Isolation and Characterization of a Low Molecular Weight Chondroitin Sulfate Proteoglycan from Rabbit Skeletal Muscle†

Narayanan Parthasarathy‡ and Marvin L. Tanzer*,§

Departments of Orthopaedic Surgery, Biochemistry, and BioStructure and Function, University of Connecticut Health Center, Farmington, Connecticut 06032

Received August 13, 1986; Revised Manuscript Received December 26, 1986

ABSTRACT: Proteoglycans may be implicated in the process of aggregation of acetylcholine receptors in the basal lamina of skeletal muscle and possibly in the mechanism of reinnervation at the neuromuscular junction. In order to further deduce the role of such proteoglycans, we have sought to isolate them and define their molecular structures. In this study, proteoglycans were extracted from rabbit skeletal muscle by using 4 M guanidine hydrochloride and were purified by sequential cesium chloride density gradient ultracentrifugation, DEAE-cellulose ion-exchange chromatography, and Sepharose CL-6B and CL-2B gel filtration under dissociative conditions. A chondroitin sulfate proteoglycan which constituted about 44% of the total hexuronic acid content of the muscle tissue was isolated. This proteoglycan was found to have an apparent molecular weight [by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)] of 95 000, consistent with its small hydrodynamic size (K_{av} = 0.8 on Sepharose CL-2B), and to consist of peptide and glycosaminoglycan in a weight ratio of 1.0/0.8. The average molecular weight of its core protein–oligosaccharide remnants is 50 000, as estimated by SDS–PAGE of the chondroitinase ABC digested proteoglycan. Alkaline NaB^3H_4 treatment of the intact proteoglycan released chondroitin sulfate chains with an average molecular weight of 21 000. Pronase digestion of the intact proteoglycan generated glycosaminoglycan–peptides with an average of two chondroitin sulfate chains per peptide. These two saccharide units account for the total glycosaminoglycans per molecule and appear to be closely spaced on the core protein. This muscle proteoglycan resembles other "small proteoglycans" recently isolated from noncartilagenous tissues in that it contains a small core protein and has relatively few glycosaminoglycan substituents. It clearly differs in size and structure from the large proteoglycans of cartilage and of embryonic muscle; it may be the major proteoglycan of adult skeletal muscle.

The extracellular matrix which surrounds skeletal muscle fibers is of considerable physiological interest, since its structural components may provide some clues to understanding the mechanisms of communication among cells and between cells and their environment. Moreover, a specialized form of this matrix, the basal lamina, by virtue of its anatomical location as a physiological and functional barrier at the neuromuscular junction, has assumed additional importance by being implicated in nerve–muscle interaction and in muscle regeneration (Sanes et al., 1978). It is generally recognized that muscle development and indeed muscle function are determined to a large extent by the molecules that constitute the extracellular matrix (Sanes, 1983), although very little is known about their molecular composition and structure. The isolation and characterization of extracellular matrix components are, therefore, one prerequisite for ascertaining their functional roles. In the present study, we have sought to isolate proteoglycans associated with the extracellular matrix of adult muscle and to define their molecular structures.

Proteoglycans are ubiquitous components of the extracellular matrix of all mammalian tissues (Heinegard & Paulson, 1984), but characterization of these glycoconjugates in muscle tissue has been limited. Chiquet and Fambrough (1984) had identified an antigen which is noncovalently linked to a chondroitin sulfate proteoglycan in muscle. In other studies, a basal lamina heparan sulfate proteoglycan was localized to the surface of skeletal muscle fibers, and it has been found to cause aggregation of acetylcholine receptors at the neuromuscular junction (Anderson & Fambrough, 1983). Recent studies by Carrino and Caplan (1984) demonstrated the presence of at least three populations of proteoglycans synthesized by skeletal muscle; one is a small proteoglycan similar to the muscle fibroblast proteoglycan, and the other two are large molecules, consisting of chondroitin sulfates which differ in their carbohydrate chain lengths. These studies have been carried out by using embryonic tissue as well as cultured cells and have depended to a great extent on metabolic radiolabeling of the newly synthesized molecules. No reports are available on direct chemical characterization of proteoglycans in mature muscle. Therefore, in the present study, proteoglycans of adult rabbit muscle have been isolated and characterized. In this tissue, a low molecular weight chondroitin sulfate proteoglycan has turned out to be the predominant proteoglycan.

Materials and Methods

Materials. Frozen rabbit muscle was purchased from Pelfreeze. Collagenase and Pronase were obtained from Sigma and Calbiochem, respectively. Ultrapure guanidine hydrochloride and urea were purchased from Schwarz/Mann; cesium chloride (CsCl) was from Var Lac Oid Chemical Co.; DE 52 (microgranular) cellulose was from Whatman; Sephadex G-50, Sepharose CL-2B, and Sepharose CL-6B were from Pharmacia Fine Chemicals. Chondroitinase ABC,

† This work was partially supported by research and training grants from the National Institutes of Health.

* Address correspondence to this author at the Department of BioStructure and Function, University of Connecticut Health Center.

‡ Department of Orthopaedic Surgery.

§ Departments of Biochemistry, BioStructure and Function, and Orthopaedic Surgery.

0006-2960/87/0426-3149$01.50/0

chondroitinase AC, and chondroitin sulfates were from Miles Laboratories; Bio-Gel P-2 and Bio-Gel P-30 were from Bio-Rad Laboratories.

Extraction of Proteoglycans from Skeletal Muscle. All the operations were carried out at 4 °C. Frozen muscle tissue (400 g) was minced finely with scissors, thawed directly into 1200 mL of 4 M guanidine hydrochloride containing 0.01 M sodium acetate, pH 6.0, 0.1 M 6-aminohexanoic acid, 0.01 M disodium ethylenediaminetetraacetate (Na_2EDTA), 1 mM benzamidine hydrochloride, and 1 mM phenylmethanesulfonyl fluoride, briefly homogenized in a cold Waring blender for 5–10 s, and extracted by stirring at 4 °C for 72 h (Hascall & Kimura, 1982). An insoluble residue was separated from the extract by centrifugation at 4 °C in a Sorvall RC 5B centrifuge using an SS-24 rotor for 30 min at 20000 rpm.

Purification of Proteoglycans by Density Gradient Centrifugation and DEAE-cellulose Chromatography. Direct dissociative CsCl density gradient ultracentrifugation of the guanidine extract was employed to separate proteoglycans from other proteins of the muscle. The density of the clarified guanidine extract was adjusted with CsCl to 1.30 g/mL, and monomer fractions (D1) were prepared directly by equilibrium density gradient centrifugation in a Beckman ultracentrifuge employing a VTi 50 rotor (45000 rpm, 44–48 h, 10 °C). The gradient fractions were collected after the tubes were punctured at the bottom. The densities in grams per milliliter for each fraction ranged from 1.40–1.45 for D1, 1.31–1.35 for D2, 1.26–1.30 for D3, and 1.20–1.23 for D4. A jellylike fraction, which occupied a substantial volume of each tube after centrifugation, was also recovered; it was probably the major proteins of muscle. The gradient fractions from different centrifugations, D1–D4, were pooled separately and stored at −20 °C until further purification by passage through DEAE-cellulose.

The density gradient fractions were extensively dialyzed against several changes of 7 M urea/0.02 M tris(hydroxymethyl)aminomethane–acetate (Tris–acetate) buffer, pH 7.0, and placed at 4 °C on columns (10-mL bed volume) of DEAE-cellulose (DE-52) equilibrated with the same buffer. After application of the sample (100–120 mg of protein), an additional volume (50 mL) of the equilibrating buffer was passed through the column. Elution of the proteoglycans was achieved with 25 mL of equilibrating buffer containing 2 M NaCl. After dialysis, aliquots of this fraction were treated with 85% (v/v) ethanol to precipitate proteoglycans and subsequently assayed for hexuronic acids (Dische, 1947) and hexoses (Spiro, 1966) to evaluate the proteoglycan content. The bottom density fractions of the gradient, D1 and D2, were analyzed directly for proteoglycans before column fractionation on DEAE-cellulose.

The DEAE-cellulose-fractionated proteoglycans derived from the D1 fraction were submitted to another DEAE-cellulose chromatography step in which 60 mL of a linear concentration gradient ranging from 0 to 1.0 M NaCl in 8 M urea/0.02 M Tris–acetate, pH 7.0, was employed on a column (2-mL bed volume). Typically, the proteoglycan sample (5 μmol of hexuronic acid) was initially dialyzed against the equilibrating buffer, 8 M urea/0.02 M Tris–acetate, pH 7.0, and after application of the sample, the column was washed with 20 mL of equilibrating buffer before the start of the gradient. A flow rate of 10 mL/h was maintained, and 1-mL fractions were collected; dried aliquots from each fraction were analyzed for hexuronic acids (Dische, 1947) by the carbazole reaction after desalting by repeated extractions with 85% (v/v) ethanol.

Gel Filtration of Proteoglycans. The proteoglycan fraction obtained by DEAE-cellulose chromatography was filtered on a Sepharose CL-6B column (1 × 110 cm) equilibrated and eluted with 4 M guanidine hydrochloride/0.05 M Tris–acetate, pH 7.0. A flow rate of 4 mL/h was maintained, and 2-mL fractions were collected. To facilitate monitoring of this fractionation, an aliquot of the sample was previously labeled by reductive methylation with [^{14}C]formaldehyde (see below) and combined with the nonradioactive material in a ratio of 1:24 (w/w) before application to the column.

Further purification of proteoglycan fractions obtained by Sepharose CL-6B gel filtration was accomplished by Sepharose CL-2B chromatography. After application of the sample, the column (1 × 110 cm) was eluted with the equilibrating buffer, 4 M guanidine hydrochloride/0.05 M Tris–acetate, pH 7.0. Aliquots of column fractions were analyzed for radioactivity.

Radiolabeling of Proteoglycans. Labeling of the proteoglycans (167 μg of protein) with ^{14}C was achieved by reductive alkylation (Jentoft & Dearborn, 1979) with [^{14}C]formaldehyde (54 mCi/mmol, New England Nuclear) as described by Okada and Spiro (1980). The specific activity of the proteoglycans was 9.7×10^7 cpm/mg of protein.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in sodium dodecyl sulfate (SDS) according to the procedure of Dreyfuss et al. (1984) on vertical polyacrylamide slab gels (1.5 mm thick). The separating gel, which consisted of a linear 5–10% acrylamide gradient, was overlaid by a 4% stacking gel. Prior to being loaded on the gel, the samples were heated for 5 min at 100 °C in 0.1 M Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Staining was accomplished with Alcian Blue (Seno et al., 1970). Gels with radioactive samples were submitted to fluorography after being soaked in ENHANCE (New England Nuclear).

Chondroitinase ABC and Chondroitinase AC Digestion of Proteoglycans. Purified ^{14}C-proteoglycans (11300 cpm) and 100 μg of chondroitin sulfate A as carrier were incubated at 37 °C with chondroitinase ABC in 0.1 M Tris–acetate/0.1 M sodium acetate, pH 7.3 (1.5 units of enzyme/mg of sample), containing protease inhibitors (Oike et al., 1980; Yanagishita & Hascall, 1983). After a 5-h digestion, 200 μL of water was added, the sample was heated for 2 min at 100 °C, cooled and lyophilized. The lyophilized material was dissolved in SDS buffer and electrophoresed as described above.

In the case of ^{3}H-labeled chains released by alkaline NaB^3H_4 treatment of the purified proteoglycan, samples (500 cpm) were treated with chondroitinase ABC or chondroitinase AC in a similar manner except after treatment and lyophilization the material was dissolved in 200 μL of 0.1 M pyridine formate, pH 5.0, prior to filtration on Sephadex G-50 columns (Vogel & Heinegard, 1985).

Preparation of Glycosaminoglycan–Peptides from Proteoglycans. Purified radiolabeled proteoglycan was mixed with 1 mg each of serum albumin and chondroitin sulfate as carriers and digested with Pronase in 0.1 M Tris–acetate, pH 8.0, containing 0.005 M calcium acetate at 37 °C for 72 h in an atmosphere of toluene. The enzyme was added initially in an amount equal to 1.0% (w/w) of the sample followed by further additions of 0.5% at 24 and 48 h. The released glycosaminoglycan–peptides were purified by fractionation on a DEAE-cellulose column (1 × 4 cm) in pyridine formate buffers (Parthasarathy & Spiro, 1982). The glycosaminoglycan–peptides, which were eluted with 2.5 M pyridine formate, pH 5.0, were further chromatographed on a column (1 × 110 cm) of Sepharose CL-6B in 4 M guanidine hydrochloride as before.

For comparative purposes, the original ^{14}C-labeled proteoglycan was also subjected to gel filtration under similar conditions.

Alkaline Borohydride Treatment of Proteoglycan and Fractionation of Released Saccharide Chains. A sample of purified proteoglycan (13.5 nmol of hexuronic acids) was incubated in 100 μL of 0.1 M NaOH containing 0.3 M NaB^3H_4 (Amersham, 0.7 mCi) (nominal specific activity 25.5 μCi/μmol) at 37 °C for 72 h (Parthasarathy & Spiro, 1984). After titration to pH 5.0 with acetic acid, the boric acid was volatilized as methyl borate in a flash evaporator by several additions of methanol. The sample was then dissolved in 0.1 M pyridine formate, pH 5.0, containing 20 μg/mL chondroitin sulfate A as a carrier and gel filtered (Parthasarathy & Spiro, 1984) on a column (1.8 × 47 cm) of Bio-Gel P-2 (200–400 mesh) equilibrated with this buffer. Fractions of 1.5 mL were collected at a flow rate of 13 mL/h, and an aliquot of each fraction was counted for radioactivity. Appropriate fractions were pooled on the basis of their radioactivity, and the buffer was removed by lyophilization. The released NaB^3H_4-reduced glycosaminoglycan chains which emerged in the excluded volume of the column were further purified by fractionation on a DEAE-cellulose column (1 × 4 cm) in pyridine formate buffers, pH 5.0, as described previously (Parthasarathy & Spiro, 1982). The glycosaminoglycan fraction, which was eluted with 2.5 M pyridine formate, pH 5.0, was further separated by gel filtration on a column (1 × 112 cm) of Bio-Gel P-30 (100–200 mesh) equilibrated with 0.1 M pyridine formate, pH 5.0, containing 20 μg/mL chondroitin sulfate as the carrier, at a flow rate of 12 mL/h. Bio-Gel P-30 gel filtration was also carried out in 4 M guanidine hydrochloride/0.05 M Tris–acetate buffer, pH 7.0.

Identification of the Nature of ^3H-Oligosaccharide Chains Released through Alkaline NaB^3H_4 Treatment. The NaB^3H_4-reduced oligosaccharides which were recovered in the included volume of the P-2 column were acid hydrolyzed (1 N HCl, 5 h, 100 °C), and the released sugar alcohols were separated into neutral and amino sugar fractions on Dowex 50 and Dowex 1 columns (Spiro, 1966); the hexosamines were submitted to N-acetylation with acetic anhydride (Spiro, 1967) prior to chromatographic separation of sugars by thin-layer chromatography on cellulose-coated plates.

Nitrous Acid Treatment of NaB^3H_4-Reduced Glycosaminoglycan Chains. Purified radiolabeled glycosaminoglycan (500 cpm) obtained after Bio-Gel P-30 chromatography was treated with 150 μL of nitrous acid reagent (Lagunoff & Warren, 1962) for 80 min at room temperature. The sample was then submitted to Sephadex G-50 chromatography in 0.1 M pyridine formate, pH 5.0, to assess the extent of degradation.

Thin-Layer Chromatography. For the resolution of sugar alcohols, thin-layer chromatography was performed for about 8 h on plastic sheets coated with cellulose (0.1-mm thickness; Merck), with a wick of Whatman 3 paper clamped to the top of the plate, in a solvent system consisting of nitromethane/acetic acid/ethanol/water (8:1:1:1) saturated with boric acid (Robyt, 1975). Components were detected by fluorography after being sprayed with ENHANCE (New England Nuclear).

Analytical Procedures. A microadaptation of the carbazole reaction (Dische, 1947) was employed for the determination of hexuronic acids, and values were expressed as glucuronic acid after correction for the contribution of neutral hexoses. For analysis of the total hexuronic acid content of the muscle tissue, glycosaminoglycans were released from the delipidated tissue by digestion with collagenase (0.1 M Tris–acetate buffer, pH 7.4, 37 °C, 24 h, 1% w/w enzyme) followed by adjustment of the pH of the digest to 8.0 with 1 M Tris, further treatment with Pronase as described before, precipitation with 5% (w/v) trichloroacetic acid, removal of acid by extensive dialysis, and determination of hexuronic acid in the dialyzed material after concentration through lyophilization.

Table I: Purification of Proteoglycan from Rabbit Skeletal Muscle by Cesium Chloride Density Gradient Centrifugation and DEAE-cellulose Fractionation

fraction	density (g/mL)	hexuronic acid[a] (μmol/100 g of wet tissue)[b]
D1 (bottom)	1.40–1.45	6.52
D2	1.31–1.35	1.85
D3	1.26–1.30	0.40[c]
D4	1.20–1.23	2.64[c]
D4 gel		ND[d]

[a] Determined by the carbozole reaction and expressed as glucuronic acid after correction for the contribution of neutral hexoses; analyses in duplicate were carried out on the proteoglycans precipitated by 85% (v/v) ethanol. [b] Total hexuronate content of the wet tissue was 11.2 μmol/100 g tissue weight. [c] Analyses were carried out on the proteoglycan fraction (7 M urea/2 M NaCl eluate) obtained by DEAE-cellulose column (bed volume, 10 mL) fractionation of the density gradient fraction. [d] ND, not determined.

Amino acid analyses were carried out on the System 6300 high-performance amino acid analyzer (Beckman) after hydrolysis of the samples (5 μg of protein) in sealed tubes under nitrogen with constant-boiling HCl for 24 h at 110 °C. Protein content was estimated by the method of Lowry et al (1951). DNA content was estimated by a microadaptation of the colorimetric diphenylamine assay (Leyva & Kelly, 1974).

Results

Isolation of Proteoglycan from Rabbit Muscle. Extraction of adult rabbit muscle with a buffer containing 4 M guanidine hydrochloride resulted in the almost complete solubilization (as discussed below) of the total hexuronic acid containing material of the tissue. The solubilized material was partitioned on a CsCl equilibrium density gradient in which a much lower starting density of 1.3 g/mL was employed instead of the conventional density of 1.5 g/mL (Carrino & Caplan, 1984) in order to avoid the possibility of low-density proteoglycans being recovered in or near the top of the gradient. Carbazole analyses performed on the bottom density gradient fractions, D1 and D2, yielded the characteristic purple color seen in hexuronic acid standards, indicating the presence of glycosaminoglycan-containing material. These fractions as well as the top fractions, D3 and D4, after dialysis against 7 M urea/0.02 M Tris–acetate, pH 7.0, were separately fractionated on DE-52 cellulose columns, and the proteoglycans were eluted with a buffer containing 2 M NaCl. Hexuronic acid measurements (Table I) indicated that the D1 fraction of the density gradient was rich in proteoglycans; they constituted 58% of the total hexuronate of the muscle tissue. Despite purification by DE-52 column fractionation, the materials present in D3 and D4 did not give a characteristic purple color during this carbazole reaction, and therefore, their hexuronic values presented in Table I might be overestimated. On the basis of the sum of the hexuronate contents of the D1 and D2 fractions, the total hexuronate which could be extracted from muscle tissue in the form of proteoglycans by 4 M guanidine hydrochloride is about 75%.

When the proteoglycan derived from the D1 fraction was further purified by DEAE-cellulose chromatography, all of the hexuronic acid containing material was bound to the column and was eluted as a peak (peak A) at a sodium chloride

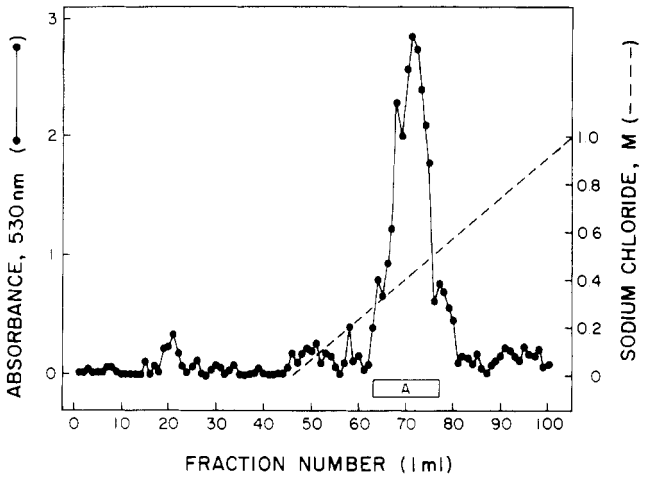

FIGURE 1: Gradient chromatography on DEAE-cellulose of rabbit muscle proteoglycan. The proteoglycan sample (5 μmol of hexuronic acid) from rabbit muscle obtained after cesium chloride density gradient centrifugation followed by DEAE-cellulose column fractionation was dialyzed against 8 M urea/0.02 M Tris–acetate buffer, pH 7.0, before being placed on a column (2-mL bed volume) of DE-52 equilibrated with the same buffer. After a wash with the equilibrating solution, a linear NaCl concentration gradient (0–1.0 M) was started at tube 45 as described under Materials and Methods. A flow rate of 10 mL/h was maintained, and aliquots of each fraction were analyzed for hexuronic acid by the carbazole reaction. Lettered areas designate fractions which were combined for further study. The recovery of hexuronic acid from the column was 91%.

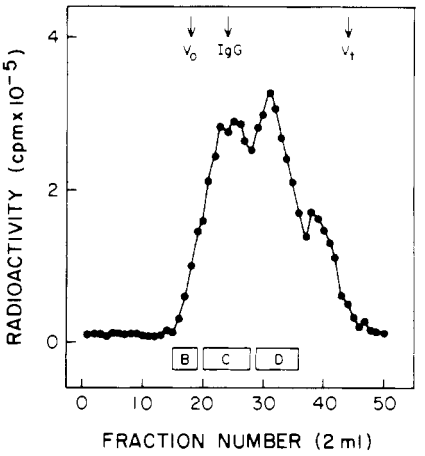

FIGURE 2: Filtration on Sepharose CL-6B of the muscle proteoglycan obtained by DEAE-cellulose chromatography. After ^{14}C labeling by reductive alkylation of an aliquot of peak A (Figure 1), it (5.8×10^7 cpm) was combined in a ratio of 1 to 24 with nonradioactive material from this proteoglycan fraction and applied to the column (1 × 110 cm), which was equilibrated and eluted with 4 M guanidine hydrochloride/0.05 M Tris–acetate, pH 7.0, at a flow rate of 4 mL/h; an aliquot from each fraction was analyzed for radioactivity. The void volume (V_0) and the total volume (V_t) of the column as well as the elution position of standard IgG (M_r 150000) are indicated by arrows. Lettered areas designate fractions which were pooled for further study. Recovery of radioactivity from the column was complete.

concentration of 0.44 M (Figure 1). Upon Sepharose CL-6B chromatography (Figure 2) of this proteoglycan (which was mixed with its unlabeled form), three radioactive fractions, peak B, peak C, and peak D, were obtained; they were present in a ratio of 0.07:0.48:0.45, respectively. Compositional analysis of peak D indicated the presence of protein but not hexuronic acid or DNA and it is believed to represent some non-proteoglycan protein labeled with [^{14}C]formaldehyde. The small peak eluted after peak D was devoid of any hexuronic acid containing material. Filtration of the major proteoglycan (peak C) on Sepharose CL-2B in 4 M guanidine hydrochloride yielded a single radioactive peak (Figure 3, top panel) eluting

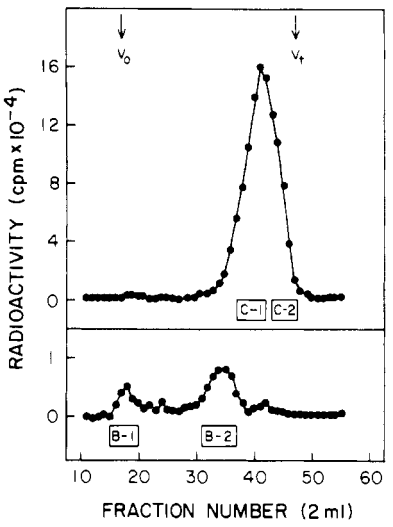

FIGURE 3: Filtration on Sepharose CL-2B of muscle proteoglycan fractions obtained by Sepharose CL-6B chromatography. (Top panel) A concentrated aliquot (7.5×10^5 cpm) of peak C (Figure 2) was applied to the column (1 × 110 cm), which was equilibrated and eluted with 4 M guanidine hydrochloride/0.05 M Tris–acetate, pH 7.0, at a flow rate of 2 mL/h; an aliquot from each fraction was counted for radioactivity. The void volume (V_0) and the total volume (V_t) of the column are indicated by arrows. Lettered areas designate fractions which were combined for further study. Recovery of radioactivity from the column was complete. (Bottom panel) A concentrated aliquot (1×10^5 cpm) from peak B (Figure 2) was applied to the column under the same conditions as described for the top panel. Recovery of radioactivity from the column was 84%.

at a K_{av} of 0.8. The fractions comprising this peak were pooled as peak C-1 and peak C-2 separately. The distribution of radioactivity between peaks C-1 and C-2, which was 67% and 33%, respectively, paralleled their hexuronic acid contents (75% and 25%). Polyacrylamide gel electrophoresis in SDS of the material in the C-2 fraction indicated the presence of some contaminating proteins (diagram not shown), and therefore, it was not characterized further. A similar finding has been reported (Vogel & Heinegard, 1985) during the purification of small molecular weight proteoglycans from adult bovine tendon.

For comparative purposes, the small leading shoulder (peak B, Figure 2) of the major proteoglycan C-1 was also gel filtered on Sepharose CL-2B (Figure 3, bottom panel). About 33% of the radiolabeled material was excluded from the column (peak B-1, Figure 3, bottom panel) while 67% eluted as a peak of K_{av} 0.60. The excluded material was found to contain mostly DNA (DNA/protein ratio = 4.0). Hexuronic acid distribution between the major (sum of C-1 and C-2) and minor (B-2) proteoglycans was found to be in the ratio of 6 to 1. Subsequent studies were confined to the purified major (peak C-1, Figure 3) proteoglycan fraction.

Electrophoresis of Proteoglycan. SDS–polyacrylamide gel electrophoresis of the proteoglycan obtained from Sepharose CL-2B filtration revealed one major radioactive broad band, which migrated to a position consistent with an average molecular weight of 95000 (range 93000–100000) (Figure 4A); the electrophoretic pattern was similar when the gel was stained with Alcian Blue (diagram not shown). A sample of this radiolabeled proteoglycan was analyzed by polyacrylamide gel electrophoresis after digestion with chondroitinase ABC (Figure 4B). The results showed that the proteoglycan changed mobility upon chondroitinase ABC digestion to a band migrating to a position of apparent M_r 50000. We observed a significant decrease in radioactivity following treatment of the proteoglycan with chondroitinase ABC (compare the peak

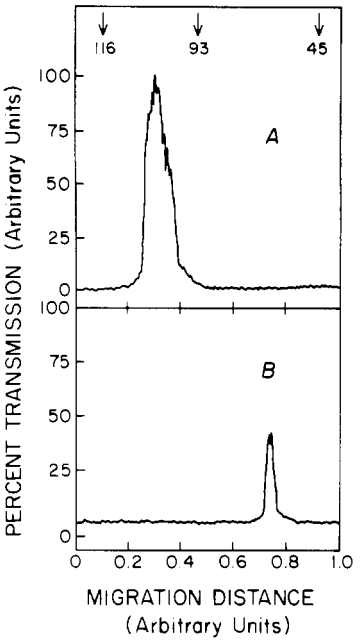

FIGURE 4: Soft laser densitometric scans of polyacrylamide gels after electrophoresis in SDS of muscle proteoglycan (A) and its core protein (B) obtained after digestion with chondroitinase ABC. (A) The purified proteoglycan (peak C-1, Figure 3) (11 300 cpm) was applied to the gel, and electrophoresis was carried out in SDS under the conditions described under Materials and Methods. The purified proteoglycan (11 300 cpm) was applied to the gel after treatment with chondroitinase ABC. The components were visualized by fluorography. The designated molecular weight markers were β-galactosidase (116 000), phosphorylase *b* (93 000), and ovalbumin (45 000). The relative scale of transmission is different between (A) and (B).

in Figure 4B with that in Figure 4A). Since it has been shown that, during reductive methylation, N^{α}-acetyllysine is readily labeled with [^{14}C]formaldehyde (Jentoft & Dearborn, 1979), we reasoned that the *N*-acetylhexosamine residues of proteoglycans may also be susceptible to this reaction. In control studies, using as substrate the glycosaminoglycan chains obtained from β-elimination reaction of a chondrosarcoma chondroitin sulfate polypeptide (a generous gift of Dr. V. C. Hascall, NIH), we found that these chains yielded a specific activity of 1.22×10^6 cpm/μmol of hexuronic acid after reductive methylation with [^{14}C]formaldehyde. Thus, the decrease in radioactivity, comparing panels A and B of Figure 4, could be attributed to enzymatic degradation of the labeled carbohydrate chains. It is unlikely that such digestion products would be detected in the electrophoretic gel.

Chemical Characteristics of Proteoglycan. Composition of the purified proteoglycan indicated (Table II) that peptide and glycosaminoglycan were present in a weight ratio of 1.0/0.8, indicating about half of the proteoglycan weight is contributed by chondroitin sulfate glycosaminoglycan; the proteoglycan was judged to be pure, devoid of any DNA contaminant. The amino acid composition of the muscle proteoglycan was comparable to a certain extent of that of small proteoglycans derived from cartilage, aorta, tendon, sclera, and bone (Heinegard et al., 1985). The muscle proteoglycan was notable for its higher content of glycine and lower contents of aspartic/glutamic acids and leucine, the key amino acids characteristic of small proteoglycans as reported by Heinegard et al. (1985).

Analysis of Glycosaminoglycans. The intact proteoglycan was treated with alkaline NaB^3H_4 in order to release, by β-elimination, single glycosaminoglycan chains and any other O-glycosidically linked carbohydrate units. The treated sample was chromatographed on a Bio-Gel P-2 column; filtration of

Table II: Composition of Proteoglycan from Rabbit Muscle

component	μmol/100 mg[a] peptide weight	residues/500 amino acid residue
aspartic acid[b]	83	50
threonine	35	21
serine	82	49
glutamic acid	97	58
proline	49	29
glycine	156	94
alanine	55	33
half-cystine	3	2
valine	36	22
methionine	9	5
isoleucine	28	17
leucine	67	40
tyrosine	22	13
phenylalanine	18	11
histidine	23	14
lysine	39	23
arginine	28	17
hexuronic acid[c]	168	100
glycosaminoglycan[d]	83[f]	
DNA	*e*	

[a] Average of two determinations. [b] Hydroxyproline and hydroxylysine were absent. [c] Determined by the carbazole reaction and expressed as glucuronic acid. [d] The glycosaminoglycan content was calculated on the basis of a repeating chondroitin sulfate disaccharide unit (M_r 495). [e] Components were below the range of detection. [f] This value is in milligrams per 100 milligrams peptide weight.

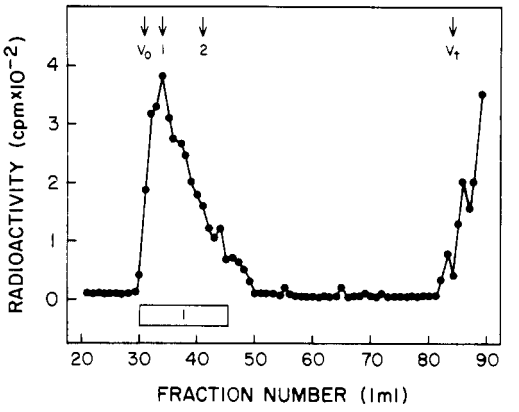

FIGURE 5: Filtration on Bio-Gel P-30 (100–200 mesh) of the glycosaminoglycan chains of the muscle proteoglycan. The purified ^{3}H-labeled glycosaminoglycan (10 900 cpm) released by alkaline NaB^3H_4 treatment of the muscle proteoglycan was applied to a column (1 × 112 cm) equilibrated with 0.1 M pyridine formate, pH 5.0, containing 20 μg/mL chondroitin sulfate as carrier. A flow rate of 12 mL/h was maintained. Each fraction was analyzed for radioactivity. Numbered areas designate fractions which were pooled for further study. The void volume (V_0) and the total volume (V_t) of the column as well as the position of elution of soybean trypsin inhibitor (M_r 20 100) (1) and cytochrome *c* (M_r 12 400) (2) are indicated by arrows. The peak of elution of standard heparin (M_r 13 500–15 000, Calbiochem) was at tube 36.

the DEAE-cellulose-purified glycosaminoglycan chains present in the material excluded from that column on Bio-Gel P-30 gave rise to two radiolabeled peaks of which glycosaminoglycan chains emerged as a peak (peak 1, Figure 5) close to the void volume of the column consistent with an average molecular weight of 21 000 while the large peak eluting at the total column volume is believed to be residual NaB^3H_4. When the Bio-Gel P-30 purified glycosaminoglycan chains (peak 1, Figure 5) were refiltered on a column of this gel which had been equilibrated with 4 M guanidine hydrochloride, they emerged as a single radioactive peak (diagram not shown) slightly after the void volume of the column ($V_e/V_0 = 1.12$); the elution profile was similar when this fraction was passed through the same column which had been equilibrated with

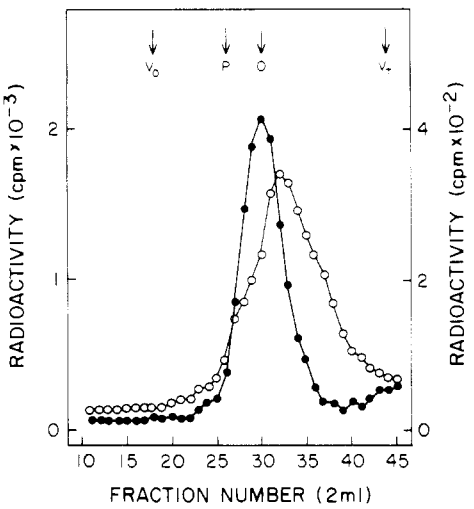

FIGURE 6: Filtration on Sepharose CL-6B of the glycosaminoglycan–peptides (●) and glycosaminoglycan chains (○) from muscle proteoglycan. The ^{14}C-labeled glycosaminoglycan–peptides (14 900 cpm) obtained from purified proteoglycan by digestion with Pronase followed by fractionation on DEAE-cellulose were placed on a column (1 × 110 cm) and equilibrated and eluted with 4 M guanidine hydrochloride/0.05 M Tris–acetate, pH 7.0, at a flow rate of 4 mL/h. The ^{3}H-labeled glycosaminoglycan chains (7500 cpm) of peak 1 (Figure 5) were applied to the column equilibrated and eluted with 4 M guanidine hydrochloride/0.05 M Tris–acetate, pH 7.0. An aliquot from each fraction was assayed for radioactivity; recovery of radioactivity from the column was complete. The void volume (V_0) and the total volume (V_t) of the column as well as the positions of elution of standard ovalbumin (O) of M_r 45 000 and the original proteoglycan (P) are indicated by arrows. The peak of elution of standard heparin (M_r 13 500–15 000, Calbiochem) was at tube 33.

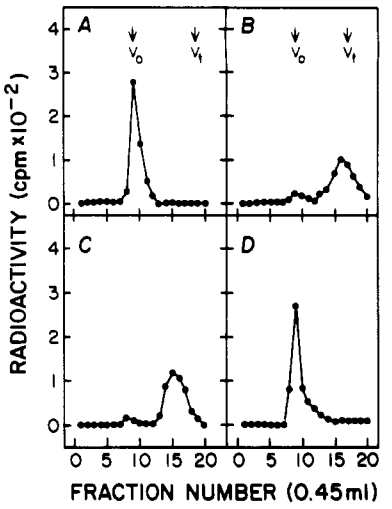

FIGURE 7: Effect of different treatments on the glycosaminoglycan chains of muscle proteoglycan as assessed by Sephadex G-50 gel filtration. Aliquots (500 cpm) of purified glycosaminoglycan chains (peak 1, Figure 5) were gel filtered on a column (0.7 × 20.0 cm) of Sephadex G-50 before (A) and after treatment with chondroitinase AC (B), chondroitinase ABC (C), or nitrous acid (D). Heparin (Calbiochem) was eluted in the excluded fractions, 8–11; after treatment with nitrous acid, it eluted as a peak at tube 13. The elution position of standard chondroitin sulfate disaccharide, -Di-6S (Miles Labs), was at tube 16.

0.1 M pyridine formate, pH 5.0. Filtration of the Bio-Gel P-30 purified glycosaminoglycan chains (peak 1, Figure 5) on Sepharose CL-6B yielded a single peak (K_{av} = 0.50) which accounted for all of the radioactivity placed on the column (Figure 6). The glycosaminoglycan chains emerged in a position ahead of the glycosaminoglycan–peptides. Calculations based on the specific activity of the Na^3BH_4 employed for the β-elimination reaction and the radioactivity recovered in peak 1 (Figure 5) from the Bio-Gel P-30 column indicated that the original proteoglycan contained 0.3 nmol of ^{3}H while chemical analysis showed a content of 13.5 nmol of hexuronic acid. The hexuronic acid to ^{3}H ratio indicated an average length of 45 disaccharide units; the approximate molecular weight is thus 22 275. This value is within experimental error of that derived from the gel filtration data.

Filtration of the glycosaminoglycan–peptides, obtained after extensive proteolytic trimming of the protein core, on Sepharose CL-6B (Figure 6) in 4 M guanidine hydrochloride yielded a radioactive peak (K_{av} = 0.46) which is substantially retarded compared to the original proteoglycan (K_{av} = 0.35). Previously, one of us reported (Parthasarathy & Spiro, 1984) a K_{av} value of 0.41 for the glycosaminoglycan–peptide which was derived from glomerular basement membrane proteoglycan and which had been shown to contain four heparan sulfate chains of M_r ~14 000. The elution profile of the glycosaminoglycan–peptides from muscle proteoglycan is consistent with a weight-average molecular weight of 45 000.

The NaB^3H_4-reduced glycosaminoglycan obtained after Bio-Gel P-30 chromatography (peak 1, Figure 5) was enzymatically digested with chondroitinase ABC or AC or was subjected to chemical deaminative cleavage with nitrous acid, and the radiolabeled products after treatment were quantitated by Sephadex G-50 chromatography. When the undegraded [^{3}H]glycosaminoglycan was filtered, all of the radioactivity was recovered in the void volume of a Sephadex G-50 column (Figure 7A). The distribution of radioactivity associated with the material eluting in the void volume, after treatment of the glycosaminoglycan with chondroitinase AC (Figure 7B) and chondroitinase ABC (Figure 7C), was 13% and 6%, respectively. Nitrous acid treatment (Figure 7D) did not alter the elution profile of the ^{3}H-labeled glycosaminoglycan, indicating the absence of heparan sulfate like chains linked to the protein core of the proteoglycan. The proportion of radioactivity (87–94%) appearing in the included volume after release by chondroitinase AC is very close to that followed by treatment with chondroitinase ABC. Furthermore, the elution profiles of the degradation products after these enzyme treatment were similar. These findings indicate that most of the glycosaminoglycans associated with the proteoglycans are chondroitin A and C sulfates and the proteoglycan is virtually devoid of dermatan sulfate or may contain a very small proportion of this glycosaminoglycan.

The Bio-Gel P-2 profile (Parthasarathy & Spiro, 1984) included ^{3}H-labeled oligosaccharides, which were acid hydrolyzed, N-acetylated, and then subjected to thin-layer chromatography. Results indicated the absence of *N*-acetylgalactosaminitol (diagram not shown), suggesting that the proteoglycan may lack short O-glycosidically linked saccharide chains terminating in *N*-acetylgalactosamine.

Discussion

Proteoglycans were solubilized in good yields from adult rabbit muscle by extraction with 4 M guanidine hydrochloride and purified by successive steps including CsCl density gradient ultracentrifugation, ion-exchange chromatography on DEAE-cellulose (Figure 1), and gel permeation on Sepharose CL-6B (Figure 2) and Sepharose CL-2B (Figure 3). From a mixture of muscle proteoglycans, a predominant proteoglycan which constituted 44% of the hexuronate of the muscle tissue was isolated. This proteoglycan, which is characterized as a chondroitin sulfate proteoglycan, is small in molecular size (M_r 95 000) (Figure 4A) and is found to contain peptide and glycosaminoglycan in almost equal proportions by weight. On the basis of the composite results of chondroitinase ABC di-

gestion (Figure 4B), protease treatment (Figure 6), and alkaline NaB^3H_4 treatment (Figure 5), the muscle proteoglycan is found to consist of two chondroitin sulfate chains with an average molecular weight of about 21 000, and these chains appear to be closely spaced on the core protein.

The large chondroitin sulfate proteoglycan [$M_r \sim(1–4) \times 10^6$] (Hascall & Sadjera, 1970) found in the cartilage is generally considered as representative of a great number of proteoglycans which are widely distributed in animal tissues (Hascall & Hascall, 1981) and which have been shown to share some common structural features such as a large protein core ($M_r \sim 2.5 \times 10^5$) (Hascall & Hascall, 1981), extensive substitution of its polypeptide by numerous glycosaminoglycan chains (Roden, 1980), diversity of saccharide chains (N and O linked) (Hascall & Rialo, 1972; DeLuca et al., 1980; Nilsson et al., 1982), and the ability to form aggregates with hyaluronate (Hascall & Sadjera, 1969). The muscle proteoglycan isolated in the present study is clearly distinct from the large proteoglycans of cartilage as well as those identified recently in embryonic muscle (Pechak et al., 1985) in that it contains a small core protein (average M_r <50 000) and has relatively few glycosaminoglycan substituents. Though the possible presence of N-linked carbohydrate units in the muscle proteoglycan is not ruled out, we did not find evidence for the presence of small O-linked oligosaccharides on the core protein. Further detailed structural investigations will be required to determine if saccharide chains other than chondroitin sulfates are linked to the polypeptide in the proteoglycan.

The low molecular weight muscle chondroitin sulfate proteoglycan isolated in the present study resembles in several respects other "small proteoglycans" recently characterized (Heinegard et al., 1985) in noncartilagenous tissues including sclera, tendon, cervix, skin, and cornea as well as those derived from bone, cartilage, and periodontal ligament. The proteoglycan fragment which remained after chondroitinase ABC digestion (Figure 4) had an average molecular weight of 50 000 which is close to the typical average molecular weight range of 45 000–48 000 reported (Heinegard et al., 1985) for the core protein–polysaccharide remnants of small proteoglycans obtained after chondroitinase treatment. Like these small proteoglycans, the amino acid composition of muscle proteoglycan was notable in its high content of aspartic/glutamic acids and leucine. However, compared to others within this group, these amino acids were present in substantially lower amounts in the muscle proteoglycan; glycine constitutes the predominant amino acid of its protein core, and its composition seems to suggest that the small proteoglycan might have been derived from the same or similar genes.

In comparing the nature of glycosaminoglycan chains of muscle chondroitin sulfate proteoglycan isolated in the present study to small proteoglycans isolated from other sources, it is of particular interest to note that the muscle proteoglycan contains galactosaminoglycans of mostly the chondroitin sulfate (A and C) type. With the exception of small proteoglycans of cartilage (Rosenberg et al, 1985) and bone (Fischer et al., 1983), most of the other proteoglycans of this class are found to contain dermatan sulfate chains (Coster & Fransson, 1981; Damle et al., 1982; Vogel & Heinegard, 1985).

Though the low molecular weight chondroitin sulfate proteoglycan isolated in the present study is a major proteoglycan of muscle, contributing to 44% of muscle hexuronate, it constitutes much less than 1% of the tissue weight. However, its potential biological function should not be underestimated. Recent [^{35}S]sulfate incorporation studies into the newly synthesized proteoglycans of embryonic muscle by Carrino et al. (1982, 1984) have indicated that there is a gradual increase in the proportion of small chondroitin sulfate proteoglycans (K_{av} = 0.77–0.81 on CL-2B) with increasing developmental age, and a similar phenomenon has been noted in heart muscle (Carrino & Caplan, 1984). However, with increasing age of the embryo, the large chondroitin sulfate muscle proteoglycans (K_{av} = 0.14–0.23 on CL-2B) decrease in their synthesis. Therefore, this small chondroitin sulfate proteoglycan obtained from muscle may be a unique or distinct one, characteristic of adult skeletal muscle. Although the nature of the mechanisms responsible for the compositional changes observed during growth is unknown, it is tempting to suggest that, by virtue of its major contribution to the proteoglycans of the muscle, the low molecular weight muscle chondroitin sulfate proteoglycan may be involved in muscle maturation.

Although chondroitin sulfate proteoglycans had been reported (Parthasarathy & Spiro, 1981; Kanwar et al., 1981; Couchman et al., 1985) to be minor proteoglycan components of basement membranes, the possible association of the small chondroitin proteoglycan isolated in the present study with the basal lamina which ensheathes muscle fibers is not ruled out. Despite our current limited knowledge about the nature of the components that constitute muscle fiber basal lamina, this ECM has been shown to play a critical role in reinnervation (McMahan & Slater, 1984) of adult muscle and in the formation of the neuromuscular junction (Anderson et al., 1984). This raises the possibility that proteoglycans, as components of the extracellular matrix (Mayne & Sanderson, 1985) might be implicated in these highly specialized functions of muscle and therefore are major candidates for future investigations of structure–function interrelationships.

ACKNOWLEDGMENTS

We thank Dr. J. H. Waite for carrying out amino acid analyses.

REFERENCES

Anderson, M. J., & Fambrough, D. M. (1983) *J. Cell Biol. 97*, 1396–1411.

Anderson, M. J., Klier, F. G., & Tanguay, K. E. (1984) *J. Cell Biol. 99*, 1769–1784.

Carrino, D. A., & Caplan, A. I. (1982) *J. Biol. Chem. 257*, 14145–14154.

Carrino, D. A., & Caplan, A. I. (1984) *J. Biol. Chem. 259*, 12419–12430.

Carrino, D. A., Pechak, D. G., & Caplan, A. I. (1984) *Exp. Biol. Med. 9*, 80–86.

Chiquet, M., & Fambrough, D. M. (1984) *J. Cell Biol. 98*, 1937–1946.

Coster, L., & Fransson, L. A. (1981) *Biochem. J. 193*, 143–153.

Couchman, J. R., Woods, A., Hook, M., & Christner, J. E. (1985) *J. Biol. Chem. 260*, 13755–13762.

Damle, S. P., Coster, L., & Gregory, J. D. (1982) *J. Biol. Chem. 257*, 5523–5527.

DeLuca, S., Lohmander, S., Nilsson, B., Hascall, V. C., & Caplan, A. I. (1980) *J. Biol. Chem. 255*, 6077–6083.

Dische, Z. (1947) *J. Biol. Chem. 167*, 189–198.

Dreyfuss, G., Adam, S. A., & Choi, Y. D. (1984) *Mol. Cell Biol. 4*, 415–423.

Fisher, L. W., Termine, J. D., Dejter, S. W., Whitson, S. W., Yanagishita, M., Kimura, J. H., Hascall, V. C., Kleinman, H. K., Hassell, J. R., & Nilsson, B. (1983) *J. Biol. Chem. 258*, 6588–6594.

Hascall, V. C., & Sadjera, S. W. (1969) *J. Biol. Chem. 244*, 2384–2396.

Hascall, V. C., & Sadjera, S. W. (1970) *J. Biol. Chem. 245*, 4920–4930.
Hascall, V. C., & Rialo, R. L. (1972) *J. Biol. Chem. 247*, 4529–4538.
Hascall, V. C., & Hascall, G. K. (1981) in *Cell Biology of Extracellular Matrix* (Hay, E. D., Ed.) pp 39–63, Plenum Press, New York.
Hascall, V. C., & Kimura, J. H. (1982) *Methods Enzymol. 82*, 769–800.
Heinegard, D., & Paulson, M. (1984) in *Extracellular Matrix Biochemistry* (Piez, K. A., & Reddi, A. H., Eds.) pp 278–328, Elsevier, New York.
Heinegard, D., Bjorne-Persson, A., Coster, L., Franzen, A., Gardell, S., Malmstrom, A., Paulsson, M., Sandfalk, R., & Vogel, K. G. (1985) *Biochem. J. 230*, 181–194.
Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.
Kanwar, Y. S., Hascall, V. C., & Farquhar, M. G. (1981) *J. Cell Biol. 90*, 527–532.
Lagunoff, D., & Warren, G. (1962) *Arch. Biochem. Biophys. 99*, 396–440.
Leyva, A., & Kelly, W. N. (1974) *Anal. Biochem. 62*, 173–179.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mayne, R., & Sanderson, R. D. (1985) *Collagen Relat. Res. 5*, 449–468.
McMahan, U. J., & Slater, C. R. (1984) *J. Cell Biol. 98*, 1453–1473.
Nilsson, B., DeLuca, S., Lohmander, S., & Hascall, V. C. (1982) *J. Biol. Chem. 257*, 10920–10927.
Oike, Y., Kimata, K., Shinomura, T., Nakazawa, K., & Suzuki, S. (1980) *Biochem. J. 191*, 193–207.
Okada, Y., & Spiro, R. G. (1980) *J. Biol. Chem. 255*, 8865–8872.
Parthasarathy, N., & Spiro, R. G. (1981) *J. Biol. Chem. 256*, 507–513.
Parthasarathy, N., & Spiro, R. G. (1982) *Arch. Biochem. Biophys. 213*, 504–511.
Parthasarathy, N., & Spiro, R. G. (1984) *J. Biol. Chem. 259*, 12749–12755.
Pechak, D. G., Carrino, D. A., & Caplan, A. I. (1985) *J. Cell Biol. 100*, 1767–1776.
Robyt, J. H. (1975) *Carbohydr. Res. 40*, 373–374.
Roden, L. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 267–371, Plenum Press, New York.
Rosenberg, L. C., Choi, H. W., Tang, L.-H., Johnson, T., Pal, S., Weber, C., Priner, A., & Poole, A. P. (1985) *J. Biol. Chem. 260*, 6304–6313.
Sanes, J. R. (1983) *Annu. Rev. Physiol. 45*, 581–600.
Sanes, J. R., Marshall, L. M., & McMahan, U. J. (1978) *J. Cell Biol. 78*, 176–198.
Seno, N., Anno, K., & Kondo, K. (1970) *Anal. Biochem. 37*, 197–202.
Spiro, R. G. (1966) *Methods Enzymol. 8*, 3–26.
Spiro, R. G. (1967) *J. Biol. Chem. 242*, 4813–4823.
Vogel, K. G., & Heinegard, D. (1985) *J. Biol. Chem. 260*, 9298–9306.
Yanagishita, M., & Hascall, V. C. (1983) *J. Biol. Chem. 258*, 12847–12856.

Hydrogen Exchange Kinetics of Bovine Pancreatic Trypsin Inhibitor β-Sheet Protons in Trypsin–Bovine Pancreatic Trypsin Inhibitor, Trypsinogen–Bovine Pancreatic Trypsin Inhibitor, and Trypsinogen–Isoleucylvaline–Bovine Pancreatic Trypsin Inhibitor†

Pamela Brandt and Clare Woodward*
Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108
Received September 5, 1986; Revised Manuscript Received January 29, 1987

ABSTRACT: Hydrogen exchange rates of six β-sheet peptide amide protons in bovine pancreatic trypsin inhibitor (BPTI) have been measured in free BPTI and in the complexes trypsinogen–BPTI, trypsinogen–Ile-Val–BPTI, bovine trypsin–BPTI, and porcine trypsin–BPTI. Exchange rates in the complexes are slower for Ile-18, Arg-20, Gln-31, Phe-33, Tyr-35, and Phe-45 NH, but the magnitude of the effect is highly variable. The ratio of the exchange rate constant in free BPTI to the exchange rate constant in the complex, k/k_{cplx}, ranges from 3 to $\gg 10^3$. Gln-31, Phe-45, and Phe-33 NH exchange rate constants are the same in each of the complexes. For Ile-18 and Tyr-35, k/k_{cplx} is $\gg 10^3$ for the trypsin complexes but is in the range 14–43 for the trypsinogen complexes. Only the Arg-20 NH exchange rate shows significant differences between trypsinogen–BPTI and trypsinogen–Ile-Val–BPTI and between porcine and bovine trypsin–BPTI.

Hydrogen isotope exchange kinetics of backbone amide protons are a measure of the dynamic structure of proteins. The perturbation of hydrogen exchange rates by ligand binding is a sensitive indicator of a shift in the conformational equilibria of interconverting substates that constitute the dynamic folded state of proteins. A decrease in exchange rates is taken as an indication of decreased conformational flexibility, and vice versa. The magnitude and direction of the ligand binding effect on hydrogen exchange kinetics vary with the system [cf. reviews of ligand effects on hydrogen exchange in Woodward

† This work was supported by NIH Grant GM26242.

0006-2960/87/0426-3156$01.50/0