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Monitoring Guanidinium-Induced Structural Changes in Ribonuclease Proteins Using Raman Spectroscopy and 2D Correlation Analysis

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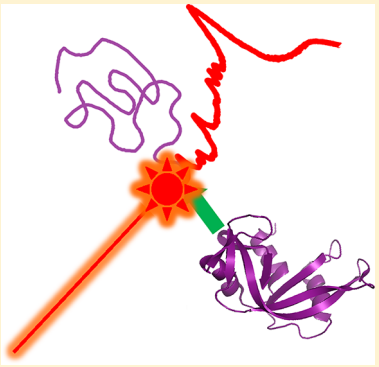
Supporting Information

ABSTRACT: Assessing the stability of proteins by comparing their unfolding profiles is a very important characterization and quality control step for any biopharmaceutical, and this is usually measured by fluorescence spectroscopy. In this paper we propose Raman spectroscopy as a rapid, noninvasive alternative analytical method and we shall show this has enhanced sensitivity and can therefore reveal very subtle protein conformational changes that are not observed with fluorescence measurements. Raman spectroscopy is a powerful nondestructive method that has a strong history of applications in protein characterization. In this work we describe how Raman microscopy can be used as a fast and reliable method of tracking protein unfolding in the presence of a chemical denaturant. We have compared Raman spectroscopic data to the equivalent samples analyzed using fluorescence spectroscopy in order to validate the Raman approach. Calculations from both Raman and fluorescence unfolding curves of $[D]_{50}$ values and Gibbs free energy correlate well with each other and more importantly agree with the values found in the literature for these proteins. In addition, 2D correlation analysis has been performed on both Raman and fluorescence data sets in order to allow further comparisons of the unfolding behavior indicated by each method. As many biopharmaceuticals are glycosylated in order to be functional, we compare the unfolding profiles of a protein (RNase A) and a glycoprotein (RNase B) as measured by Raman spectroscopy and discuss the implications that glycosylation has on the stability of the protein.



It is widely known that changes in the tertiary, three-dimensional structure of a protein-based pharmaceutical can directly affect drug activity and may also induce protein aggregation.[1] Therefore it is essential that the stability of a biopharmaceutical product is well characterized. In this study we investigate Raman spectroscopy as an alternative to the current gold standard analytical methods for monitoring protein unfolding: fluorescence spectroscopy and differential scanning calorimetry (DSC).[2,3]

The tertiary structure of a protein depends largely on its solvent environment. When in aqueous solutions at neutral pH, most proteins will adopt an extremely ordered "native" conformation. For most protein biopharmaceuticals, this native tertiary structure is the biologically active conformation. When the solvent environment is perturbed by extreme temperatures, pH, or the addition of a chemical denaturant, the three-dimensional protein structure will unfold into a disordered or denatured state. Guanidine hydrochloride (GuHCl) is one of the most commonly used chaotropic agents for protein unfolding studies. However, despite its widespread use as a chemical denaturant the mechanism of action is still unclear, although it is thought to involve the formation of hydrogen bonds between the denaturant and the peptide backbone and also an increase in the solubility of both polar and nonpolar amino acid side chains.[4]

As it has also been reported that the stability of a protein molecule is enhanced by the addition of a glycan group,[5] we will in this study compare the unfolding behavior of a protein and its glycosylated equivalent. The addition of an oligosaccharide increases the internal noncovalent forces which hold the protein in its folded form by decreasing exchange rates of the backbone amide protons and hence increasing the concentration of denaturant required.[6] Ribonuclease (RNase) A and its glycosylated equivalent RNase B were chosen as model proteins for this study, mainly because of their similarity in secondary and tertiary structure, but these proteins are also appropriate as the structure and stability of both proteins are well characterized in the literature.[6−8]

Raman spectroscopy has a rich history as a technique for the characterization of proteins.[9−11] Much of this work has investigated structural differences in protein molecules by taking advantage of the sensitivity of the amide I region to conformational changes, in particular monitoring changes which occur during acid induced unfolding of proteins by UV resonance Raman.[10,12−14] The ability to monitor chemical induced denaturation of proteins using Raman spectroscopy

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has been previously demonstrated in a number of proteins; however, these studies focused on the analysis of spectral features which arise from aromatic amino acid residues rather than those due to vibrations of the peptide backbone.[15,16] The thermal unfolding of RNase A has also been measured previously by Raman spectroscopy; however, this approach centered on analysis of the C−H stretching modes in the 3000 cm^{-1} region.[17] Raman spectroscopy has also been used to characterize various glycoproteins, including RNase B,[18−20] but these studies did not investigate unfolding but rather the detection of the glycan.

In this paper we report the use of Raman spectroscopy to monitor the unfolding of Ribonuclease proteins in the presence of GuHCl. Through the use of unfolding curves and 2D correlation analysis, we compare the results derived from Raman spectroscopy to those obtained by a conventional fluorescence unfolding experiment. We also acquired Raman and fluorescence data from the glycosylated equivalent of RNase A, RNase B, in order to evaluate how the addition of a complex glycan affects the stability and unfolding of RNase proteins.

MATERIALS AND METHODS

Ribonuclease A (RNase A), Ribonuclease B (RNase B), guanidine hydrochloride (GuHCl), and phosphate buffered saline (PBS) tablets were all of analytical grade and purchased from Sigma Aldrich (Dorset, U.K).

For fluorescence spectroscopy 10 mg/mL solutions of RNase A and RNase B were made up in PBS solution (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl; pH 7.4). A 7 M stock solution of GuHCl in PBS was made and subsequently diluted with the RNase solutions into three substocks of 2, 4, and 6 M GuHCl. Substocks were then diluted into the desired GuHCl range (between 0 and 6 at 0.2 M intervals, a total of 28 concentrations), where the final protein concentration was 0.1 mg/mL and the final sample volume 1 mL. Samples were then incubated at 37 °C overnight before analysis. Each unfolding experiment was performed in triplicate. For Raman spectroscopy initial solutions of 400 mg/mL RNase A and B were made up in PBS. The above method was then employed so that final concentration of proteins so that the final protein concentration was 10 mg/mL within a sample volume of 400 μL.

Fluorescence Spectroscopy. Fluorescence spectra were obtained on a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu Biotech, Manchester, U.K.) equipped with a 150 W xenon lamp and a holographic grating with 1300 grooves/mm. The excitation wavelength range was 220−990 nm and the measurement range was 220−750 nm. Shimadzu Pop Up Scan software was used for instrument control. For this study, the excitation wavelength was set at 280 nm and the excitation slit was set at 3 μm. The emission slit was set at 5 μm with an emission wavelength range of 220−550 nm. Samples were analyzed in a quartz cell with a fast scanning speed and sensitivity set to high.

Raman Spectroscopy. Raman data were collected using a Renishaw 2000 Raman microscope (Renishaw Plc., Old Town, Wotton-under-Edge, Gloucestershire, U.K.) with a low-power (27 mW) near-infrared 780 nm diode laser with power at the sampling point between 2 and 4 mW and a spectral resolution 6 cm^{-1}. The instrument was wavelength calibrated with a silicon wafer focused under a 50× objective and collected as a static spectrum centered at 520 cm^{-1} for 1 s. The GRAMS WiRE software package (Galactic Industries Corp., 395 Main St., Salem, NH) running under Windows 95 was used for instrument control and data capture. A volume of 100 μL of each sample was pipetted into a 96 well plate, and a 20× objective lens was focused on the top of the solution. Spectra were collected over a spectral range of 200−2000 cm^{-1}, over five accumulations with 120 s exposure time.

Data Analysis. Raman Spectroscopic data were exported from the instrument software into Matlab 7.6 (The MathWorks, Inc., Natick, MA) where data preprocessing was performed, in order to allow direct comparison of the data. 2D correlation calculations were performed using 2D Shige freeware (http://science.kwansei.ac.jp/~ozaki/index-e.html) and moving window contour plots were plotted in Matlab. Spectral figures and peak fitting were plotted/calculated in GRAMS Ai software (Galactic Industries Corp., 395 Main St., Salem, NH).

The protein unfolding curves drawn from both fluorescence and Raman data were used to calculate $[D]_{50}$ values, by first calculating the fractions of folded (ff) and unfolded (fu) protein molecules at each given GuHCl concentration. This was achieved using eqs 1 and 2, in which X is the observed signal, X_N is the signal observed from the native protein and X_U is the signal observed from the unfolded protein.

$$\mathrm{fu} = \frac{X - X_N}{X_U - X_N} \tag{1}$$

$$\mathrm{ff} = 1 - \mathrm{fu} \tag{2}$$

The calculated folded and unfolded fractions were then used to calculate the Gibbs free energy (ΔG) of the protein molecules at each denaturant concentration according the eq 3 as detailed in ref 21.

$$\Delta G = -RT \ln\left(\frac{\mathrm{fu}}{\mathrm{ff}}\right) \tag{3}$$

where R is the ideal gas constant and T is the temperature in K.

RESULTS AND DISCUSSION

Fluorescence Spectroscopy. The fluorescence spectra of RNase A (7 μM) at a number of different guanidine hydrochloride concentrations are shown in Figure 1A. It can be seen from these spectra that fluorescence emission increases as the concentration of the denaturant increases; this is due to fluorescent, hydrophobic groups such as tryptophan and tyrosine being exposed as the protein unfolds into a less ordered state. The intensity of the fluorescence emission at the approximate peak center (345 nm) was calculated for each GuHCl concentration and plotted as an equilibrium curve in Figure 2A. It is possible from this plot to calculate the fractions of folded and unfolded protein molecules at each GuHCl concentration and from this estimate the concentration of denaturant which is needed to unfold half of the protein molecules, the $[D]_{50}$. The $[D]_{50}$ calculated for RNase A for this study was 3.1 M, which is consistent with the $[D]_{50}$ quoted in the literature for RNase A as 3.1−3.2 M.[22,23]

Raman Spectroscopy. Raman spectra of RNase A (700 μM) over a similar concentration gradient of GuHCl were recorded along with a control spectrum of GuHCl in PBS at each concentration; the latter was used since GuHCl denaturant itself has a Raman spectrum and this needs to be compensated for. Thus prior to data analysis, the control spectrum at each concentration was subtracted from the corresponding spectrum of protein and GuHCl; the resulting

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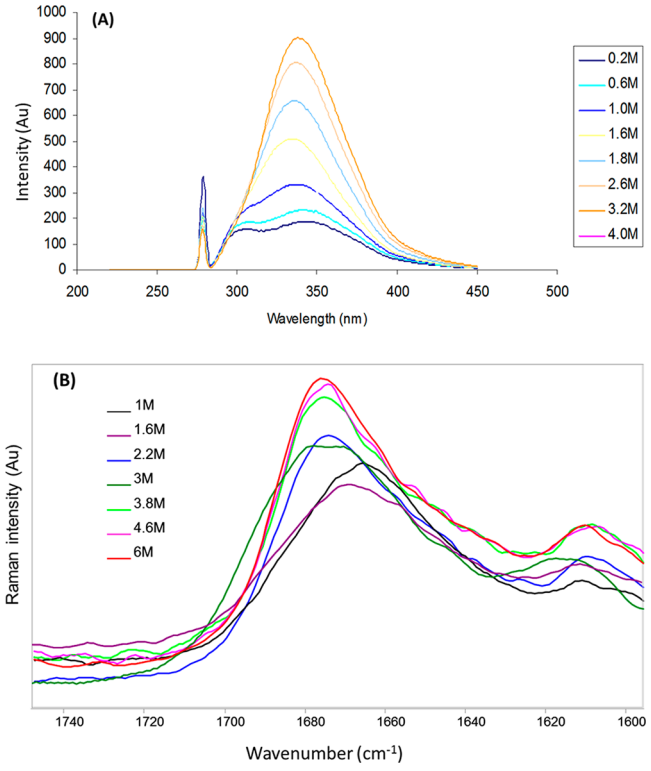

Figure 1. (A) Fluorescence spectra of 7 μM RNase A at different GuHCl concentrations and (B) amide I region of the Raman spectra of 700 μM RNase A at various GuHCl concentrations after subtraction of the control GuHCl spectra, smoothing, and baseline correction.

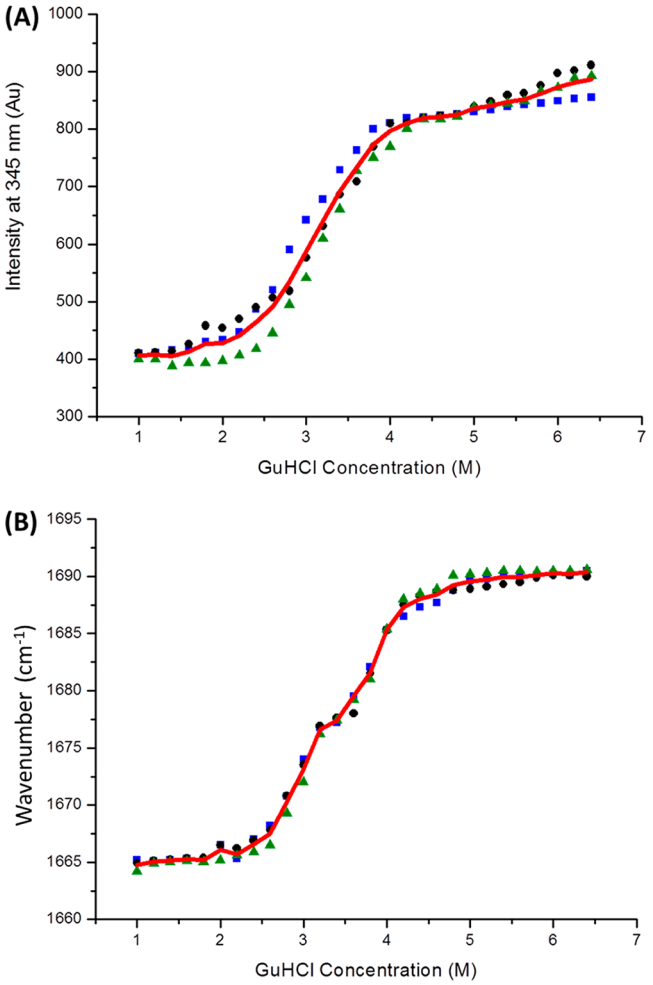

Figure 2. (A) Protein unfolding curve for RNase A drawn from fluorescence data (intensity at 345 nm). The red line indicates the mean of the triplicate measurements taken and (B) protein unfolding curve for RNase A drawn from Raman spectroscopy data (peak center of amide I band). The red line indicates the mean of the triplicate measurements taken.

spectra are shown in Figure 1B, which focuses on the amide I region (1600−1700 cm^{-1}). We choose to focus on this particular spectral region, which is mainly comprised of C═O stretching modes from the carbonyl groups in the protein backbone, due to its known sensitivity to changes in and loss of secondary structure.[9,12,24] A notable shift in this band as the concentration of denaturant increases can easily be observed along with change in the band shape. In order to find the peak center of the band, a Gaussian curve fit was applied to the amide I region, using the peak fitting function in GRAMS Ai. Peak center values were then plotted as a function of denaturant concentration giving a traditional protein stability curve drawn from the Raman data (Figure 2B).

Method Comparison. In order to validate the use of Raman spectroscopy for monitoring denaturant-induced unfolding in proteins we compare the results gained from the Raman experiments to those obtained by "traditional" fluorescence spectroscopy. We start by simply comparing the protein unfolding equilibrium curves drawn by each method; it can be seen easily that the curves compare favorably with each other, both indicating that main unfolding events take place between GuHCl concentrations of 2.5−4 M. In order to compare both methods directly, the data from both curves are plotted against each other in Figure 3. If the two methods produced identical results a linear trend would be seen in the graph and the best-fit line would indicate this. While the general shape of this curve does increase concomitantly and confirms that the Raman results are comparable with the traditional fluorescence based method, it is evident when comparing the stability curves in Figure 2 that the curve drawn from the Raman data appears to hint toward a two stage transition, whereas the fluorescence data shows only one. This could be due to an inaccuracy in the Raman method, but as this trend is observed consistently over three independent measurements it is more likely that the Raman spectroscopy data are able to highlight smaller conformational changes not observable by fluorescence spectroscopy. This Raman method is therefore able to separate out two distinct transitions which overlap in the fluorescence data to appear as one transition, which has never before been reported for RNase A.

We have also compared our Raman method to the more traditional Raman based approach for detecting conformational changes in proteins; this involve measuring changes in intensity of the tryptophan vibration centered at ∼875 cm^{-1}, a band which will decrease in intensity as the protein unfolds and tryptophan residues become more exposed.[25,26] This method, like the fluorescence method, shows only one unfolding transition which occurs at ∼3.1 M GuHCl (data in the Supporting Information, Figure S1).

The $[D]_{50}$ values calculated from each of the triplicate measurements and the average $[D]_{50}$ for each method are shown in Table 1 for RNase A. The values calculated from the Raman data are in very good agreement with the fluorescence

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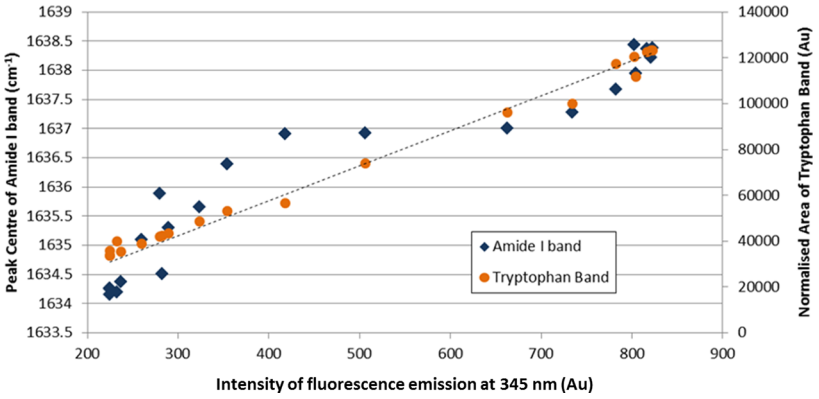

Figure 3. Graph to compare RNase A unfolding data obtained by Raman and fluorescence spectroscopy.

Table 1. Comparison of $[D]_{50}$ and ΔG Values for RNase A and RNase B Obtained from the Fluorescence and Raman Methods[a]

	$[D]_{50}$	ΔG
	RNase A	
Raman	3.14 (0.08)	−10971.47 (30.74)
fluorescence	3.13 (0.04)	−11043.58 (12.98)
	RNase B	
Raman	3.51 (0.06)	−3866.82 (41.83)
fluorescence	3.56 (0.04)	−4037.50 (15.91)

[a]Values are the mean of triplicate repeats, and the standard deviation is provided in parentheses. ΔG values quoted are the free energy at the $[D]_{50}$ concentration: for RNase A this was at 3.1 and at 3.5 for RNase B.

results calculated from this study, and most encouragingly both values fall within the literature values for the $[D]_{50}$ of RNase A: 3.1−3.2 M GuHCl.[22,23] The standard deviation of the triplicate measurements are also given in Table 1, and these show Raman spectroscopy to be a reproducible method for tracking protein unfolding and calculating $[D]_{50}$ values. Furthermore, it is also possible to calculate the Gibbs free energy (ΔG) of a protein at each given denaturant concentration from the unfolding curve.[21] The ΔG values for RNase A at the $[D]_{50}$ concentration (3.1 M) were calculated for both the Raman and fluorescence methods (Table 1) and were also found to be in good agreement.

As an extra comparison step and also to probe the two step transition seen in the Raman unfolding profiles further, we have applied 2D correlation moving windows analysis to the data. 2D correlation analysis is a method of visualizing a set of spectra by applying a cross-correlation analysis to the data and then plotting the results in the form of a contour map.[27] In 2D moving windows analysis, the data are split into smaller sets or "windows" prior to analysis, in order to locate key transition points. The data are then plotted as a contour map which relates the spectral changes to the perturbation. 2D correlation analysis was initially performed on the full spectral range (not shown), where it was found that the majority of variance was occurring in the 1630−1740 cm^{-1} region, reaffirming our previous selection of this spectral feature. The results of the 2D correlation moving windows analysis on the fluorescence and Raman data are shown in parts a and b of Figure 4, respectively. Like with the stability curves, the 2D contour plots show that both methods indicate that unfolding transitions occur between guanidine concentrations of 2.8 and 4 M. The regions where the most changes are occurring in the spectra, indicated by the red contours, correspond well with the calculated $[D]_{50}$ values. Interestingly, as with the Raman equilibrium curve (Figure 2b), Figure 4b also shows the unfolding transition to be comprised of two separate events (and all three repeats show the same transitions; data not shown). Figure 4c shows the 2D correlation moving windows contour plot for the 860−900 cm^{-1} region of the Raman spectra, showing that the changes occurring in the tryptophan band are in one single transition. These results confirm that not only is this Raman spectroscopic

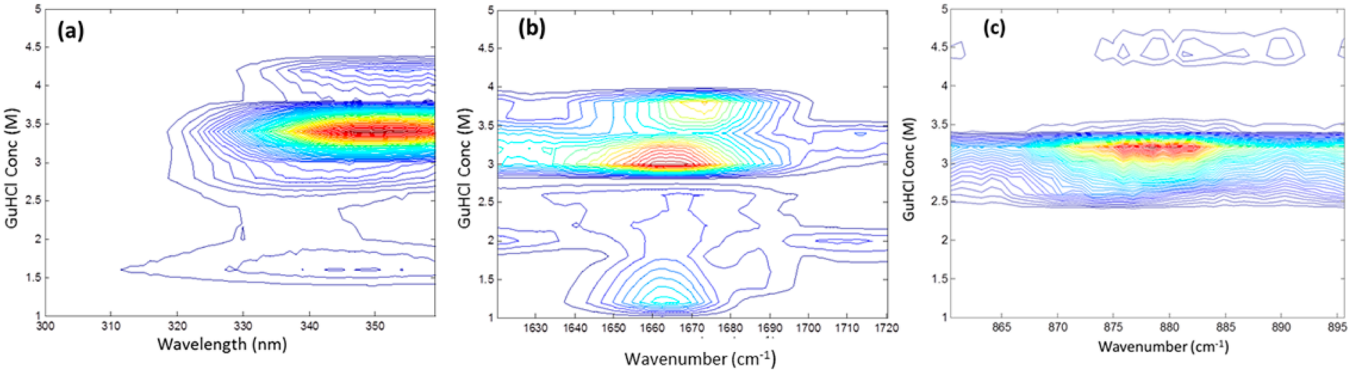

Figure 4. 2D moving window contour plots as a function of the average translating window of GuHCl concentration of (a) fluorescence spectra of RNase A, (b) amide I region (1620−1720 cm^{-1}) of the Raman spectrum of RNase A, and (c) tryptophan region (860−900 cm^{-1}) of the Raman spectrum of RNase A.

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method a suitable alternative to fluorescence spectroscopy for probing protein structural changes but that it can also provide additional information on more subtle changes which are not observed by the fluorescence method.

Comparing the Stability of RNase A and B. We used the Raman spectroscopy method detailed and tested above to monitor the unfolding of RNase B. RNase B is a glycosylated form of RNase A, with a single N-linked glycan at Asn^{34} that comprises 2 linear *N*-acetylglucosamine residues, followed by 3−10 mannose sugars, which are branched into 2 or 3 links. Both proteins have identical primary and secondary structure and very similar tertiary structures, with the differences that do occur being due to the addition of the glycan group. Therefore an additional aim of this work was to investigate whether Raman spectroscopy can be used to detect differences in protein stability which are brought about by glycosylation.

The Raman spectra of RNase B at various GuHCl concentrations are shown in the Supporting Information, as with the RNase A data an upward shift in the position of the amide I band as the denaturant concentration increases (Figure S2a in the Supporting Information). Using the method described previously, protein unfolding curves were generated from triplicate measurements and the average curve from RNase B is shown in Figure S2b in the Supporting Information and is compared to the average RNase A curve. It is clear in these stability curves that the concentration of GuHCl needed to denature RNase A is significantly lower than that needed for RNase B, confirming that the presence of a sugar group does indeed increase stability in RNase proteins. The equilibrium curve was used to find the $[D]_{50}$ for RNase B (Table 1), which was calculated as 3.5 M. Compared to 3.1 M GuHCl $[D]_{50}$ for RNase A, it is confirmed that the glycoprotein is, indeed, the more stable molecule; unfortunately, there are no literature values to confirm this, but the fluorescence measurements of RNase B shown in Table 1 do corroborate the Raman data. In addition, we can compare the ΔG values of each protein at the same GuHCl concentration, as ΔG will decrease (become negative) as the stability of a protein decreases. Therefore, comparing ΔG at 3.0 M GuHCl gives further proof that RNase B is indeed the more stable system as ΔG for RNase B is -1002.7 J mol^{-1} compared with for RNase A is -9905.5 J mol^{-1}.

CONCLUSION

We have described a novel Raman spectroscopy-based method for monitoring the unfolding of proteins in the presence of a chemical denaturant and have shown the potential of this technique through comparisons with fluorescence spectroscopy. Through the use of unfolding curves and $[D]_{50}$ calculations we have shown that the results obtained from Raman spectroscopy are very comparable to those obtained by a conventional fluorescence unfolding experiment. By employing 2D correlation moving windows analysis, we have been able to demonstrate that Raman spectroscopy is more sensitive to smaller conformational changes than fluorescence emission data. We have demonstrated successfully that in this model system, tracking changes in the amide I region of the Raman spectra has proved more sensitive than the traditionally used vibrational modes (*viz.*, the tryptophan at 860−900 cm^{-1} and tyrosine ratio 829 cm^{-1}/850 cm^{-1}). It is hoped that this methodology can be transferred to more complex systems, although this univariate strategy may need to be adapted into a more robust multivarate approach as the complexity of spectral changes increases. Finally, using RNase A and B as model proteins, we have shown that this Raman method is capable of evaluating increases that occur in stability when this protein is glycosylated.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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