Comparisons of Antibody Reactivity and Enzyme Sensitivity between Small Proteoglycans from Bovine Tendon, Bone, and Cartilage*

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Preparations of small proteoglycans from bovine tendon, bone, and cartilage have been compared for sensitivity to various enzymes and reactivity with different polyclonal antibodies. Chondroitinase ABC digestion of all proteoglycans generated a core protein preparation that migrated similarly in sodium dodecyl sulfate-polyacrylamide electrophoresis as a doublet band with Mr ≈ 45,000. The small proteoglycans of cartilage were divided into two populations based upon electrophoretic migration of the intact molecules (Rosenberg, L. C., Choi, H. U., Tank, L. H., Johnson, T. L., Pal, S., Webber, C., Reiner, A., and Poole, A. R. (1985) J. Biol. Chem. 260, 6304–6313). The core preparations of tendon, bone, and the faster-migrating (PG II) proteoglycans of cartilage all interacted in Western blot/enzyme-linked immunosorbent assay analysis with polyclonal antibody raised against either the tendon or bone proteoglycans. The slower-migrating (PG I) proteoglycans of cartilage did not react with these antibodies. Digestion of the tendon small proteoglycan with Staphylococcus aureus V8 protease released glycosaminoglycan chains from the molecule and generated a 40-kDa protein fragment that was resistant to further rapid degradation by the enzyme. This large digestion fragment was also prominent following V8 protease digestion of the faster-migrating (PG II) population of small cartilage proteoglycans, but not the small proteoglycan of bone. The N-terminal amino acid sequence of the tendon (PG II) proteoglycan was determined. These observations provide additional evidence for heterogeneity among the chemically similar small proteoglycans from different tissues.

A population of small proteoglycans has been identified in a number of collagenous connective tissues including tendon, bone, skin, cartilage, aorta, cornea, and sclera (for review see Refs. 1 and 2). These proteoglycans generally have Mr ≈ 100,000, elute from Sepharose Cl-4B at Kav ≈ 0.5, and have a core protein that migrates in various gel systems with an apparent molecular weight of 40,000–50,000 after chondroitinase ABC digestion. In highly fibrous tissues bearing tensional loads these small proteoglycans are the predominant proteoglycan whereas in tissues bearing compressional loads, such as cartilage, they are a minor proportion of the total proteoglycan due to the high level of larger proteoglycans. For instance, small proteoglycans comprise 90% by weight of the proteoglycans found in the fibrous proximal portion of bovine tendon (3) but only 2% of the proteoglycan in articular cartilage (4, 5). These small proteoglycans are of particular interest because both biochemical (6, 7) and morphological (8, 9) evidence indicates that at least some of them are able to interact with collagen fibrils and thus may play specific and important roles in the structural organization and material properties of the tissue.

A recent comparison of small proteoglycans from several bovine tissues has indicated that although similar in terms of overall size, length of glycosaminoglycan chains, amino acid content, and apparent molecular weight of the core preparations after chondroitinase ABC digestion, there are nonetheless differences in structure that can be detected by comparison of trypsin-generated fragments and by inhibition ELISA immunological assays (10). Rosenberg et al. (5) recently demonstrated that the small proteoglycans of cartilage can be divided into two populations that share basic chemical characteristics but nonetheless do not share immunologic identity and can be separated by SDS-polyacrylamide gradient gel electrophoresis. By similar methods Fisher (11) separated two populations of small proteoglycans from bone and determined that they differ in amino acid composition.

The present study was undertaken to assess certain biochemical and immunological characteristics of small proteoglycan preparations that appeared similar by published accounts but had not actually been subjected to direct comparisons in one laboratory. These preparations were from bovine tendon (3), bone (2) and cartilage (5). The study confirms and extends observation of biochemical distinctions between these very similar molecules.

**MATERIALS AND METHODS**

Proteoglycan Preparations—Preparation of the small proteoglycan of tendon was as described by Vogel and Heinesgard (3). In summary, the proteoglycans were extracted from the fibrous proximal region of adult bovine tendon with 4 M guanidine hydrochloride and subjected to CsCl density-gradient centrifugation. The low-density fractions (density < 1.36 g/ml) were combined and the proteoglycans highly purified by ion-exchange chromatography in 7 M urea.

The small proteoglycan of bone was prepared as described by Fisher et al. (12). In summary, midshaft subperiosteal bone slices (1–2-mm deep from fetal bovine femurs and tibias were extracted with 4 M guanidine HCl, 0.5 M EDTA and highly purified by ion-exchange chromatography in 7 M urea. The smaller (PG I) population was separated from the larger (PG II) population by three successive passes through a Sepharose CL-6B column eluted with 4 M guanidine HCl.

* The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PTH, phenylthiouridactoin.

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The small proteoglycans of cartilage were prepared as described by Rosenberg et al. (5). In summary, the proteoglycans were extracted from adult bovine articular cartilage in 4 M guanidine HCl and subjected to associative CsCl density-gradient centrifugation (0.15 M sodium acetate). Fractions A2–4 (density 1.42–1.53 g/ml) were combined, fractionated by CsCl density gradient centrifugation in 4 M guanidine HCl, and the lower-density fractions (density < 1.45 g/ml) were further purified by ion-exchange chromatography in 6 M urea followed by Sepharose CL-4B chromatography in 4 M guanidine HCl. The small proteoglycans were dialyzed into 0.5 M sodium acetate and concentrated to 15 mg/ml. Different populations were then separated by Sepharose CL-4B chromatography in 0.5 M sodium acetate, PG II eluted with K0, = 0.28; PG II and a small amount of PG I eluted with K0, = 0.50.

Enzymatic Digestions—Chondroitinase ABC and Staphylococcus aureus protease, V8, were obtained from Miles Scientific. Chondroitinase ABC digestions were performed by combining 25 μg of proteoglycan in 25 μl of 0.1 M Tris acetate buffer, pH 7.3, with 0.01 unit of enzyme and incubating at 37 °C for 1 h. V8 protease digestions were performed by combining 25 μg of proteoglycan in 25 μl of 0.05 M sodium phosphate buffer, pH 8.0, with 0.05 μg of enzyme and incubating at 37 °C for times from 5 min up to 66 h. When both enzymes were used in sequence, the samples were dissolved in 0.05 M sodium phosphate buffer, pH 8.0. The digestions were stopped by addition of 25 μl of gel sample buffer containing 2-mercaptoethanol. The mixtures were then heated to 100 °C for 5 min.

Alkaline Cleavage—To remove glycosaminoglycan chains from the proteoglycans 80 μg of proteoglycan was incubated in 80 μl of 0.05 M NaOH, 1 M NaBH4 at 45 °C for 48 h. The mixture was neutralized by addition of 4 μl of glacial acetic acid, dialyzed against distilled water, and mixed with gel sample buffer for electrophoresis.

Electrophoresis and Western Blot Procedures—Linear 4–20% polyacrylamide gradient-sodium dodecyl sulfate gels with a 3% stacking gel were run in a Hoefer SE-600 apparatus as described by Fisher et al. (16) using the Laemmli buffer system with doubled concentrations of Tris and glycine in the electrode buffers. After overnight electrophoresis at 8 mA/gel, 4 °C, the separated components were transferred to nitrocellulose paper in a Bio-Rad Trans-blot apparatus by application of 100 V for 20 min in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol (13). Three copies of the gels were made, and the remaining gel was then stained with both Coomassie Blue and Alcian Blue, as described previously (12). Molecular weight standards were purchased from Pharmacia P-L Biochemicals.

Polyonal antisera against the small proteoglycan of adult tendon was raised in rabbits by subcutaneous injection of 0.5 mg of purified proteoglycan (the D12.B2.Cs2SO4 preparation described in Ref. 3) mixed with Freund's complete adjuvant, as described (10). The antigen was PG II. The reactivity of this antisera with the antigen was identical when either intact proteoglycan or chondroitinase ABC-treated proteoglycan was utilized in an inhibition ELISA assay, indicating that reactivity was against antigenic determinants on the core protein. No cross-reactivity of this antisera with large proteoglycans from tendon could be detected using this assay (10). The antisera against purified one-chain (PG II) proteoglycans of bone was raised in a similar manner and showed no interaction with the large proteoglycans of bone (12). In addition, antisera reactive with an A1D1 preparation of large cartilage proteoglycans showed no reaction with these small proteoglycans. Immunodetection of the transferred proteins was performed using peroxidase-conjugated anti-rabbit IgG second antibody (Cappel Labs, T1000) as described (14).

Amino Acid Sequence Analysis—A sample of purified small dermatan sulfate proteoglycan from adult bovine tendon (D12.B2.Cs2SO4, Ref. 3) was dissolved in 0.1 M acetic acid and applied to a BioBrene-coated glass filter of an Applied Biosystems, Inc. model 470A gas phase protein sequenator. The released anilinothiobenzoine-amino acids were converted to PTH-derivatives using the trifluoroacetic acid conversion program. The PTH-derivatives were analyzed on a Beckman 346 high pressure liquid chromatograph using the procedure described by Lazure et al. (15).

RESULTS

Different Populations of Small Proteoglycans

Small proteoglycans (M, ~100,000) can be separated from large proteoglycans (M, ~106) by a combination of procedures taking advantage of both the smaller hydrodynamic volume of the small proteoglycans and their generally lower buoyant density in CsCl gradients. When small proteoglycan preparations were run in a 4–20% gradient gel electrophoresis system it was possible to distinguish two distinct populations, one running in a broad band with apparent M, of about 100,000 and a second with apparent M, of about 200,000. Following the terminology suggested by Rosenberg et al. (5) in describing the dermatan sulfate proteoglycans of bovine articular cartilage, the slower-migrating population of small proteoglycans is called PG I and the more rapidly-migrating population PG II (Fig. 1). In every case digestion of these small proteoglycans by chondroitinase ABC produced a doublet core preparation migrating with a similar apparent M, of about 45,000 (Fig. 1). This core preparation was characterized by a major band and a slightly faster-migrating minor band.
**Immunoreactivity of the Small Proteoglycans**

Antiserum raised against PG II of tendon showed strong interaction with the core preparations resulting from chondroitinase ABC digestion of small proteoglycan populations from tendon, bone, and cartilage when PG II was present (Fig. 1). A similar result was seen using anti-bone PG II antiserum (data not shown). However, the core protein of isolated PG I from cartilage was not reactive with either of these antibodies. This indicates that the core protein of cartilage PG I is immunologically distinct from the core of PG II, even though similar in size. The immunoblot of chondroitinase ABC-digested proteoglycans shows that both the major and the minor PG II core protein bands reacted with the antibodies. It cannot be determined from this analysis whether the small amount of reaction product formed in the region of the PG I core preparation indicates a very low level of interaction between the PG I core protein and the antibody or the low level of PG II present in this preparation.

**Peptide Fragments of the Small Proteoglycans**

**Digestion with Chondroitinase ABC**

The immunoreactive fragments generated by unidentified proteases (17) present in chondroitinase ABC digests of small proteoglycans from tendon, bone, and cartilage are compared in Fig. 2. Seven fragments between M, 20,000 and 40,000 were resolved and appeared very similar for proteoglycans from the three different tissues. Although there were apparent tissue distinctions in terms of the intensity of certain bands, there was no clear case where an immunoreactive peptide fragment of the proteoglycan from one tissue was lacking in another. The patterns were similar when replicate blots were stained with either anti-tendon or anti-bone antiserum.

**Digestion with S. aureus V8 Protease**

**Tendon**—Digestion of the small proteoglycan of tendon with a low level (1:500, w/w) of the *S. aureus* protease, V8, produced a doublet core preparation having a major peptide fragment with M, = 40,000 and a minor band that migrated slightly faster. Both of these fragments reacted strongly with anti-tendon antiserum. By staining the gel with Alcian blue, it was possible to see the glycosaminoglycan chains as a broad band in the region from M, 25,000-80,000; migration of the glycosaminoglycans was independent from migration of the core protein fragment and clearly different from migration of the intact proteoglycan. Thus, the action of V8 protease resulted in a core preparation that was somewhat smaller but otherwise similar to the core preparation produced by removal of the glycosaminoglycan chains with chondroitinase ABC. These results are shown diagrammatically in Fig. 3.

The amount of 40-kDa fragment present in the digestion mixture was slightly diminished by 4 h of digestion, but substantial amounts of the fragment were still present even after 8 h of digestion. Immunoreactive fragments were also produced, and the smallest of these migrated in a clear band that was slightly above the position of the smallest fragments stained by Coomassie Blue (Fig. 4, arrows). Prior digestion of the proteoglycan with chondroitinase ABC had no effect upon the result of V8 digestion (Fig. 4, right side). These results indicate that the glycosaminoglycan chain of this small proteoglycan must be located on a terminal portion of the core protein. Furthermore, the major portion of the core protein that does not contain any glycosaminoglycan is highly resistant to V8 protease degradation.

**Cartilage**—V8 protease digestion of the cartilage preparation containing both PG I and PG II also resulted in a 40-kDa fragment of the core protein that was highly resistant to further V8 degradation (Fig. 5). The pattern of minor peptide fragments generated from cartilage proteoglycans by the V8 protease also appeared similar to that of the tendon proteoglycan preparation, and both of these were very similar to the smaller peptide fragments produced by chondroitinase ABC treatment of these proteoglycans, as shown in Fig. 2. It is clear from Fig. 5, however, that V8 digestion of the mixture of small proteoglycans from cartilage did not readily remove all glycosaminoglycan chains. After 4 h of treatment with V8 protease a proteoglycan band migrating intermediate to the initial positions of PG I and PG II was still present.

This was further investigated by 1 h of digestion of cartilage PG I alone. This treatment divided the initial population into two parts, the original proteoglycan and a new band migrating faster than the intact PG I proteoglycans yet significantly slower than intact PG II (Fig. 5, right lanes). In addition, a number of peptide fragments were produced. The 40-kDa fragment, if present, was not prominent, and none of these fragments interacted with the anti-tendon antibody. From these results we conclude that 1) the prominent 40,000 frag-

![Fig. 2. Immunostaining of the core preparation and fragments of small proteoglycans after treatment with chondroitinase ABC.](image1)

![Fig. 3. Representative drawings of intact, chondroitinase ABC, and S. aureus V8 protease-digested tendon PG II. Typical staining patterns are shown. Dotted areas are the turquoise-colored Alcian blue-staining regions of the intact proteoglycans (none) and free glycosaminoglycan chains (V8). The other bands represent proteins stained with Coomassie Blue.](image2)
Comparisons of Small Proteoglycans

Hrs of V8  V8 post ABC' 2 Q)

10.5 1 2 4 8 20 661

PG I-
PG II-
PG Core-

PG Core-

PG Core-

Anti-Proteoglycan

Alcian & Coomassie Blue

FIG. 4. Gradient gel electrophoresis (top) and the identical electroblot (bottom) of tendon small proteoglycan treated with S. aureus V8 protease without or following treatment with chondroitinase ABC. Tendon small proteoglycans (PG) were incubated with V8 protease and aliquots (25 µg) removed at the times indicated (lanes 1–8). Similar incubation was carried out after prior digestion with chondroitinase ABC (lanes 11–16). A nitrocellulose blot of the gel was developed using anti-tendon PG II antiserum (bottom); material remaining on the gel was stained with Alcian blue and Coomassie Blue (top). Molecular weight standards are shown in lane 9 (see Fig. 1; 14K, α-lactalbumin); samples of each enzyme are shown in lanes 17 and 18.

FIG. 5. Gradient gel electrophoresis of small proteoglycans from tendon, cartilage, and bone after digestion with V8 protease. Samples of small proteoglycan from tendon (PG II), cartilage (PG I + PG II), and bone (PG II) were digested with V8 protease for 0, 0.1, and 4 h. Cartilage PG I proteoglycans were digested with V8 protease for 0 or 1 h. A nitrocellulose blot of the gel was stained using anti-tendon PG II antiserum (bottom); material remaining on the gel was stained with Alcian blue and Coomassie Blue (top). See Fig. 1 for molecular weight standards.

ment generated by V8 treatment of the small cartilage proteoglycans came uniquely from PG II, and 2) V8 treatment of PG I gradually clips these proteoglycans into somewhat smaller glycosaminoglycan-containing fragments. Treatment of the PG I/PG II mixture with chondroitinase ABC first, followed by V8 protease, resulted in two Coomassie blue-stained core preparations with M, of 45,000 and 40,000 (data not shown). The immunoblot of this gel showed definite staining at the 40-kDa position but only a slight hint of staining at the position of the 45-kDa core. This result strengthens the conclusion that the core protein of only the PG II proteoglycan is clipped by V8 protease in a manner which removes the glycosaminoglycan and produces a 40-kDa fragment that is resistant to further V8 digestion.

A population of cartilage small proteoglycans prepared by Heinegard et al. (4) was also tested for interaction with the anti-tendon PG II antiserum and enzyme susceptibility. The chondroitinase ABC-digested core preparation showed poor cross-reaction with the anti-tendon antiserum by Western blot analysis, as had been previously reported by inhibition ELISA analysis (10). In addition, these proteoglycans migrated to a position high in a 4–20% gradient SDS-polyacrylamide gel, and no prominent 40-kDa fragment was produced by V8 protease treatment. These results are similar to results expected from a PG I-rich preparation.

Bone—Treatment of the PG II preparation of bone proteoglycan with V8 protease for 1 h did not produce a prominent 40-kDa fragment although migration of the bone proteoglycan in the gradient gel system was clearly altered (Fig. 5). The Alcian blue-stained material remained high in the gel, indicating that the glycosaminoglycan chain was still attached to some protein fragment. That the material remaining after V8 protease digestion of the bone PG II was not free glycosaminoglycan chains is shown in Fig. 6. The migration positions of glycosaminoglycan chains generated by alkaline cleavage of bone and tendon proteoglycans were essentially identical. However, the product resulting from V8 protease treatment of the bone proteoglycan remained significantly higher in the gel than the free chains. It has been previously shown by column chromatography that the glycosaminoglycan chains of the tendon and bone proteoglycans are of similar size (M, ≈ 40,000 (3, 11)).

When chondroitinase ABC treatment preceded V8 protease
digestion of this bone proteoglycan, neither the expected 45-kDa chondroitinase-generated core preparation nor a 40-kDa fragment were observed, suggesting that the bone proteoglycan core protein without attached glycosaminoglycan was at least partially degraded by V8 protease. Only when the duration of digestion of the intact proteoglycan by V8 protease was reduced to 0.1 h was it possible to detect a small amount of the 40-kDa fragment by immunoreaction (Fig. 5). This result was distinctly different from the effect of V8 protease on PG II of either tendon or cartilage. The fact that a 40-kDa fragment was observed only after brief digestion suggests that there may be two different populations of bone PG II. That is, 1) a small amount of tendon/cartilage-like proteoglycan that yields a 40,000 fragment from which glycosaminoglycan chains were released, and 2) a larger amount of proteoglycan that is degraded into a variety of large fragments to which the glycosaminoglycan is still attached.

If V8 protease treatment of the bone proteoglycan was producing a single large resistant core fragment to which glycosaminoglycan was still attached, this fragment should be released by subsequent digestion with chondroitinase ABC. Treatment of the bone proteoglycan with V8 followed by chondroitinase did not reveal any such distinct fragment (data not shown). A similar sequence of digestions on the tendon proteoglycan resulted in some 40-kDa fragments.

During preparation, the bone proteoglycan sample was briefly subjected to reducing conditions (11, 12) whereas none of the preparative procedures for the other proteoglycans included this step. It was considered possible that brief reduction could have altered the susceptibility of bone proteoglycan to V8 protease. This possibility was investigated by adding 1% 2-mercaptoethanol to 100 μg of the tendon proteoglycan in 7 M urea and then immediately starting dialysis against water. This reduction under denaturing conditions is similar to that seen by the bone proteoglycan during preparation. A control sample, to which reducing agent was not added, was dialyzed in a separate container. When digested either with chondroitinase ABC or V8 protease, the nonreduced tendon proteoglycan sample behaved as reported above, indicating that the dialysis steps had not affected its enzyme susceptibility. On the other hand, treatment of the briefly reduced tendon proteoglycan sample with V8 generated only a small amount of the 40,000 fragment, and numerous smaller peptide fragments were detected by immunostaining (data not shown). The glycosaminoglycans were shifted to a low position on the gel, comparable to that of free glycosaminoglycan chains. Although this result indicates that brief reduction made the tendon proteoglycan core protein more susceptible to V8 protease cleavage, it does not provide a complete explanation for the difference in the cleavage pattern seen between tendon and bone PG II proteoglycans. Unlike the reduced tendon PG II, the glycosaminoglycans of the reduced bone proteoglycan remained high in the gradient gel after V8 protease treatment, indicating that they were still attached to a large protein fragment. Resolution of this question requires the availability of bone proteoglycans isolated without use of reducing agents. Due to the extensive and random disulfide exchange in bone, this has not been possible (18).

N-Terminal Amino Acid Sequence of Tendon Proteoglycan

The small proteoglycan of tendon was submitted to automated Edman degradation. A unique sequence was derived which was as follows, H₂N-Asp-Glu-Ala-X-Gly-Ile-Gly-Pro-Glu-Glu-His-Ph-Pro-Glu. While more than 1.5 nmol of PTH-alanine in cycle 3 and of PTH-glycine in cycle 5 were released during the degradation, no PTH-amino acid could be identified in the 4th cycle at the 100-pmol level. This sequence for the tendon proteoglycan is identical to that determined for a small proteoglycan of bovine skin by Pearson et al. (19). These workers have demonstrated that the 4th residue of the skin proteoglycan is a serine residue which serves as a site of attachment for the dermatan sulfate chain (20). The fact that no residue could be identified at position 4 in our sequence suggests that this is also the case in our preparation, since glycosylated residues are known to yield blank cycles (21).

DISCUSSION

Preparations of small proteoglycans from three different bovine tissues, tendon, cartilage, and bone, have been compared in these studies. The proteoglycan preparations are similar to one another in that they all have been extracted from the tissue by dissociating solvents (4 M guanidine HCl) and highly purified by ion exchange chromatography. The intact proteoglycans from each preparation equilibrate in low-density fractions during CsCl density-gradient centrifugation and elute from Sepharose CL-4B at Kᵥ = 0.5 under dissociating conditions. Although published estimates of the molecular weight of the chondroitinase-digested core proteins from these proteoglycans ranged from 38,000 to 48,000 Da (3, 5, 12), simultaneous electrophoresis demonstrated that a doublet core protein preparation with similar apparent Mᵥ = 45,000 was generated from the small proteoglycans of each tissue. The differences noted initially must, therefore, have been due to differences in the gel systems and molecular weight markers being used in different laboratories. Each core protein is estimated to have only 1 rather long (Mᵥ = 35–40 kDa) glycosaminoglycan chain attached, but the composition of these glycosaminoglycan chains is different. The small proteoglycan of tendon is dermatan sulfate high in iduronic acid (70% (3)); the cartilage proteoglycan has lower idurionate content, about 35% (5), while the glycosaminoglycans of the bone proteoglycan are chondroitin sulfate (i.e. contain no
iduronate (12)). The preparations also differed in that adult tissue was utilized to prepare proteoglycans from tendon and cartilage whereas bone proteoglycans were extracted from fetal to neonatal tissue.

Two populations of small proteoglycans have now been observed in both bone and cartilage as defined by their migration on 4-20% gradient gels. Analytical considerations led Fisher (11) to conclude that the slower-migrating population of PG I from bone differed from PG II by containing two, rather than one, glycosaminoglycan chains. On the other hand, the two cartilage populations have been separated based upon distinctions in self-aggregation characteristics at high proteoglycan concentration. The small proteoglycans extracted from the fibrous portion of tendon were virtually all of the faster-migrating (PG II) type. It is of interest, however, that preliminary analysis of proteoglycans from the more distal fibrocartilaginous portion of tendon indicates that both slower- and faster-migrating populations of small proteoglycans may be present (22). In the present study we have confirmed the observation that PG I of cartilage does not cross-react with antibodies raised against PG II proteoglycans (11) and, in addition, that antibodies raised against the PG II proteoglycans of one tissue will recognize other PG II core proteins. Thus, we can say that the PG II proteoglycan populations from tendon, cartilage, and bone appear to be immunologically similar. Antiserum raised against PG I proteoglycans is not available, and PG I populations from tendon or bone have not yet been characterized by these methods. Thus, it should not be concluded that these populations will necessarily turn out to be similar to the PG I from cartilage. For reasons that remain unclear, several workers have found it difficult to raise antiserum against PG I from a variety of tissues and species.

In addition to demonstrating a doublet core preparation of similar apparent molecular weight, digestion with chondroitinase ABC also demonstrated that a similar pattern of immunologically determined peptide fragments could be generated from all of the PG II core proteins. Even though chondroitinase ABC was utilized at a low level (10 million units/25 μg of proteoglycan) the enzyme preparation obviously contained a small amount of unidentified protease activity. This rather fortuitous observation provided us with a unique epitope mapping technique. By this method the core proteins of immunoreactive small proteoglycans from tendon, cartilage, and bone were similar. The peptides generated by chondroitinase ABC digestion represent a small amount of degradation, detectable only through the extreme sensitivity of the ELISA technique. It should be noted that the major core preparation generated by this enzyme was the same size as that generated by using even lower amounts of enzyme (1 or 0.1 million units/25 μg of proteoglycan) or the same amount of enzyme for longer periods of time. This indicates that even in the presence of this contaminant protease activity, the major 45-kDa core preparation represents the undegraded protein.

Removal of both a small portion of the tendon small proteoglycan core protein and the glycosaminoglycan chain by V8 protease digestion indicates that the glycosaminoglycan of this proteoglycan must be linked to the core protein near one terminus. Pearson and co-workers (19, 20) have now determined that N-terminal residue 4 of the small proteoglycan of bovine skin is occupied by serine and is the site of glycosaminoglycan chain attachment. Since the N-terminal sequence of the tendon proteoglycan is identical to that of the skin proteoglycan we can conclude that the portion of core protein removed from the tendon proteoglycan by V8 protease digestion is located near the N-terminal end of the molecule. The S. aureus V8 protease is reported to cleave peptide bonds on the carboxyl-terminal side of aspartic acid and glutamic acid residues (23). Like other workers, however, we have observed a distinct hierarchy of susceptible cleavage sites. There would appear to be two potential sites for cleavage in the sequenced region of the tendon proteoglycan. It is also possible that the major sensitive site(s) has not yet been sequenced since the molecular weight of the core preparation was reduced by 3-5 kDa, a substantially greater reduction than would occur with removal of only 10 amino acids. From amino acid composition determinations we know that the core protein of the tendon small proteoglycan is rich in aspartic acid/asparagine and glutamic acid/glutamine (3). It is thus certain that the 40-kDa fragment that remains after V8 protease digestion is not resistant to further degradation due simply to lack of acidic amino acid sites.

These considerations are presented in the model shown in Fig. 7. We suggest that the tendon and cartilage PG II molecules possess a V8-sensitive site on the C-terminal side of the glycosaminoglycan chain that is rapidly cleaved. Additional sensitive sites within the remaining core protein are cleaved much more slowly. For the bone proteoglycan this rapid-cleavage site is blocked in a major proportion of the molecules. The N-terminal amino acid sequence has not yet been determined for the bone proteoglycan core protein so it is not possible to say whether the different cleavage pattern of bone proteoglycan is due to small differences in primary structure of the core proteins, perhaps leading to a different attachment site of the glycosaminoglycan chain. The structural basis for the distinctions we have noted here should be resolved once the gene structure for these proteins is determined. Although reduction does not change the electrophoretic mobility of the tendon core preparation produced by chondroitinase ABC digestion,2 reduction did render the molecule more susceptible to degradation by V8 protease. It is reasonable to suggest that the 40-kDa fragment's resistance to V8 degradation results from a tertiary structure that has made the potentially sensitive sites inaccessible to the enzyme.

Attempts to distinguish structural variations among the small proteoglycans from different tissues are of interest because of the possibility that these proteoglycans perform different functions in the tissue. The small proteoglycan of tendon has been shown to have a unique ability to inhibit

![Fig. 7. Diagrammatic representation of small proteoglycans (PG II) from tendon, cartilage, and bone. Left, the PG II small proteoglycans of tendon and cartilage possess an N-terminal site sensitive to rapid cleavage by S. aureus V8 protease (large arrow). Cleavage at this site removes the glycosaminoglycan from the molecule and leaves a large intact peptide fragment. The small arrows have been arbitrarily positioned to represent the presence of additional V8 protease-sensitive sites within this fragment; these are cleaved much more slowly. Right, for most of the PG II small proteoglycans of bone the site of rapid cleavage by V8 protease is blocked (solid circle). Thus, the glycosaminoglycan of the bone proteoglycan remains attached to the core protein or core fragments much longer. The possible importance of tertiary structure in this cleavage pattern is not included in this diagram.](image-url)

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2 L. W. Fisher and A. R. Poole, personal communication.
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Acknowledgments—We would like to express our appreciation to Dr. Larry Rosenberg, Montefiore Medical Center, Bronx, NY, for giving us his preparations of small proteoglycans from cartilage and tendon for the studies of fibrillogenesis (4) were not like the PG I1 proteoglycans used in our fibrillogenesis investigation were more like the PG I cartilage proteoglycans utilized in this study. That is, the cartilage small proteoglycans used in our fibrillogenesis investigation were more like the PG I cartilage proteoglycans analyzed in the present study. In addition, the V8 protease sensitivity described in this report suggests that there may be additional subtle differences between the core protein of bone and tendon PG II small proteoglycans that have not been detected by polyclonal antibodies or trypsin fragmentation (10). Only a small percentage of the PG II proteoglycans from bone demonstrated rapid sensitivity to this enzyme, suggesting that different tissues may not only have different proportions of PG I and PG II small proteoglycans, but also different proportions of distinct types of PG II. It remains to be determined whether specific sensitivity to the V8 protease, resulting in a resistant 40-kDa fragment, will correlate with the ability of other small proteoglycans to interact with collagen.

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fibrilogenesis of collagen in an in vitro assay (6). Small proteoglycan preparations from cartilage and aorta did not show this inhibitory capacity, and it now appears that the core protein of the small cartilage proteoglycans utilized in the studies of fibrillogenesis (4) were not like the PG II molecules utilized in this study. It is remains to be determined whether specific sensitivity to the V8 protease, resulting in a resistant 40-kDa fragment, will correlate with the ability of other small proteoglycans to interact with collagen.