁻¹ arising from an extended amide III mode. This mode was assigned in earlier work to ₃₁₀-helix (Wilson *et al.*, 1996; Barron *et al.*, 1996; Teraoka *et al.*, 1998), but later studies, including the present one, suggest that in fact the positive ~1342 cm⁻¹ band is characteristic of a highly hydrated form of α -helix, and that the positive ~1300 cm⁻¹ band is characteristic of α -helix in a more hydrophobic environment. Side-chain ROA bands can also be identified. These include those in the range ~1400-1480 cm⁻¹, which originate mainly in CH₂ and CH₃ deformations of the aliphatic side-chains, and the positive bands at ~1555 cm⁻¹ shown by M13 and IKE which originate in the single tryptophan residues of their major coat protein subunits. Since it contains no tryptophan residue, Pf1 shows no ROA band at ~1555 cm⁻¹. Non-aromatic side-chains may also influence some of the details of the α -helix ROA bands, as found in the conventional Raman spectra

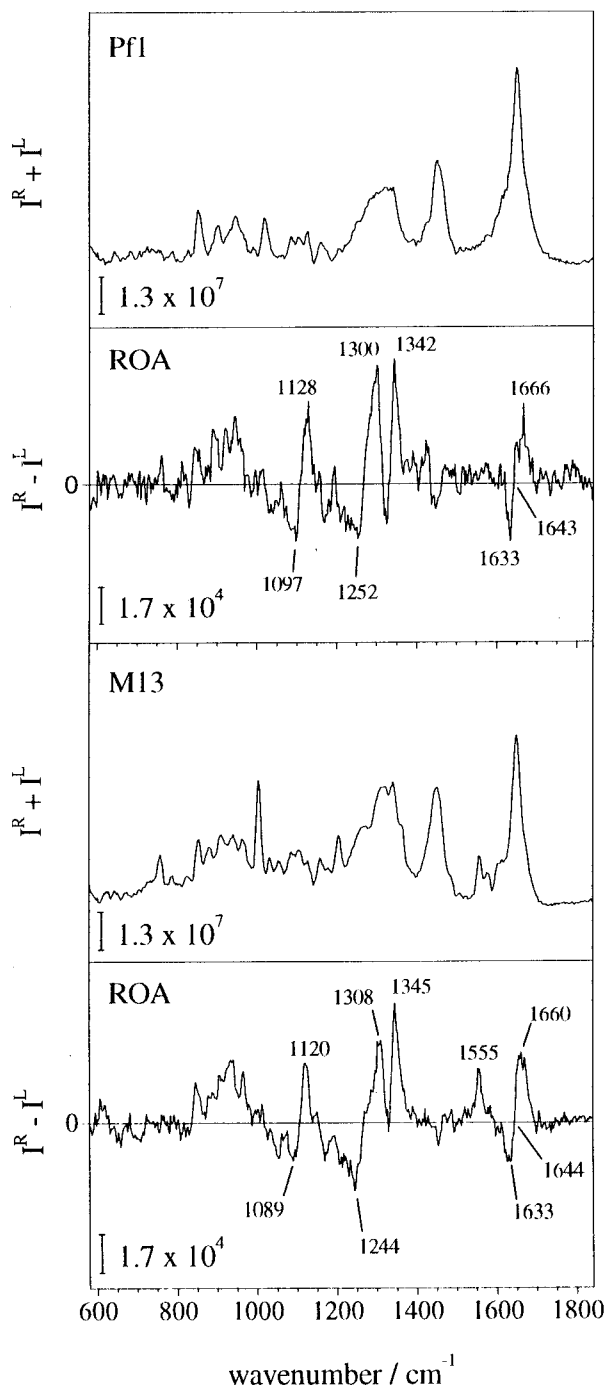


Figure 1. The backscattered Raman ($I^R + I^L$) and ROA ($I^R - I^L$) spectra of filamentous bacteriophages Pf1 (top pair) and M13 (bottom pair) in 150 mM NaCl, 15 mM Tris-HCl (pH 7.6) measured at room temperature ($\sim 20^\circ\text{C}$). The concentrations were ~ 10 mg/ml for Pf1 and 18 mg/ml for M13. The viruses were purified from infected suspension cultures of their host strains (*Pseudomonas aeruginosa* strain K for Pf1 and *Escherichia coli* Hfr 3300 for M13mp18) according to the procedures summarized by Kostrikis *et al.* (1994); viral concentrations were estimated from UV absorbance measurements (Day *et al.*, 1988a; Kostrikis *et al.*, 1994). The instrument used was configured similar to that described by Barron *et al.* (1996). Instrumental conditions: laser wavelength 514.5 nm, laser power ~ 700 mW at the sample, spectral resolution ~ 10 cm^{-1} , recording time ~ 48 hours for each

of α -helical assemblies (Overman & Thomas, 1999). Because of their low DNA contents, no bands directly attributable to the DNA can be discerned in the ROA spectra of the three viruses in H_2O solution.

Figure 2 displays the Raman and ROA spectra of Pf1 (top pair) and M13 (bottom pair) in $^2\text{H}_2\text{O}$, measured several weeks after the solutions were prepared. ROA spectra are usually more difficult to measure in $^2\text{H}_2\text{O}$ than in H_2O . Consequently some of the details of these first bacteriophage ROA spectra in $^2\text{H}_2\text{O}$ are not as reliable as those in H_2O . As we have found with peptides and proteins, the intensities over much of the ROA spectra are generally weaker in $^2\text{H}_2\text{O}$ than in H_2O solution. Compared to the ROA spectrum of Pf1 in H_2O , in $^2\text{H}_2\text{O}$ the positive ~ 1342 cm^{-1} ROA band has vanished completely and the positive ~ 1300 cm^{-1} band is reduced by $\sim 50\%$. This shows that N-H deformations of the peptide backbone make a significant contribution to the generation of the ~ 1342 cm^{-1} ROA band, and that the associated structural elements are highly exposed to the solvent. The influence of $^2\text{H}_2\text{O}$ on the ROA spectrum of M13 is more complex: for example, although the positive ~ 1342 cm^{-1} band is again completely eliminated, some positive intensity is present in the range ~ 1310 - 1325 cm^{-1} , and the positive ~ 1300 cm^{-1} band is more degraded than in Pf1. Overall, it appears that the amide hydrogen atoms in one substantial fraction of each of the coat proteins exchange rapidly and those in another substantial fraction exchange very slowly. Previous hydrogen-deuterium exchange studies of these viruses using IR linear dichroism (Fritzsche *et al.*, 1981, 1986) and conventional Raman spectroscopy (Williams *et al.*, 1984; Overman & Thomas, 1998b) reached a similar conclusion.

Recently, Overman & Thomas (1998a,b) used an elegant isotopic editing method to prepare variants of filamentous bacteriophage fd containing ^{13}C -labelled carbonyl groups and ^2H -labeled C^α -H groups at selected main chain sites which they studied using conventional Raman difference spectroscopy. Isotopic shifts were used to identify new bands, one of which near 1345 cm^{-1} was

ROA spectrum. No changes were observed in the parent Raman spectra over the duration of the measurements. The Raman and ROA intensities for M13, and for the spectra shown in Figures 2 and 3, have been scaled with respect to the concentration and recording time used for the room temperature spectra of Pf1 in H_2O in order that a direct comparison of band intensities can be made. The amino acid residue sequence of the major coat protein of Pf1 is: GVIDTSAVESAITDGQGDMKAIGGYIVGALVILAVAGLIYSMLRKA, and that of M13 is: AEGDDPAKAAFNSLQASATEYIGYAWAMVVVIVGATIGIKLFKFKFTSKAS, where hydrophobic residues, identified by Chou *et al.* (1997), have been underlined.

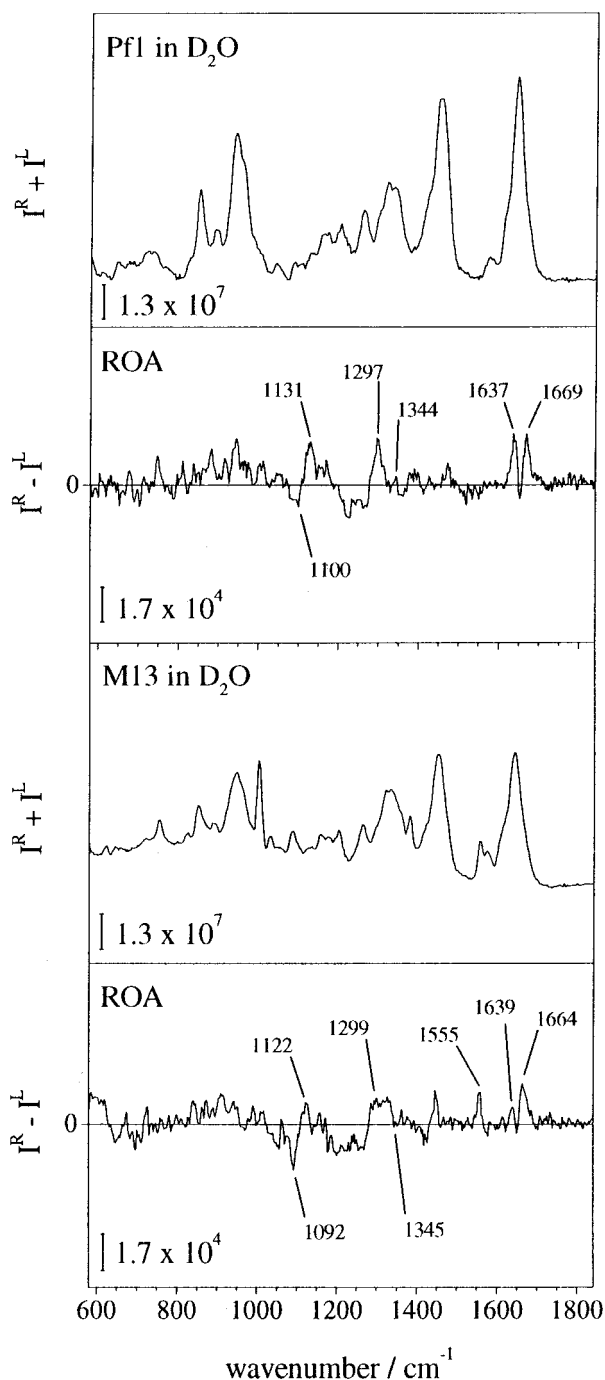


Figure 2. The backscattered Raman and ROA spectra of Pf1 (top pair) and M13 (bottom pair) in a $^2\text{H}_2\text{O}$ version of the H_2O buffer described in the legend to Figure 1.

assigned to $\text{C}^\alpha\text{—H}$ bending plus $\text{C}^\alpha\text{—C}$ stretching within an assumed α -helix conformation based on the structure model of Marvin *et al.* (1994) for the fd virus they used. These authors emphasized that other internal coordinates may also contribute. One such coordinate could be amide N—H deformations since, according to their data, the Raman

band decreases significantly in $^2\text{H}_2\text{O}$ solution (Overman & Thomas, 1998b). It is likely that the $\sim 1345\text{ cm}^{-1}$ band identified by difference Raman spectroscopy is the parent Raman band of the positive $\sim 1342\text{ cm}^{-1}$ ROA band reported here.

From an analysis of the hydration of α -helices in a set of protein X-ray crystal structures, Sundaralingam & Sekharudu (1989, 1990) and Sekharudu & Sundaralingam (1993) identified three types of hydrogen bonding interactions between water molecules and the peptide backbone which may help to explain some of our observations. In *external* hydration, the $\text{C}=\text{O}_i$ group already engaged in intrachain helix hydrogen bonding to NH_{i+4} forms a hydrogen bond with an external water molecule. In *three-centred* hydration, both the carbonyl group and the amide group hydrogen bond with water, hence participating in a bifurcated or three centred hydrogen bond. In *water-inserted* hydration, the water molecule inserts itself between the $\text{C}=\text{O}_i$ and NH_{i+4} groups, replacing the $i \rightarrow i+4$ α -helix hydrogen bond with a hydrogen-bonded bridge. The external type of backbone hydration 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 (Blundell *et al.*, 1983; Barlow & Thornton, 1988; Parthasarathy *et al.*, 1995). Water-inserted hydration of α -helices 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.

Our data suggest that the positive $\sim 1342\text{ cm}^{-1}$ virus ROA bands arise from a highly hydrated form of α -helix, and the positive $\sim 1300\text{ cm}^{-1}$ bands from a less hydrated form. In the highly hydrated form, the peptide amide groups as well as the carbonyl groups are probably hydrogen-bonded to water molecules, as in the three-centred and water-inserted types of hydration described above; whereas the less hydrated form may be completely hydrophobic or may have just the external type of hydration with only the carbonyl group hydrogen-bonded to water. Evidence from other studies indicates that the highly hydrated form is more likely to contain the three-centred type of hydration, or something similar, than the water-inserted type. For example, although the ROA spectrum of the α -helical protein human serum albumin is dominated by a strong positive $\sim 1340\text{ cm}^{-1}$ $^2\text{H}_2\text{O}$ sensitive ROA band (Barron *et al.*, 1996; Teraoka *et al.*, 1998), well-refined X-ray crystal structures reveal mainly standard α -helix with very little 3_{10} -helix (69.2% α and 1.7% 3_{10} according to PDB structure 1ao6; 67.9% α and 3.6% 3_{10} according to PDB structure 1bj5). Assuming the hydration adopted in solution is preserved in the protein crystals, a large amount of 3_{10} -helix or related conformations would have been

expected in the X-ray structures if the water-inserted type of hydration were favoured. Hydrogen bonds between the peptide backbone and hydrophilic side-chains in place of water molecules may be present in some residues. This is known to occur when hydrophilic residues are present in α -helices in a highly hydrophobic environment as in membranes (Gray & Mathews, 1984). However, this is not likely to be the source of the positive $\sim 1342\text{ cm}^{-1}$ ROA bands in the viruses, since amide hydrogen-deuterium exchange would be slow in this situation.

In Pf1 a stretch of purely hydrophobic residues extends from Ala21 to Tyr40, whereas the equivalent stretches in M13 and IKE, although mainly hydrophobic, do contain a few hydrophilic residues. This may account for the $\sim 30\%$ weaker intensities of the positive $\sim 1300\text{ cm}^{-1}$ ROA bands relative to the $\sim 1342\text{ cm}^{-1}$ bands in M13 and IKE compared with Pf1, for which the two bands have roughly equal intensities. It also suggests that M13 and IKE have more non-helical structure than Pf1 which may be located in this part of the sequence. For simplicity we referred, in the foregoing, to Pf1, M13 and IKE as all having a positive ROA band at $\sim 1300\text{ cm}^{-1}$, but in fact only that of Pf1 peaks at exactly this wavenumber. These bands in M13 and IKE actually peak nearer $\sim 1308\text{ cm}^{-1}$, and that in M13 shows more degradation in $^2\text{H}_2\text{O}$ than does that in Pf1 (we did not measure the ROA of IKE in $^2\text{H}_2\text{O}$). One possible explanation for this shift is that, whereas residues Ala21 to Tyr40 in Pf1 may support α -helix in a fully hydrophobic environment, the equivalent sections in M13 and IKE may contain segments with the external type of hydration described above in which the peptide backbone carbonyl, but not the N—H, is hydrated.

Since the positive $\sim 1342\text{ cm}^{-1}$ ROA band has similar intensity in Pf1, M13 and IKE, it may originate primarily in the helices at the N-terminal ends of the major coat proteins where the first ~ 20 residues in all three are mixed with respect to hydrophobic and hydrophilic character. The presence of long stretches of highly hydrated α -helices at the N-terminal ends could explain the enhanced flexibility necessary to form the "stem and loop" structures seen in some micrographs of Pf1 (Kostrikis *et al.*, 1991). As Parthasarathy *et al.* (1995) have emphasized, the α -helix is not a rigid straight rod but a deformable molecular cylinder which can undergo either a regular bend, sharp kink or non-regular distortions depending on the amino acid sequence of the helical segment and its environment in the protein. The bends and distortions known to be present in α -helix in contact with water (Blundell *et al.*, 1983; Barlow & Thornton, 1988; Sundaralingam & Sekharudu, 1989, 1990; Sekharudu & Sundaralingam, 1993; Parthasarathy *et al.*, 1995) may well be found to be present when the structures of the fully hydrated viruses are eventually established.

Pf1 shows a structural phase transition near 10°C , such that oriented specimens prepared at

higher temperatures produce X-ray fibre diffraction patterns differing from those for specimens prepared at lower temperatures (Wachtel *et al.*, 1976). It has been proposed that the transition involves a change in protein shell symmetry from 27 subunits/5 turns to 71 subunits/13 turns (Makowski & Caspar, 1981; Nave *et al.*, 1981; Marvin *et al.*, 1992). The ROA spectrum measured at $\sim 1^\circ\text{C}$, displayed in Figure 3, shows only small changes from that at room temperature (Figure 1). The dominant α -helix bands at ~ 1300 and 1342 cm^{-1} are unaltered, consistent with the conclusions from thermal difference UV CD (Casadevall & Day, 1988) and from X-ray studies that there is no detectable difference between the two forms as regards protein conformation. However, difference spectra (not shown) of these Raman and ROA data for the transition reveal changes at wavenumbers, indicating changes in DNA structure and in a tyrosine located at the DNA-protein interface consistent with those deduced from the UV CD study of Casadevall & Day (1988). It is intriguing that one of the difference ROA bands is at $\sim 1640\text{ cm}^{-1}$, close to where a new strong sharp positive band appears in the ROA spectrum of Pf1 in $^2\text{H}_2\text{O}$ (Figure 2). Since some signals from base stretching modes were found to be boosted by up to an order of magnitude in the ROA spectra of polyribonucleotides in $^2\text{H}_2\text{O}$ compared with H_2O solution (Bell *et al.*, 1997a), these new bands may arise from DNA base vibrations (see also Wen *et al.*, 1999). However, in view of the unusual DNA confor-

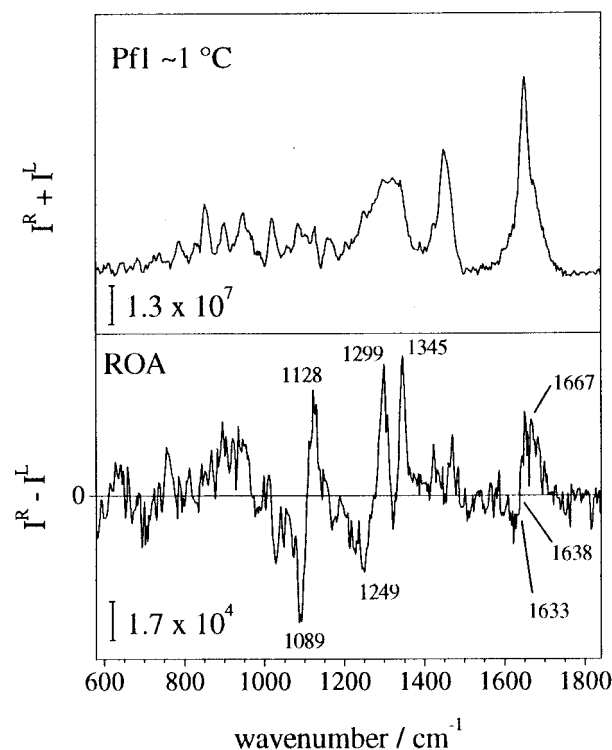


Figure 3. The backscattered Raman and ROA spectra of Pf1 measured at $\sim 1^\circ\text{C}$.

mation present in Pf1 (Day *et al.*, 1988a,b; Liu & Day, 1994; Allemand *et al.*, 1998; Welsh *et al.*, 1998; Wen *et al.*, 1999), and hence the absence of corresponding reference spectra, together with a requirement for ROA spectra of higher quality than the preliminary ones obtained in this first study, further work is necessary before firm conclusions can be made about changes in DNA conformation.

In conclusion, ROA measurements on Pf1, M13 and IKe indicate that the major coat proteins of all three contain long stretches of highly hydrated α -helical elements and stretches of lower hydration. They also reveal small but significant differences in protein conformation and hydration between the three strains. Furthermore, ROA confirms that there is little difference in protein conformation between the high and low temperature forms of Pf1, but does suggest there may be differences in DNA structure. The ability of ROA to provide such information is expected to be useful in structural studies of other viruses and nucleoproteins.

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