Selective Hydrolysis of Chondroitin Sulfates by Hyaluronidase†

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ABSTRACT: Chondroitin 4-sulfate and chondroitin 6-sulfate were incubated with testicular hyaluronidase in the presence of excess β-glucuronidase. The β-glucuronidase caused rapid removal of the nonreducing terminal β-D-glucuronosyl residues from the oligosaccharides formed by the action of the hyaluronidase, destroying the oligosaccharide acceptors required for the transglycosylation activity of hyaluronidase and releasing free D-glucuronic acid at a rate that was equal to the rate of the hyaluronidase-catalyzed hydrolysis. When hyaluronidase was assayed at 37 °C in the presence of 0.05 M NaCl, 0.05 M Na_2SO_4, and 0.1 M sodium acetate at pH 5, chondroitin 4-sulfate was hydrolyzed at 1.5 times the rate found for chondroitin 6-sulfate. When hyaluronidase was assayed at 45 °C in 0.06 M sodium acetate at pH 6, chondroitin 4-sulfate was hydrolyzed at 8 times the rate observed for chondroitin 6-sulfate. Under the pH 5 conditions, the chondroitin 4-sulfate was converted to a mixture of tri- and pentasaccharides, while the chondroitin 6-sulfate was converted primarily to a mixture of penta- and heptasaccharides, with only a small amount of trisaccharide. Under the pH 6 conditions, the chondroitin 4-sulfate was converted to a mixture of penta- and heptasaccharides, with only a small amount of trisaccharide, but the products from chondroitin 6-sulfate were a mixture of oligosaccharides ranging in degree of polymerization from 7 to 25 monosaccharides per oligosaccharide. End-group analyses of the products formed at pH 6 showed that both substrates were cleaved preferentially at the glycosidic bonds of the 4-sulfated disaccharides. The use of the enhanced substrate selectivity of hyaluronidase at the higher pH in the analysis of chondroitin sulfate structures is discussed.

Testicular hyaluronidase converts hyaluronic acid and chondroitin sulfate to oligosaccharides that have *N*-acetyl-D-hexosamine residues at their reducing terminals and D-glucuronic acid residues at their nonreducing terminals (Meyer et al., 1960; Meyer, 1971). The enzyme has been used extensively to prepare oligosaccharides for studies of the metabolism and the structures of these polymers. However, several aspects of the activity of hyaluronidase limit its usefulness in such studies. First, the enzyme catalyzes transglycosylation as well as hydrolysis (Weissmann, 1955; Hoffman et al., 1956; Highsmith et al., 1975) and thus may generate oligosaccharides having sequences that were not present in the original substrates. Second, because the available assay procedures do not give equivalent responses with all of the substrates of hyaluronidase (Meyer, 1947; Meyer & Rapport, 1952; Bowness & Tan, 1968; Zaneveld et al., 1973; Gorham et al., 1975; Seno et al., 1975) and because transglycosylation may occur at different rates for different substrates, it has not been possible to obtain a true measure of the selectivity of the enzyme for its different substrates.

In the present work, hyaluronidase was assayed in the presence of excess β-glucuronidase to prevent the transglycosylation. With this assay, conditions were found that give marked differences in the rates and extents of hydrolysis of commercial samples of chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitin, hyaluronic acid, and of chondroitin sulfate synthesized by cultured chick embryo chondrocytes. This procedure, which allows isolation of oligosaccharides containing sequences present in the original polymeric substrate, has been used to demonstrate sequences that contain both 4-sulfated and 6-sulfated disaccharides in all chondroitin sulfate preparations examined.

Experimental Procedures

Substrates. Chondroitin sulfates, type A, from whale cartilage, and type C, from shark cartilage, were obtained from Sigma Chemical Co. These were analyzed for their uronic acid content by the modified carbazole method of Bitter & Muir (1962) and for their disaccharide composition by the high-performance liquid chromatography procedure described previously (Delaney et al., 1980). $^{35}SO_4$-Labeled heavy proteochondroitin sulfate was isolated from the culture medium of chick embryo tibial chondrocytes that were labeled for 24 h with 50 μCi/mL $H_2{}^{35}SO_4$ (43 Ci/mmol, New England Nuclear) by the procedure used previously (Kim & Conrad, 1977, 1980, 1982). The disaccharide composition of this fraction was determined by radiochromatographic analysis of its chondroitinase digestion products (Kim & Conrad, 1974). Hyaluronic acid was obtained from Sigma Chemical Co. Chondroitin was prepared by desulfation of chondroitin sulfate by the method of Kantor & Shubert (1956), and the nondialyzable fraction of the chondroitin was used. Analysis of the chondroitinase digestion products of this material showed that 93% of the disaccharide units were unsulfated.

Enzyme Assays. For hyaluronidase, two assay conditions were used. One, the pH 5 assay, was typical of the hyaluronidase assay procedures used most commonly in the literature (Meyer, 1947; Meyer & Rapport, 1962; Bowness & Tan, 1968; Zaneveld et al., 1973; Gorham et al., 1975). The other, the pH 6 assay, was developed here as an assay that gave maximal differences in the rates of hyaluronidase action on chondroitin 4-sulfate and chondroitin 6-sulfate. In these assays, 1 mg of chondroitin sulfate was incubated with a mixture of 110 turbidity reducing units of ovine testicular hyaluronidase (Sigma, type V, 1100 units/mg) and 900 Fishman units of bovine liver β-glucuronidase (Sigma, type B-10, 9000 units/mg) in 1.0 mL of pH 5.0 buffer at 37 °C or in 1.0 mL of pH 6.0 buffer at 45 °C. The pH 5 buffer contained 0.05 M NaCl and 0.05 M Na_2SO_4 in 0.1 M sodium acetate. The pH 6 buffer was 0.06 M sodium acetate. D-

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[^{14}C]Glucose (New England Nuclear, 267 mCi/mmol) was added to each assay as an internal standard at a concentration of 2×10^7 dpm/assay.

Assay tubes were placed in a thermostated water bath, and after 15 min, 20 μL of each assay mixture was transferred to a 500-μL plastic centrifuge tube containing 2.5 μL of 1 M Na_2CO_3 to stop the reaction. The sample was reduced by adding 10 μL of an 0.25 M solution of NaB^3H_4 (Amersham Radiochemicals, 424 mCi/mmol) in 0.1 N NaOH and heating the mixture at 50 °C for 45 min in the fume hood (Conrad et al., 1973). Excess NaB^3H_4 was destroyed by addition of 10 μL of 0.5 M H_2SO_4, and the sample was assayed for L-[^{3}H]gulonic acid (NaB^3H_4-reduced D-glucuronic acid) by radiochromatography as previously described (Conrad et al., 1973; Conrad, 1980). Under the counting conditions used, 1 nmol of D-glucuronic acid gave 9615 cpm (10000 cpm ≡ 1.04 nmol). Rates of D-glucuronic acid release in these assays were linear for 15 min but fell off slowly as the less active smaller oligosaccharide substrates began to accumulate in the assay mixture.

β-Glucuronidase was assayed by measuring the rate of 4-methylumbelliferone formation when enzyme was incubated with 4-methylumbelliferyl β-D-glucuronide in the appropriate buffer (Glaser & Conrad, 1979b).

Gel Filtration of Hyaluronidase/β-Glucuronidase-Generated Oligosaccharides. Aliquots (0.5 mL) of each digest of the unlabeled substrates were mixed with an aliquot of $^{35}SO_4$-labeled trisaccharide, added as an internal standard, and the mixtures were separated on a 1 × 208 cm column of Sephadex G-25 equilibrated and run at room temperature in 1 M NaCl. The flow rate was maintained at 12 mL/h, and 1-mL fractions were collected. Aliquots of each fraction were assayed by the carbazole method (Bitter & Muir, 1962) or by scintillation counting in a Triton–xylene-based scintillation fluid (Anderson & McClure, 1973). For further characterization, oligosaccharide peak fractions were combined and desalted on a 2 × 22 cm column of Sephadex G-10 run in water. Aliquots of the digests of the $^{35}SO_4$-labeled chondroitin sulfate were chromatographed in the same manner but without the internal standard. Effluent fractions were analyzed by scintillation counting. The degree of polymerization of each oligosaccharide was determined as reported elsewhere (Delaney et al., 1980).

Chondroitinase and Chondrosulfatase Digestion. Stock solutions of chondroitinase ABC, chondroitinase AC, chondro-4-sulfatase, and chondro-6-sulfatase, all from Sigma Chemical Co., were prepared in a 1:10 dilution of enriched Tris[1] buffer (Saito et al., 1968). The concentrations of the chondroitinases were 10 units/mL while those of the chondrosulfatases were 5 units/mL. For digestions with the chondroitinases alone, a 100-μL aliquot of the aqueous oligosaccharide solution was mixed with 10 μL of enriched Tris buffer and 10 μL of each of the chondroitinase ABC and chondroitinase AC stock solutions. When the chondroitinases and chondrosulfatases were used together, the incubation mixtures were supplemented with 10 μL each of the two chondrosulfatase stock solutions. Samples were incubated at 37 °C for 3 h and the products were analyzed as described below. The $^{35}SO_4$-labeled proteochondroitin sulfate was digested with the chondroitinases, after pretreatment with thermolysin to digest the core protein (Kim & Conrad, 1974). For aldehyde-reduced oligosaccharides, more stringent conditions (Glaser & Conrad, 1979b) were used for complete chondroitinase digestion.

Aldehyde Reduction of Oligosaccharides. Oligosaccharides were reduced with ^{3}H-labeled (424 mCi/mmol, Amersham) or unlabeled $NaBH_4$ under mildly alkaline conditions developed to minimize their alkaline degradation (Glaser & Conrad, 1979b) and analyzed directly by paper chromatography, paper electrophoresis, or slab gel electrophoresis. Labeled oligosaccharides were separated after chromatography or electrophoresis on 1 × 22.5 in. strips of Whatman No. 3 chromatography paper; the strips were cut into 0.5-in. segments, which were counted in 5 mL of a scintillation fluid containing 4 g of 2,5-diphenyloxazole/L of toluene. When separations required a preliminary separation on one strip followed by a further separation on a second strip, the paper segments containing the unresolved components were counted and then rinsed with toluene to remove the diphenyloxazole, air-dried, and eluted with water. The eluate was transferred to the origin of the second strip, which was chromatographed or electrophoresed to complete the separation. Paper chromatograms were developed with 1-butanol, glacial acetic acid, and 1 M ammonia in a volume ratio of 2:3:1 (system 1) or with ethyl acetate, pyridine, and 0.05 M sodium borate in a volume ratio of 3:2:1 (system 2). Paper electrophoresis was carried out at 1400 V (25 V/cm, 2 mA/strip) in a pH 1.7 buffer containing 88% formic acid, glacial acetic acid, and water in a volume ratio of 2:7:70.

Slab Gel Electrophoresis. Gel electrophoresis of ^{3}H-labeled oligosaccharides was run by a modification of the procedure described by Maxam & Gilbert (1977). The gels were composed of 25% (w/v) acrylamide (Bio-Rad), 0.83% (w/v) *N,N'*-methylenebis(acrylamide), 50 mM Tris–borate, pH 8.3, 1 mM EDTA, and 1.63 mM ammonium persulfate. The gel solution (40 mL) was polymerized with 20 μL of *N,N,N',N'*-tetramethylethylenediamine, and the polymerized gel was allowed to stand for 1–12 h before removal of the well-forming comb and addition of the 50 mM Tris–borate–1 mM EDTA electrophoresis buffer. The gel was preelectrophoresed at a constant voltage of 500 V (12 mA) for 3 h before the sample was loaded.

Prior to electrophoresis, the NaB^3H_4-reduced samples were desalted by paper chromatography in system 1 for 10 h. The labeled oligosaccharides, which remained in the first 3 in. of the chromatogram, were eluted with water, and the eluate was evaporated to dryness and redissolved in water. An aliquot containing 2×10^6 dpm of a mixture of oligosaccharides, or 4×10^5 dpm of a standard oligosaccharide, was transferred to a 6 × 50 mm test tube, evaporated to dryness, and redissolved in 10 μL of sample buffer containing 5 mM Tris–borate, pH 8.3, 5 mM EDTA, 30% glycerol, and 0.1 mg/mL each of phenol red and bromphenol blue. Each sample or standard was layered at the bottom of a sample well, and electrophoresis was run at 800 V (9.5 mA) in a water-cooled electrophoresis apparatus.

The labeled oligosaccharides were detected by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975) with sodium salicylate as a fluor (Chamberlain, 1979). The gel was gently

[1] Abbreviations: GalNAc, *N*-acetyl-D-galactosamine; $GalNAc_R$, *N*-acetyl-D-galactosaminitol; DP, degree of polymerization, in monosaccharide units per nonreducing terminal; Di-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose; Di-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; $\Delta Di\text{-}4S_R$, $\Delta Di\text{-}6S_R$, and $\Delta Di\text{-}0S_R$, aldehyde-reduced forms of ΔDi-4S, ΔDi-6S, and ΔDi-0S, respectively; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

shaken for 30 min in 10 volumes of 1 M sodium salicylate in 65% ethanol and then sandwiched between two pieces of gel slab backing (Bio-Rad) and dried under reduced pressure at 65 °C. The bands were detected by exposing the dried gel to preflashed (Laskey & Mills, 1975) X-Omat AR film (Eastman Kodak Co.) for 2–6 days at −70 °C.

Test for Transglycosylation. [^{3}H]Chondroitin and chondroitin [^{35}S]sulfate were prepared by incubation of monolayers of chick embryo tibial chondrocytes (Kim & Conrad, 1977) with labeled precursors in the presence of *p*-nitrophenyl β-D-xyloside (Kim & Conrad, 1980). ^{3}H-Labeled chondrotin, in which less than 10% of the disaccharide units were sulfated, was prepared by incubation of cultures with D-[^{3}H]glucosamine in the presence of 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid to prevent SO_4^{2-} uptake by the cells (J. J. Kim and H. E. Conrad, unpublished results). Cells were grown to a density of 2.5 × 10^6 cells/60-mm dish in DME(2) medium containing 10% fetal calf serum (Kim & Conrad, 1976). The culture medium was removed, and the cells were washed twice with 0.5 mL of Tris–saline and then incubated for 30 min with 5 mL of Tris–saline so that the cells would utilize or release most of their intracellular SO_4^{2-}. The cells were then incubated for 24 h at 37 °C with 5 mL of SO_4^{2-}-free DME(2) containing 0.5% fetal calf serum, 1 mM β-xyloside, 0.5 mM 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, and 375 μCi of D-[^{3}H]glucosamine (30.3 Ci/mmol, Amersham). After removal of the culture medium, the cells were washed twice with 0.5 mL of Tris–saline, and the combined culture medium and washes were saved for recovery of the [^{3}H]chondroitin.

Chondroitin[^{35}S]sulfate in which more than 85% of the disaccharide units were sulfated was prepared by incubating an identical chondrocyte culture for 24 h at 37 °C with 5 mL of DME(2) containing 0.5% fetal calf serum, 1 mM β-xyloside, and 25 μCi of $H_2{}^{35}SO_4$ (43 Ci/mmol, New England Nuclear). The medium and washes were combined as above for recovery of the chondroitin [^{35}S]sulfate. An identical culture in which the $H_2{}^{35}SO_4$ was replaced by 100 μCi of [^{3}H]glucosamine was used to obtain an [^{3}H]chondroitin sulfate preparation that was analyzed to determine the distribution of 4-, 6-, and unsulfated disaccharides in the chondroitin [^{35}S]sulfate preparation formed under these labeling conditions.

All media were dialyzed overnight in the cold against 50 mM Na_2SO_4 and then six changes of cold distilled water. The dialyzed preparations were lyophilized and redissolved in 1 mL of H_2O. An aliquot of each ^{3}H-labeled sample was used for analysis of the disaccharide composition, and the remainder was used in the tests for transglycosylation, which were carried out as follows. The [^{3}H]chondroitin (150 000 dpm), the chondroitin [^{35}S]sulfate (30 000 dpm), and a mixture containing 150 000 dpm of [^{3}H]chondroitin and 30 000 dpm of chondroitin [^{35}S]sulfate were incubated with hyaluronidase/β-glucuronidase under the pH 5 and the pH 6 assay conditions for 24 h in a total volume of 160 μL/assay. The entire volume of each incubation was spotted for paper electrophoretic analysis.

Results

Effect of Assay Conditions on Hyaluronidase Activity. The rates of chondroitin 4-sulfate and chondroitin 6-sulfate hydrolysis, measured as the rate of appearance of free D-glucuronic acid in the β-glucuronidase-coupled reaction, were determined at pH 5 under conditions typical of those normally used for hyaluronidase assays and at pH 6 under conditions that were developed to obtain maximal differences in the rates of hydrolysis of these two substrates. Under these conditions

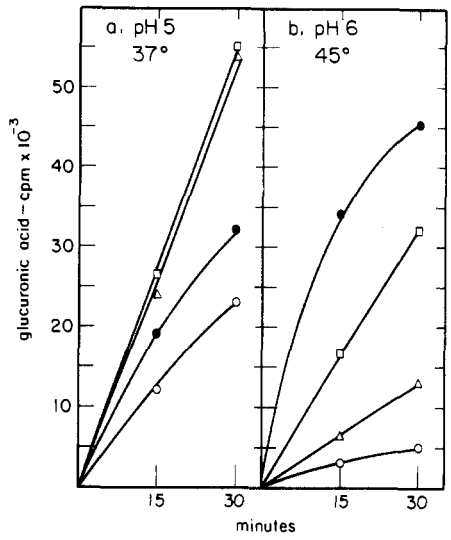

FIGURE 1: Comparison of rates of hyaluronidase/β-glucuronidase hydrolysis of chondroitin 4-sulfate (●), chondroitin 6-sulfate (○), chondroitin (△), and hyaluronic acid (□) under the pH 5 and the pH 6 assay conditions. For panel a, assays were carried out at pH 5 and 37 °C in 0.1 M sodium acetate, 0.05 M NaCl, and 0.05 M Na_2SO_4. Panel b assays were carried out at pH 6 and 45 °C in 0.06 M sodium acetate.

the rate of D-glucuronic acid release in the presence of 900 Fishman units of β-glucuronidase was proportional to the number of hyaluronidase units up to 22 turbidity reducing units. Also, in the presence of 11 turbidity reducing units of hyaluronidase, the rate of D-glucuronic acid formation did not change as the β-glucuronidase level was increased from 450 to 1800 Fishman units. The β-glucuronidase retained full activity for at least 24 h under all of the assay conditions used below (Knudson, 1982). Figure 1 shows a comparison of the rates observed under the two assay conditions. Under the pH 5 assay conditions, the rates for the sulfated substrates were similar, and the unsulfated substrates, hyaluronic acid and chondroitin, were hydrolyzed at similar rates, which were higher than those observed for the sulfated substrates. However, at pH 6, the rates of hydrolysis of these unsulfated substrates were quite different from each other, and both rates were intermediate between those of the two sulfated substrates.

Test for Transglycosylation. Under both assay conditions, the rate of D-glucuronic acid liberation from the hyaluronidase substrates was directly proportional to the amount of hyaluronidase present in the assay mixture (Knudson, 1982). This implied that the β-glucuronidase was in such excess that oligosaccharides having nonreducing terminal D-glucuronic acid residues did not accumulate in the assay mixture and that transglycosylation, therefore, could not occur. As a further demonstration that transglycosylation did not occur, assays were performed with a mixture of [^{3}H]chondroitin and chondroitin [^{35}S]sulfate as substrate. Analyses of these polymeric substrates showed that they contained 6-sulfated, 4-sulfated, and unsulfated disaccharide units in ratios of 5.7:1.3:93 and 66:20:14 for [^{3}H]chondroitin and chondroitin [^{35}S]sulfate, respectively. If transglycosylation were to occur upon incubation of a mixture of these two substrates with hyaluronidase in the presence of excess β-glucuronidase, doubly labeled oligosaccharides having an intermediate degree of sulfation would be formed. The paper electrophoretic behaviors of the products of the digestions of these two substrates are shown in Figure 2. Electrophoresis of the pH 5 digest of a mixture of the ^{3}H- and $^{35}SO_4$-labeled polymers (Figure 2e) separated a mixture of highly sulfated, rapidly moving $^{35}SO_4$-labeled oligosaccharides from the slower-moving peak

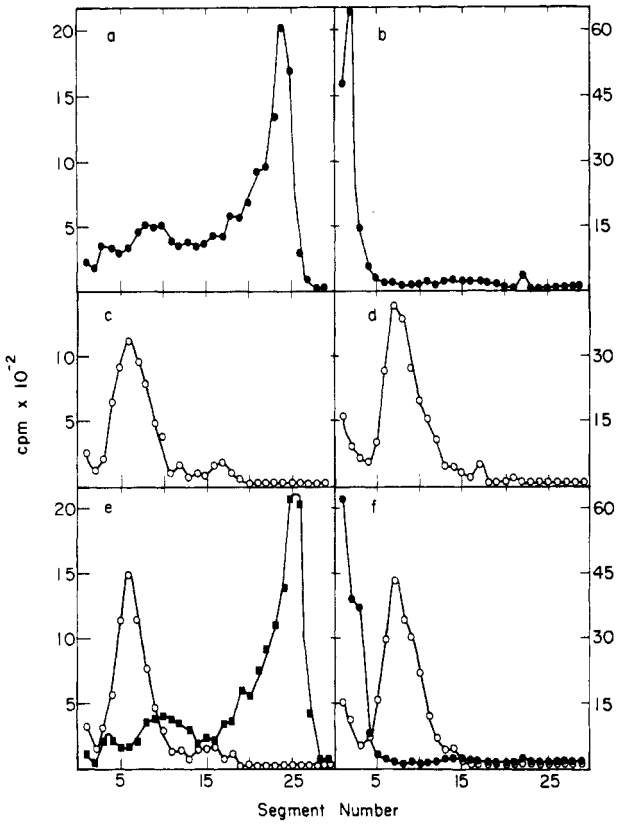

FIGURE 2: Paper electrophoretic profiles of products of hyaluronidase/β-glucuronidase digestion of chondroitin [^{35}S]sulfate at pH 5 (a), chondroitin [^{35}S]sulfate at pH 6 (b), [^{3}H]chondroitin at pH 5 (c), [^{3}H]chondroitin at pH 6 (d), a mixture of chondroitin [^{35}S]sulfate and [^{3}H]chondroitin at pH 5 (e), and a mixture of chondroitin [^{35}S]sulfate and [^{3}H]chondroitin at pH 6 (f). The 24-h digests were paper electrophoresed for 2.5 h, and the electrophoretograms were analyzed for ^{3}H cpm (○) or $^{35}SO_4$ cpm (●) by scintillation counting.

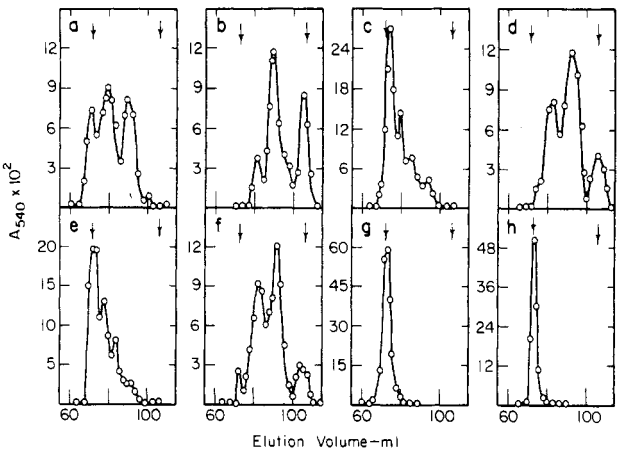

FIGURE 3: Sephadex G-25 elution profiles of oligosaccharide mixtures formed by hyaluronidase/β-glucuronidase hydrolysis of chondroitin 4-sulfate and chondroitin 6-sulfate. Elution profiles for pH 5 digests are shown in the top panels for chondroitin 4-sulfate after 1 (a) and 24 h (b) and for chondroitin 6-sulfate after 1 (c) and 24 h (d). Profiles for the pH 6 digests are shown in the bottom panels for chondroitin 4-sulfate after 1 (e) and 24 h (f) and for chondroitin 6-sulfate after 1 (g) and 24 h (h). In each panel, the first arrow shows the position of the void volume of the column, while the second arrow shows the elution position of $^{35}SO_4$-labeled trisaccharide internal standard. The carbohydrate peaks were detected by the carbazole assay (Bitter & Muir, 1962).

of the ^{3}H-labeled, minimally-sulfated oligosaccharides. Panels a and c show the electrophoretic profiles of the products formed when chondroitin [^{35}S]sulfate and [^{3}H]chondroitin, respectively, were digested alone under the same pH 5 conditions. Analogous profiles for the pH 6 digests are shown in panels b, d, and f. In the latter case, the higher molecular weight oligosaccharides formed from the chondroitin [^{35}S]sulfate (see below) did not migrate from the origin, but the [^{3}H]-chondroitin products were clearly separated from the $^{35}SO_4$-labeled products formed when the mixture of substrates was digested. When the major oligosaccharide peaks were eluted from the paper electrophoretograms and analyzed by paper chromatography, no evidence was found for doubly labeled oligosaccharides with an intermediate degree of sulfation. This experiment was repeated with a mixture of the same chondroitin [^{35}S]sulfate and a preparation of [^{3}H]-hyaluronic acid, kindly furnished by Dr. Bryan Toole, with identical results. Thus, it was concluded that transglycosylation did not occur under either of the assay conditions.

Size of Hyaluronidase/β-Glucuronidase Digestion Products. Chondroitin 4-sulfate and chondroitin 6-sulfate were digested with hyaluronidase/β-glucuronidase for 1 and for 24 h, and the digests were chromatographed on Sephadex G-25 as shown in Figure 3. The positions of the void volume and of an $^{35}SO_4$-labeled chondroitin sulfate trisaccharide, added to each sample as an internal standard, are indicated by arrows in each panel. The peaks of the digestion products were identified on the basis of the elution positions of previously characterized oligosaccharides (Delaney et al., 1980) and by comparison of their gel electrophoretic migrations with those of standards (below). The profiles of the pH 5 digestion products, given in panels a–d, show that chondroitin 4-sulfate was converted largely to penta- and heptasaccharides after 1 h and to tri- and pentasaccharides after 24 h, while chondroitin 6-sulfate yielded larger fragments after 1 h and primarily penta- and heptasaccharides after 24 h. The pH 6 digests of both substrates gave elution profiles (panels e–h) that differed from their corresponding pH 5 digests. Exhaustive digestion of chondroitin 4-sulfate at pH 6 yielded primarily penta- and heptasaccharides, while the oligosaccharides from chondroitin 6-sulfate remained almost totally in the excluded volume of the column. These profiles were not altered by longer digestion times, even when more enzyme was added.

Figure 4a shows slab gel electrophoretograms of the oligosaccharides formed by digestion of chondroitin 4-sulfate and chondroitin 6-sulfate for 24 h at the two pHs. In this experiment, electrophoresis was continued until the trisaccharides ran off of the gel in order to maximize the resolution of the higher oligosaccharides. The digests gave a series of bands, each of which differed from its adjacent bands by one disaccharide unit. The lighter bands, which appeared as shadows trailing the dark bands of some of the smaller oligosaccharides, represent oligosaccharides that contain one or more unsulfated disaccharide units (T. W. Chu and H. E. Conrad, unpublished results). The bands in lanes 1, 2, and 4 show oligosaccharides present in proportions similar to those seen in the corresponding gel filtration profiles in Figure 3. Lane 5 shows that the products of the pH 6 digestion of chondroitin 6-sulfate, which were excluded from the Sephadex G-60 column, are a mixture of relatively large oligosaccharides with degrees of polymerization ranging to more than 25 monosaccharides per oligosaccharide. Figure 4b shows the profiles of the oligosaccharides formed in the early stages of the digestion of the two substrates at pH 6.

Substrate Cleavage Sites. In the presence of excess β-glucuronidase, each cleavage of chondroitin sulfate by hyaluronidase generates one new oligosaccharide with a sulfated GalNAc reducing terminal and a second new oligosaccharide with a nonreducing terminal sulfated GalNAc. The structures

Table I: Terminal Residues of Oligosaccharides Formed by Hyaluronidase/β-Glucuronidase Digestion at pH 6 and 45 °C

substrate	% composition ΔDi-4S	% composition ΔDi-6S	digestion time (min)	oligosaccharide characterization: av DP	% of nonreducing terminals: $GalNAc_R$-4-SO_4	% of nonreducing terminals: $GalNAc_R$-6-SO_4	% of reducing terminals: Δ-4S_R	% of reducing terminals: Δ-6S_R
chondroitin 4-sulfate	78	22	30	14.9	89	11	90	10
			60	11.4	88	12	90	10
chondroitin 6-sulfate	18	82	30	25.3	35	65	34	66
			60	24.6	34	66	41	59

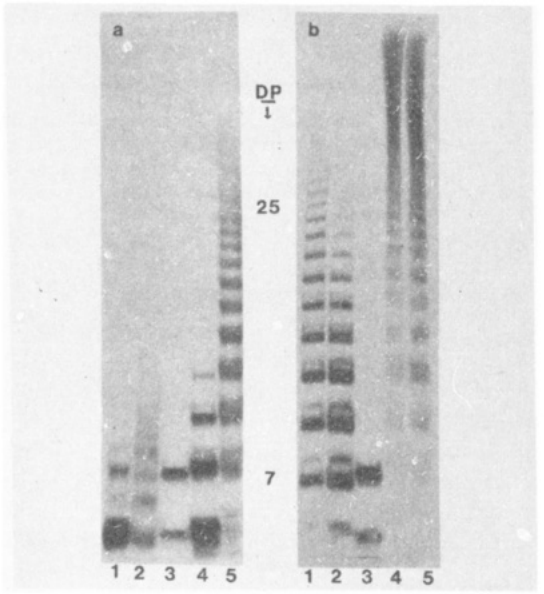

FIGURE 4: Slab gel electrophoresis of chondroitin sulfate digests. Panel a shows 24-h hyaluronidase/β-glucuronidase digests of chondroitin 4-sulfate at pH 5 (lane 1), chondroitin 4-sulfate at pH 6 (lane 2), chondroitin 6-sulfate at pH 5 (lane 4), and chondroitin 6-sulfate at pH 6 (lane 5). Lane 3 contains chondroitin 6-sulfate penta- and heptasaccharide standards. Panel b shows pH 6 digests of chondroitin 4-sulfate after 30 (lane 1) and after 60 min (lane 2) and the corresponding digests of chondroitin 6-sulfate after 30 (lane 4) and after 60 min (lane 5). Lane 3 contains chondroitin 6-sulfate penta- and heptasaccharide standards. Both gels were electrophoresed until the pentasaccharides reached the bottom of the gel and the trisaccharides ran off of the gel. DP's of the heptasaccharide standard and the 25-mer are indicated.

of the substrate at the sites most susceptible to hyaluronidase were defined by identifying the sulfated GalNAc residues at the terminals of the mixture of oligosaccharides generated in the early stages of substrate hydrolysis in the following manner. Each digest was treated with NaB^3H_4 to convert the reducing terminals of the oligosaccharides to [^{3}H]$GalNAc_RSO_4$ residues. The reduced oligosaccharides were then treated with a mixture of chondroitinases and chondrosulfatases. The chondroitinase action released GalNAc-4-SO_4 and GalNAc-6-SO_4 from the nonreducing terminals, ΔDi-4S and ΔDi-6S from the internal disaccharide units of the oligosaccharides, and [^{3}H]Δ-4S_R and [^{3}H]Δ-6S_R from the reducing terminals. The chondrosulfatases in the digestion mixture attacked ΔDi-4S and ΔDi-6S as they were released from the internal disaccharide units, converting them to ΔDi-0S and inorganic SO_4^{2-}. However, the chondrosulfatases do not act on the $GalNAcSO_4$'s or on [^{3}H]Δ-4S_R or [^{3}H]Δ-6S_R. Consequently, the only sulfated products remaining in the chondroitinase/chondrosulfatase digest were those derived from the terminal units of the oligosaccharides formed in the hyaluronidase/β-glucuronidase reaction. The chondroitinase/chondrosulfatase reaction mixture was then reduced with

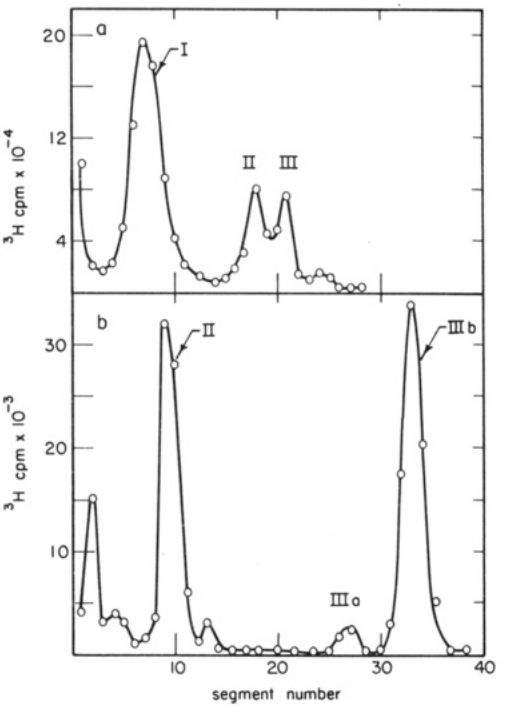

FIGURE 5: Analysis of average DP and reducing and nonreducing terminals of oligosaccharides formed from unlabeled chondroitin sulfate substrates. Chondroitin 4-sulfate was hydrolyzed with hyaluronidase/β-glucuronidase for 30 min at pH 6 and 45 °C. An aliquot of the digest was reduced with NaB^3H_4, treated with a mixture of chondroitinases and chondrosulfatases, and reduced a second time with NaB^3H_4 as described under Experimental Procedures. The resulting mixture was paper electrophoresed for 150 min, and segments of the electrophoretogram were counted to obtain the profile in panel a. Peaks II and III in panel a were eluted together from the paper segments, concentrated, and paper chromatographed in system 2 for 10 h, and the segments of the chromatogram were counted to obtain the profile in panel b. Peaks were identified by comparison with standards: (I) Δ-0S_R; (II) a mixture of Δ-4S_R and Δ-6S_R; (III) a mixture of $GalNAc_R$-6-SO_4 (IIIa) and $GalNAc_R$-4-SO_4 (IIIb). Peak II was eluted from the chromatogram and analyzed for its 4- and 6-sulfated components by high-performance liquid chromatography (Delaney et al., 1980).

NaB^3H_4 a second time to label the GalNAc-4-SO_4, the GalNAc-6-SO_4, and the ΔDi-0S and separated by the procedures shown in Figure 5. The number of ^{3}H cpm in each product was used to calculate (1) the distribution of 4- and 6-sulfated residues obtained from the reducing and nonreducing terminals and (2) the average degree of polymerization (DP) of the oligosaccharide mixture, as follows:

$$DP = [\text{total } ^3\text{H cpm in monosaccharides} + 2(\text{total } ^3\text{H cpm in dissacharides})]/(\text{total } ^3\text{H cpm in monosaccharides})$$

Data showing the composition of the hyaluronidase/β-glucuronidase digestion products and the chondroitin sulfate substrates from which they were derived are shown in Table I.

Table II: Characterization of $^{35}SO_4$-Labeled Oligosaccharides

oligosaccharide	chondroitinase digestion products (mol/mol of nonreducing terminal)				degree of polymerization			
	ΔDi-6S	ΔDi-4S	GalNAc-6-SO_4	GalNAc-4-SO_4	overall	C4S[a]	C6S[a]	6-SO_4/4-SO_4[b]
trisaccharide	0.59	0.34	0.72	0.28	2.9	3.5	2.6	2.1
pentasaccharide	1.39	0.51	0.82	0.18	4.8	6.7	4.4	3.2
heptasaccharide	2.52	0.42	0.82	0.18	6.9	5.6	7.2	5.5

[a] C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate. [b] The ratio of 6-sulfated to 4-sulfated GalNAc residues in the $^{35}SO_4$-labeled proteochondroitin sulfate.

Hyaluronidase/β-Glucuronidase Digestion of Chondroitin Sulfate Synthesized by Chick Embryo Chondrocytes. $^{35}SO_4$-Labeled proteochondroitin sulfate was digested for 4 h with hyaluronidase/β-glucuronidase under the pH 5 digestion conditions, and the tri-, and penta-, and heptasaccharide fractions were isolated by the gel-filtration procedure shown in Figure 3. Each fraction was analyzed by treatment with chondroitinase to convert the oligosaccharide to a mixture containing GalNAc-4-SO_4 and GalNAc-6-SO_4 from the nonreducing terminals and ΔDi-4S and ΔDi-6S from the remaining disaccharide units of the oligosaccharide. The products were separated (Glaser & Conrad, 1979a), and the total $^{35}SO_4$ cpm in each peak were used to calculate the overall DP for each oligosaccharide by using the following relationship:

$$DP = [^{35}SO_4 \text{ cpm in monosaccharides} + 2(^{35}SO_4 \text{ cpm in disaccharides})]/(^{35}SO_4 \text{ cpm in monosaccharides})$$

The overall DP values, given in Table II, are close to the values expected for the tri-, penta-, and heptasaccharides. These same data were then used to calculate two additional DP values—one using only the 4-sulfated mono- and disaccharides and a second using only the 6-sulfated mono- and disaccharides. The latter DP values deviated significantly from the overall DP values of the fractions. This indicates that each oligosaccharide fraction is a mixture of oligosaccharides, all of which have the same DP but some of which are hybrid structures containing both 4- and 6-sulfated GalNAc residues (see Discussion). This was confirmed by the demonstration that each fraction could be partially resolved into two or more peaks by paper chromatography or by high-performance liquid chromatography (Delaney et al., 1980; Knudson, 1982).

Discussion

The β-glucuronidase-coupled hyaluronidase assay used here converts all hyaluronidase substrates to the same product, D-glucuronic acid, at a rate dependent on the hyaluronidase action and under conditions where the transglycosylation reaction was prevented. Several observations confirm the effectiveness of the excess β-glucuronidase in preventing transglycosylation. First, the rate of D-glucuronic acid formation was proportional to the amount of hyaluronidase in the assay and was not increased by increasing the amount of β-glucuronidase. Second, in both the early (Figure 3, Table I) and the late (Figure 3, Table II) stages of the hyaluronidase/β-glucuronidase reaction, the only nonreducing terminals found among the reaction products were GalNAc-SO_4 residues. Chondroitinase treatment of the hyaluronidase/β-glucuronidase-generated products released no detectable levels of Di-4S or Di-6S, which would be formed from the nonreducing terminals if the D-glucuronic acid residues were not completely removed. Finally, digestion of a mixture of [^{3}H]chondroitin and chondroitin [^{35}S]sulfate did not yield doubly-labeled oligosaccharide products.

In this coupled assay, the selectivity in the cleavage of chondroitin sulfates by hyaluronidase is apparent at pH 5 and 37 °C and is accentuated by raising the pH to 6.0 and the temperature to 45 °C and by lowering the ionic strength of the assay mixture. At pH 5, the selectivity manifests itself in the differential initial rates of hydrolysis of chondroitin 4-sulfate and chondroitin 6-sulfate, in the size of the limit oligosaccharides that are formed by exhaustive hyaluronidase/β-glucuronidase digestion of the two substrates, and in the ratios of 6- to 4-sulfated GalNAc residues in the tri-, penta-, and heptasaccharide fractions formed from the $^{35}SO_4$-labeled polymer (Table II). Dorfman & Matalon (1976) noted a difference between the ratios of 4- and 6-sulfated GalNAc residues in chondroitin sulfate and the oligosaccharides formed when the polymer was digested with bovine testicular hyaluronidase and suggested that this may have resulted from a previously undescribed selectivity in the action of hyaluronidase but did not investigate the hyaluronidase reaction further.

The pH 6 assay conditions enhance the selectivity of hyaluronidase, resulting in markedly different rates of hydrolysis of chondroitin 4-sulfate and chondroitin 6-sulfate and in the accentuation of the differences in the sizes of the limit dextrins formed from the two substrates. At pH 6, the combined action of hyaluronidase and β-glucuronidase converts chondroitin 4-sulfate to a mixture of tri-, penta-, and heptasaccharides. In contrast, the limit dextrins formed from chondroitin 6-sulfate have DP's ranging from 7 to 25 monosaccharides per oligosaccharide, with the most abundant products having DP's from 9 to 13.

An attempt to explain the selectivity of hyaluronidase for its substrates requires a consideration of the structural features of the substrates, the cleavage products, and the enzyme itself. A growing body of evidence indicates that chondroitin 4-sulfate and chondroitin 6-sulfate are actually hybrid polymers that are highly enriched in 4-sulfated and 6-sulfated disaccharides, respectively (Seno et al., 1975; Michelacci & Dietrich, 1976; Faltynek & Silbert, 1978). In the present study, the presence of hybrid sequences in the chondroitin sulfate chains is confirmed by several observations. As shown in Table I, one-fifth of the disaccharides in the chondroitin 4-sulfate substrate were 6-sulfated, and one-fifth of the disaccharides in the chondroitin 6-sulfate substrate were 4-sulfated. At pH 6, exhaustive digestion of the chondroitin 4-sulfate yielded a mixture of tri-, penta-, and heptasaccharides but no higher oligosaccharides. Under the same conditions, the chondroitin 6-sulfate gave higher oligosaccharides but almost no tri-, penta-, and heptasaccharides. If each of these substrates were simply a mixture of totally 4-sulfated chains and totally 6-sulfated chains, hyaluronidase/β-glucuronidase digestion of each substrate should have given tri-, penta-, and heptasaccharides in proportion to the amount of 4-sulfated polymer present and higher oligosaccharides in proportion to the amount of 6-sulfated polymer present. The failure to observe both sizes of oligosaccharide products in either digest is an indication

that both of these substrates contain hybrid structures. The finding that the numbers of cleavage sites observed for chondroitin 4-sulfate and chondroitin 6-sulfate after exhaustive digestion at pH 6 are more or less proportional to the percentage of 4-sulfated disaccharides in the substrates suggests that cleavage occurs primarily at the 4-sulfated disaccharide residues. The data in Table I are consistent with this suggestion. If this is the case, then the average sizes of the oligosaccharide products formed at pH 6 suggest that the 4-sulfated and 6-sulfated disaccharides in these chains are distributed randomly rather than in block sequences, in agreement with the conclusion of Michelacci & Dietrich (1976). Similar structural features may be ascribed to chondroitin [^{35}S]sulfate obtained from chondrocyte cultures. Digestion of this substrate at pH 6 did not yield any of the smaller oligosaccharides typical of chondroitin 4-sulfate sequences.

A second indication that the chondroitin [^{35}S]sulfate is a hybrid of 4- and 6-sulfated disaccharides is that the tri-, penta-, and heptasaccharides formed by exhaustive digestion at pH 5 are mixtures of hybrid oligomers, as indicated by the differences of the DP's calculated for the 4-sulfated or the 6-sulfated chondroitinase products from the overall DP calculated for the same fraction. For example, chondroitinase digestion of a trisaccharide fraction containing a mixture of totally 4-sulfated trisaccharides and totally 6-sulfated trisaccharides should yield equimolar amounts of GalNAc-4-SO_4 and ΔDi-4S plus equimolar amounts of GalNAc-6-SO_4 and ΔDi-6S. Thus, the same DP value would be obtained in calculations with the 4-sulfated products alone, the 6-sulfated products alone, or both the 4- and 6-sulfated products. This would not be the case if hybrid structures were present in the mixture, except by coincidence (e.g., if equal amounts of complementary hybrid structures were present). The same argument applies to the penta- and heptasaccharide fractions or, in fact, to mixtures of oligosaccharides having a range of DP's (Glaser & Conrad, 1979a). Thus, the data in Table II prove that the oligosaccharides derived from chondroitin [^{35}S]sulfate are mixtures of hybrid structures.

The substrates may also be described in terms of their secondary structures. The polymer conformations of chondroitin 4-sulfate and chondroitin 6-sulfate have been examined both by X-ray diffraction analysis of the solid polymers (Adkins et al., 1974; Arnoff et al., 1975; Rees, 1975; Comper & Laurent, 1978) and by solution studies (Scott & Tigwell, 1978; Heatley et al., 1982; Scott et al., 1981; Cowman et al., 1981; Welti et al., 1979; Morris et al., 1980; Scott & Heatley, 1979) and appear to be identical in both the solid and the solution states. The data indicate that the polysaccharide chains of chondroitin 4-sulfate and chondroitin 6-sulfate are coiled identically over the pH range used in this study, with the glycosidic bonds close to the helix axis and the sulfate ester groups extending well beyond the normal periphery of the helix. The primary difference in the polymer conformations of the two substrates lies in the greater distance of the 6-sulfate ester groups from the helix center then the 4-sulfate esters and in the higher rotational freedom of the 6-sulfate groups resulting from their attachment to exocyclic carbons. Since at pH 6 the substrates appear to be cleaved only in the regions of the 4-sulfated disaccharides, this suggests that the projecting 6-sulfate groups prevent contact between the enzyme active site and the glycosidic bonds of the 6-sulfated *N*-acetyl-D-galactosamine residues.

The striking change in hyaluronidase specificity observed when the pH is raised from 5 to 6 is most likely due to a change in the structure at the active site of the enzyme rather than to altered substrate structures since neither the conformation of the substrates nor the ionization of their uronic acid carboxyl groups (Mathews, 1961) is altered in this pH range. The ovine enzyme used in this study is closely related to the bovine testicular hyaluronidase (Meyer et al., 1960; Meyer, 1971), which appears to have active site amino acid side chains with pK_a's of 4.5 and 6–7 (Highsmith et al., 1975). This suggests the possibility of an electrostatic repulsion between a catalytic side chain of the enzyme and the sulfate ester groups exposed on the surface of chondroitin 6-sulfate. Consistent with this suggestion is the finding that raising the ionic strength of the pH 6 assay medium increases the rate of hydrolysis of chondroitin 6-sulfate but has relativley little effect on the rate of hydrolysis of chondroitin 4-sulfate (data not shown). However, further studies are required for definitive characterization of these enzyme–substrate interactions.

It is not clear whether the pH effects on hyaluronidase activity might be of physiological significance. However, it may be possible to take advantage of these pH effects and the methodology described here in the structural characterization of chondroitin sulfates from various sources.

Acknowledgments

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Registry No. Chondroitin 4-sulfate, 24967-93-9; chondroitin 6-sulfate, 25322-46-7; hyaluronidase, 9001-54-1.

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Fluorescence of Fluorescein Attached to Myosin SH_1 Distinguishes the Rigor State from the Actin-Myosin-Nucleotide State†

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ABSTRACT: It has been found that the fluorescence intensity of 5-(iodoacetamido)fluorescein (5-IAF) attached to the SH_1 of myosin subfragment 1 (S-1) increases 3-fold on formation of the rigor complex. On adding Mg^{2+}-ADP, light scattering indicates no dissociation, but the fluorescence increment disappears. Thus, this fluorescence signal can distinguish the rigor state from other states, especially from ternary complexes such as actin-myosin-nucleotide. We demonstrate that by using this signal we can measure spectroscopically several kinetic parameters of acto-S-1-nucleotide interaction: In the presence of 20 mM KCl, 2 mM $MgSO_4$, and 10 mM TES (pH 7.5) at 22 °C, Mg^{2+}-ADP binds to acto-S-1_5* (S-1_5* denotes 5-IAF-labeled S-1) with a $K_a = 2 \times 10^6$ M^{-1}, and Mg^{2+}-PP_i binds to acto-S-1_5* with two apparent affinities, $K_a = 8 \times 10^4$ M^{-1} and 1.4×10^3 M^{-1}; the association rates of Mg^{2+}-ADP and Mg^{2+}-ATP for acto-S-1_5* are 10^7 s^{-1} M^{-1} and 4×10^6 s^{-1} M^{-1}, respectively, and the dissociation rate of Mg^{2+}-ADP for acto-S-1_5* is 5 s^{-1}. In contrast to the fluorescence intensity of the dye, the lifetime and the absorbance are essentially unaffected by complex formation with F-actin or nucleotides. Therefore, we conclude that there must be a static quencher such as Trp, Tyr, or Met in the neighborhood of the attached dye and that the contact between dye and quencher is modulated by actin-induced or nucleotide-induced conformational changes in S-1.

Since muscle contraction is brought about by the cyclic interaction of actin-myosin-ATP, it is quite important to have fast indicators sensitive to intermediate states of this interaction. If the responses of such indicators (chemical signal) are available, we can apply them in vivo and try to correlate the chemical states of myosin cross-bridges with a physiological parameter such as tension or with a microscopic parameter such as cross-bridge inclination. Especially, we can then study such correlations in the time domain and deduce kinetic parameters (Morales, 1982). Although tension transients induced by quick stretch or release of isometrically contracting fibers have been studied (Huxley & Simmons, 1971, 1973), no one has yet tried to see directly and simultaneously the transients of chemical states along with the tension transients. Recently, a new type of fiber transient has been introduced by Goldman et al. (1982), by employment of flash photolysis of "caged ATP". The tension transient following quick conversion of caged ATP into ATP has been observed in the absence and presence of Ca^{2+}. But direct observation of chemical states of myosin cross-bridges was not made in that study. Rotational motion of myosin cross-bridges has been studied by analyzing

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