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Studying the isolated glycan moiety of RNase B by means of Raman and Raman optical activity

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Raman and Raman optical activity (ROA) spectroscopy was applied to study the solution phase structure of the glycan moiety of the protein RNase B. Spectral data of the isolated, yet intact glycan moiety of RNase B was achieved by subtracting high quality spectral data of RNase A, the non-glycosylated form of the RNase, from the spectra of the glycoprotein. The remaining difference spectra were compared to spectra generated from Raman and ROA data of the constituent disaccharides of the RNase glycan, achieving very convincing spectral overlap. The results show that ROA spectroscopy is able to extract detailed spectral data of the glycan moieties of proteins, provided the non-glycosylated isoform is available. Furthermore, the good comparison between the full glycan spectrum and the regenerated spectra based on the disaccharide data lends great promise to ROA as a tool in the detailed, solution phase structural analysis of this structurally elusive class of biomolecules.

The structural and functional characterisation of glycoproteins is challenging, yet highly essential considering the importance of this class of proteins as e.g. biopharmaceuticals.¹ Nevertheless, classical structural characterisation tools, such as X-ray crystallography and NMR often fail to provide insight into glycoprotein structure. In the past, Raman and Raman optical activity (ROA) spectroscopy have been shown to provide rich and valuable information on *i.a.* the solution structure of proteins,² carbohydrates³ and glycoproteins.^{3c, 4}

Conventional Raman bands arise from the 3N-6 vibrational modes in biomolecules, where N denotes the number of atoms. ROA, which is detected as a small difference in the circularly polarized components of Raman scattered radiation (using unpolarized incident laser light), cuts through this complexity of the Raman spectra and samples the most rigid and chiral elements in (bio)molecules. The ROA spectrum thus displays characteristic features of the conformation of the biomolecule. Protein ROA spectra show distinct patterns dependent on secondary and tertiary structure.^{2,5} ROA spectra of carbohydrate samples contain information on the ring conformation, relative disposition of hydroxyl groups around the ring, the absolute configuration and axial or equatorial

orientation of groups attached to the anomeric carbon and the exocyclic CH₂OH conformation.^{2,3} As proteins and carbohydrates can be studied in detail with ROA spectroscopy, combining this sensitivity to these two major groups of biomolecules in the study of glycoproteins is natural.

Brewster et al have previously employed conventional Raman spectroscopy to study aglycosyl bovine ribonuclease A (RNase A) and its monoglycosylated form, bovine ribonuclease B (RNase B) in order to showcase the monitoring of the glycosylation state.⁶ These proteins are typical model systems in glycoprotein research as they have identical peptide sequences but RNase B contains a N-linked glycan attached to asparagine 34 (Asn34). Directly comparing the X-ray structures of RNase A and RNase B shows no statistically significant differences in the peptide.⁷ Electron density can be observed for the glycan extending away from the peptide, but suggests the glycan is disordered or mobile within the crystal.⁷ The glycan structure varies, consisting of two N-acetylglucosamine (GlcNAc) residues and between five to nine mannose residues.⁸ The glycosylation of RNase A affects the dynamic stability, functional activity and resistance to proteolysis,^{8b} but detailed structural analysis of this moiety has so far been elusive.

In this study, both the Raman and ROA spectra of the glycan moiety of intact RNase B in aqueous solution were obtained by subtracting the respective RNase A spectra, showcasing the potential of ROA as a structural tool in glycobiology.

Figure 1. Raman (top) and ROA (bottom) spectra of RNase A (black) and RNase B (red)

The Raman and ROA spectra of RNase A and RNase B are presented in Figure 1 and are found to be almost identical. In the Raman spectrum of RNase A, the large band observed

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at $\sim 982\text{ cm}^{-1}$ can be assigned to the symmetric stretch of sulphate ions and will result in an artefact upon subtracting the two spectra. Considering the high level of similarity of the ROA spectra of RNase A and RNase B, it can be concluded that the protein structure in solution is not significantly altered by the glycosylation at Asn34, which is in agreement with X-ray⁷ and NMR studies.⁹

In Figure 2, the difference Raman and ROA spectra are displayed in comparison to the reconstructed Raman and ROA spectra of the isolated glycan (see Experimental Section for details). The comparison shows that by subtracting the aglycosyl form, RNase A, from the glycosylated form, RNase B, spectra emerges that can visually be identified as carbohydrate Raman and ROA spectra. As the protein structure does not change significantly upon glycosylation at a single site, the spectral subtraction eliminates the protein bands and retains solely the bands originating from the glycan moiety. In this fashion, the structure and stereochemistry of the glycan can be studied as it is bonded to the protein, information that is extremely difficult to access by other methods. In general, while the subtracted ROA spectrum is considerably noisier than the reconstructed ROA spectrum, both the two sets of Raman and ROA spectra compares very well, illustrating two main findings: The spectral data of the glycan moiety of RNase B has clearly been isolated from the bulk protein spectra, and as comparison with the weighted average of isolated disaccharide spectra is quite convincing, the conformation of these constituent parts does not change significantly upon inclusion into the glycan moiety.

Figure 2. Reconstructed Raman (A) and ROA (C) spectra of the glycan in RNase B and difference Raman (B) and ROA (D) spectra of RNase B and RNase A. The base lining artefacts in spectrum (B) are due to slightly differential scattering of water when measuring Raman.

The region below 800 cm^{-1} was previously shown to be instrumental in identifying carbohydrate contributions to the ROA spectrum.^{4a} The 1–2–3–4 pattern in the ROA spectrum figure 2 (D) is highly similar to the pattern indicated by Zhu et al. to be originating from N,N'-diacetylchitobiose in bovine α_1 -acid glycoprotein (AGP), for example.^{4b} Furthermore, the main features in the regions $800\text{--}1200\text{ cm}^{-1}$ and $1300\text{--}1500\text{ cm}^{-1}$, previously shown experimentally to be sensitive to linkage distribution and orientation of the $\text{-CH}_2\text{OH}$ groups in carbohydrates, respectively^{3a}, are highly conserved when comparing the two ROA spectra. This clearly indicates, as

previously suggested in the study of yeast invertase^{4a} that the constituent disaccharide fragments of high mannose glycoproteins retain their conformation when isolated.

A detailed discussion of the observed spectral features lies outside the scope of this preliminary study. It is nonetheless clearly shown that the carbohydrate spectrum can be isolated from the RNase B protein spectrum, and furthermore that the constituent disaccharide spectra of the glycan can reproduce the spectral features of this compound. Comparing the difference spectra to the averaged sugar spectra, could in the future be a way to study the conformations and structure of the carbohydrate. However, differences will also arise due to interactions between the glycan and the peptide and hydration and hydrogen-bonding networks, why one should exercise some caution in making these assignments. Nonetheless, detailed assignments could offer important information on the conformation and dynamics of the glycan in the intact glycoprotein. With expanding experimental observations and the development of suitable quantum mechanical methods to study Raman and ROA signatures of peptide and carbohydrate spectra¹⁰, even with elaborate hydration shells¹¹, detailed assignments of glycan and glycoprotein spectra will be feasible in the future. Such detailed analyses may provide stereochemical and conformational insight in glycoprotein structure.

Experimental Section

Protein samples were obtained from Sigma-Aldrich BE and used without further purification. The Raman and ROA spectra were measured at ambient temperature in water using the previously described ChiralRAMAN-2X instrument (BioTools, Inc.¹²). The Raman spectra are displayed as the circular intensity sums ($I_R + I_L$) and the ROA spectra as the circular intensity differences ($I_R - I_L$) with I_R and I_L denoting the scattered Raman intensities with right- and left-circular polarization, respectively. The instrument operates at a laser wavelength of 532 nm ; laser power at the source of 400 mW ; spectral resolution of 7 cm^{-1} ; and an acquisition time of 100 hours for both RNase A and RNase B (in order to achieve high signal to noise). Solvent water spectra were subtracted from the Raman spectra after which the baseline correction procedure by Boelens et al was applied.¹³ The ROA spectra were smoothed using a second order, 15 point Savitzky-Golay filter. The Raman and ROA spectra were normalized based on the amide I region in the Raman spectra prior to subtracting RNase A from RNase B. The carbohydrate spectra used to generate the weighted glycan spectra were previously published by Johannessen et al.^{4a} These latter Raman and ROA spectra, were constructed by averaging the spectra of $\alpha\text{-D-Manp-(1}\rightarrow\text{2)-}\alpha\text{-D-Manp-OMe}$ (M2M), $\alpha\text{-D-Manp-(1}\rightarrow\text{3)-}\alpha\text{-D-Manp-OMe}$ (M3M), $\alpha\text{-D-Manp-(1}\rightarrow\text{6)-}\alpha\text{-D-Manp-OMe}$ (M6M) and N,N'-diacetylchitobiose spectra in 1/2/2/1 proportions to reasonably mimic the shortest glycan moiety in RNase B, which, as mentioned in the main text, contains a varying amount of mannose units in bulk solution.

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The glycan moiety of RNase B is studied *in situ* on the surface of the protein using Raman and Raman optical activity. We show that by subtracting the background protein spectra, it is possible to study the full length glycan moiety in detail in solution. This study provides a much needed tool for the structural analysis of complex carbohydrates and glycans in biological systems.

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