4. INTRODUCTION

DSPG is another major proteoglycan of the corneal stroma. It contains one chondroitin/dermatan sulphate linked to the core protein through an O-glycosidic linkage and one to three N-linked oligosaccharides (Midura and Hascall, 1989). The core protein has a molecular weight of 40,000 Da (Schrecengost et al., 1992) and amino acid analysis indicates it to be rich in leucine (Heinegard et al., 1985). Antibodies raised against decorin, a proteoglycan present in connective tissues such as skin, tendon, bone and cartilage react with the corneal DSPG, while biglycan, another universally present proteoglycan, also reacts with the corneal DSPG but to a lesser extent (Fisher et al., 1989). Hence the DSPG of the cornea is suggested to be decorin. The amino acid sequence of chick (Li et al., 1992), bovine (Day et al., 1987) and human (Krusius and Ruoslahti, 1986) decorin reveals striking homologies. Based on these, a structural model of decorin has been proposed (Li et al., 1992). The core protein has three repeat units each of which each has three conserved regions, as shown in Figure 4.1. It has a 15 amino acid long signal peptide at the N-terminal end which contains one Trp residue, followed by a pre-peptide, 14 amino acids long. These two regions are however not present in proteoglycans extracted from the extracellular matrix (Sawhney et al., 1991).

Most studies on DSPG have been biochemical or immunological in nature. However, its molecular structure has not been probed in its entirety. Structural studies of proteoglycans using optical spectroscopic methods such as far-UV circular dichroism and Raman spectroscopy, in general, are complicated as the spectral bands of the amide chromophores of the dermatan sulphate chain overlap with the amide chromophore of the protein backbone (Stone, 1971), confounding unequivocal analysis. In this context, fluorescence spectroscopy would be the method of choice, as the molecule has a lone Trp at the C-terminal end of the core protein, which can serve as an intrinsic spectral probes. In the earlier chapter, we focused on the conformational features of KSPG using a similar approach. This chapter aims at probing the conformation of DSPG using steady state and time resolved emission spectroscopy. Denaturation studies carried out give us information regarding the stability of the molecule.
Figure 4.1: A structural model for decorin. S, O-glycosylation site; inverted Y, potential N-linked glycosylation site and dashed line, signal peptide. Open box, LPPSLTELXLXXNKISKI; Closed box, LXNLXXLXLXXNXI; Dotted box, LXXLXLXXNXL.
4.1. MATERIALS AND METHODS

4.1.1. Purification of dermatan sulphate proteoglycan (DSPG)

The proteoglycan fraction obtained by DEAE chromatography (as described in Chapter II) was further purified on either Superose 6 FPLC or a Sepharose CL-4B, equilibrated with 4 M GdmCl, 20 mM Tris, pH 6.8 (Hassell et al., 1979; Coster and Fransson, 1981). The elution position of proteoglycans was assessed colorimetrically using the dimethyl methylene blue binding assay. The proteoglycan peak was pooled, dialysed and lyophilized. The lyophilized proteoglycan was dissolved in 0.2 M sodium acetate, pH 5.8, and digested using keratanase for 4-6 h at 37 °C, as described earlier (Rada et al., 1993). The digested proteoglycans were dialysed against 7 M urea containing 0.15 M NaCl, 50 mM Tris, pH 6.8 and loaded on a DEAE Sephadex column equilibrated in the same buffer. The keratan sulphate proteoglycan core protein was collected as the pass-through fraction while DSPG was eluted with 2 M NaCl in urea, dialysed against water and lyophilized. The purity of DSPG was checked by digestion of GAG chains with chondroitinase ABC. All the studies of DSPG were carried out in 50 mM Tris, 100 mM NaCl, pH 7.4.

4.1.2. Enzymatic deglycosylation of DSPG

DSPG was subjected to enzymatic digestion using 0.1 U of chondroitinase ABC (Sigma Chemicals Co.) at 37 °C for 3 h, which removes the chondroitin/dermatan sulphate (CS/DS) side chains thereby converting those proteoglycans with CS/DS side chains to core proteins. The change in the molecular weight of the proteoglycan can be visualized by separation on a 10 % SDS-PAGE.

4.1.3. Dimethyl methylene blue binding assay

The presence of proteoglycan in the chromatographic fractions were monitored using the DMB binding assay as described by Chandrashekar et al. (1987). The fractions were added to 0.05 M acetate buffer, pH 6.9, in a microtitre plate to which DMB reagent was added. The absorbance was read as a ratio of the value at 550 nm to that at 610 nm.
4.1.4. Steady-state fluorescence measurements

Fluorescence emission spectra, and CsCl quenching of Trp were carried out as described in Chapter II. All experiments were performed with 1 mg/ml DSPG in 50 mM Tris, 100 mM NaCl, pH 7.4. In quenching experiments, the fluorescence intensities were read at 334 nm and 342 nm following excitation at 295 nm.

4.1.5. Denaturation studies

DSPG was added, from a concentrated stock to a final concentration of 0.2 mg/ml, to the solutions of GdmCl whose concentrations ranged between 0 and 4.5 M. Urea was added (from a 10 M stock) to a 0.1 mg/ml DSPG solution to final concentrations ranging between 0 and 6 M. The solutions were incubated for 1-2 h and the fluorescence emission maxima of Trp recorded.

Thermal denaturation of 0.33 mg/ml DSPG was carried out and the Trp emission maxima at different temperatures recorded as described in Chapter III.

4.1.6. Life-time measurements and time-resolved fluorescence spectroscopy

Emission life-time measurements and time-resolved emission spectra (TRES) were carried out with a Photon Technology International (Canada) LS-100 luminescence spectrometer, using the time correlated single photon counting mode. The emission was fixed at 334 nm or 342 nm with a bandpass of 4 nm. To optimize the signal to noise ratio, 5000 photon counts were collected in the peak channel. A double exponential fit was used to define the life times.

TRES was obtained by exciting the sample at 296 nm and accumulating data for decay between emission wavelengths of 310 and 360 nm at every 5 nm intervals and reconstructing the emission spectra from the deconvoluted decay curves, as described in Chapter III.
4.2. RESULTS AND DISCUSSIONS

4.2.1. Purification of DSPG

The proteoglycan fraction obtained after DEAE ion exchange chromatography is essentially a mixture of KSPG and DSPG, along with a 20 KDa contaminating protein. It was subjected to gel filtration chromatography under denaturing conditions to remove the contaminating 20 KDa protein from the proteoglycans. Figure 4.2A shows the Superose 6 gel filtration chromatogram of the proteoglycan fraction obtained following DEAE ion exchange. The proteoglycan peak was monitored using the DMB binding assay. This fraction was subjected to enzymatic digestion using keratanase to remove KSPG from the proteoglycan mixture, and separated on a Mono-Q FPLC column to remove the core protein of KSPG, which comes as the unbound fraction (Figure 4.2B). The purity of the DSPG preparation was checked by subjecting the purified protein to enzymatic digestion with chondroitinase ABC and separated on SDS-PAGE. Figure 4.3 shows the shift in the DSPG band from the > 90 KDa position (lane 1) to 40 KDa following enzymatic digestion (lane 2), indicating the protein to be free from proteoglycans having GAG chains other than dermatan/chondroitin sulphate. Lane 1 shows weaker staining than lane 2 as GAG are known to interfere with the staining.

4.2.2. Steady state fluorescence

Figure 4.4 shows the Trp fluorescence emission spectra of DSPG. The spectrum shows two emission maxima at 334 and 342 nm, with the 334 nm peak having relatively high intensity. Generally, Trp in proteins gives a single broad emission band but, such structured emission bands of Trp are known to occur in azurin, whose emission spectrum in the 310 nm region is very similar to that of indole in hexane (Konev, 1967). This is thought to arise from the dual emission from \(^1L_a\) and the \(^1L_b\) states of Trp. The \(^1L_a\) transition is more solvent-sensitive than the \(^1L_b\) transition and they are shifted to lower energies in polar solvents. Hence it is thought that the lone Trp residue is completely buried in the interior of the protein and is surrounded by nonpolar amino acid residues (Lakowicz, 1983).

Native \(\delta\)-crystallin exhibits a similar doublet emission band at excitation 295 nm with emission at 315 and 325 nm (Horwitz and Piatigorsky, 1980). Fluorescence quenching studies
Figure 4.2 A: High performance gel permeation chromatography of 2 M PG fraction on a Superose 6 column equilibrated with 4 M Gdm.Cl and 50 mM Tris, pH 7.4. Protein absorbance at 280 nm (---); keratan sulfate as detected by the dimethyl methylene blue assay (---). B: High performance ion-exchange chromatography of proteoglycans after keratanase treatment on a Mono Q column equilibrated with 7M urea, 50 mM Tris, pH 6.8. KSPG core protein was eluted as unbound fraction and bound DSPG was eluted with a linear gradient of 0-0.15 M NaCl. The gradient was then increased to 2 M NaCl to remove any bound proteins from the column.
Figure 4.3: DSPG separated on 10% SDS-PAGE after deglycosylation with chondroitinase ABC for 4 h at 37 °C. Lane 1, untreated DSPG; lane 2, DSPG after treatment with chondroitinase ABC.
Figure 4.4: Trp fluorescence emission spectra of DSPG showing the doublet emission.
indicated the lone Trp residue to be buried. The doublet emission in this case was thought to arise due to the location of Trp in the subunit interface (Rao et al., 1994). These results on azurin and chick δ-crystallin are relevant to the fluorescence spectral features of DSPG. It is clear, however, that the Trp in DSPG is in a more polar milieu.

4.2.3. Fluorescence quenching studies

Quenching studies using the polar quencher CsCl was carried out to check the fraction of Trp accessible for quenching at both the 334 nm and 342 nm emission maxima. Figure 4.5 shows the modified Stern-Volmer plot for Cs+ Quenching at 334 nm and 342 nm. The fraction available for quenching is around 0.24 for 334 nm peak while that of 342 nm peak is 0.33. This further points towards the microenvironmental differences between the Trp residues leading to emission at two different wavelengths.

4.2.4. Life-time analysis

This was carried out at the emission wavelengths of 335 and 340 nm and an excitation wavelength of 296 nm, in both water and D2O, so as to selectively monitor the Trp residues of DSPG. D2O was used to monitor the isotope effect on the two emission peaks. The emission of Trps in DSPG could be fitted using a three-exponential decay fit at both the emission wavelengths, with the three life times of 0.25, 2 and 7.4 ns at 335 nm and 0.3, 2.6 and 7.5 ns at 340 nm. In D2O, the Trp in DSPG was also found to decay triexponentially, with life-times of 0.2, 0.7, and 4.5 ns at 335 nm and 0.6, 2.1 and 7.4 at 340 ns (Table 4.1). The life-time of Trp in DSPG is considerably reduced in the presence of D2O indicating the fluorophore to be accessible to the isotope (Ricci, 1970). To gather more information about the dynamics of DSPG and its excited state interactions, time resolved emission spectra were recorded.

Figure 4.6 shows the three dimensional TRES plot of DSPG in aqueous buffer. In the early periods of the time scale, two prominent peaks can be seen, one at 335 nm and the other at 349 nm, each with a minor shoulder at 339 and 355 nm respectively. The intensities of both the peaks gradually increase and stabilize at 2.3 ns after which they decay. A peak at 324 nm gradually increases in its intensity between 4.7 and 5.3 ns, stabilizes at 5.5 ns after which it decays. The gradual rise and fall of the peaks indicate the fluorophore to be hydrated and
Figure 4.5: Stern-Volmer plot of the Cs⁺ quenching of Trp fluorescence of DSPG. CsCl was added to a 1 mg/ml protein solution in increasing amounts to a final concentration of 0.7 M. The changes in the fluorescence intensity was monitored at 334 nm (open circles) and at 342 nm (closed circles).
<table>
<thead>
<tr>
<th>Sample</th>
<th>$\alpha_1$</th>
<th>$\tau_{1,\text{ns}}$</th>
<th>$f_1$</th>
<th>$\alpha_2$</th>
<th>$\tau_{2, \text{ns}}$</th>
<th>$f_2$</th>
<th>$\alpha_3$</th>
<th>$\tau_{3, \text{ns}}$</th>
<th>$f_3$</th>
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</thead>
<tbody>
<tr>
<td>DSPG $H_2O$</td>
<td>0.051</td>
<td>2.111</td>
<td>0.257</td>
<td>0.938</td>
<td>0.246</td>
<td>0.550</td>
<td>0.011</td>
<td>7.352</td>
<td>0.192</td>
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<td>(335 nm)</td>
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<tr>
<td>DSPG $H_2O$</td>
<td>0.897</td>
<td>0.316</td>
<td>0.457</td>
<td>0.088</td>
<td>2.575</td>
<td>0.366</td>
<td>0.015</td>
<td>7.478</td>
<td>0.177</td>
</tr>
<tr>
<td>(340 nm)</td>
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<tr>
<td>DSPG $D_2O$</td>
<td>0.363</td>
<td>0.715</td>
<td>0.526</td>
<td>0.614</td>
<td>0.214</td>
<td>0.267</td>
<td>0.023</td>
<td>4.510</td>
<td>0.207</td>
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<tr>
<td>(335 nm)</td>
<td></td>
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<tr>
<td>DSPG $D_2O$</td>
<td>0.077</td>
<td>2.108</td>
<td>0.306</td>
<td>0.914</td>
<td>0.324</td>
<td>0.559</td>
<td>0.010</td>
<td>7.444</td>
<td>0.135</td>
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<tr>
<td>(340 nm)</td>
<td></td>
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Table 4.1: Life time distribution of Trp in DSPG.

$\alpha$ gives the pre-exponential (individual intensity), $\tau$ the life time in nanoseconds (ns), $f$ represents the fractional contribution of each component to total fluorescence.
Figure 4.6: A three dimensional representation of time-resolved emission spectra of DSPG. The sample was excited at 296 nm, corresponding to the peak of the nitrogen lamp, and the spectra recorded in a single photon counting mode. A total of 5000 counts were collected at each emission wavelength, the TRES reconstructed from the decay data by triple-exponential analysis and plotted at an angle of 45°.
hence able to undergo solvent dipole reorientation around the excited fluorophore (Easter et al., 1976, Lakowicz, 1983, Sommer et al., 1990). The above results rule out the possibility of the doublet band arising from vibronic structure; emission maxima indicate the Trp to be in a polar environment, and the contact of indole nucleus with polar solvent molecules is known to quench the structured emission and to broaden the absorption band (Lakowicz, 1983).

4.2.5. Structural stability of DSPG

The thermal denaturation curve described in Figure 4.7 reveals that DSPG undergoes a sharp transition around 54 °C. The 335 nm Trp emission is red-shifted to around 350 nm, a value that is typical of Trp residues in denatured proteins, where they are exposed to the solvent. In contrast, the 340 nm band is affected less dramatically and more monotonically. Beyond 55 °C, both the bands merge into a single band around 350 nm. This dual behaviour of Trp fluorescence reiterates the suggestion that the Trp residues in DSPG experience two different environments, one more polar than the other; heating appears to open up the conformation of DSPG in a manner that removes this difference and place the fluorophores in contact with water. The thermal transition is sharp, with no apparent intermediate states. This led us to analyze the transition in terms of a two-state model using van’t Hoff plots; with the midpoint of transition around 53 °C. The enthalpy of the transition was found to be around 30 kcal mol\(^{-1}\), while the entropy of the transition was estimated to be 92 e.u. These values are comparable, but lower than the values of \(\Delta H\) 128 kcal mol\(^{-1}\) and \(\Delta S\) 380 e.u. in the case of the avian lens protein \(\delta\)-crystallin (Rao et al., 1994), which has been alluded to earlier. This suggests that DSPG might not be a conformationally robust molecule. Also, its thermal transition is not reversible. Since the solution, which becomes opalescent upon heating to beyond 55 °C, does not clear upon cooling and the doublet emission of Trp is not restored, it is likely that intermolecular mixed disulphide crosslinks are formed when DSPG unfolds at high temperatures.

In order to further investigate the conformational stability of DSPG, we monitored its behaviour towards chemical denaturants such as GdmCl and urea. Figure 4.8A shows that GdmCl is able, at concentrations beyond 2.5 M, to unfold DSPG. Here too, the 334 nm band shows a steeper transition compared to the 342 nm band. The two bands merge beyond 2.5M
Figure 4.7: Thermal denaturation profile of 0.3 mg/ml DSPG in 50 mM Tris, 100 mM NaCl, pH 7.4. Changes in the Trp emission maxima at 334 nm (open circles) and 342 nm (filled circles).
Figure 4.8: Changes in emission maxima of the Trp doublet of DSPG in 50 mM Tris, 100 mM NaCl, pH 7.4, monitored at 334 nm (open circle) and 342 nm (filled circle) as a function of guanidinium chloride concentration (A) and urea (B).
GdmCI and red-shift to about 348 nm at concentrations exceeding 4 M. The effect of increasing concentration of this denaturant, is reversible, since dilution of the solution regenerates the original fluorescence bands. The other chemical denaturant, urea, has a similar effect (Figure 4.8B), though not as strong as GdmCI. The doublet emission of DSPG merges into a single band around 3 M urea, which gradually red shifts to a final value of 348 nm at 6 M urea. Denaturation with urea is a reversible effect since dilution of a solution in high urea concentration regenerates the original doublet emission.

Conformational transitions of multimeric proteins often expose hydrophobic surfaces e.g., α- and δ-crystallins (Walsh et al., 1991; Rao et al., 1994). This is easily studied using the extrinsic probe ANS, which binds to hydrophobic surfaces, with a consequent blue shift and intensification of emission (Stryer, 1965). Figure 4.9 shows ANS binding to DSPG, as the latter is unfolded in increasing concentrations of urea. ANS binds to native DSPG at an apolar site, revealed by its emission around 470 nm. With increasing addition of urea, ANS emission steadily red-shifts as DSPG is unfolded; apparently the unfolding of the proteoglycan does not expose any apolar surface to which the extrinsic probe ANS binds.

These studies collectively indicate that the doublet emission of Trp is abolished upon denaturation, hence the native structure is important for such an emission to occur. Unlike δ-crystallin where the Trp doublet emission band arises due to the presence of Trp at the subunit interface, DSPG does not seem to have any specific subunit arrangement as denaturation by urea and probing by ANS does not result in enhanced binding of ANS. This would result in a blue shift in the emission maxima, as is expected of a protein with subunit association (Rao et al., 1994). The doublet emission of DSPG can hence be thought to be a result of Trp being present in two different environments. Glycosylation is known to alter and stabilize the conformation of peptides (Urge et al., 1992). Depending on the extent of glycosylation in DSPG, various conformations can be expected, leading to heterogeneity in the Trp environment.

Fluorescence spectroscopy can thus be used as a handy tool for studying the conformation of proteoglycans. We have applied this approach to the study of KSPG to study its conformation, stability and interactions as described in Chapter III. The properties of DSPG here are quite different from the fluorescence properties of KSPG. Proteoglycans seem
Figure 4.9: A plot of the emission maxima of ANS-bound DSPG as a function of urea concentration.
to be a class of proteins with diverse conformational features in spite of similarities in the primary structure, sharing common features such as leucine-rich repeats. Such a diversity could be due to the presence of GAG chains. It would be worthwhile to study the conformational features of other proteoglycans which would provide insights into their diverse functional roles in the extracellular matrix.