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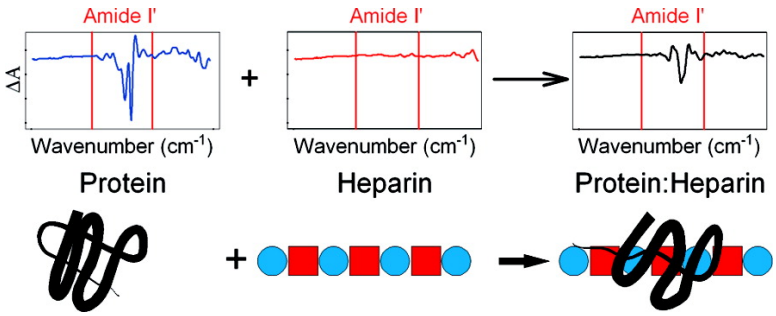

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Selective Detection of Protein Secondary Structural Changes in Solution Protein−Polysaccharide Complexes Using Vibrational Circular Dichroism (VCD)

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The challenge of storing and transmitting the vast quantities of information required for intercellular communication in living organisms was met by employing complex carbohydrates. The structural differences of the constituent sugars and manifold modes of connecting them to form oligo- and polysaccharides resulted in structural diversity with vast potential information content. Conformational differences of carbohydrates are brought to bear through interactions with proteins, often accompanied by protein conformational changes following binding, resulting in altered activity. Examples include the binding of monosaccharides by lectins, oligosaccharides by selectins and larger polysaccharides to a host of growth factors, serine proteases, and other proteins.[1−6] Other examples of the influences of carbohydrates on protein structure and activity include N- and O-linked glycoproteins, which often exhibit markedly different activities following removal of the carbohydrate portion.[6,7] However, observing and accurately quantifying the consequences of carbohydrate (particularly polysaccharides) binding to proteins in solution is difficult, especially for carbohydrates containing chromophores, notably *N*-acetyl groups, that overlap those spectral features usually employed for protein secondary structural analysis. These features occur between ∼170 and 250 nm in electronic CD ($n-\pi^*$ transitions in carbonyl chromophores) and the amide I (C═O stretching ∼1650 cm^{-1}), Amide II (N−H bending and C−N stretching ∼1550 cm^{-1}), and amide III (coupled C−N stretching and N−H bending ∼1300 cm^{-1}) bands in FTIR. Furthermore, the high molecular weight, consequent low mobility, and anisotropic motion of polysaccharides effectively preclude NMR studies of many protein−polysaccharide complexes.

These problems are acute for many biologically relevant polysaccharides such as the glycosaminoglycans (GAGs) that include heparan sulfate (HS), heparin, chondroitin sulfates (CS-A, CS-B, and CS-C and hyaluronic acid), and others such as colominic acid (CA); α(2,8)-linked poly-*N*-acetyl neuraminic acid, and *Escherichia coli* K5; α(1,4)-linked poly(D-GlcA β(1,4) D-GlcN). We have also recently shown that the CD spectra of heparin polysaccharide and its derivatives change in both position and intensity as a function of substitution pattern, cation form, and conformation.[8] NMR studies of GAGs and their derivatives, employing $^1J_{CH}$, $^3J_{HH}$, and NOE measurements, have revealed that the conformation of the glycosidic linkages and the equilibrium of iduronate ring conformers between the $^1C_4$,$^4C_1$, and $^2S_0$ forms change as a function of substitution pattern[9] and counterion identity,[8] providing an explanation for the variation in their CD spectra. This challenges the contention that protein secondary structure changes following binding of these molecules can be analyzed accurately by CD either by subtraction or spectral deconvolution. The residual CD spectrum following subtraction of the polysaccharide spectrum from the protein−polysaccharide complex (Figure 1) could be due to any combination of conformational changes in either the protein or polysaccharide. This prompted us to search for a spectroscopic technique which was sensitive to the structural changes taking place in the protein component of protein−polysaccharide complexes while being insensitive to changes in the polysaccharide component, thereby allowing dissection of the structural changes.

Vibrational circular dichroism (VCD; CD in the IR region) arises from the differential interaction and adsorption of left and right circularly polarized light (characterized by a $\pm\pi/2$ phase lag of the electric and magnetic components) with the electric and magnetic transition moments of chiral molecules.[10] The magnitude of VCD is defined by the dissymmetry factor $\Delta\epsilon/\epsilon = 2(\epsilon_L - \epsilon_R)/(\epsilon_L + \epsilon_R)$, where ϵ_L and ϵ_R represent the molar absorption coefficients of left and right circularly polarized light and $\Delta\epsilon/\epsilon$ is proportional to $1/\lambda$, where λ is the wavelength, explaining why the effect is weaker in the IR (10^{-4}−10^{-5}) than that in the UV−visible range (10^{-3}).[10] VCD is a potentially powerful spectroscopic technique in the analysis of protein secondary structure. The short-range nature of VCD phenomena gives rise to characteristic spectra for secondary structures,[11] which contribute proportionally to the final spectrum, unlike electronic CD, in which α-helical structures tend to dominate. In addition, α-helical and β-sheet structures exhibit characteristic VCD signals with both positive and negative spectral features in the amide I′ region (Figure 3, ConA), improving their differentiation and assisting assignment, an advantage particularly over transmission FTIR, in which signals from secondary structural elements can be misassigned because of variation in their spectral positions.

However, for polysaccharides that obscure CD (180−250 nm)[8] and FTIR spectral features (amide I′ band; 1600−1700 cm^{-1}) of the protein component of protein−polysaccharide complexes, their VCD spectra differ from those of polypeptides in two respects; the first is that the amide chromophores are usually less well-represented in polysaccharides. The second, more fundamental difference is that the *N*-acetyl groups, which are appended to the carbohydrate backbone structure, bear little regular geometric relationship to each other, unlike that between one chromophore and its neighbor in a polypeptide chain of defined secondary structure giving rise to characteristic VCD features in the amide I′ region.[12] In contrast, for carbohydrates containing *N*-acetyl groups, VCD spectra are devoid of features in the amide I′ region compared to transmission FTIR spectra (Figure 2). This is the case for biologically important carbohydrates ranging from N-linked glycans containing *N*-acetyl glucosamine and *N*-acetyl neuraminic acid (sialic acid) to polysaccharides that include heparin as well as more heavily N-acetylated polysaccharides such as HS, CS, and CA (see Supporting Information). This suggests that the contrast enhancement property of VCD in the amide I′ region can be exploited,

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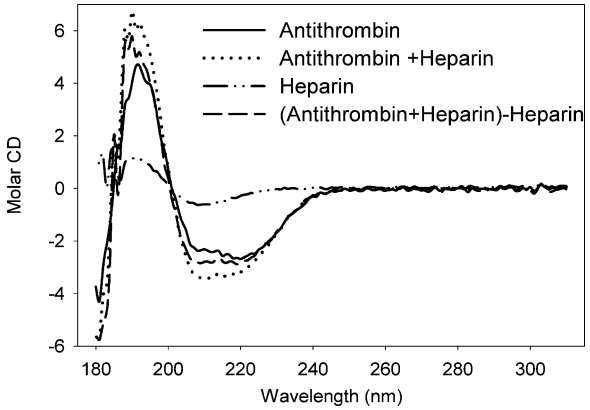

Figure 1. CD spectra of antithrombin and antithrombin bound to heparin polysaccharide. The spectrum of heparin cannot be subtracted from that of the complex (i.e., (antithrombin + heparin) − heparin ≠ antithrombin). A conformational change has occurred; however, it is not possible to attribute this change to particular components of the complex. Molar CD (M^{-1}·cm^{-1}) = $[\theta]/3298$, where $[\theta]$ is molar ellipticity.

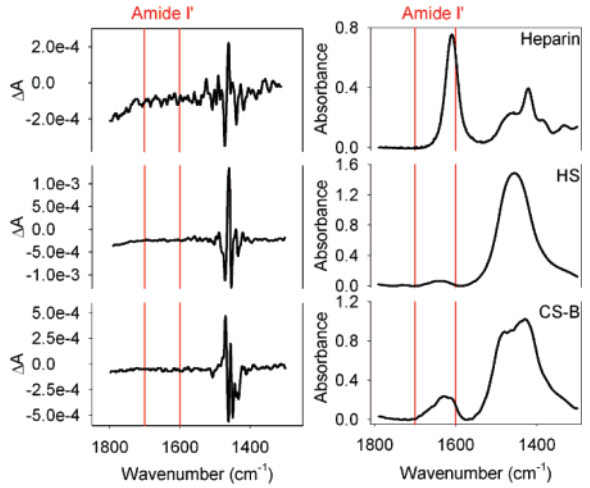

Figure 2. VCD (left) and transmission (right) IR spectra of heparin (upper), HS, and CS-B (lower) (all samples at 25 mg/mL). The amide I′ region is essentially featureless in VCD.

allowing protein secondary structural changes following binding to these carbohydrates to be selectively observed in solution.

VCD spectra were recorded (6 × 1000 scans, 4 cm^{-1} resolution, BaF_2 window) in D_2O to reduce the heavy absorption of IR radiation by water in the amide I′ region. The *N*-acetyl chromophores of heparin were effectively invisible in the amide I′ region of the VCD spectra of both bovine serum albumin (BSA) (see Supporting Information) and concanavalin A (ConA) complexes (Figure 3), while the protein chromophores were selectively observed. The VCD spectrum of ConA is typical for the β-sheet secondary structure and undergoes substantial changes when complexed with heparin (Figure 3).

The main drawback of VCD is the weakness of the signal (typically $\Delta\epsilon/\epsilon = 10^{-4}-10^{-5}$), but the technique has been employed successfully in both the solid[12] and solution[13] states and, most notably, in the analysis of protein secondary structure.[14−16] The work reported here extends the utility of solution VCD, exploiting its ability to selectively enhance the contrast between protein and carbohydrate (*N*-acetyl) signals in the amide I′ region. This allows the selective secondary structure analysis in solution of the protein component of protein−polysaccharide and protein−oligosaccharide

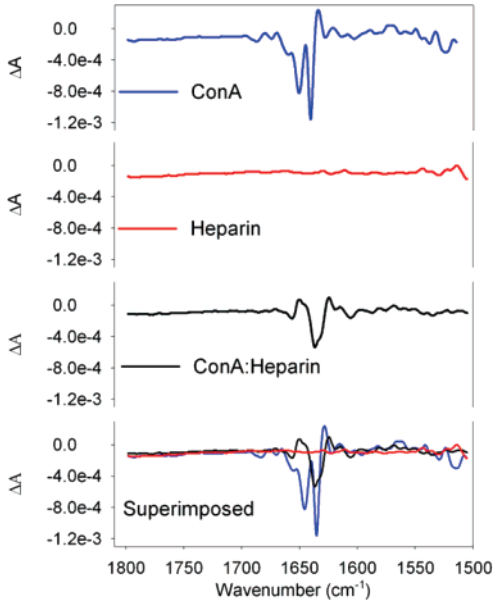

Figure 3. VCD spectra of ConA, heparin, and ConA bound to heparin; ConA is at 100 mg/mL, and heparin is present at molar equivalence.

complexes, which are currently problematic, to be undertaken. The recent advent of high-intensity mid-IR sources worldwide[17] now makes high-resolution and high-sensitivity VCD on many more proteins (allowing a comprehensive reference library of spectra to be compiled), glycoproteins, and protein−carbohydrate complexes an attractive possibility.

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Supporting Information Available: Experimental information and VCD/transmission spectra of GAGs and other polysaccharides. This material is available free of charge via the Internet at http://pubs.acs.org.

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