New Evidence for Conformational Flexibility in Cyclodextrins from Vibrational Raman Optical Activity

Alasdair F. Bell,* Lutz Hecht and Laurence D. Barron

Abstract: Conformational flexibility in cyclodextrins (CDs) as a function of methylation, solvent interaction and the extent of inclusion complex formation has been studied by using vibrational Raman optical activity (ROA). The work exploited the sensitivity of ROA to skeletal mobility by comparing the intensity of the glycosidic ROA couplet between about 850 and 970 cm^{-1} in maltoheptose (MH), β-cyclodextrin (β-CD), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) in buffered aqueous solution, in DMSO and with sodium benzoate and benzoic acid as guests in buffered aqueous solution. Increases in couplet signal strength were interpreted in terms of a reduction in conformational flexibility of the CD ring. In buffered aqueous solution the ROA intensity order MH < TM-β-CD < β-CD < DM-β-CD was observed. The linear molecule MH is expected to be the most flexible of the four oligosaccharides studied, while the changes registered for the three CD macrocycles may be related to the degree of intramolecular hydrogen bond formation and its influence on conformational flexibility. In DMSO, the same ROA intensity order is observed, but with an approximately constant increase relative to the values obtained in aqueous solution. This can be explained by the tighter binding of DMSO in the CD cavities compared with H_2O. For the inclusion complexes, our results indicate that the tighter the guest is bound, the larger is the reduction in the conformational flexibility of the CD macrocycle. The residual mobility sensed by ROA in CDs is similar to that sensed in proteins; this provides further insight into their analogous ligand-binding and catalytic properties.

Keywords
carbohydrates · conformation analysis · cyclodextrins · inclusion compounds · Raman optical activity

Introduction

It is now generally accepted that conformational flexibility is an important element in describing the behaviour of di-, oligo- and polysaccharides, which *in turn* is crucial to understanding how they function in biological systems.[1–3] In particular, there is a growing appreciation that cyclodextrins (CDs) are flexible systems and not the rigid entities sometimes portrayed in the literature. Until now flexibility in CDs has mainly been investigated by molecular mechanics (MM) and molecular dynamics (MD) calculations.[4–11] Here we apply the novel experimental technique of Raman optical activity (ROA) to this problem, since it has become apparent from recent studies on proteins[12–14] that ROA displays a remarkable sensitivity to conformational mobility.

ROA can be measured as a small intensity difference in Raman scattered radiation from chiral samples in right and left circularly polarized incident light.[15–19] Over the past few years a series of instrumental advances[20, 21] have improved the sensitivity of ROA measurements to the point where the spectra of aqueous solutions of biological molecules, such as proteins, nucleic acids and polysaccharides, can be recorded routinely.[22] A number of previous ROA studies on carbohydrates[23–29] have revealed that this technique provides information on the central stereochemical features of these molecules. In particular, studies on di- and polysaccharides have demonstrated that ROA can probe the type and conformation of the glycosidic link.[24, 25]

CDs are cyclic, nonreducing oligosaccharides usually composed of six, seven or eight D-glucose residues that are bonded through α-(1 → 4) glycosidic linkages and classified as α-, β- and γ-CDs, respectively.[30–35] The CDs have the general shape of a hollow, truncated cone, whereby the secondary hydroxyl groups project from the wider rim, and the primary hydroxyl groups from the narrower rim. The size of the central CD cavity depends on the number of D-glucose residues in the ring, and as all the C–H groups face inwards, these molecules have a hydrophobic centre. A remarkable property of CDs is their ability to form inclusion complexes with a wide variety of molecules in both aqueous solution and the solid state, which, among other things, is exploited in their use as enzyme mimics and in the construction of nanoscale devices.[30–35] The extent of complexation is determined by a number of factors, including the size of the guest molecule, hydrophobic forces, van der Waals interactions and hydrogen bonding; but it is also evident that a

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certain degree of flexibility is required in the CDs so that they can accommodate guests of different sizes and shapes.

An early ROA study on α-, β- and γ-CD in aqueous solution[29] revealed an enormous couplet centred at about 915 cm^{-1}, approximately an order of magnitude larger than those typically encountered, which was assigned to vibrations involving the glycosidic link. In this first study, a strongly alkaline solution was used to increase the otherwise low solubility of β-CD. However, thanks to the dramatic increase in the sensitivity of our ROA instrument achieved since then, we have been able to obtain excellent spectra with much more dilute samples (0.01–0.015 M) at neutral pH.

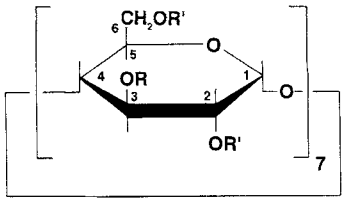

Figure 1. The structural formula of β-CD (R = R′ = H), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD; R = H, R′ = Me) and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD; R = R′ = Me).

Our results for β-CD and the methylated derivatives DM-β-CD and TM-β-CD, the structural formulae of which are depicted in Figure 1, as well as those for host–guest inclusion complexes with sodium benzoate and benzoic acid, indicate that the intensity of the glycosidic ROA couplet near 915 cm^{-1} is a sensitive probe of conformational flexibility in CD systems.

Results and Discussion

The Raman and ROA spectra of β-CD in buffered aqueous solution between 600 and 1500 cm^{-1} are presented in Figure 2. The focus of this study is the glycosidic ROA couplet centred around 915 cm^{-1}, so that, although there are some other interesting features in the ROA spectra, for the other samples we shall only display a small wavenumber region around the glycosidic couplet, and for simplicity will omit their conventional Raman spectra. In Figure 3, the ROA spectra of β-CD at 20 and 40 °C between about 850 and 970 cm^{-1} are compared. The ROA spectra of maltoheptose (MH), β-CD, DM-β-CD and TM-β-CD between about 850 and 970 cm^{-1} in buffered aqueous solution and DMSO are presented in Figures 4 and 5, respectively. As an example of the effect of inclusion-complex formation, the ROA spectra of β-CD with different initial molar ratios of sodium benzoate as a guest in buffered aqueous solution between about 850 and 970 cm^{-1} are shown in Figure 6.

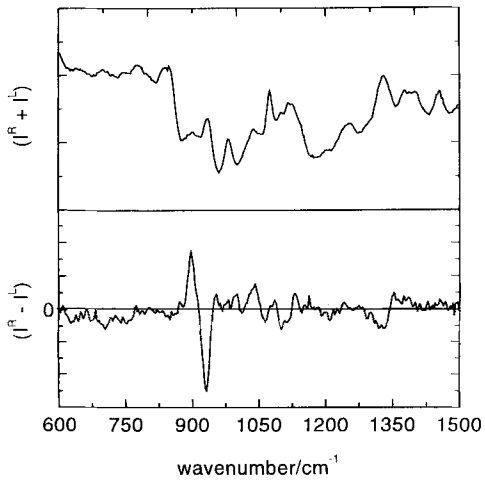

Figure 2. The Raman ($I^R + I^L$) and ROA ($I^R - I^L$) spectra of β-CD at 0.01 M between 600 and 1500 cm^{-1} in sodium phosphate buffer (pH 7, 0.02 M).

MH is a linear molecule composed of seven α-(1 → 4)-linked D-glucose residues, and it was chosen to provide a direct comparison between linear and cyclic molecules with the same number of D-glucose residues. TM-β-CD was included in this study because, as a result of the methylation of both the O(2) and O(3) groups of each D-glucose residue, this molecule is unable to form the intramolecular hydrogen bonds that are thought to stabilise CD ring structures.[30–35] On the other hand, DM-β-CD can form intramolecular hydrogen bonds, but only with the O(3) hydroxyl group as the donor, and the O(2′) hydroxyl group on an adjacent D-glucose residue as the acceptor. Methylation of the primary and secondary hydroxyl groups increases the size of the hydrophobic cavity and alters physical properties, such as solubility and binding specificity towards guest molecules, relative to β-CD.

The conventional Raman bands associated with the glycosidic ROA couplet of α-(1 → 4)-linked carbohydrates have been assigned to C–C and C–O skeletal ring stretches coupled with motions of the glycosidic link.[36–40] Earlier ROA studies[25, 26] found that the D-glucose monomer does not produce a couplet at around 915 cm^{-1}, but that the dimer D-maltose does. The intensity of this ROA couplet is found to increase incrementally in the corresponding linear D-glucose trimer, tetramer, pentamer and hexamer molecules (unpublished results), and this provides strong evidence that the generation of the glycosidic ROA couplet around 915 cm^{-1} in α-(1 → 4)-linked di- and oligosaccharides of D-glucose involves the vibrational coordinates of the glycosidic link. This glycosidic ROA couplet is also present in β-CD, DM-β-CD and TM-β-CD in H_2O, but with approximately thirty- to sixtyfold intensity increase relative to D-maltose.

Upon deuteration of the exchangeable hydroxyl hydrogen atoms, this couplet collapses into a relatively weak, broad, positive ROA signal at about 880 cm^{-1} for β-CD and DM-β-CD (but not for TM-β-CD, which lacks any exchangeable hydrogen atoms). This suggests that C-O-H deformations play an important role in generating the ROA signal. However, TM-β-CD lacks any C-O-H groups yet still exhibits an intense glycosidic ROA couplet in H_2O. Therefore, an alternative explanation for the observed differences between the spectra in H_2O and D_2O must be sought. A possibility is that C-O-D deformations, which are known to occur at 942 cm^{-1} in deuterated methanol,[41] couple with the C–C and C–O stretching coordinates to create a new set of normal modes that do not generate an intense glycosidic ROA couplet.

The ROA intensities as well as the wavenumber for the maxima, crossovers and minima of the glycosidic ROA couplet for MH, β-CD, DM-β-CD and TM-β-CD in buffered aqueous solution, in DMSO, and with sodium benzoate or benzoic acid as guests in buffered aqueous solution are listed in Table 1. In what

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follows we demonstrate that these results can be interpreted in terms of changes in conformational flexibility. A good starting point is X-ray data on CDs, both with and without guest molecules, which reveal variations of up to $\pm 30^{\circ}$ for the glycosidic link torsion angles, but only variations of up to $\pm 7^{\circ}$ for the ring torsion angles.[42] These values suggest that the conformational flexibility in CD structures resides mainly in the glycosidic links. This is also the conclusion from a number of molecular mechanics (MM) and molecular dynamics (MD) simulations, which also revealed that, contrary to earlier expectations, CDs do in fact enjoy a substantial degree of conformational freedom around the glycosidic links and that this conformational flexibility is a crucial factor in their ability to form inclusion complexes.[4-11]

Origin of the Sensitivity of ROA Signals to Conformational Flexibility: A qualitative explanation for the remarkable sensitivity of ROA signals to conformational mobility in heteropolypeptides has been proposed on the basis of a simple two-group model for the generation of ROA intensity.[12] Basically, this sensitivity originates in the dependence of ROA on absolute chirality, which leads to cancellation of contributions from enantiomeric structures, such as two-group units with equal and opposite torsion angles, which can arise as the mobile structure explores the accessible conformational space. In contrast, contributions to observables which are "blind" to chirality, such as conventional Raman band intensities, tend to be additive and hence much less sensitive to this type of mobility.

For the CDs (and other carbohydrates) a number of chiral two-group structures can be identified around the glycosidic link (e.g. O(5)C(1)O(1)C(4′), C(2)C(1)O(1)C(4′), C(1)O(1)C(4′)C(5′) and C(1)O(1)C(4′)C(3′) for an α-(1 → 4) linkage). Each thermally accessible torsion angle contributes to the overall ROA intensity, so that, as a result of its $\sin 2\theta$ dependence on the two-group torsion angle θ for stretching coordinates,[15] significant changes in the overall ROA intensity might be expected even for fairly small changes in the accessible torsion angles.

Temperature Dependence of the Glycosidic ROA Couplet in β-CD: On raising the temperature from 20 to 40 °C, the intensity of the glycosidic ROA couplet decreases by almost 20% (Figure 3). A comparison of the ^{13}C NMR spectrum of α-CD at 30 °C with that of the frozen solution at −40 °C revealed a broadening of signals on freezing, but no change in the chemical shifts; these observations were attributed to a reduction in the range of thermally accessible conformations.[43] This supports the conclusion that the intensity increases discussed below can be related to reduced flexibility in the CD rings.

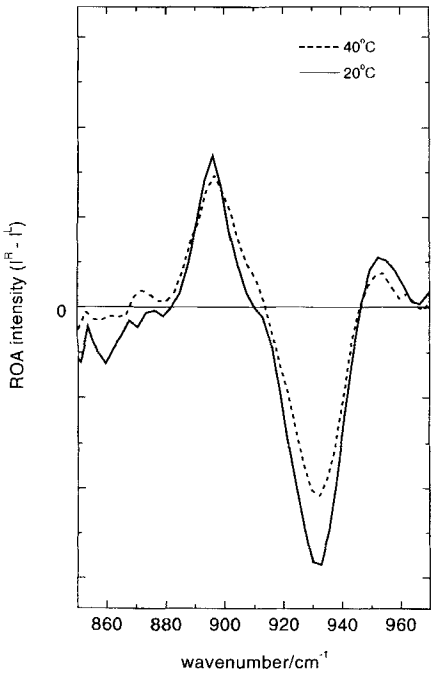

Figure 3. The ROA spectrum of β-CD between about 850 and 970 cm^{-1} in sodium phosphate buffer (pH 7, 0.02 M) at 20 and 40 °C.

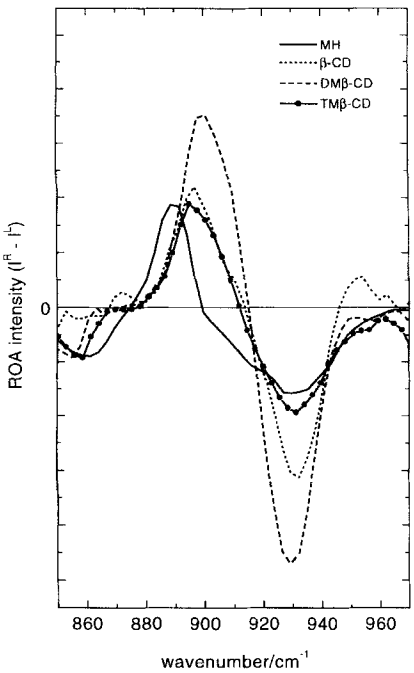

Figure 4. The ROA spectra of MH, β-CD, DM-β-CD and TM-β-CD between about 850 and 970 cm^{-1} in sodium phosphate buffer (pH 7, 0.02 M).

Uncomplexed CDs and MH in H_2O: From Table 1 and Figure 4 it is clear that in buffered aqueous solution the glycosidic ROA couplet intensity increases in the order MH < TM-β-CD < β-CD < DM-β-CD. For β-CD and its two methylated derivatives, cyclisation restricts the conformational flexibility around the glycosidic linkages as certain conformations are ruled out on steric grounds.[42] Thus it seems reasonable to expect MH, which is a linear molecule, to have a greater degree of conformational flexibility than the CDs and, therefore, a lower glycosidic couplet intensity. The wavenumbers of the maximum, crossover and minimum of the MH couplet differ from those of the CD couplets, all of which have remarkably similar wavenumber values. This observation, together with the intensity increase, may be regarded as an indicator of the difference between this class of cyclic molecules and their linear counterparts.

The fact that the glycosidic ROA couplet is also considerably weaker in TM-β-CD than in either β-CD or DM-β-CD suggests that it is considerably more flexible. Indeed, a number of solu-

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Table 1. Position of the maxima, minima and crossover points and the corrected integral intensities (I) of the glycosidic ROA couplets.

Oligosaccharide	Solvent	Max. [cm^{-1}]	Crossover [cm^{-1}]	Min. [cm^{-1}]	I [a] ($\times 10^{-6}$)
maltoheptose	H_2O [b]	890	899	930	0.76
β-CD	H_2O [b]	896	914	932	2.37
DM-β-CD	H_2O [b]	900	915	929	2.92
TM-β-CD	H_2O [b]	896	912	931	1.64
maltoheptose	DMSO	886	912	951	1.07
β-CD	DMSO	894	908	928	2.68
DM-β-CD	DMSO	902	915	925	3.21
TM-β-CD	DMSO	898	910	924	1.98
β-CD + sodium benzoate (1:1)	H_2O [b]	897	914	932	2.55
β-CD + sodium benzoate (1:5)	H_2O [b]	895	912	933	2.64
β-CD + sodium benzoate (1:50)	H_2O [b]	896	913	932	3.29
DM-β-CD + sodium benzoate (1:5)	H_2O [b]	900	915	930	2.95
DM-β-CD + sodium benzoate (1:50)	H_2O [b]	898	915	928	2.98
TM-β-CD + sodium benzoate (1:5)	H_2O [b]	898	914	931	1.67
TM-β-CD + sodium benzoate (1:50)	H_2O [b]	897	912	927	1.88
β-CD + benzoic acid (1:1)	H_2O [c]	896	915	931	3.02
DM-β-CD + benzoic acid (1:1)	H_2O [c]	899	915	930	3.50
TM-β-CD + benzoic acid (1:1)	H_2O [c]	899	916	925	2.00

[a] ROA intensity corrected for acquisition time, concentration and laser power. [b] Phosphate buffer, pH 7, 0.02 M. [c] Glycine buffer, pH 2.8, 0.1 M.

tion NMR,[44–47] UV circular dichroism,[48, 49] and X-ray studies[34, 50] have revealed a remarkable amount of distortion of the TM-β-CD macrocycle compared to β-CD and DM-β-CD. This increased flexibility can at least be partially explained by the fact that TM-β-CD cannot form any intramolecular hydrogen bonds since all three hydroxyl groups are methylated. In the solid state such intramolecular hydrogen bonds are known to form between the O(2) and O(3′) hydroxyl groups of adjacent residues and help to stabilise the ring structure and contribute to the rigidity of the macrocycle.[32, 33] However, in aqueous solution intramolecular hydrogen bonding may be weaker due to competition from intermolecular hydrogen bonds to the solvent. Indeed, MD simulations on α-CD incorporating water molecules indicate that the intramolecular hydrogen bonds may only be present for approximately 30% of the time.[6] There is, however, evidence from NMR and IR studies for intramolecular hydrogen-bond formation in [D_6]DMSO solution.[51–53] Thus, it seems probable that a significant fraction of the intramolecular hydrogen bonds will be formed in CDs in aqueous solution at any given time. Another factor in the increased flexibility of TM-β-CD are the two bulky methyl groups on the secondary hydroxyl rim, which force the molecule to adapt its conformation to avoid unfavourable steric clashes between these two methyl groups.

On going from β-CD to DM-β-CD there is again an increase in intensity for the glycosidic couplet in H_2O. One possible explanation for this increase is the stronger intramolecular hydrogen bonds formed by DM-β-CD compared to to β-CD, as revealed by NMR studies.[52, 53] These studies used [D_6]DMSO as the solvent, but as can be seen in Table 1, the same trend in the ROA intensities is observed for both H_2O and DMSO, indicating that the same mechanism for the increase may be responsible in the two solvents. Another possibility is that methylation of O(2) in some way restricts the rotation around the glycosidic link. This position is also methylated in TM-β-CD, but this derivative cannot form intramolecular hydrogen bonds and so does not provide a direct comparison.

Comparison of Uncomplexed CDs and MH in H_2O and in DMSO: In DMSO we observe the same intensity order for MH and the CDs as in H_2O (Table 1, Figure 5). Comparison of the ROA intensity data in H_2O with those in DMSO reveals a marked increase in couplet intensity and that the magnitude of this increase is approximately constant for the four oligosaccharides studied. One obvious explanation for this observation is that the increased degree of intramolecular hydrogen bonding expected in a less polar solvent leads to a decrease in flexibility. However, since we also observe this increase in TM-β-CD, which is incapable of intramolecular hydrogen bonding, this is certainly not the only factor.

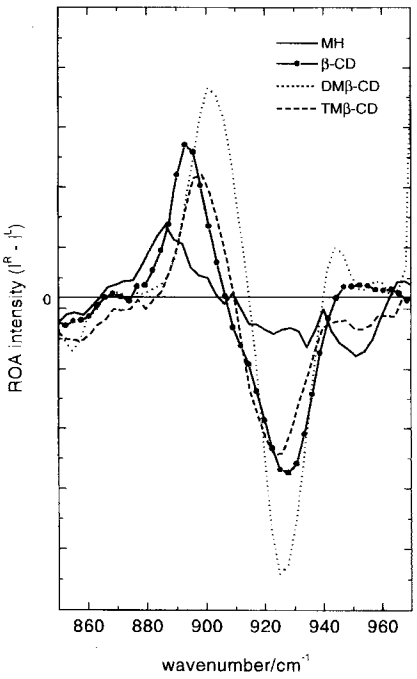

Figure 5. The ROA spectra of MH, β-CD, DM-β-CD and TM-β-CD between about 850 and 970 cm^{-1} in DMSO.

Another consideration is inclusion-complex formation between the CDs and DMSO molecules. It is known that in aqueous solution DMSO forms only very weak complexes with

β-CD (association constant $K_a = 1.44$[54]). However, as there are no other potential guest molecules present, and the cavity cannot remain empty, DMSO molecules must be included. The fact that DMSO exhibits an association constant greater than unity means that it binds more tightly than H_2O. One consequence of this tighter binding is that DMSO would be expected to cause a greater reduction in the conformational flexibility of the host. Thus, although the difference in binding between DMSO and H_2O is small, a moderate intensity increase is observed since every CD cavity will have DMSO molecules included. Furthermore, the almost constant value of this increase suggests that the difference in binding between H_2O and DMSO is similar for the three CDs studied here. In addition, it has been demonstrated that low molecular weight chains of α-(1 → 4)-linked D-glucose residues, such as MH, form helical wormlike chains in DMSO solution.[55] Such helix formation is likely to reduce the flexibility of these chains and thus result in an increase in the couplet strength which might explain the observed intensity increase for MH.

Comparison of Uncomplexed and Complexed CDs in H_2O: The glycosidic ROA couplet intensity data for β-CD, DM-β-CD and TM-β-CD complexed with sodium benzoate and benzoic acid in buffered aqueous solution are compared with the corresponding data for the uncomplexed CDs in Table 1. The pK_a of benzoic acid increases from 4.06 to 5.1 when it is complexed with β-CD.[56] Therefore, to ensure that either benzoic acid or sodium benzoate was the dominant species complexed, the solutions were buffered at pH 2.8 and 7.0, respectively. Due to the far greater solubility of the conjugate base, a larger range of molar ratios could be investigated. We also measured the glycosidic ROA couplet intensity of uncomplexed β-CD, DM-β-CD and TM-β-CD at pH 2.8 (not shown) and, with the exception of DM-β-CD for which a moderate increase was observed, found no significant difference to the values obtained at pH 7.0.

We found that all the CD complexes studied, with the exception of DM-β-CD with sodium benzoate, exhibit an increase (of varying magnitude) in the glycosidic ROA couplet intensity, whereas MH shows no such increase. This we interpret in terms of inclusion-complex formation, which is expected to reduce the flexibility of the CDs as they must adapt to provide the best fit for the guest molecules. This result is consistent with MM and MD calculations on CD inclusion complexes that describe a "freezing" of the glycosidic torsion angles upon complex formation.[9–11] Such calculations have also predicted an increase in intramolecular hydrogen bonding upon complexation.[6]

Specifically, for β-CD with sodium benzoate as a guest we observe a progressive increase in the glycosidic ROA couplet intensity as the starting conditions are altered to produce a greater percentage of complexed CD molecules (i.e. increasing the initial molar ratio in favour of the guest; Figure 6). TM-β-CD also exhibits an increase in couplet strength upon complex formation, although the observed change is smaller than for β-CD. However, as mentioned above, for DM-β-CD with sodium benzoate as a guest, the value is almost constant over the range of initial molar ratios examined, which may indicate that DM-β-CD forms only weak complexes with sodium benzoate. Complex formation of the three CDs with benzoic acid is also shown by the increase in couplet intensities listed in Table 1. The

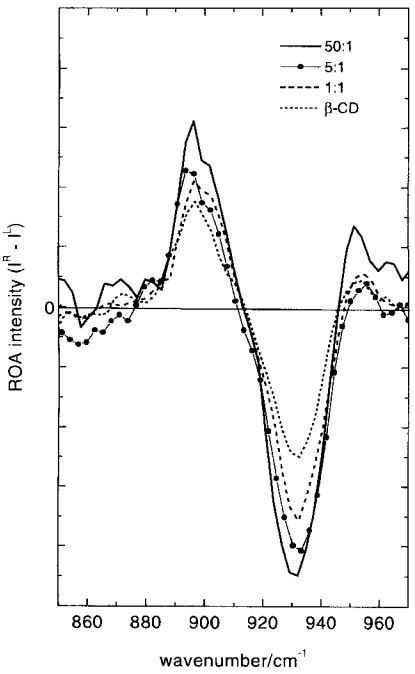

Figure 6. The ROA spectra of β-CD with sodium benzoate as guest between about 850 and 970 cm^{-1} in sodium phosphate buffer (pH 7, 0.02M) with different initial molar ratios (sodium benzoate:β-CD).

size of these increases, for 1:1 inital molar ratios, are much larger in each case than for the corresponding complexes with sodium benzoate. This observation reflects the larger K_a values for benzoic acid with CDs. For example, the K_a values for β-CD complexed with benzoic acid and sodium benzoate are 600 and 60, respectively.[57] These values fit our ROA data if it is considered that tighter guest binding results in a greater reduction in macrocycle flexibility. Similarly, for TM-β-CD bound to benzoic acid a K_a value of 200 is found,[57] and the corresponding change in ROA intensity upon complexation is smaller than that observed for β-CD, which binds more tightly to this particular guest. These results suggest that it may be possible to obtain association constants for CD complexes from ROA data.

Concluding Remarks

Our ROA measurements provide experimental evidence for flexibility in CD systems that supports, at least in a qualitative manner, the results of a number of MM and MD calculations.[4–11] Such flexibility can account for the wide range of guests that can be incorporated, since guests must be allowed to enter and leave the CD cavity, and the CDs must adapt to changes in rotational orientation of the guest within the cavity, which have been observed in NMR experiments.[58] Our results also suggest that the main cause of this reduced flexibility is an increase in the intramolecular hydrogen bonding.

The present ROA study on CDs, together with the previous ROA studies on proteins,[12–14] provides further insight into the analogous behaviour of the two types of molecules with respect to ligand binding and catalytic activity. In both cases residual mobility within the links connecting a framework of rigid elements allows rapid sampling of a range of conforma-

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tions without loss of the overall three-dimensional integrity of the structure (the native tertiary fold in the case of proteins).

In extending our earlier ROA work on the glycosidic link in di- and polysaccharides[24, 25] to CDs, we have discovered a correlation between the flexibility of these systems and the intensity of the glycosidic couplet at about 915 cm^{-1}. This reinforces our earlier conclusion that ROA is a valuable new source of information on the glycosidic link, which is often the most important factor in determining the conformation of di-, oligo- and polysaccharides.

Experimental Section

All the samples used in this study were supplied by Sigma Chemical Corporation. MH was used without further purification; β-CD and its two methylated derivatives were recrystallised from water. The samples for ROA measurement were prepared by dissolving a carefully weighed quantity of carbohydrate into 200 μL of the appropriate aqueous buffer or DMSO (Sigma, 99.9%). These solutions were then passed through 0.45 μm Millipore membrane filters into quartz microfluorescence cells and centrifuged for approximately 10 min. Samples of DM-β-CD and TM-β-CD as well as the four samples run in DMSO had moderately high fluorescence backgrounds which were removed by photobleaching the samples overnight in the laser beam prior to ROA acquisition. The inclusion complexes were prepared by dissolving the CDs in phosphate buffer (pH 7.0, 0.02 M) or glycine buffer (pH 2.8, 0.1 M) before adding an appropriate quantity of sodium benzoate or benzoic acid to give the required molar ratio. The resulting solutions were then stirred for at least 1 h and left overnight to equilibrate.

The unpolarised, backscattering Raman and ROA spectra of all the samples presented here were recorded on the ROA instrument, GUROAS3. This is an upgraded version of the GUROAS1 instrument previously described in detail elsewhere.[20] The most important difference between the two is the introduction of a new $f/1.4$ stigmatic spectrograph based on a novel transmissive diffraction grating (Kaiser, Holospec) which, like the GUROAS1 astigmatic single grating $f/4.1$ spectrograph, is equipped with a Peltier cooled back-thinned charge coupled device (CCD) detector for a virtually shot noise limited detection of Raman-scattered photons, and a high optical density holographic notch filter (OD > 8) for an efficient suppression of the Rayleigh line. This new spectrograph required some modifications to the optical system[21] but results in roughly a five-fold increase in the speed of ROA measurements with slightly improved resolution. In addition, the aluminium coated 45° mirror has been replaced with an enhanced silver-coated mirror (Balzers, Silflex) and a new temperature-stabilised electro-optic modulator (EOM) has been employed. These last two components have considerably improved the artifact suppression in this new instrument.

In this study we have concentrated on the raw ROA intensities $I^R - I^L$ rather than on the dimensionless Δ values $\Delta = (I^R - I^L)/(I^R + I^L)$, where I^R and I^L are the Raman scattered intensities for right and left circularly polarised incident light, respectively, because of the difficulties inherent in correctly measuring accurate Raman intensities. One problem is separating the background from the signal (although this can be alleviated by using solvent subtraction techniques), but more important in this instance is the problem of separating the two or more closely spaced bands found in the conventional Raman spectra in the range of the glycosidic ROA couplet. In contrast, there are no background or deconvolution problems associated with measuring the ROA intensities. However, there is the possibility of small baseline offsets, which we have combatted by adding together the modulus of the integrated intensities of the positive and negative components of the glycosidic couplet with a Labcalc (Galactic) array basic program, which was also used for all the spectral manipulations.

Laser powers at the sample between 250 and 700 mW (at 514.5 nm) were used. The width of the entrance slit was 100 μm, which corresponds to a spectral resolution of approximately 10 cm^{-1}. Concentrations of CDs ranged between 0.01 and 0.15 M, depending upon the solubility of the CD derivatives in particular. All pH measurements were made with a micro-combination electrode (Ingold). For the ROA measurements on β-CD at 40 °C, hot dry air was blown over the sample cell with an FTS systems model TC-84 AirJet Crystal Cooler.

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