Tracking conformational changes in phosvitin throughout a crowding-agent-based titration

Tracking conformational changes in phosvitin throughout a crowding agent based titration

Evelien Van de Vondel[a], Wouter Herrebout[a], Christian Johannessen*[a]

Abstract: In this paper, the sensitivity of Raman optical activity (ROA) towards small conformational changes is explored by tracking the structural changes in an intrinsically disordered protein, phosvitin, caused by different concentrations of crowding agent. It is shown that ROA is capable of tracking small conformational changes towards beta-sheet and alpha-helical secondary structural properties of the protein. Furthermore, it is indicated that the influence of the crowding agents Ficoll 70 and dextran 70 used on the structural properties of phosvitin differs significantly, with the structural changes induced by the presence of Ficoll 70 being more pronounced and being already visible at a lower concentration. The data also suggests that some spectral changes do not arise from a change in the secondary structure of the protein, but are related to differences in interaction between the phosphorylated residues of the protein and the sugar-based crowding agent.

Introduction

Proteins, an important group of biomacromolecules, show a wide variety in structure and function. It is imperative to understand the behavior of proteins in realistic cellular environments, due to this diversity but also because of the link between malfunctioning proteins and high-profile diseases, such as Alzheimer's and Parkinson's diseases. The activity of proteins *in vitro* is traditionally measured in molecular biology under so-called physiological conditions, e.g. very low protein concentrations. However, the crowding theory states that 'traditional' protein studies do not take the overall high concentration of biomacromolecules in the cytosol into account. This concentration can be as high as 300-400 mg mL^{-1}, depending on the cell type. This background concentration consists of biomacromolecules that occupy a space that is mutually impenetrable. This assumption induces nonspecific steric repulsion, rendering part of the intracellular volume unavailable for other macromolecules. The number, sizes and shapes of the macromolecules in this "molecular background" determine the size of this excluded volume. This exclusion of volume could force some molecules with a flexible structure, such as proteins, into a more compact structure.[1–3] The exclusion of volume can be mimicked in experiments with so called crowding agents, macromolecules with a high water-solubility. These crowding agents should not interact with the studied system by any interaction different from steric repulsion, to ensure that the observed effect is purely originating in the exclusion of volume.[3] However, it was suggested that in some cases, the effect Ficoll 70 (F70), a highly popular crowding agent, is based on selective interactions and differs from the effect induced by other crowding agents. [4,5]

In one of these studies, Raman optical activity (ROA), a technique with a high sensitivity towards conformational changes in intrinsically disordered proteins[4,6,7], was used to explore the conformational effect of crowded environments on proteins. ROA is a chiroptical spectroscopic technique that exhibits unprecedented sensitivity towards distinguishing and identifying local structure in proteins by means of band patterns in the spectra.[7–16]

In the current study, the level of sensitivity of ROA towards small conformational changes was explored, by monitoring the effect of crowded environments on the structural properties of a protein as a function of the concentration of two crowding agents, F70 and dextran 70 (D70), enabling us to determine differences between the two induced effects on the structure of the protein. [4] It was opted to use a disordered protein as the studied protein, as it should be easier to observe the induction of structural changes when there is little to no structure to begin with. Phosvitin, a phosphoglycoprotein and the major protein component of egg yolk, has been shown to behave as a disordered protein.[17] Due to its high solubility, it is a prime candidate for ROA-studies. From the presented data, it is suggested that ROA is capable of closely monitoring small structural changes, indicating that the sensitivity of the technique is even greater than expected, especially when using a CID plot to identify structural contributions. In a CID plot, the CID-value per wavenumber is calculated as $|I_R-I_L|/(I_R+I_L)$ and plotted against the corresponding wavenumber. As the expected value for the CID is 10^{-3}, this enables the identification of individual structural contributions as well as the identification of artefacts, rising from the subtraction method used in this study. Furthermore, it is once more indicated that the influence of F70 on the structure of the protein differs significantly from the influence of D70, suggesting that the observed effect is selective and thus inherently not (only) the crowding effect, hence validating the results obtained from the experiment with previous experiments.[4,5]

Results and Discussion

A first set of results obtained with ROA, measured on samples of phosvitin with different concentrations of F70, can be found in figure 1. The bottom spectrum in this figure provides information on the structure of phosvitin solely in solution. The disordered nature of the protein is indicated by the signal in the amide I region at 1677 cm^{-1} [18] and the PPII-signal in the amide III region, at 1319 cm^{-1}.[19] Moreover, the skeletal stretch region shows virtually no signals, further strengthening the rationale that this is a disordered protein.

[a] E. Van de Vondel, W. Herrebout, C. Johannessen
Molecular Spectroscopy Group, Department of Chemistry
University of Antwerp
Groenenborgerlaan 171
Christian.johannessen@uantwerpen.be

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No major changes in the spectrum can be observed upon the addition of 50 mg mL^{-1} F70, as is visible in the second spectrum from the bottom in figure 1. Only a slight change can be observed in the amide III region, where the main couplet has been split in two distinct signals, one at 1307 cm^{-1} and one at 1321 cm^{-1}. The latter can be contributed to the PPII-signal[20,21], but the former could be attributed to the tightening of the protein conformation. An extra signal appearing in the skeletal stretch region at 974 cm^{-1} could also indicate this tightening. However, it has previously been reported that features in this region could also originate from vibrations of the sugar rings and the phosphate backbone of ribonucleotides.[18] It is therefore possible that these features stem from an interaction between the phosphoserine, present in the phosvitin sample, and F70, a sucrose polymer.

The spectrum of phosvitin in the presence of 100 mg mL^{-1} F70 is shown as the third spectrum from the bottom in figure 1. Here, the amide III region is clearly broader, containing an extra feature at 1339 cm^{-1} that could indicate the addition of alpha-helical content on top the still remaining PPII-structure, pinpointed by the feature at 1316 cm^{-1}.[18,19,21] The tightening of the protein structure is also visible in the skeletal stretch region, where an additional signal around 976 cm^{-1}, although it is hard to distinguish from the noise-level.

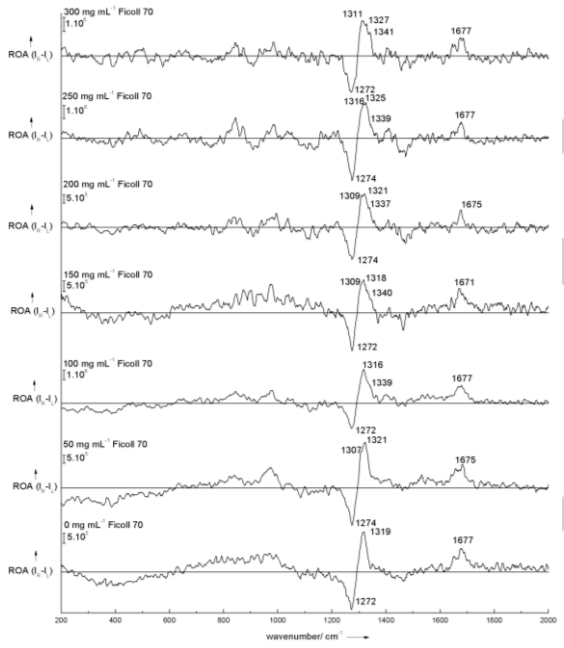

Figure 1. ROA spectra of phosvitin (60 mg mL^{-1}) in the presence of different concentrations of Ficoll 70. The concentrations of the crowding agent range between 50 and 300 mg mL^{-1}. Spectra were accumulated for 72 hours with laser power at source of 500 mW, a laser wavelength of 532 nm and a spectral resolution of 7 7 cm^{-1}.

The effect of the presence of 150 mg mL^{-1} F70 on the spectrum of phosvitin can be found in the middle of figure 1. Here, the broadening of the amide III region is still apparent, with an extra feature at 1340 cm^{-1},[18,21–23] indicating alpha-helical content, the PPII-signal at 1318 cm^{-1} [19,21] and an extra contribution at 1309 cm^{-1}, possibly indicating further tightening of the structure of the protein. The skeletal stretch region of this spectrum is the nosiest of all, possibly indicating that this is a tipping point between different conformations of the protein, with the structural changes being induced by the growing concentration of F70.

The third spectrum from the top in figure 1 comes from a solution of phosvitin in the presence of 200 mg mL^{-1} F70. The extra contribution, around 1340 (1337) cm^{-1}, reappears, similar to the spectra of the solutions with 100 and 150 mg mL^{-1} F70. This feature once more indicates the presence of alpha-helical structure.[18,23] The feature at 1309 cm^{-1} also remains, indicating the on-going tightening structure of the protein. The amide III region still contains the PPII-marker at 1321 cm^{-1}.[19,21] The skeletal stretch regions is in this case significantly less noisy, suggesting that there are less different conformations present in the solution.

The second spectrum from the top is of phosvitin in the presence of 250 mg mL^{-1} F70. The broadening is still visible in the amide III region, with the extra feature at 1339 cm^{-1}, indicating alpha-helical content. The main feature seems to be split at 1325 cm^{-1} and 1316 cm^{-1}, which could indicate the presence of beta-sheet.[24,25] Although this feature cannot be attributed to structural properties with certainty, it strengthens the hypothesis that the amount of secondary structural content in the protein differs with the concentration of crowding agent.

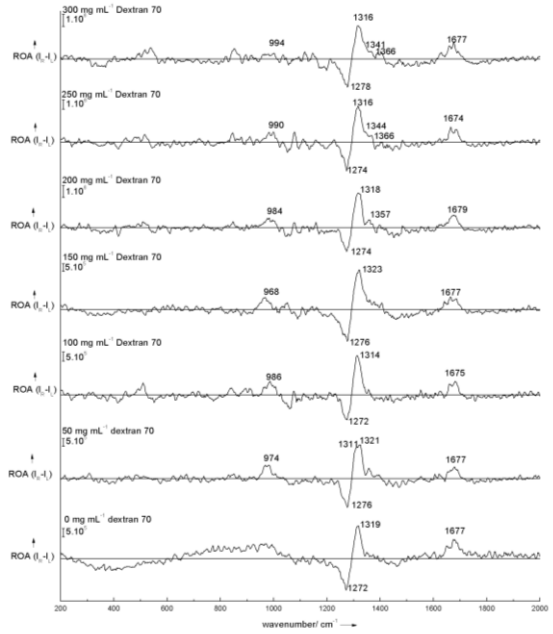

Figure 2. ROA spectra of phosvitin (60 mg mL^{-1}) in the presence of different concentrations of dextran 70. The concentrations of the crowding agent range between 50 and 300 mg mL^{-1}. Spectra were accumulated for 72 hours with laser power at source of 500 mW, a laser wavelength of 532 nm and a spectral resolution of 7 7 cm^{-1}.

Finally, the top spectrum belongs to phosvitin in the presence of 300 mg mL^{-1} F70. The amide III region of this solution is even more complicated, with a distinct feature at 1341 cm^{-1}, indicating the presence of alpha-helical content. Furthermore, the main feature seems once more split, with two maxima at 1327 cm^{-1} and 1311 cm^{-1}. This could signify that the difference between the different secondary structural contents becomes more distinct, or that the amount of beta-sheet structural content increases. In order to emphasize the induced structural change, the spectral difference induced by the presence of 250 and 300 mg mL^{-1} F70 is illustrated in figure S5 and S6.

From the presented data, it is clear that an increase in the concentration of F70 present in the solution increases the secondary structural content of phosvitin. The nature of this induced secondary structure depends on the concentration F70 present, as at lower concentrations alpha-helical content is induced, while the spectra of phosvitin in the presence of higher concentrations of F70 suggest also the presence of beta-sheet structural content. As it has been shown that F70 should be used as a crowding agent with caution due to the possibility of specific interactions[4,5], the experiments were repeated with D70 as a crowding agent, to determine whether the discussed structural effects are due to a specific interaction between F70 and phosvitin. It is shown by the results of this study in figure 2 that the structural change induced by the presence of the crowding agent differs for D70 and F70.

The spectrum of a pure solution of phosvitin has been repeated on the bottom in figure 2, as the benchmark of its original disordered nature of the protein, indicated by the disordered signal in the amide I region at 1677 cm^{-1} [18], the PPII-signal in the amide III region at 1319 cm^{-1} [19], and the lack of features in the skeletal stretch region.

The spectra of phosvitin in the presence of 50 and 100 mg mL^{-1} D70 show a similar lack of real difference in structural content compared to the first spectrum. Therefore, they will be discussed together. The amide I region appears unperturbed in these cases, although it contains a bit more noise. In the amide III region, the PPII-signal is conserved, while an extra small contribution around 1310 cm^{-1} could be distinguished. The skeletal stretch region contains but one feature, a positive contribution at 974-988 cm^{-1}, which is present in all spectra in the presence of D70. This feature is found in the region 900-1150 cm^{-1}. Once more, it is possible that this feature does not arise from tightening of the protein, but originates from an interaction between the phosphorene, present in the phosvitin sample, and D70, a glucose polymer.

The middle spectrum in figure 2 originates from a solution of phosvitin in the presence of 150 mg mL^{-1} D70. A broadening of the amide III is visible in this spectrum, a trend that is also observed in the spectra of phosvitin in the presence of 200, 250 and 300 mg mL^{-1} D70. This is due to an extra contribution at 1357 cm^{-1} in the spectrum of phosvitin in the presence of 200 mg mL^{-1}, which evolves towards two distinct features, one at 1344 cm^{-1}, indicating the presence of alpha-helical structure, and one at 1366 cm^{-1} in the spectra of 250 and 300 mg mL^{-1} D70. The first feature suggests that phosvitin adopts alpha-helical structure elements in the presence of very high concentrations of D70. This alpha-helical structural content is also induced by the presence of F70, although the effect of D70 on the structural content of phosvitin is less pronounced and also only onsets at higher concentrations. As an example, an alpha-helical contribution can already be found in the spectrum of phosvitin in the presence of 100 mg mL^{-1} F70, where the spectrum of phosvitin in the presence of 100 mg mL^{-1} D70 lacks any indication of structural difference based on the amide III region. This claim is visually supported by figure 3.

From these results, it is apparent that F70 induces different spectral changes, and thus conformational changes, than D70, as suggested before by the study with alpha-casein.[4] However, the induced structural differences differ for both proteins, as for example, in the case of alpha-casein extra contributions around 1380 cm^{-1} [4] were reported, which are not visible in the spectra, reported in the current study. In order to further investigate the differences in the amide III and skeletal stretch regions of phosvitin, induced by the presence of the crowding agents, the CID-value per wavenumber was calculated as $|I_R-I_L|/(I_R+I_L)$.

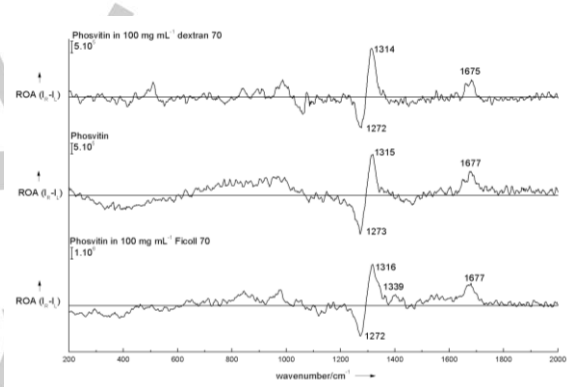

Figure 3. ROA spectra of phosvitin in the presence of 100 mg mL^{-1} Ficoll 70 (bottom spectrum), solely in solution (middle spectrum) and in the presence of 100 mg mL^{-1} dextran 70 (upper spectrum).

In the past, the CID has been used as a method to determine the similarity between calculated and experimental ROA spectra[26,27]. However, in this study, it will be used as a method to validate the claims about the amount of structural elements present in the described samples, as deviating CID intensities might indicate the presence of artefacts in the spectra, originating in the subtraction method used during these experiments. The results of this analysis for the amide III region are visible in figure 4. It is important to note that the scale of the figure accommodates the 'normal' intensity range of the CID-values to be inspected for ROA signals (10^{-3}). It is clear that are there significantly more maxima in the CID-plots than assigned features in the ROA spectra. This could be due to the fact that the ROA-spectra show a superposition of different features, thus making the exact positioning of the features difficult. Thus, the CID-plot can be considered an extra tool employed to validate claims about the amount of structural elements present in the described samples.

The two bottom CID plots in figure 4 contain information on the structure of phosvitin solely in solution.

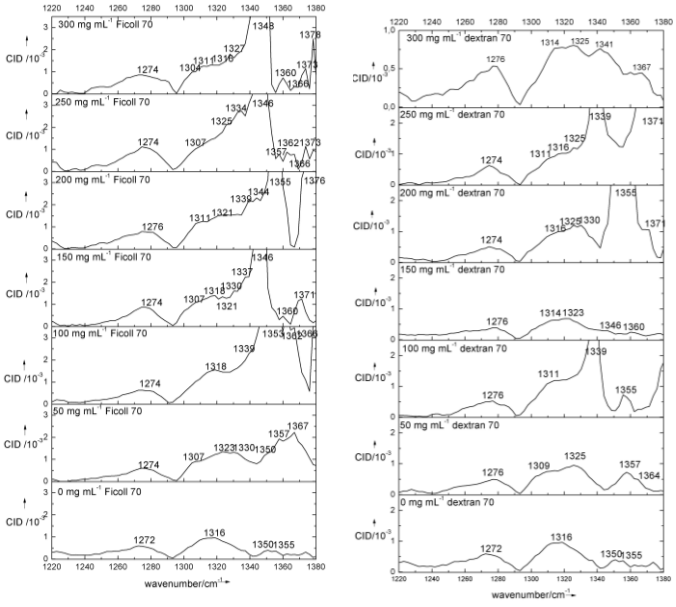

Figure 4. CID per wavenumber of the ROA spectra of phosvitin in the presence of different concentrations of Ficoll 70 (left) and dextran 70 (right).

The largest CID-values in this spectrum coincide with the minimum and the maximum of the distinct couplet in the ROA-spectrum that could be linked to PPII-structure. Surprisingly, there are also two local maxima at 1350 and 1355 cm^{-1}, suggesting that the structure is not completely disordered, but shows some tendency towards beta-strand content.

The plots for phosvitin in the presence of 50 mg mL^{-1} crowding agent are shown second from the bottom in figure 4. Although the ROA-spectra seem to be highly comparable to the spectrum of phosvitin solely in solution, the CID-plots suggest extra contributions, next to the markers of the PPII-couplet, with maxima at 1274-1276 and 1323-1325 cm^{-1}. Both spectra gain extra maxima at 1357 cm^{-1} and 1364-1367 cm^{-1}, strengthening the hypothesis that phosvitin shows a tendency towards beta-sheet structural content. This tendency is even stronger pronounced in the presence of F70, with an extra maximum at 1350 cm^{-1}. Furthermore, both spectra show an extra maximum at 1307-1309 cm^{-1}, which could be linked to a dormant contribution of the double maximum that beta-sheets cause in the ROA-spectrum. The third CID plots from the bottom in figure 4 originate in solutions of phosvitin and 100 mg mL^{-1} F70 and D70. The difference between the influence of F70 and D70 on the spectral properties of the protein is here apparent, as the absolute maximum in the presence of D70 can be found at 1339 cm^{-1}, indicating alpha-helical content, while the F70 sample has an absolute maximum at 1353 cm^{-1}, indicating the presence of beta-sheet in the structural content. Both CID plots contain both markers, though the relative intensity differs significantly, indicating that the crowding agents induce a different kind of structural change onto the protein. Moreover, the presence of beta-sheet is confirmed in the F70 spectrum by additional beta-sheet markers at 1362 and 1366 cm^{-1} that are not present in the D70 spectrum.

The CID plots of phosvitin in the presence of 150 mg mL^{-1} D70 and F70 can be found in the middle of figure 4. In comparison to the spectrum of phosvitin solely in solution, both spectra contain an extra contribution at 1360 cm^{-1}, suggesting the presence of beta-sheet structural content, as well as a contribution at 1346 cm^{-1}, suggesting alpha-helical content. It has to be noted that the intensity of the latter is far more pronounced in the spectrum in the presence of F70 than in the spectrum in the presence of D70. Both spectra one more contain a contribution at 1321-1323 cm^{-1}, indicating the remaining presence of PPII-helical structure. The presence of beta-sheet structural content is confirmed in the amide III region by the maxima at 1314 cm^{-1} in de D70 sample and 1307 and 1319 cm^{-1} in the case of the F70 sample. Also in this case, it seems that the presence of F70 induces far more structural change in the protein, as the CID plot has additional maxima at 1330 and 1337 cm^{-1}, suggesting alpha-helical content, and at 1371 cm^{-1}, once more suggesting the presence of beta-sheet structural content. These observations indicate once more that the presence of F70 induces more structural change than the presence of D70, suggesting that this structural effect is not only originating from the crowding effect.

Finally, this hypothesis seems to be validated by the top plots in figure 4, which contain the CID plots of phosvitin in the presence of 300 mg mL^{-1} crowding agent. Both plots still suggest the presence of PPII-helical structures, with maxima at 1325-1327 cm^{-1} and at 1274-1276 cm^{-1}, beta-sheet structural content, with maxima at 1314-1316 and 1366-1367 cm^{-1}, and alpha-helical content, indicated by the maximum at 1341-1348 cm^{-1}. It is once more indicated that the spectrum in the presence of F70 is far richer in structural information, as local maxima at 1304, 1311, 1360 and 1373 cm^{-1} are also apparent.

A similar analysis was performed for the skeletal stretch region of the ROA spectra. The results of this study can be found in the supplementary information. In this case, new maxima arise in the CID-plots in the region 900-1150 cm^{-1}. Although it is possible that the changes in this region can originate in structural changes in the protein, ROA features in this region, have also been linked to vibrations of the sugar rings and a phosphate backbone.[18] More than half of the sequence of phosvitin consists of serines (123 out of 216 amino acids), and most of these are phosphorylated. Therefore, it is not unreasonably to assume that at least a part of the features in this region are linked to interactions between the crowding agent and the phosphorylated residues. As the change in the Raman spectra might be less pronounced, it is possible that there is no difference observable in the Raman spectra of the crowding agent. As the CID values are based on a subtracted Raman spectrum, this might explain the unexpected high CID values seen in the plots.

The information, derived from the CID plots validates the conclusions, based on the ROA spectra. However, it is clear that the analysis of a CID plot provides more detailed information, even when only applied to experimental data, and enables the

determination of the origin of some of the features in ROA spectra, resulting from a subtraction method. In table 1, the structural features of phosvitin over the course of the crowding agent titrations, as observed in the ROA spectra and CID plots, are summarized.

Table 1. Overview of the structural changes in phosvitin induced by crowding.

Concentration of crowding agent	Structural properties of phosvitin in the presence of F70	Structural properties of phosvitin in the presence of D70
0 mg mL^{-1}	PPII-helix, disorder	PPII-helix, disorder
50 mg mL^{-1}	-PPII-helix, disorder -Tightening of the structure	PPII-helix, disorder
100 mg mL^{-1}	-PPII-helix, disorder -Tightening of the structure -alpha-helical structure	PPII-helix, disorder
150 mg mL^{-1}	-PPII-helix, disorder -Tightening of the structure -alpha-helical structure	-PPII-helix, disorder -Broadening of amide III feature indicates presence alpha-helical structure
200 mg mL^{-1}	-PPII-helix, disorder -Tightening of the structure -alpha-helical structure	-PPII-helix, disorder - alpha-helical structure
250 mg mL^{-1}	-PPII-helix, disorder -Tightening of the structure -alpha-helical structure -beta-sheet structure	-PPII-helix, disorder -alpha-helical structure
300 mg mL^{-1}	-PPII-helix, disorder -Tightening of the structure -alpha-helical structure -beta-sheet structure	-PPII-helix, disorder -alpha-helical structure

Conclusions

In conclusion, it has been shown by this data that a CID plot is a useful tool for the interpretation of complicated ROA data, as it enables the extraction of individual contributions to the spectrum. This will enable the measurement of more complicated samples, opening the door to 'real biological' samples. However, it has to be noted that an interpretation of these plots needs to happen with a critical eye, as it is easy to over-interpret this data. Furthermore, it has been shown that the sensitivity of ROA towards small conformational changes in proteins is so precise that tracking of changes throughout a titration is possible, and gives rise to additional valuable structural information. Once more, it is clear that ROA is a vital tool in understanding the behavior of proteins in solution, as the information content in the spectra gives unparalleled access to detailed interpretation of structural changes.

With respect to the crowding effect, the conclusion of the previous studies is validated[4,5], as there is a clear difference between the influence of F70 and D70 on the structural properties of the protein. In the case of phosvitin, the presence of a relative low concentration of crowding agent can already induce alpha-helical contributions, visible in the ROA spectra, and strengthen the tendencies towards beta-sheets, as derived from the CID plots. At higher concentrations of crowding agent, the alpha-helical contributions are really apparent, both in the ROA spectra as in the CID plots. However, the structural changes induced by the presence of F70 are more pronounced than those induced by D70, and are already visible at lower concentrations. Furthermore, it has to be noted that the structural changes, visible in the range 900-1150 cm^{-1}, might not originate in a change in secondary structure of the protein, but in the interaction between the phosphorylated residues of the protein and the sugar-based crowding agent. We therefore suggest that future research into the crowding effect on the structural properties of phosphorylated proteins, non-sugar based crowding agents, such as PEG, are taken into account.
Finally, however, it is clear that environmental factors can have a distinct effect on the structural properties and thus the behavior of a protein in solution. Therefore, one should always try to determine these properties in an environment that mimics the biological situation as closely as possible, to ensure the relevance of the findings. Due to its sensitivity, ROA could be a tool to perform such studies.

Experimental Section

Phosvitin, F70 and D70, were supplied by Sigma-Aldrich. The Raman and ROA spectra were measured at ambient temperature in deionized water using the previously described ChiralRAMAN-2X instrument (BioTools, Inc.),[28] which employs the scattered circular polarization (SCP) measurement strategy. The ROA spectra are presented as intensity differences ($I_R - I_L$), with I_R and I_L denoting the Raman intensities with right- and left-circular polarization, respectively, whereas the Raman spectra are presented as the sum of these polarized components ($I_R + I_L$). The Raman spectra can be found in the supplementary information. The spectrometer is wavelength-calibrated by measuring a neon-arc lamp and band aligning to premeasured standard. The sample concentrations were 60 mg mL^{-1} for phosvitin and between 50 and 300 mg mL^{-1} for F70 and D70. Experimental conditions: laser wavelength 532 nm; laser power at the source 500 mW; spectral resolution 7 cm^{-1}; acquisition times 72 h for each sample. Cosmic ray spikes were removed from the ROA spectra by means of a median filter, after which a spectrum of the mixture of crowding agent and protein was aligned with a spectrum of the crowding agent alone in solution by normalizing to a non-variable sugar band. Next, the spectrum of the crowding agent was subtracted from the spectrum of the crowding agent and protein together in solution. An example of this subtraction procedure can be found in the supplementary information (Figure S4). The spectra, resulting from this subtraction, were filtered by a third-order nine-point Savitzky–Golay filter. All of these treatments were always critically evaluated in MATLAB. The CID values were calculated by dividing the absolute intensity of the ROA signal for the wavenumber by the intensity of the corresponding Raman signal for the same wavenumber over a range.($\frac{I_R - I_L}{I_R + I_L}$)

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Keywords: Biochemistry • Crowding • Disorder • Raman optical activity • Structural biology

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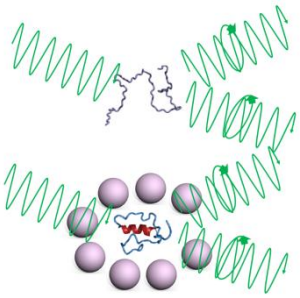
Entry for the Table of Contents (Please choose one layout)

Layout 1:

ARTICLE

The sensitivity of Raman optical activity (ROA) towards small conformational changes is explored by tracking the structural changes in an intrinsically disordered protein, phosvitin, caused by different concentrations of crowding agent.



*Author(s), Corresponding Author(s)**

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