Distinct Secondary Structures of the Leucine-Rich Repeat Proteoglycans Decorin and Biglycan: glycosylation-dependent conformational stability

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Summary

Biglycan and decorin, closely related small leucine-rich repeat proteoglycans, have been overexpressed in eukaryotic cells and two major glycoforms isolated under native conditions: a proteoglycan substituted with glycosaminoglycan chains; and a core protein form secreted devoid of glycosaminoglycans (A.M. Hocking, R.A. Strugnell, P. Ramamurthy, D.J. McQuillan, J. Biol. Chem. 271: 19571-19577, 1996; P. Ramamurthy, A.M. Hocking, D.J. McQuillan, J. Biol. Chem. 271: 19578-19584, 1996). A comparative biophysical study of these glycoforms has revealed that the overall secondary structures of biglycan and decorin are different. Far-UV CD spectroscopy of decorin and biglycan proteoglycans indicates that, although they are predominantly β-sheet, biglycan has a significantly higher content of α-helical structure. Decorin proteoglycan and core protein are very similar, whereas the biglycan core protein exhibits closer similarity to the decorin glycoforms than to the biglycan proteoglycan form. However, enzymatic removal of the chondroitin sulfate chains from biglycan proteoglycan does not induce a shift to the core protein structure, suggesting that the final form is influenced by polysaccharide addition only during biosynthesis. Fluorescence emission spectroscopy demonstrated that the single tryptophan residue, which is at a conserved position at the C-terminal domain of both biglycan and decorin, is found in similar microenvironments. This indicates that, at least in this specific domain, the different glycoforms do exhibit apparent conservation of structure. Exposure of decorin and biglycan to 10 M urea resulted in an increase in fluorescent intensity, which indicates that the emission from tryptophan in the native state is quenched. Comparison of urea-induced protein unfolding curves provided further evidence that decorin and biglycan assume different structures in solution. Decorin proteoglycan and core protein unfold in a manner similar to a classic two-state model, in which there is a steep transition to an unfolded state between 1-2 M urea. The biglycan core protein also shows a similar steep transition. However, biglycan proteoglycan shows a broad unfolding transition between 1-6 M urea, probably indicating the presence of stable unfolding intermediates.
INTRODUCTION

Decorin and biglycan are small proteoglycans comprising chemically similar core proteins substituted at the N-terminal end with one or two chondroitin/dermatan sulfate chains, respectively. Despite the presumed structural similarity between biglycan and decorin (1), they have distinct patterns of temporal and spatial expression suggesting different functions. They are members of a family of glycoproteins grouped together on the basis of their presence in the extracellular matrix, and by virtue of a leucine-rich motif that dominates the core protein (for review, see (2)). Most of the members of this family exist in tissues as proteoglycans and have been labeled the small leucine-rich repeat proteoglycans (SLRPs).

The protein core of decorin (Fig. 1a) and biglycan (Fig. 1b) can be divided into distinct domains, based on amino acid sequence and specific post-translational modifications. A signal sequence targets the nascent polypeptide to the secretory route; a short propeptide of highly charged amino acids undergoes differential tissue- and cell-specific cleavage; an N-terminal glycosaminoglycan attachment region containing one (decorin) or two (biglycan) Ser-Gly dipeptide consensus sequences; a leucine-rich repeat (LRR) domain that represents more than two thirds of the core protein is flanked by highly conserved disulfide bonded cysteine clusters; and a short C-terminal domain. The core protein of decorin has three consensus sites for N-linked oligosaccharides; two of these sites are conserved in biglycan.

The post-translational modifications of decorin and biglycan are complex and variable, wherein differentially glycosylated forms of these molecules have been isolated from tissues and cells. Decorin core protein devoid of a glycosaminoglycan chain has been isolated (3,4), although it is
yet to be demonstrated whether this is due to post-secretory cleavage of the GAG attachment
domain, or synthesis and secretion of core protein that bypasses the GAG synthetic machinery.

There is evidence that members of the SLRP family may be “part-time” proteoglycans.

Overexpression of decorin and biglycan yields significant amounts of secreted core protein devoid
of glycosaminoglycan chains (5,6), the proportion of which appears to be cell type-dependent
and related to the endogenous activity of xylosyltransferase (the enzyme that catalyzes the first
sugar transfer reaction initiating chondroitin sulfate polymerization on a core protein substrate).

The decorin core protein is differentially substituted with N-linked oligosaccharides (5,7,8) with
two and three sites utilized.

The leucine-rich repeat (LRR) is a structural motif first identified by Patthy (9) and subsequently
refined by Kobe and Deisenhofer (10), that is usually present in tandem array and has been
described in an increasing number of proteins, giving rise to a LRR superfamily. It is likely that
the conserved residues of each LRR motif define the secondary structure, while the intervening
residues determine specificity of interaction with ligands. In 1993, Kobe and Deisenhofer solved
the crystal structure of ribonuclease inhibitor (11), a LRR protein that consists of fifteen repeats.

This remains the only report of the detailed structure of a LRR protein and may provide a
prototype for all LRR proteins. Each LRR consists of a β-sheet parallel to an α-helix forming a
hairpin structure which is aligned parallel to a common axis resulting in a non-globular horseshoe
shaped protein. Binding of the ligand to the concave face (i.e. β-strands) results in a
conformational change of the entire structure and increases the available surface area for binding.
The LRR domain of the SLRP family members is unique within the superfamily in that it is flanked by cysteine clusters; at the N-terminal end of the LRR domain there are four similarly spaced cysteine residues in a twenty amino acid stretch which are involved in disulfide bonds; and at the COOH-terminal end there are two cysteine residues also believed to form an intrachain disulfide bond. The 24 amino acid LRR consensus for members of the SLRP family is x-x-I/V/L-x-x-x-F/P-L-x-x-L/P-x-x-L-x-L/I-x-L-x-x-N-x-I/L, where x is any amino acid, and in the case where more than one amino acid is noted, the first occurs most often (2).

Attempts have been made to predict the structure of decorin (and related molecules) based on the crystal structure of ribonuclease inhibitor. Computer modeling, constrained by parameters established by the structure of ribonuclease inhibitor, have suggested that the decorin core protein forms an arch-shaped protein with the glycosaminoglycan chain and N-linked oligosaccharides situated on the same side of the molecule (1). High magnification rotary shadowing electron micrographs of scleral decorin reveal a similarly “horseshoe-shaped” molecule (12) consistent with the computer modeling prediction. However, the ribonuclease inhibitor is composed entirely of LRRs, whereas the N- and C- termini of SLRPs have extended non-LRR containing domains. The inhibitor lacks cysteine clusters flanking the LRR domain which, through intramolecular disulfide bond formation (13), might provide points of stabilization at either end of the LRR domain. The length of the decorin repeat motif is 24 residues, which is shorter than for ribonuclease inhibitor and may result in a restricted β-sheet. Furthermore, the extensive glycosylation of SLRPs may influence the folding of the LRR domain.
In the current study, our data indicate that recombinant human decorin and biglycan have different secondary structures in solution and marked differences in conformational stability, as assessed by circular dichroism and fluorescent spectroscopy, which is inconsistent with the utility of modeling based solely on the structure of ribonuclease inhibitor. Furthermore, we provide evidence that both the conformation and stability of these molecules is variably influenced by whether they are synthesized with or without a glycosaminoglycan chain, whereas removal of polysaccharides after secretion has no appreciable influence on conformation or stability.
**EXPERIMENTAL PROCEDURES**

**Materials** – Ultrapure urea was obtained from Ambion Inc. (Austin, TX). Ultrafree-15 centrifugal filtration devices were from Millipore Corp. (Bedford, MA). All other materials were obtained as previously described (6).

**Protein purification** - Recombinant decorin and biglycan glycoforms were expressed and purified using the vaccinia virus/T7 bacteriophage expression system, as previously described (5,6). Briefly, recombinant proteins were purified on a column of iminodiacetic acid immobilized on Sepharose 6B. Proteoglycan and core protein forms were resolved and eluted by a linear gradient of imidazole in column buffer (0.5 M NaCl, 20 mM Tris-HCl, pH 8.0). Pooled fractions were dialyzed against phosphate-buffered saline (PBS) pH 7.4 and concentrated on Ultrafree-15 centrifugal filter devices. Protein concentrations were determined by the molar extinction coefficient (14).

**Stock solutions** – Urea stock solutions (10 M) in a buffer of 50 mM NaH₂PO₄/K₂HPO₄, pH 7.0 (15), were prepared daily for each experiment and filtered (0.22-μm pore) prior to use. The urea concentration of each stock solution was calculated by weight and by refractive index (15). The buffer solution without urea is referred to as “phosphate buffer”.

**Circular dichroism spectroscopy** - CD spectra of protein samples were recorded on a Jasco 720 spectropolarimeter using a 2 mm path-length quartz cell at room temperature at a concentration of 10 μM in phosphate buffer. The recorded spectra (190-250 nm) were the average of 10 scans and were corrected to background (phosphate buffer alone). Sample cuvettes were sealed with a Teflon stopper so that no evaporation occurred. The CD signal (mdeg) was converted to molar ellipticity (θ) by the following equation:

\[ \theta \text{ (deg.cm}^2\text{.dmol}^{-1}) = \frac{m \text{deg} \times 0.1}{\text{molarity(M)} \times \text{pathlength(cm)} \times \text{amino acid residues}} \]
**Fluorescence emission spectroscopy** - Fluorescence emission was measured using a FluoroMax2 spectrofluorometer; the pathlength of the cuvette was 1 mm and the signal averaged for 50 seconds. The excitation wavelength for tryptophan emission was 296 nm in all experiments. Emission scans were collected from 300-500 nm.

**Equilibrium unfolding curves** - Equilibrium unfolding experiments were done as previously described (15). Briefly, stock solutions were prepared in phosphate buffer to be 10 times the desired final protein concentration. Phosphate buffer, urea from the 10 M stock solution, and 100 μl of stock protein solution to give a final volume of 1 ml were mixed; this yielded final urea concentrations of 0-8 M and a final protein concentration of 10 μM. Protein samples were gently mixed and equilibrated for 18 h at 25°C. Unfolding was monitored using circular dichroism spectroscopy and fluorescence emission spectroscopy. The CD signal was determined at 220 nm for the proteoglycan and core protein forms of biglycan and decorin.
RESULTS AND DISCUSSION

Recombinant biglycan and decorin were expressed in the vaccinia/T7 bacteriophage system (5,6). Both biglycan and decorin were synthesized as two glycoforms: a proteoglycan form, and a core protein form that was secreted devoid of glycosaminoglycan chains.

Circular dichroism spectroscopy- Far-UV CD spectra were measured for native recombinant biglycan and decorin glycoforms (Fig. 2). Biglycan proteoglycan had spectra with minima at 220 nm (Fig. 2a, solid circles); the CD spectra of decorin proteoglycan was different, with a minima at 218 nm and a broader curve (Fig. 2c, solid circles). Several deconvolution computer programs were used to facilitate an objective comparative analysis of the CD spectra, including SELCON (16), VARSELEC (17,18), CCA (19), and Contin (20) using file conversion software (SOFTSEC™: File Conversion for Windows obtained from Softwood Company). The estimated contribution of different structural motifs (i.e. β-sheet, β-turn, α-helix, random coil) varied between different deconvolution programs (data not shown), but it was nevertheless consistently determined that biglycan and decorin assumed different structures in solution. For instance, the method of Sreerama and Woody (16) predicted that the secondary structure of biglycan to be 30% α-helix, 14% β-sheet, 15% β-turn and 46% random coil, whereas decorin comprised 8% α-helix, 44% β-sheet, 14% β-turn and 33% random coil.

After equilibration of recombinant biglycan and decorin in 10 M urea for 16 hours, samples were analyzed by CD spectroscopy (Fig. 2, open circles). The CD spectra for both biglycan (Fig. 2a, open circles) and decorin (Fig. 2c, open circles) in 10 M urea reflected a loss of CD signal typical of an absence of secondary structure.

CD spectroscopy was also used to examine the secondary structure of the core protein glycoforms of biglycan and decorin (Fig. 2). The CD spectra for the biglycan core protein in phosphate buffer
had a minima at 215 nm (Fig. 2b, solid circles), with a significantly broader curve than seen for the proteoglycan form (Fig. 2a). Computer deconvolution analysis predicted that the secondary structure of biglycan core protein was significantly different to the proteoglycan form (13% α-helix, 36% β-sheet, 19% β-turn and 37% random coil (16)). Thus the biglycan core protein, synthesized devoid of glycosaminoglycan chains, had a secondary structure that is different to the proteoglycan form. The effect of chondroitin sulfate chain addition on the secondary structure of biglycan and decorin core proteins was assessed by digestion with chondroitinase ABC (to remove the glycosaminoglycan chains). There was no detectable difference between the spectra generated from undigested and digested proteoglycans. Glycosaminoglycan chains, at 10-fold higher concentration than in samples analyzed in Fig. 2, did not contribute to the CD spectra (data not shown). Therefore, removal of the glycosaminoglycan chains from secreted proteoglycan does not significantly influence secondary structure.

Comparison of the CD spectra of decorin core protein (Fig. 2d, solid circles) with the spectra of decorin proteoglycan (Fig. 2b, solid circles) showed essentially identical curves with the minima of both glycoforms at 218 nm. The CD scans clearly show that decorin and biglycan have distinct secondary structures. Furthermore, biglycan synthesized and secreted devoid of chondroitin sulfate chains assumes a different structure to biglycan substituted with chondroitin sulfate. However, removal of the bulk of the chondroitin sulfate mass from biglycan after purification had no measurable influence on the structure. Biglycan appears to have more α-helical content in its secondary structure relative to biglycan core and decorin glycoforms, which are primarily β-sheet in structure. Decorin, on the other hand, appears to form the same structure in solution irrespective of substitution with chondroitin sulfate.

**Fluorescence spectroscopy** - The mature core protein of biglycan and decorin both have a single tryptophan residue situated between the two conserved cysteine residues at the C-terminal end of
the core protein (Fig. 3a). Peptide sequencing of bovine biglycan has shown that these cysteines form an intramolecular disulfide bond (13). Comparison of the amino acid sequence in this region reveals that biglycan and decorin share 65% amino acid identity. Fluorescence spectroscopy was used to analyze the environment of this tryptophan in native and denatured biglycan and decorin.

The fluorescent intensity for all four glycoforms increased in the presence of 10 M urea (Fig. 3b-e, closed circles) relative to the intensity in phosphate buffer (Fig. 3b-e, solid circles). These data indicate that the emission from the tryptophan in the native glycoforms is quenched. Biglycan proteoglycan in PBS had a maximum emission wavelength of 342 nm (Fig. 2b, open circles) and after denaturation the peak emission wavelength was shifted to 352 nm (Fig. 2b, closed circles). The maximum emission wavelength of decorin proteoglycan in PBS was 345 nm (Fig. 2d, solid circles); in 10 M urea, the emission wavelength shifted to 354 nm (Fig. 2d, open circles). These results suggest that the tryptophan in native biglycan and decorin is partially buried; in denatured biglycan and decorin, the tryptophan is exposed to a polar environment.

The magnitude of the peak emission wavelength shift for the native and denatured core protein forms of biglycan (Fig. 3c) was similar to that observed for the proteoglycan form (Fig. 3b). The intrinsic fluorescence spectra for biglycan core protein revealed that the native protein had an emission maxima at 341 nm (Fig. 3c, solid circles) which shifted to 353 nm for the denatured protein (Fig. 3d, open circles). Therefore, it appears that the tryptophan is in a similar environment in both glycoforms of biglycan. The spectra for native decorin core protein had a peak emission wavelength maxima of 350 nm (Fig. 3e, solid circles). The maxima for the fluorescence spectra of decorin core protein in 10 M urea was 355 nm (Fig. 3e, open circles). The peak emission wavelength for decorin is different between core protein (350 nm) and proteoglycan (345 nm) which indicates that the microenvironment of the tryptophan in these two glycoforms may be different. The decorin core protein (Fig. 3e) is more solvent exposed in the native state relative to
the decorin proteoglycan (Fig. 3d). However, taken together these subtle differences suggest that
the C-terminal domain is structured similarly among all four glycoforms.

**Urea denaturation curves** - To further characterize structural differences between biglycan and
decorin, the conformational stability was investigated. Urea denaturation curves were determined
for each glycoform of biglycan and decorin. The CD spectra (shown in Fig. 2) revealed the
maximal CD signal difference between native and denatured proteoglycan was at 220 nm, and this
wavelength was used to monitor changes in the CD signal as unfolding occurred. The denaturation
curves generated for the proteoglycan forms of biglycan and decorin are complex (Fig. 4), but
highly reproducible, and were not amenable to curve fitting algorithms. The denaturation curve for
biglycan proteoglycan indicates the protein is very susceptible to urea denaturation based on the
limited pre-transition baseline from 0-0.5 M urea (Fig. 4a). However, the transition from the folded
to unfolded state occurs gradually from 1-6 M urea, and it is not clear where the post-transition
baseline begins. The unfolding of biglycan proteoglycan probably proceeds through stable
intermediates reflecting sequential disruption of domains. Unfolding of decorin proteoglycan
follows a simpler, possibly two-state, mechanism (Fig. 4b), with a pre-transition baseline from 0-1.0
M urea, a sharp transition between 1.0-2.0 M urea, and an apparent post-transition baseline with
increasing urea concentration.

The urea-induced unfolding of the core protein glycoforms of biglycan and decorin was also
examined. Biglycan core protein (Fig. 4c) had a pre-transition between 0-1.5 M urea, a sharp
transition between 1.5-2.5 M, followed by a slowly increasing post-transition above 3.5 M urea.
The denaturation curve for decorin core protein was similar to both the biglycan core protein and
the decorin proteoglycan glycoforms, with a pre-transition region at 0-1.0 M, a transition from 1.0-
2.0 M urea, and a similar post-transitional baseline at higher urea concentrations (Fig. 3d).

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Reversibility of unfolding - Reversibility of unfolding is an important parameter when defining conformational stability. All four glycoforms were equilibrated in 10 M urea and then diluted to a urea concentration of 1.0 M urea, at a final concentration of 10 μM. This preparation was compared with glycoforms (10 μM) that had been equilibrated directly in 1 M urea or 10 M urea. The effect on secondary structure was examined by far-UV CD spectroscopy (Fig. 5). All of the refolding profiles (Fig. 5, triangles) demonstrate that none of the glycoforms were able to refold to their original conformation after exposure to 10 M. The biglycan proteoglycan in PBS (Fig. 2a, solid circles) and 1.0 M urea (Fig. 4a, solid circles) had similar CD profiles, with a minima of 215 nm. After exposure to 10 M urea and subsequent dilution to 1 M urea, the minima shifted to 213 nm and the curve was significantly broader (Fig. 4a, triangles). Decorin proteoglycan equilibrated in 1 M urea overnight had a similar spectra to native proteoglycan (Fig. 4c, closed circles). When the urea concentration was diluted back to 1 M urea, the secondary structure was reproducibly different to the CD spectra of the native proteoglycan, exhibiting a minima at 214 nm and a significantly broader curve (Fig. 4c, triangles). The biglycan core protein in PBS and in 1 M urea (Fig. 4b, squares) had a minima ~ 218 nm, which was shifted to 209 nm in the refolded spectra (Fig. 4b, triangles). The spectra for decorin core protein in 1.0 M urea (Fig. 4d, circles) had a minima at 216 nm, and the refolded protein (Fig. 4d, triangles) had a spectra with a minima at 214 nm. These spectra clearly demonstrate these refolded glycoforms do not appear to assume the same secondary structure as in the native state, under the conditions used. Indeed, it is possible that the refolded form has a molten globule-like structure that may regain a secondary structure but does not resemble the native folded protein (21).

In summary, comparison of the biophysical properties of biglycan and decorin indicates that these small leucine-rich repeat proteoglycans have different overall secondary structures, as assessed by circular dichroism spectroscopy. However, fluorescence spectroscopy indicates that the conserved tryptophan in the C-terminal di-sulfide bonded domain is in a similar environment for both biglycan
and decorin. A qualitative analysis of conformational stability revealed the possibility of multiple transitions during urea-induced unfolding of biglycan proteoglycan, in contrast biglycan core protein and the decorin glycoforms appear to follow a two-state unfolding mechanism. Glycosylation also had differential effects on the structure and stability of biglycan, but not decorin. The core protein form of biglycan is more stable than the proteoglycan, and they appear to assume different structures in solution. This is in contrast to the decorin core protein, which assumes a similar conformation independent of substitution with a glycosaminoglycan chain.

Glycosaminoglycans are long extended polymers of repeating disaccharide units. It is not unreasonable to speculate that these large polysaccharides can influence the structural conformation or stability of a protein. In this study, the chondroitin sulfate chains of biglycan may have a critical role in stabilizing the secondary structure of the whole protein. Significant differences in the CD spectra of the native biglycan proteoglycan and core protein provides evidence that the presence of glycosaminoglycan chains can alter structure. It is unclear how the glycosaminoglycan affects the secondary structure, but it should be noted that this is not a general role in proteoglycans as the glycosaminoglycan chains of decorin had no detectable effect on structure or conformational stability. This observation once again confirms that biglycan and decorin have different folded conformations. Furthermore, this study clearly indicates that there are inherent flaws in applying generalizations from the structure of one member of the LRR superfamily to other distantly related proteins. It further emphasizes the need for the structure of one or more members of the SLRP family to be solved, and this will impact significantly on the biology of these molecules with respect to their role in collagen fibrillogenesis (22,23), modulation of TGF-β activity (24-28), and interaction with cell surface receptors (29-31).
Acknowledgements

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REFERENCES

FOOTNOTES

1. **Abbreviations used:** SLRP, small leucine-rich repeat proteoglycans; LRR, leucine-rich repeat; GAG, glycosaminoglycan; PBS, phosphate buffered saline; CD, circular dichroism.

2. Hocking, A.M., Seo, N-S., and McQuillan, D.J., manuscript in preparation
**FIGURE LEGENDS**

**Figure 1.** Schematic representation of (a) decorin and (b) biglycan, indicating major domains, including: signal sequence (sig), propeptide (pro), glycosaminoglycan attachment (GAG), putative di-sulfide bonds (boxed S), ten leucine-rich repeats (numbered boxes), and N-linked oligosaccharide attachment sites.

**Figure 2.** Far-UV CD spectra of (a) biglycan proteoglycan, (b) biglycan core protein, (c) decorin proteoglycan, and (d) decorin core protein, in phosphate buffer (●) and after equilibration in 10 M urea (O). The vertical dashed line is in an identical position in all panels and is provided to assist in curve comparison.

**Figure 3.** Fluorescence emission spectra of recombinant glycoforms. (a) Amino acid sequence spanning the C-terminal di-sulfide bonded cysteine domain of decorin (upper sequence) and biglycan (lower sequence). Conserved residues are indicated by vertical lines, and the conserved tryptophan (W) is boxed. Fluorescence emission spectra are shown for (b) biglycan proteoglycan, (c) biglycan core protein, (d) decorin proteoglycan, and (e) decorin core protein, in phosphate buffer (●) and after equilibration in 10 M urea (O). The vertical dashed line is in an identical position in all panels and is provided to assist in curve comparison.

**Figure 4.** Urea induced unfolding of (a) biglycan proteoglycan, (b) biglycan core protein, (c) decorin proteoglycan, and (d) decorin core protein. Glycoproteins (10 μM) were equilibrated overnight in increasing concentrations of urea, and unfolding monitored by far-UV circular dichroism spectroscopy.
Figure 5. Refolding of (a) biglycan proteoglycan, (b) biglycan core protein, (c) decorin proteoglycan, and (d) decorin core protein, after urea denaturation. Far-UV CD spectra of glycoprotein (10 μM) equilibrated overnight in: 1 M urea (●); 10 M urea (✓); and 10 M urea for 6 h then diluted into 1 M urea and equilibrated for 12 h (▲).
Fig. 1 Krishnan et al.

GAG chain

1-10 leucine-rich repeats

N-linked oligosaccharide
Far UV CD-spectra

Fig. 2, Krishnan et al.
Fig. 3, Krishnan et al.

(a) C-terminal cysteine domain

\[
\begin{align*}
321 & \text{CPMGFGVKRAYNGISLFLNNPVPY} & \text{EVQPATFRC}^{354} \\
313 & \text{CPPGHTKASYSVLSLFSNPVQY} & \text{EIQPSTFRC}^{346}
\end{align*}
\]

Fluorescence emission spectra

(b) biglycan proteoglycan
- PBS
- 10 M urea

c) biglycan core protein

d) decorin proteoglycan

e) decorin core protein

Intensity (arbitrary units)

Wavelength (nm)
Protein unfolding with urea

Urea concentration (M)

CD signal (mdeg)

biglycan proteoglycan

decorin proteoglycan

biglycan core protein

decorin core protein

Fig. 4, Krishnan et al.
Refolding after urea denaturation

Fig. 5 Krishnan et al.