Far-Ultraviolet Circular Dichroism of Solutions, Gels, and Films of Chitins

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Abstract: Circular dichroism spectra are reported for hexafluoro-2-propanol solutions of chitin (to 160 nm) and chitan (to 180 nm), films of chitin and chitan cast from hexafluoro-2-propanol (to 140 nm), films of chitin cast from HCl (to 160 and 140 nm), gels of chitin and chitan (to 188 nm), and films of 2-acetamido-2-deoxy-D-glucosamine cast from hexafluoro-2-propanol (to 150 nm). We associate the polymer CD observed in the solid state (films and gels) with conformations reported previously for chitin and chitan mats. A trans amide conformation and intermolecular hydrogen bonding are proposed as the important determinants of the observed solid-state CD.

The optical activity of the amide group in peptides has been the subject of many experimental and theoretical investigations of peptide conformation. That chromophore is also present in numerous components of glycolipids, glycoproteins, and proteoglycans as the acetamide functional group of carbohydrate moieties. Optical activity studies of mono- and oligosaccharides have demonstrated the high sensitivity of acetamide $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ CD[2] to solvent, configuration, and conformation,[3] and acetamide CD has been observed to reflect polymer secondary structure of glycosaminoglycans, the polysaccharide members of the connective tissue proteoglycans.[4]

Glycosaminoglycan CD is complicated by the presence of two chromophoric groups with overlapping electronic transitions, the carboxyl and acetamide substituents. In a comparison of the solution and solid-state CD of sodium hyaluronate[4b] and in studies of conformational transitions in solutions of hyaluronic acid and chondroitins[4c,d] some major CD changes were tentatively attributed to the amide chromophore. The ability to assign features of the CD to individual chromophoric groups is a prerequisite to extraction of the specific conformational information which CD can potentially provide.

It is therefore desirable to investigate the CD of a polysaccharide containing no carboxyl groups but otherwise closely related in structure to the glycosaminoglycans. The β-1,4-linked 2-acetamido-2-deoxy-D-glycosamines chitin and chitan are two such naturally occurring polysaccharides. In both glycosaminoglycans and chitins the acetamide group is an equatorial substituent at the C-2 position of a pyranose residue in an unbranched polymer (Figure 1).

Chitin is found in anthropods, green algae, yeast, and fungi, and, like the glycosaminoglycans, it is often associated with protein. Chitan comprises the extracellular fibers of the diatom, *Thalassiosira fluviatilis*. The production of these fibers increases the viscosity of the diatom media.

The CD of both chitin and chitan in three states (solution, gel, and film) were measured; the use of a vacuum UV CD instrument permitted observation of the high-energy wavelength regions.[5]

Experimental Section

Chitin and chitosan were Seikagaku Kogyo Co. special grade polysaccharides; the chitin source was Taraba crab and the manufacturers report a molecular weight range of 29 000–85 000. Such samples are not completely acetylated and typically contain about 12% free amine groups.[6] The sodium D line rotation of the chitin sample after 40 min (*c* 0.1, concentrated HCl) was −19; the value reported for another crustacean sample was −21 (*c* 1.03, 10 N HCl) after 30 min.[7]

Chitan was a gift from Dr. A. G. McInnes of the Atlantic Regional Laboratory of the National Research Council, Halifax, Nova Scotia. The chitan molecular weight is approximately 30×10^6. Chitan is 100% acetylated at the C-2 nitrogen.[7] The sodium D line rotation reported for chitan is −19 (*c* 1.04, 10 N HCl) after 30 min.[7] HFIP, Sequanal grade, was purchased from the Pierce Chemical Co. The 2-acetamido-2-deoxy-D-glucosamine was from Calbiochem, grade A.

The vacuum UV CD instrument has been previously described.[5] Spectra were recorded with effective bandwidths of 1.6 or 3.2 nm, a time constant of 30 s, and a scan speed of 0.5 or 1.0 nm/min. Absorbance, optical rotation, and supplementary CD measurements were obtained on a Jasco Model ORD-UV-5 optical rotatory dispersion recorder equipped with a CD attachment (Sproul Scientific SS 15-2).

Weighed samples of chitin and chitan were dissolved in HFIP to known total volumes. After overnight sitting at room temperature some insoluble material occasionally remained; this was removed by filtration with 3-μ pore size Millipore filters. Optical measurements on solutions and gels were made in fused silica cells of 0.05-, 0.1-, 1-, and, in the case of sodium D line rotation meausrements, 50-mm path lengths. Molar ellipticities were determined from the solutions in which there was no precipitation, and were calculated on the basis of an average monosaccharide residue molecular weight of 198 for chitin and 203 for chitan.

The CD of polymers in the solid state was measured on films formed by evaporation on CaF_2 crystals of solvent from a known volume of solution. Chitin and chitan films from HFIP were evaporated over Drierite; films from HCl solutions were formed more quickly in evacuated desiccators. Chitin is insoluble in H_2O; films termed "chitin, H_2O" resulted from the addition of H_2O to films cast from HCl solutions. Any films which showed a dependence of their CD signal upon orientation in the light path were discarded. Chitan showed a greater tendency than chitin to form such oriented films; lower concentrations of casting solution resulted in orientation-free films.

Film molar ellipticities were calculated from the mole per unit film area. The area of the film and the volume and concentration of casting solution determine this number; variations in film thickness introduce uncertainty into the molar ellipticity value.

Results

Chitin and chitan are insoluble in most solvents; previous solution studies have consisted of observation of their hydrolysis in HCl through nuclear magnetic resonance and optical rotation. Hydrolysis does not occur in HFIP as evidenced by the constancy of sodium D line rotations of these solutions (−58, *c* 0.1).

The solution spectrum of chitin in HFIP (Figure 2) is different from that of the related monomer (methyl-2-acetamido-2-deoxy-β-D-glucopyranoside) and dimer [2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose, chitobiose] in HFIP. It is, however, similar to that of 2-acetamido-2-deoxy-D sugars differing from those compounds (and from chitin) in configuration at C-4 or C-1 or in linkage position.[3d]

The CD spectrum of films cast from chitin solutions in HFIP shows generally increased molar ellipticities and a sign change

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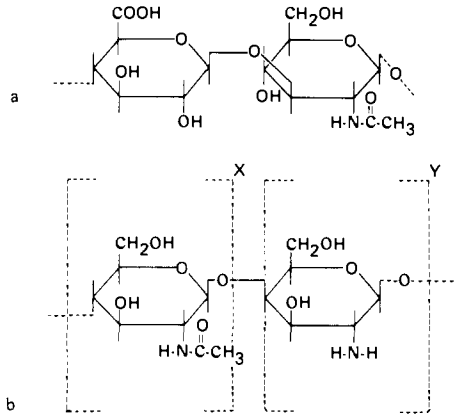

Figure 1. (a) Hyaluronic acid. (b) Chitan ($Y = 0$), chitin ($X \gg Y$), and chitosan ($X \ll Y$).

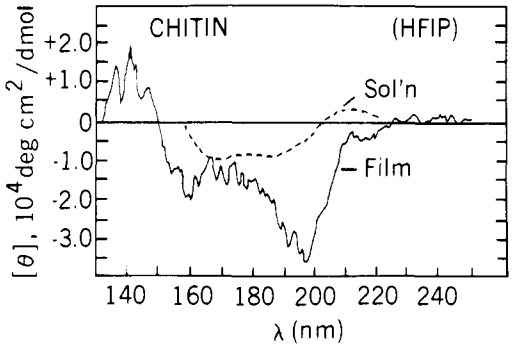

Figure 2. CD of chitin, in HFIP solution (dashed line) and as a film cast from HFIP (solid line, actual tracing of spectrum).

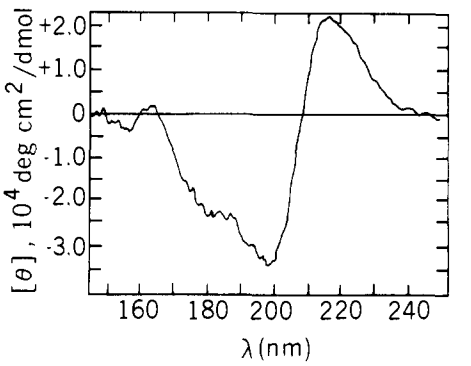

Figure 3. Vacuum UV CD of a 2-acetamido-2-deoxy-D-glucosamine film cast from HFIP (actual tracing of spectrum).

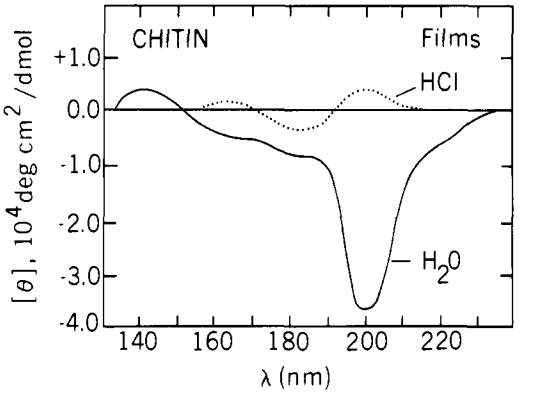

Figure 4. CD of chitin, film cast from HCl (dotted line) and film formed after water is added to HCl film (solid line).

in the n→π* band (Figure 2). Absorption spectra show a shift of maximum absorbance ($\epsilon \sim 10^4$) from 186 nm in solution to 194 nm in film. This confirms the assignment of the 197-nm film CD extremum to a π→π* transition red shifted relative to solution. Similar shifts in both CD and absorbance were observed upon formation of films from solutions of 2-acetamido-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-glucose and from the latter in H_2O.[8] An example of the CD of a monomer film is shown in Figure 3.

To minimize hydrolysis in HCl work, films were cast and solution CD measurements recorded shortly after addition of solvent to chitin samples. The smallness of the increase in sodium D line rotation at the time of casting or recording of spectra, and the insolubility in H_2O of the films, indicated a low degree of degradation. Addition of H_2O converted the acid film (Figure 4, dotted curve) to the water film (Figure 4, solid curve); addition of HCl to the water film gave back the acid film spectrum. Negative CD bands near 205 nm were found for HCl solutions of 2-acetamido-2-deoxy-D-glucose and chitin; solvent absorbance prevented detection of higher energy bands.

The CD of chitan in HFIP solution and film are presented in Figure 5. Comparison of chitin and chitan spectra shows that the free amine groups of chitin do not affect the shape of the CD curve; any effect on the magnitude of film molar ellipticities is less than the variation in these values found for each polymer. Again, red shifts of the π→π* absorption maxima accompanied film formation.

Chitan solutions in HFIP gelled upon sitting at concentrations of as low as 1 mg/mL; gelation was also induced through addition of water. Gel formation was never observed for chitin in pure HFIP, but was induced by addition of methanol, H_2O, or ether to the HFIP solution. A chitin gel in formic acid has been described; its preparation required an initial dispersion of chitosan in formic acid followed by acetylation to chitin.[9] The chitan gel in mixed HFIP/H_2O is a possible model system for the viscous extracellular mucilage of diatom cultures.

The CD and absorption spectra of these gels resemble those of HFIP films,[10] although the intensities and positions of the negative π→π* band vary more widely than among the films. Figure 6 (dotted curve) shows the CD of a chitan gel which formed from a 1.5 mg/mL solution in HFIP. The CD changes accompanying gelation occurred gradually. Various spectra intermediate between the solution spectrum (Figure 5) and the gel spectrum were found, and we expect that molar ellipticities greater than those of Figure 6 might be observed. The dashed curve in Figure 6 is typical of that found for chitan gels containing H_2O; magnitudes as large as −90 000 cm²/dmol were observed. The CD of the gel formed from 1 mg/mL chitin in 1:4 H_2O/HFIP is shown as the solid curve in Figure 6; the observed values varied by as much as 30% from these.

Discussion

The CD of hyaluronic acid in forms likely to be highly ordered (e.g., sodium hyaluronate films and hyaluronic acid at low pH in mixed solvents)[4b-d] is similar in some respects to that reported here for chitin and chitan films and gels. In all three instances a red shift of a band in the π→π* region is observed in the CD spectra, and is accompanied by an increase in molar ellipticity relative to single solvent, solution conditions. The chitin/chitan case provides strong evidence for the origin of the π→π*-region effect in hyaluronic acid to be the amide π→π* transition,[11] and offers the opportunity to discuss the relationship of the observed amide CD with molecular structures likely under the experimental conditions.

The solid-state structures of chitan and chitin mats have

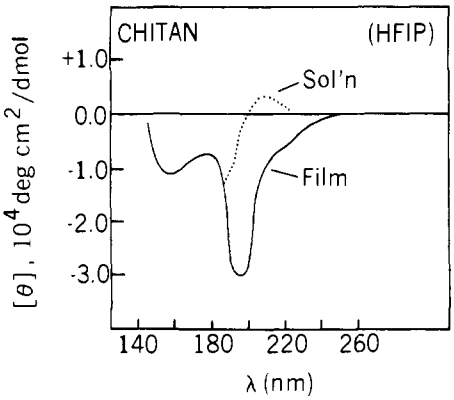

Figure 5. CD of chitan, HFIP solution (dotted line), recorded on Jasco instrument, and as a film cast from HFIP (solid line).

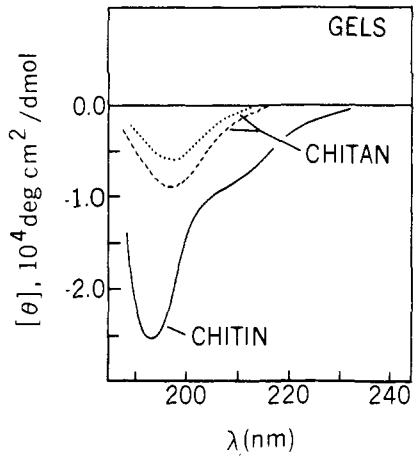

Figure 6. CD of chitan gel formed from HFIP solution (dotted line), CD of chitan gel formed from HFIP plus H_2O (dashed line), and CD of chitin gel formed from HFIP plus H_2O (solid line).

been characterized by X-ray diffraction and infrared spectroscopy.[6,12] Three forms have been identified; the proposed structures share most features and differ in the sense of alignment of neighboring chains (parallel, antiparallel, and mixed). The α and β structures consist of sheets made of intermolecularly hydrogen bonded chains with both the carbonyl and nitrogen protons of each amide participating in the hydrogen bonds. The amide groups are in the trans conformation and the amide plane is nearly perpendicular to the ring "plane" (amide oxygen eclipsing C-2 H). There are intramolecular hydrogen bonds across the glycosidic linkage between O-5′ and C-3 OH.

The similarity of the CD in the HFIP and H_2O chitin films (Figures 2 and 4, solid curves) and in the chitan and chitin HFIP films (Figures 2 and 5, solid curves) suggest a favoring in the film solid state of whichever conformational features determine the solid-line spectrum of Figure 2; it is quite possible that these are the features common to the three infrared and X-ray diffraction structures. Thus, we tentatively associate the solid-line CD pattern of Figure 2 with the conformations found in chitin and chitan mats.

It may be that in the HCl films (Figure 4, dotted line) the highly hydrogen-bonded network is disrupted as intermolecular hydrogen bonds are replaced by protonation of the amides in the formation of the hydrochloride. The large blue shift of the $n\rightarrow\pi^*$ in the HCl film spectrum supports that interpretation.

Since the CD spectrum of Figure 2 is also found for chitin and chitan gels (Figure 6), the conformational constraints which determine the CD may be common to both gel and film conformations. If so, factors influencing gelation may indicate the nature of those constraints. Conformational transitions have been found to play important roles in gelation of several polysaccharides,[13] and the use of CD as a monitor of such transitions has been demonstrated for agarose.[14]

Addition of cosolvents to chitin solution in HFIP induces gelation. The availability of HFIP is decreased, and solvation by HFIP may be replaced by interaction with the cosolvent, intramolecular interactions, or intermolecular solute–solute interactions. It is unlikely that solvation by cosolvent could promote gelation. Increasing intramolecular interactions, such as O-5′ to C-3 OH hydrogen bonding, would imply a lesser solvation at these sites by water than by HFIP. This is unlikely in view of the insolubility of many unsubstituted polysaccharides in HFIP which are soluble in H_2O. HFIP is a good amide solvent in which chitosan, the deacetylated polymer of chitin, was found to be insoluble. It is therefore presumed that the acetamide groups of chitin and chitan in these solutions are strongly solvated.[15] Removal of the HFIP–amide interaction increases the likelihood of intermolecular solute–solute interactions, such as hydrogen bonding between amides. Such intermolecular interactions could provide a network necessary for gelation.

HFIP solutions of chitan can gel even in the absence of a cosolvent. Chitan is distinguished from chitin by the absence of free amine groups and its greater molecular weight; the greater propensity for gelation of chitan must lie in either or both of these factors. Postulation of intramolecularly hydrogen-bonded species as promoters of gelation does not explain the influence of these two factors. The replacement of amine with acetamide substituents should not affect the strength of an O-5′ to C-3 OH hydrogen bond and, at the glycosidic angle ranges permitting such a bond, the contacts between acetamide atoms and groups on neighboring residues would be at least as sterically unfavorable as those involving the smaller amine group. In low molecular weight species one might expect increasing chain length to favor transitions to ordered forms in which cooperative interactions such as the O-5′ to C-3 OH hydrogen bond are involved. The molecular weight of the chitin sample is great enough, however, that no further increase should affect such a transition.

It is expected, though, that the absence of amine groups and longer chains would enhance gelation in mechanisms determined by intermolecular interactions. An increase in the percentage of acetamido groups increases the opportunities for formation of intermolecular amide–amide hydrogen bonds. Likewise longer chains are more effective in network formation. Thus, while intramolecular hydrogen bonding with constraint of C-3 OH orientation and of glycosidic angles may occur in the gelled chitin and chitan as well as in the films, it is the implication of our work that it is intermolecular interactions between amide groups rather than intramolecular interactions that is the more important factor in gel formation.

Only the trans amide conformation would permit amide groups to participate in two hydrogen bonds as both donors and acceptors. The orientation of the amide planes with respect to the ring would not be limited to that found in the solid-state conformation. Therefore a trans amide conformation and intermolecular hydrogen bonding are suggested as the important determinants of the solid-state CD in Figure 2. Information on the acetamide conformation in sodium hyaluronate films is available from X-ray diffraction studies. It is not known to which of the several observed diffraction patterns the hyaluronate film of the CD study most closely corresponds. In all of the proposed diffraction structures, however, the amide groups are trans, and oriented at 20–40° from eclipsed positions of the C-2 hydrogen.[16]

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References and Notes

(1) Department of Biochemistry, University of Wisconsin, Madison, Wis. 53706.
(2) The following abbreviations are used: CD (circular dichroism), HFIP (hexofluoro-2-propanol), UV (ultraviolet).
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(10) The chitan and chitin gels contain more solvent than do the films and resemble the films only in their transparency. The HFIP films are very thin and "dry" in appearance. Any residual HFIP is so tightly bound that neither water rinsing nor several hours at $<10^{-5}$ Torr changes the appearance or optical properties of the films.
(11) Changes in the lower energy CD of hyaluronic acid have been assigned to the carboxyl $n\rightarrow\pi^*$ band; measurements on carboxyl-containing model compounds indicate that the $\pi\rightarrow\pi^*$ transition of this group occurs near 182 nm in both solutions and films.[8]
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Effect of External Pressure on the Spectroscopic and Conformational Properties of the Visual Chromophores

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Abstract: The effect of external pressure (3900–23 700 psi) on (1) the spectroscopic properties of *all-trans*- and 13-*cis*-retinal and (2) the conformational equilibrium between the 12-s-cis and 12-s-trans conformers of 11-*cis*-retinal is analyzed in methylcyclohexane solvent. The latter data are interpreted using a pressure and solvent effect formalism which explicitly includes the contributions of dispersive and electrostatic interactions, and the effect of external pressure on those interactions, in the calculation of the total free energy change with applied external pressure. The experimental results indicate that the 12-s-trans conformer is preferentially stabilized at high external pressure despite the fact that this conformer is predicted to have the larger molecular volume in solution. This rather unusual experimental result is explained in terms of a significant preferential electrostatic stabilization of the 12-s-trans species with increased pressure due to the larger dipole moment of this conformer and the increase in the solvent reaction field with external pressure. The 12-s-trans conformer is calculated to have a dipole moment at least 1.6 D larger and a cavity radius at least 0.012 Å larger than the 12-s-cis conformer. The 12-s-cis conformer is calculated to have a vacuum free energy approximately 0.5 kcal/mol lower than that of the 12-s-trans conformer.

I. Introduction

The initial molecular event of vertebrate vision is postulated to involve a photochemical isomerization of the polyene chromophore, 11-*cis*-retinal, which is bound to the opsin protein of rhodopsin via a protonated Schiff base linkage.[1-5] Although the crystal conformation of 11-*cis*-retinal has been demonstrated to have a distorted 12-s-cis geometry,[6] in ambient temperature solution this molecule populates two energetically similar conformers with 12-s-cis and 12-s-trans geometries (see Figure 1).[7-18] Investigators have studied the conformational properties of 11-*cis*-retinal using a variety of experimental[7-13] and theoretical[14-18] techniques. Nevertheless, the conformational properties of this molecule are still a subject of debate (see, for example, ref 7 and 10). Furthermore, the conformation of the chromophore in rhodopsin is still not unequivocally assigned, although spectroscopic data suggest that a 12-s-trans geometry is likely.[19-21]

A recent solvent effect investigation of 11-*cis*-retinal predicted that the 12-s-trans conformer occupies a larger solvent cavity in solution than the 12-s-cis conformer.[7] The difference in cavity radii of the two conformers was calculated to be approximately 0.03 Å,[7] and this relatively large difference encouraged us to investigate the conformational properties of 11-*cis*-retinal using external pressure.[22] We expected to spectroscopically observe an increase in the population of the smaller conformer with increasing external pressure. Our data, however, leads us to the surprising conclusion that applied external pressure stabilizes the larger, 12-s-trans conformer of 11-*cis*-retinal in methylcyclohexane. A detailed analysis of the pressure effect data using the formalism described in the theoretical section provides an explanation for this interesting pressure-induced conformational equilibrium. In particular, our results indicate that pressure-induced electrostatic stabilization is a very important variable in determining the effect of external pressure on the conformational equilibria of polar compounds even in nonpolar solvents. Our calculations indicate that the 12-s-trans conformer has a dipole moment at least 1.6 D larger, a cavity radius at least 0.012 Å larger, and a vacuum free energy approximately 0.5 kcal/mol larger than the 12-s-cis

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