THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 38, Issue of September 23, pp. 23617–23624, 1994
Printed in U.S.A.

Intramolecular Self-cleavage of Polysialic Acid*

(Received for publication, May 20, 1994, and in revised form, July 12, 1994)

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Polysialic acid (PSA) is an unusual homopolymer of sialic acid (Sia) found on a limited number of animal glycoproteins and in the capsules of certain pathogenic bacteria. The biological properties of PSA are known to vary markedly with the length of the polymer. We confirm here that while the primary linkage unit of PSA (Siaα2–8Sia) is more stable than commoner Sia linkages, PSA with >3 Sia units is substantially more labile. A "limit digest" of PSA yields fragments of degree of polymerization (DP) = 2 and 3 and little monomeric Sia. In keeping with this, the fragmentation of PSA of DP 4 is non-random, with the internal glycosidic bond being more labile than those at the two ends. The accelerated breakdown of PSA involves an intramolecular mechanism that is not explained by lactone formation, cation effects, or specific secondary structural features. However, it is dependent upon the intactness of internal carboxyl groups, which have an anomalously high pK_a. Thus, the instability of PSA appears to result from intramolecular self-cleavage of the glycosidic bonds of internal Sia units, in which the adjacent carboxyl group with a high pK_a acts as a proton donor for general acid catalysis. This lability of PSA is seen under mildly acidic conditions that can be encountered in various physiological and pathological situations and thus has potential implications for neuronal adhesion, embryogenesis, and bacterial pathogenicity.

Sialic acid (Sia)[1] is a common terminal monosaccharide on many animal glycoconjugates (1). Polysialic acid (PSA) is a rare homopolymer of Sia found only on a few animal glycoproteins and in certain bacterial polysaccharides (2–6). In animals, PSA with the structure (Siaα2–8Sia)$_n$ (where n = 3 to >50) has been extensively studied as a component of the neural cell adhesion molecule (N-CAM), wherein it modulates neural development and plasticity (2, 3, 7–12). In certain bacteria, pathogenicity depends upon capsular PSA (2, 6, 13–15), which is non-immunogenic and prevents host complement activation. PSA is known to have some unusual length-dependent properties. NMR and molecular dynamics studies indicate that PSA with a degree of polymerization (DP) of >3 develops a distinctive tertiary structure (13, 16–20), wherein the Sia units at each end have a conformation different from that of the inner residues (16). Also, several monospecific antibodies recognize PSA only when DP is >8–10 (13, 20–23), and two highly specific microbial enzymes selectively recognize PSA of extended length (5, 24–26).

Of all the glycosidic linkages found in mammalian oligosaccharides, those of Sias are the most labile, being relatively easily cleaved by heating in mild acid (1). However, scattered statements in the literature suggest that linkages in PSA may be even more labile than those of single Sia residues when they exist as the terminal units of heterogenous oligosaccharide chains. Thus, PSA on N-CAM is easily degraded during preparation for SDS-polyacrylamide gel electrophoresis gels (*i.e.* boiling in pH 6.5 sample buffer) (27, 28), PSA breaks down even during prolonged freezer storage,[2] and PSA is sometimes deliberately degraded into fragments for analysis by incubation in very mild acid (29) or by boiling at neutral pH (27, 28, 30). The primary glycosidic linkage in PSA (Siaα2–8Sia) also occurs in certain glycolipids (gangliosides). In striking contrast to the situation with PSA, the very same linkage in gangliosides is reported to be very difficult to cleave quantitatively by acid hydrolysis without degradation of the monosaccharides themselves (31). In fact, some early work specifically states that α2,8-linked Sia units are more stable than other types of Sia linkages in nature (32). Here, we explore these apparently contradictory statements about the stability (or lack thereof) of the Siaα2–8Sia linkage. We show that long PSA (DP >3) is substantially more unstable than PSA of DP 2 or DP 3 (hereafter called "oligosialic acid" or OSA),[3] pursue the mechanisms responsible for this difference, and provide indications of its biological relevance.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: PSA of DP ~100, EY Labs; colominic acid (PSA of DP ~12), hyaluronic acid, chondroitin sulfate, heparin, and sialyllactose, Sigma; deuterated water, dimethyl sulfoxide-d_6, and tetramethylsilane, Aldrich; Neu5Ac, Kantoishi Chemicals (Japan). [^{3}H]Neu5Ac, [^{3}H]Neu5Ac-labeled fetuin, and [^{3}H]PSA labeled at the reducing end were prepared as previously described (33). ^{3}H-Labeled and unlabeled OSAs containing 2–10 Sia residues were isolated by HPLC as described below. The *Escherichia coli* K1 strain was provided by Dr. Richard Silver (FDA), and PSA isolated from *E. coli* K92 (containing alternating α2,8/α2,9 linkages) was a generous gift of Dr. William Vann (FDA). All other chemicals were of reagent grade or better and were obtained from commercial sources.

Sialic Acid Assays—The hydrolysis of each α2,8 glycosidic linkage found in PSA is accompanied by the appearance of a new reducing end as well as the appearance of a new nonreducing end in equimolar ratio.

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[1] The abbreviations used are: Sia, sialic acid, type unspecificied; DP, degree of polymerization; PSA, polysialic acid (DP >3); OSA, oligosialic acid (DP 2 and DP 3); HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; N-CAM, neural cell adhesion molecule.

[2] H. Higa and A. Varki, unpublished observations.

[3] Traditionally, the term PSA has been used for molecules with even the smallest degree of polymerization (*i.e.* even DP 2). Since we show here that polymers of DP 2 or DP 3 are substantially less labile than those with DP >3, we suggest that the former be called "oligosialic acids" (OSA). This suggestion also takes into account the finding that PSA begins to attain a different conformation as soon as the DP becomes >3 (16).

Thus, the degradation of PSA was quantitated using either a reducing end assay or by a periodate:formaldehyde assay for the detection of terminal Sias (34). The amount of Sia detected by these assays is equivalent to the amount of α glycosidic linkage hydrolyzed. To permit comparisons, the rate of hydrolysis of glycosidic linkages was always expressed as percent of total available linkages. For each experiment, total α2,8 linkages in the sample was quantitated by measurement of free Sia after hydrolysis with 0.1 M H_2SO_4 at 80 °C for 90 min. In some cases, the release of monomeric Sia was quantitated using the thiobarbituric acid assay (32).

The size of PSA was analyzed by two different methods. Using colorimetric analysis, the size was determined by dividing the total Sia content of the sample by the amount of total reducing end or nonreducing end. With a mixture of sizes, the value obtained represents an average. Thus, PSA purchased from Sigma "colominic acid" was found to be a mixture with an average size of 12 ± 3 Sia residues, whereas the PSA from EY labs was of DP ~100. The size of labeled PSAs was determined from elution time on a Mono-Q HR 5/5 anion exchange HPLC column (the size of PSA is proportional to its total charge). This analysis was carried out as described (35) with modifications to improve resolution of PSA species in a linear gradient. Best separations were obtained when the column was first equilibrated in 10 mM Tris/HCl, pH 7.4, at 1 ml/min, and elution was accomplished with a linear gradient from 0 to 0.2 M sodium sulfate in the same buffer. After sample application, the column was maintained in 10 mM Tris/HCl, pH 7.4, for 5 min, and the gradient was applied over a period of 25 min. The column was then maintained at 0.2 M sodium sulfate in 10 mM Tris/HCl, pH 7.5, for an additional 5 min and finally returned to the starting buffer over a period of 5 min. Unlabeled PSA was monitored using a UV detector at 205 nm, and labeled PSA was monitored with an on-line flow radioactivity detector (Flo-One Beta).

Isolation of DP 2, DP 3, and DP 4 Sialic Acid Polymers—PSA (200 μmol total sialic acid) was partially hydrolyzed in 0.1 M sodium acetate buffer, pH 4.5, at 48 °C for 20–22h. The hydrolysate was dried down, dissolved in 5 ml of 20 mM Tris HCl, pH 8.0, and applied onto a column (15 × 1.5 cm) of DEAE-Sephadex A-25 equilibrated in 20 mM Tris HCl, pH 7.2. The column was washed with 45 ml of equilibration buffer, and the OSAs were eluted with a 160-ml linear gradient from 0 to 0.4 M NaCl in the same buffer. Fractions were collected and aliquots assayed for Sia content by the tetrabutylammonium assay after hydrolysis in 0.1 M H_2SO_4 at 80 °C for 90 min. Complete resolution of PSA oligomers of size DP 2–DP 4 was obtained.

Preparation of Metabolically Labeled E. coli K1 Capsular PSA—E. coli K1 was grown overnight in 10 ml of L broth medium with 5 mM Neu5Ac at 37 °C to induce expression of the Sia permease (36). The cells were pelleted by centrifugation at 10,000 × *g*, resuspended in fresh media (10 ml) containing 2.5 × 10^6 cpm of [^{3}H]Neu5Ac, and incubated overnight at 37 °C. The uniformly labeled PSA from the medium was precipitated with 0.3% cetyltrimethylammonium bromide, dissolved in 1 M $CaCl_2$, and reprecipitated with 80% ethanol (33).

Assay for the Release of Labeled Capsular Polysialic Acid from E. coli K1—E. coli K1 cells were metabolically labeled as described above with [^{3}H]Neu5Ac, which is exclusively incorporated into the PSA capsule (37). Labeled cells were washed twice with 1 ml of 0.1 M NaCl, and aliquots (12,000 cpm each) were incubated in 50 mM MOPS, pH 6.0, at 37 °C on a rotary agitator. At various times, duplicate samples were removed and centrifuged at 7000 × *g* for 7 min; the supernatant medium and pellet was monitored for radioactivity (all radioactivity remained in high molecular weight PSA; data not shown).

Synthesis of Carboxylic Acid Derivatives of PSA—The carboxyl groups of PSA (Sigma) were partially converted into the methylamide by activation with EDC, followed by treatment with methylamine. For preparation of the fully lactonized form of PSA, we followed the procedure of Lifely *et al.* (38) using the large polymer obtained from EY labs. The precipitated lactonized polymer was extensively washed with water, lyophilized, and then dissolved in dry dimethyl sulfoxide. Tetrabutylammonium borohydride was added to reduce the lactones, and the reaction was allowed to proceed at room temperature for 5 days with constant stirring. A control was set up without addition of tetrabutylammonium borohydride. Both samples were extensively dialyzed against distilled water, lyophilized, redissolved in water, and further desalted by gel filtration on a Sephadex G-50 column. Fractions positive for Sia eluted at the void volume of the column and were pooled for further studies. Aliquots of the intact lactone were also treated with base (10 mM NaOH at 37 °C for 0.5 h) to restore the molecule to the native state with carboxyl groups.

NMR—^{1}H and ^{13}C NMR spectra were obtained using a Varian Unity 500 MHz NMR spectrometer with the temperature maintained at 23 °C. The water-soluble polymers, such as PSA, OSAs, PSA alcohol, and PSA amide derivatives, were submitted to deuterium exchange by repeated solubilization in 98% D_2O followed by lyophilization. Between 10 and 60 mg of each sample, dissolved in 0.4 ml of 99.9% D_2O, were studied. Acquisition times ranged from overnight to 1 h for ^{13}C experiments to obtain an adequate signal to noise ratio. Chemical shifts were calculated relative to tetramethylsilane (measured relative to internal acetone at 31.07 ppm ^{13}C and 2.225 ppm for ^{1}H NMR). Water-insoluble polymers, such as PSA lactone, were analyzed after dissolving in dimethyl sulfoxide-d_6, and chemical shifts were, in these cases, measured relative to the dimethyl sulfoxide methyl signal (39.5 ppm).

Microtitration of OSA and PSA—OSA and PSA samples were dissolved in water and passed through a Dowex AG 50-H^+ column to convert all carboxylic groups to the protonated form. After washing the sample through the column with water, the final solution was immediately taken to full volume in graduated flasks (5 ml) and promptly analyzed. The final concentrations ranged from 2–12 mM (in carboxylic acid). A 1-ml aliquot was titrated with a 0.1 M NaOH solution, the concentration of which had been previously checked with benzoic acid using the same microtitration apparatus. The solution was placed at the bottom of a 20-ml glass tube with a side arm into which a magnetic bar and a pH meter electrode were introduced through the top. Nitrogen was continuously flushed through a thin tubing also introduced from the top of the glass tube. A 10-ml syringe containing the NaOH solution was placed in a syringe pump on the right side of the tube. The syringe was equipped with a 25-cm needle that was bent and introduced through the side arm of the glass tube. The syringe pump was previously calibrated to monitor the number of nanoliters of base delivered per second. Titration was performed by constant addition of the base using the pump at constant speed and registering the pH and the time. Data were graphed (pH *versus* volume), and pK_a values were calculated from the first derivative of the curves.

RESULTS AND DISCUSSION

Basic Assumptions Used for the Study of PSA Lability—As shown below, the glycosidic bonds within PSA are quite labile, even in conditions of pH and temperature encountered in some physiological situations. For practical reasons, the hydrolysis of Sia glycosidic linkages in various molecules was compared under the more acidic of these conditions, wherein results could be obtained in shorter time frames. In each experiment, the cleavage rate of Sia linkages in different compounds is expressed as percent hydrolysis of total available Sia glycosidic linkages in the sample (total determined after complete hydrolysis of an aliquot; see "Experimental Procedures"). This assumes that all Sia glycosidic bonds in the sample are equally susceptible to hydrolysis and negates any differences due to the concentration of the sample. Expressing the results in this manner also makes it possible to directly compare the breakdown rates of homopolymers of different size.

The Lability of the α2–8 Linkage of PSA Is Size-dependent—The α2–8 linkages of PSA of average DP ~14 break down very easily in mildly acidic conditions (Fig. 1*A*) at a rate much faster than that for monomeric Sia in α2-6 linkage to *N*-linked oligosaccharides on a glycoprotein (Fig. 1*B*). In contrast to PSA, the α2-8 linkage in OSA of DP 2 required much harsher conditions for breakdown (Fig. 1*C*). In fact, cleavage of DP 2 requires such harsh conditions that yield is lowered by some destruction of the Sia units themselves. The rates under milder conditions appear to be size-dependent, with DP 4 breakdown being faster than that of DP 2 but less than that of DP ~8 (Fig. 1*D*). The hydrolysis rate difference between the short OSA and longer PSA was so great that conditions could not be found to directly compare their breakdown. For example, mild conditions that cleaved 72% of the linkages in PSA of DP >100 gave no detectable cleavage of OSA of DP 2 (data not shown). Thus, the identical chemical bond (Siaα2–8Sia) shows a markedly different acid stability in PSA and OSA.

A "Limit Digest" of PSA Yields OSA and Little Monomeric Sia—Uniformly labeled PSA of DP >100 was obtained from metabolically labeled *E. coli* K1 (see "Experimental Proce-

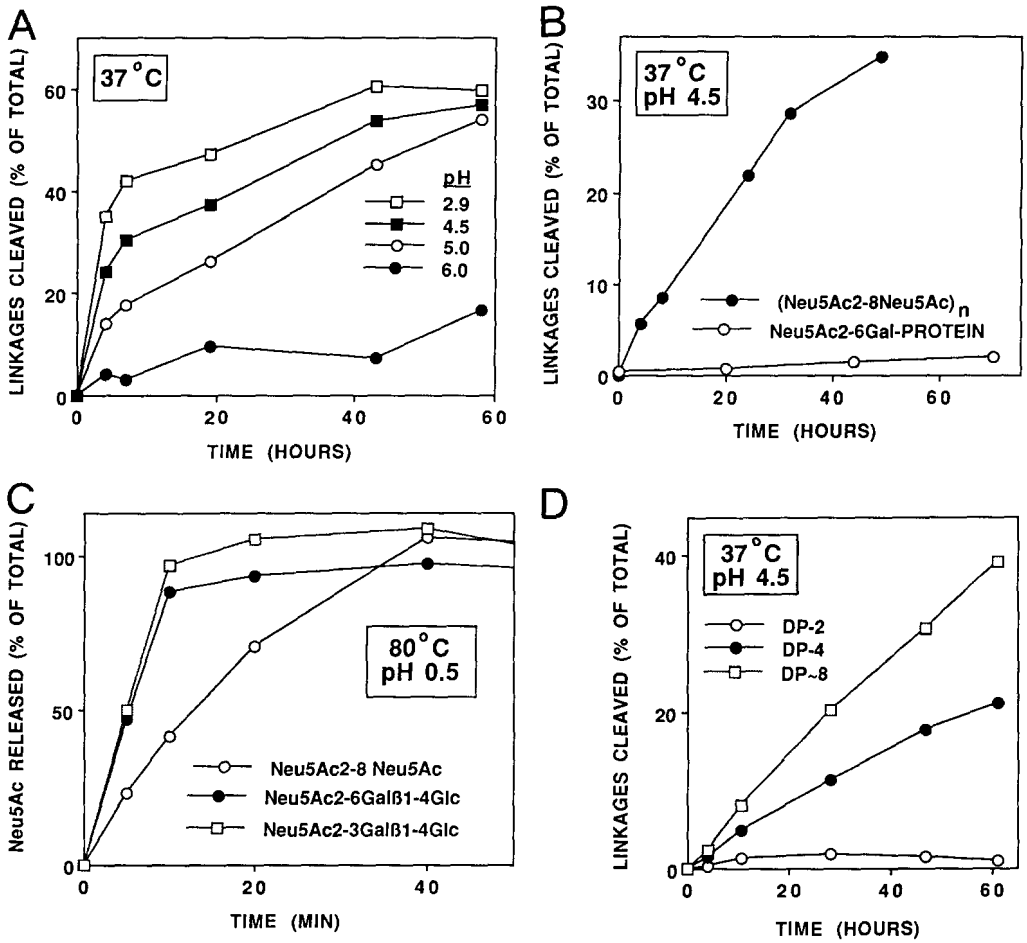

FIG. 1. **Hydrolysis of glycosidic bonds in mono-, oligo-, and polysialic acids.** *A*, reactions (200 µl) containing PSA (1.6 µmol of total Sia, average DP ~14) in 0.1 M sodium acetate buffer were incubated at 37 °C at differing pH. At the indicated times, duplicate aliquots were assayed for reducing end sugar. *B*, hydrolysis of PSA at pH 4.5 was monitored, as in *A*. Release of α2-6-linked Sia (Neu5Ac, *N*-acetylneuraminic acid) under identical conditions was monitored by following release of free [^{3}H]Neu5Ac from asialofetuin resialylated with rat liver α2-6-sialyltransferase and CMP-[^{3}H]Neu5Ac. *C*, the three oligosaccharides shown in the figure were treated with 0.1 M H_2SO_4 at 80 °C, and aliquots were monitored at various times for release of free Neu5Ac by the thiobarbituric acid assay (32). The 100% values for each were determined on separate aliquots subjected to complete acid hydrolysis. *D*, polymers of DP 2, DP 4, and DP ~8 were isolated from partially hydrolyzed PSA, and breakdown at pH 4.5 was monitored exactly as in *A*. Note that DP 2 is the same as Neu5Acα2-8Neu5Ac in *C*.

dures"), and the kinetics of hydrolysis were analyzed under mildly acidic conditions. The untreated polymer had too high a charge density to elute from an anion exchange column (Fig. 2). After exposure to mild acid, fragments could be eluted and resolved. At 12 h, very little monomer appears, and the major species are oligomers in the DP 2–DP 5 range. Further hydrolysis primarily yields DP 2 and DP 3, still with very little monomer (data not shown). Thus, the terminal linkages of the fragments (at both ends) must be much more stable than the internal linkages. Interestingly, uniformly labeled DP 3 and DP 4 do not resolve well in this anion exchange chromatography system (Fig. 2). This phenomenon has been previously noted (29, 35) and attributed to possible differences in tertiary structure. However, it may actually relate to differences of pK_a between the internal and terminal bonds of DP 4 (see below).

Fragmentation of DP 4 Is Non-random—A mixture of uniformly labeled DP 3 and DP 4 (from an experiment like that in Fig. 2) was used to compare their breakdown under mildly acidic conditions. Under conditions where substantial breakdown of DP 4 to DP 2 has occurred, very little cleavage of bonds in DP 3 is seen (Fig. 3*A*), and the time course of monomer appearance is much slower (Fig. 3*B*). Thus, DP 4 molecules undergo selective cleavage of their internal bonds while the terminal bonds of both DP 3 and DP 4 are much more stable. Similar studies with uniformly labeled DP 5 to DP 8 also

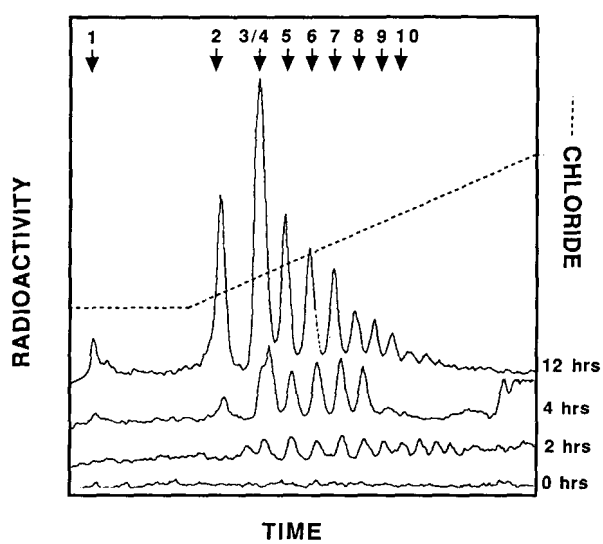

FIG. 2. **Products of PSA breakdown.** Uniformly labeled PSA isolated from the medium of K1+ *E. coli* cells fed with [^{3}H]Neu5Ac was hydrolyzed at pH 4.5 and 48 °C. At various times, aliquots were analyzed for breakdown using anion exchange HPLC, as described under "Experimental Procedures." The effluent was monitored for radioactivity with an on-line detector.

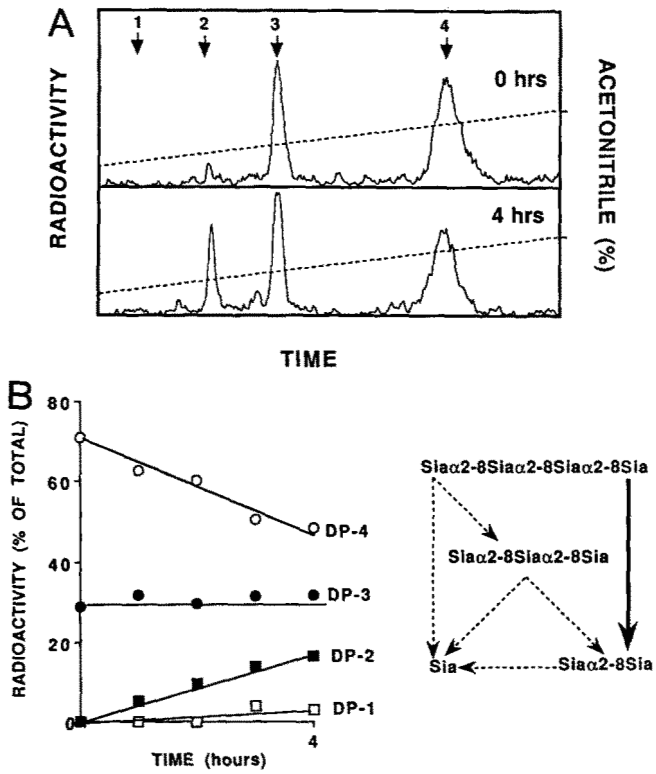

FIG. 3. **Kinetics of breakdown of DP 3 and DP 4.** *A*, a mixture of uniformly labeled OSA of DP 3 and DP 4 (see Fig. 2) was fractionated by HPLC on a Varian Micropak AX-5 column with a gradient of 50–35% acetonitrile over 30 min in 70 mM NaH_2PO_4 at a flow of 1 ml/min. The effluent was monitored for radioactivity with an on-line detector. *B*, the time course of breakdown of DP 3 and DP 4 was monitored as shown in *A*. The major (*heavy arrow*) and minor (*dotted arrows*) pathways of breakdown are indicated in the *inset*.

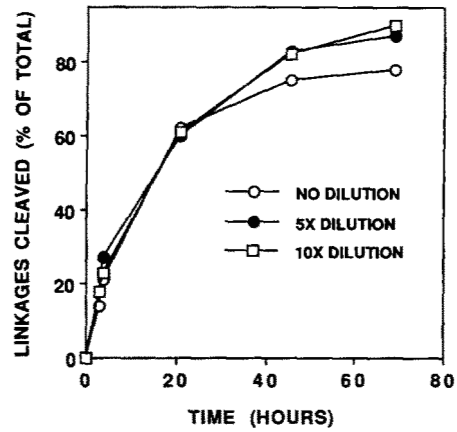

FIG. 4. **Lack of effect of dilution upon the rapid breakdown of PSA.** Three reaction mixtures (200, 1000, and 2000 μl) containing 1.6 μmol of PSA in 0.1 M sodium acetate, pH 4.5, were incubated at 47 °C. At indicated times, proportional fractions of each mixture were assayed in duplicate for the appearance of new nonreducing ends.

showed accumulation of dimers and trimers with little monomer formation (data not shown). Thus, in all cases, the glycosidic linkages of the two terminal Sia units are very stable while the internal linkages are labile. This can explain the observation that DP 2 and DP 3 (no internal linkages) are stable while all molecules with DP >3 are labile. Thus, the apparent increase in lability between DP 4 and DP ~8 (Fig. 1*D*) mainly reflects the increased proportion of labile internal bonds in the latter.

The Accelerated Breakdown of PSA Involves an Intramolecular Mechanism—Extensive dilution of PSA does not affect the hydrolysis rate as long as the pH is held constant (see example in Fig. 4). Also, addition of excess unlabeled PSA does not affect the breakdown rate of tracer amounts of radioactively labeled PSA (data not shown). Furthermore, when unlabeled PSA is mixed with tracer amounts of radioactive monomeric Sia on proteins, no cleavage of the latter occurs (data not shown). Taken together, these results indicate that PSA breakdown involves an intramolecular self-cleavage reaction.

Possible Mechanisms for the Accelerated Breakdown of PSA—The relative instability of the Siaα2–8Sia linkage of PSA is first apparent when DP = 4. This is the minimum polymer size with a glycosidic bond not at one end or the other of a chain, and it is this internal bond that is more labile. Why is this so? Based upon prior knowledge of PSA, three possibilities can be considered. First, PSA is known to develop size-dependent changes in tertiary structure (13, 17–20), which might render the internal bonds more labile. Second, internal lactone formation (between the carboxyl group and the 9-hydroxyl) can occur in α2-8-linked Sia under mildly acidic conditions (38), and this could possibly further destabilize the tertiary structure mentioned above. Conversely, preferential lactone formation in DP 2 and DP 3 could be responsible for selectively stabilizing these short oligomers. Finally, prior analyses of synthetic acidic homopolymers (*e.g.* polymethacrylic acid) have shown that an increasing pK_a of carboxyl groups occurs with increasing DP (39). If this phenomenon also occurs in PSA, an unusual situation would result, wherein at relatively high pH values an internal glycosidic linkage oxygen would be immediately adjacent to a carboxyl group with a potential donor proton.

Lactone Formation Is Not Responsible for Stabilization of Oligosialic Acids—A freshly prepared "limit hydrolysate" of PSA (as in Fig. 2, containing mainly DP 2 and DP 3) was directly injected onto an AX-5 HPLC column (as in Fig. 3*A*). Under the analytical conditions used (pH <6), lactones are stable, and lactonized DP 2 and DP 3 are separated from their parent compounds. No significant lactonization of DP 2 or DP 3 was seen (data not shown), indicating that their stability cannot be attributed to lactone formation. On the other hand, while PSA forms lactones more easily under mildly acidic conditions (38), the degree of lactonization at pH 4–5 is low (38), and it is difficult to envisage a mechanism wherein a few lactones would explain the overall destabilization. Furthermore, 9-*O*-acetylation of PSA (which would abolish lactone formation) does not stabilize the molecule (see below). Thus, if lactone formation is involved in destabilization, it does not play a major role.

Limited Effects of Denaturants and Cations upon PSA Breakdown—Heating to 89 °C or adding 5 M urea at pH 7.5 had no significant effect on breakdown rate when the pH was subsequently adjusted to 4.5. However, heating to 89 °C in 5 M urea at pH 7.5 prior to pH adjustment decreased the rate ~3-fold (data not shown). This small effect is reversible upon dialysis, indicating a lack of unwanted side reactions. These results could be explained by loss of a conformation required for intramolecular self-cleavage or by disruption of critical hydrogen bonds involved in the reaction. In fact, urea is known to eliminate electrostatic interactions and to lower the pK_a of carboxyl groups (40). Similar results were obtained with guanidine hydrochloride (data not shown). Since PSA is a polyanion, cations could also affect the rate of intramolecular self-cleavage either by disrupting tertiary structure or by interfering with the carboxyl group charge. Monovalent cations at high concentrations (0.75 M) caused a slight but consistent inhibition of breakdown while some (but not all) divalent cations also had a slight inhibitory action (data not shown). Taken together, these results suggest that the carboxyl group and its ionization state might

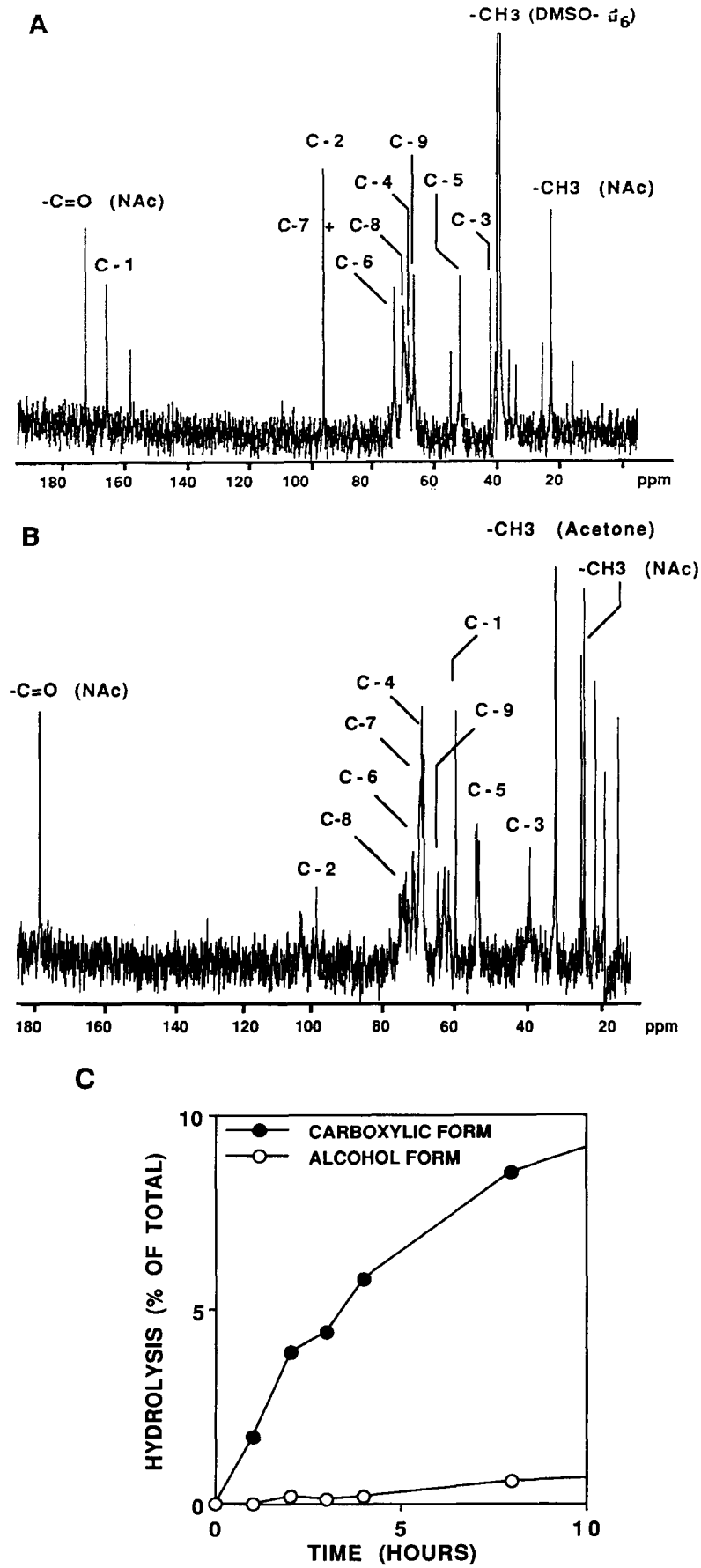

FIG. 5. **Reduction of carboxyl groups stabilizes PSA.** Perlactonized PSA was prepared as described under "Experimental Procedures" and characterized by ^{13}C NMR (*A*). Aliquots were reduced with tetrabutylammonium borohydride in dimethyl sulfoxide (*DMSO*) (alcohol form) or restored to the original state by base treatment (carboxylic acid form) as described under "Experimental Procedures." Conversion to the alcohol form was confirmed by ^{13}C NMR (60), which showed disappearance of the lactone carbon signal (δ = 167.60 ppm), with appearance of the hydroxylmethyl carbon signal (δ = 60.88 ppm) (*B*). No residual lactone *N*-acetyl carbonyl signal (δ = 174.3 ppm) was observed, indicating a remaining proportion of lactone of <5% (detection limit). The alcohol form and carboxylic acid form were analyzed for breakdown at pH 4.5 and 48 °C as described in the legend to Fig. 1*A* (*C*).

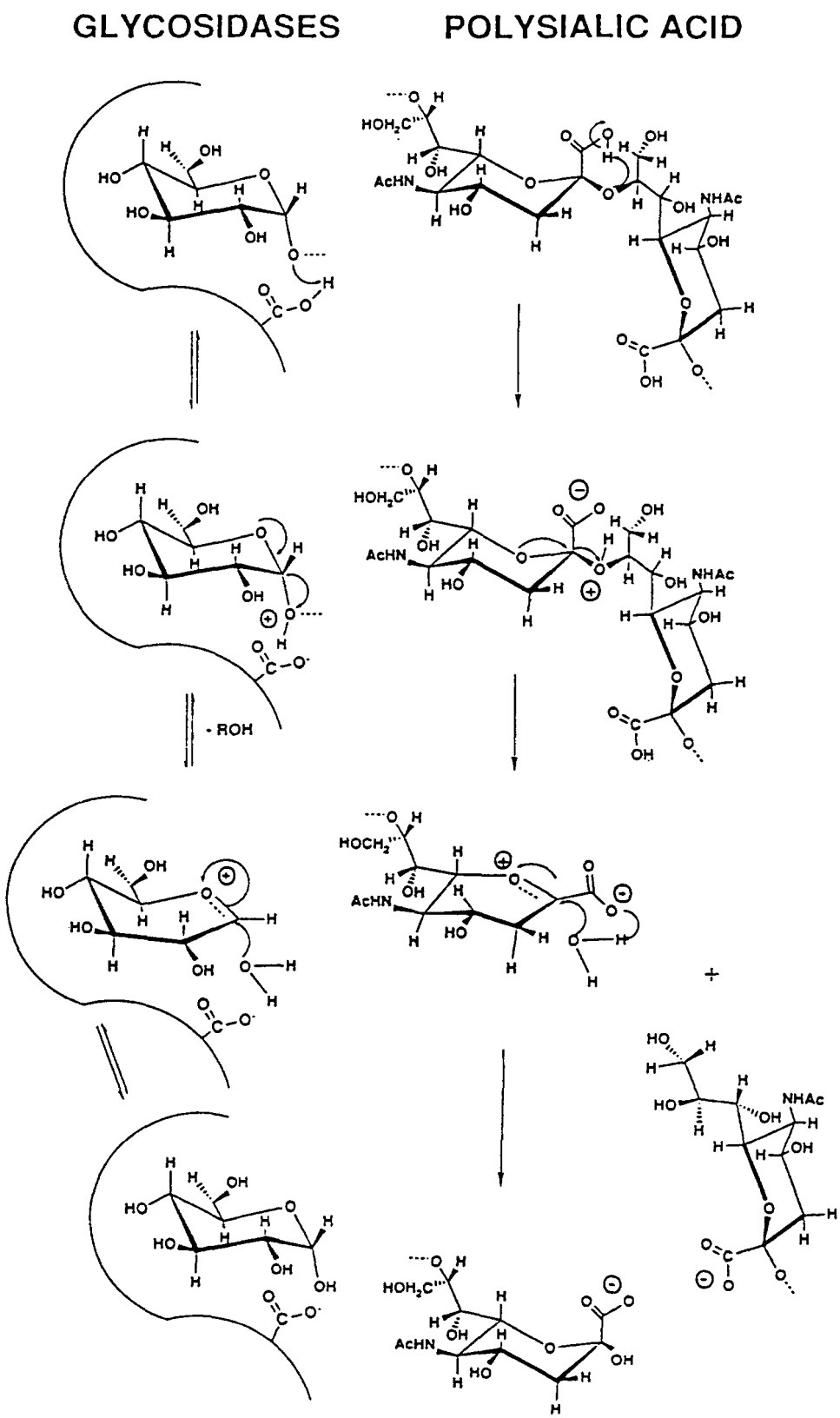

FIG. 6. **Proposed mechanism of intramolecular self-cleavage of PSA.** The mechanism for self-cleavage of PSA (*right side*) probably involves a protonated carboxyl group with an anomalously high pK_a immediately adjacent to the glycosidic bond that is eventually cleaved. The *left side* shows the well accepted mechanism of many glycosidases, in which the first step involves a proton donor (usually a carboxyl group) with an anomalously high pK_a.

be important in the reaction mechanism.

Effects of Selective Modifications of PSA Structure on the Rate of Intramolecular Self-cleavage—The *E. coli* K92 (2) capsular polysaccharide (an alternating polymer of α2-8- and α2-9-linked Sia residues) showed a rate of spontaneous breakdown similar to that of the α2-8-linked homopolymer (data not

shown), indicating that lability is not a unique feature of the specific linkage type. Homopolymeric PSA containing *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, or ketodeoxynonulosonic acid all show a similar degree of instability in mildly acidic medium (29), indicating that the type of substitution at the 5 position is not important. Adding 7- or 9-*O*-acetyl esters to ~45% of the Sia units of PSA with a specific *O*-acetyl-transferase from *E. coli* (26) had no detectable effect upon the rate of hydrolysis (data not shown). Since the bulky 7- or 9-*O*-acetyl esters adjacent to the glycosidic linkage at the 8 position would substantially change the conformation of the molecule, these results further indicate that precise tertiary structure is not critical for destabilization. In contrast, modifications of the carboxyl group had striking effects. Conversion of ~40% of the carboxyl groups into methylamides (proved by ^{1}H NMR analysis) slowed the reaction by ~75% (data not shown). Near complete conversion of the carboxyl groups to primary alcohols (>95%, proven by ^{13}C NMR analysis; see Fig. 5, *A* and *B*) reduced the rate of hydrolysis by >90% (Fig. 5*C*). Thus, the carboxyl groups of Sia residues must play a critical role in the intramolecular self-cleavage of PSA.

The Carboxyl Groups of PSA Have an Anomalously High pK_a, Which Can Explain the Instability of the Adjacent Glycosidic Linkages—The pK_a of the carboxyl group of monomeric Sia is known to be rather low for a carboxylic acid, being in the range of 2.2–3.0 in various studies (1). Since the acid lability of monomeric Sia linkages markedly increases as the pH approaches this pK_a (41), it was suggested that a 5-membered ring might be formed, involving the protonated carboxyl group and the adjacent glycosidic oxygen, thus destabilizing the glycosidic linkage (41). In unrelated studies using model compounds such as *o*- and *p*-carboxyphenyl β-glucosides, the proximity of a carboxyl group to a glycosidic oxygen was found to accelerate markedly the rate of breakdown of the glycosidic bond under acidic conditions (42). In this case, it was suggested that intramolecular general acid catalysis occurs, with the carboxylic proton being directly donated to the glycosidic oxygen via the formation of an oxycarbonium ion intermediate (42, 43). Thus, if the internal carboxyl groups of PSA had a high pK_a, this would cause protonation at a relatively high pH, favoring a similar reaction at the immediately adjacent glycosidic oxygen (Fig. 6). We measured the pK_a of monomeric Sia (Neu5Ac, *N*-acetylneuraminic acid), OSA, and PSA by microtitration in comparable conditions: equivalent concentration of protonated carboxyl groups, absence of salts, and defined temperature (detailed data not shown). Values obtained for the pK_a of DP 2 and DP 3 were in a similar range (3.6–3.8) and slightly higher than those measured for Neu5Ac (2.9–3.2). In contrast, the measured pK_as of PSAs of average DP ~100 were much higher (Mean of seven observed values, 4.83; range, 3.91–5.53). The pK_a of polyanions is notoriously difficult to establish precisely, being highly dependent on concentration, presence of cations, type of cation, etc. (39, 44). Also, as a polycarboxylic acid is neutralized with base, negatively charged carboxylate ions form and repel neighboring charged groups, leading to coil expansion and increasing the difficulty of neutralizing the remaining carboxyl groups because of electrostatic interactions (45). Thus, the values we measured for PSA are approximate and represent composite averages of many internal carboxyl groups of the polymer. Regardless, the difference in pK_a between the OSA and PSA is of such a magnitude (up to almost 2 pH units) that it is a likely candidate to explain the differential stability of the two classes of molecules. To more closely address this, we studied the pK_a of DP 4, which has two stable terminal bonds and one unstable internal bond. This molecule contains one carboxyl group that remains protonated up to pH 4.1. This could explain the difference in behavior between OSA of DP 3 and DP 4 toward the hydrolysis of glycosidic linkages at pH 4.8.

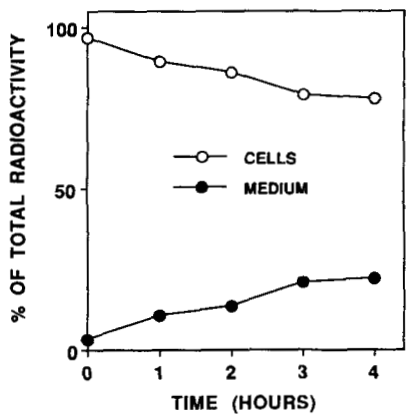

FIG. 7. **Release of PSA from cell surfaces.** K1 *E. coli* were uniformly labeled with [^{3}H]Neu5Ac, which is exclusively incorporated into the PSA capsule. Labeled cells were washed and reincubated as described under "Experimental Procedures." At the indicated times, duplicate samples were removed and centrifuged, and the supernatant medium and pellet was monitored for radioactivity (all radioactivity remained in high molecular weight PSA; data not shown). The background in the supernatant prior to start of the assay was 0.37% of total radioactivity. In a separate incubation at pH 8.0 (data not shown), this level remained unchanged over a period of 18 h. The pH of the medium did not alter significantly under either incubation condition.

Proposed Mechanism for the Reaction—The above data, taken together with previous information on model compounds (42, 43) and naturally occurring glycosidase reactions (46–48), leads us to propose a mechanism involving the internal carboxyl groups of PSA (Fig. 6). Intramolecular glycosidic cleavage at mildly acidic pH is likely to result from the direct donation of a proton from an internal carboxyl group with an unusually high pK_a to the adjacent glycosidic oxygen, favoring general acid catalysis via the formation of an oxycarbonium ion intermediate (Fig. 6).

Is the Rate of PSA Breakdown Sufficient To Be of Biological Relevance?—The conditions used for many of the present studies (pH 4–4.8, temperatures of 37–48 °C) were chosen to maximize the efficiency of the experiments. To directly monitor the loss of PSA from living cell surfaces, we fed [^{3}H]Neu5Ac to K1+ *E. coli*, resulting in surface radioactivity exclusively in the PSA polysaccharide (37). The cells spontaneously "shed" labeled capsular material into the medium under mildly acidic conditions (Fig. 7), all of which was in large fragments, with no detectable monomers (data not shown). In other studies of purified PSA incubated at pH 6.8 and 37 °C, we noted that internal bonds were broken at the rate of ~0.5%/h (data not shown). At first glance, this might seem too slow to be of physiological relevance. However, PSA in biological systems is found attached to cell surfaces in polymers ranging in size from 3 to 200 (2). Because of this location, a single internal cleavage would be sufficient to cause irreversible release of a large portion of a long chain. Also, extracellular pH can be significantly acidic in organs such as the kidney, where PSA is found. In the brain, extracellular and cerebrospinal fluid pH is tightly controlled in the near neutral range (49–52). However, under abnormal conditions such as ischemia, uncontrolled seizure activity, or infection, significantly acidic pH values can occur (49–55). Furthermore, it is conceivable that in the immediate microenvironment of cell surfaces or in restricted extracellular spaces, the pH may fall low enough to cause significant loss of PSA. Likewise, the acidic extracellular environment of many tumors could affect the expression of PSA on cancer cells (56). Within a cell, organelles such as endosomes and lysosomes certainly

have pH values low enough (57, 58) to cause rapid breakdown of PSA. Thus, during the terminal degradation of N-CAM in lysosomes, there may not be a necessity for an endosialidase enzyme. Also, if N-CAM is internalized into endosomes and then recycled to the cell surface, the drop in pH could affect the size of PSA attached to its oligosaccharide. In this regard, the well known "embryonic to adult" transition of N-CAM involves a change in the size of PSA from DP >10 to short "stubs" of 3–4 units (12). While it has been suggested that the transition involves the synthesis of new molecules of N-CAM (59), exposure to lower pH is another possibility that should be considered.

Acknowledgments—We thank Hudson Freeze, Leland Powell, Claudio Schteingart, and Willie Vann for helpful discussions and for many useful reagents.

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