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Common Cleavage Pattern of Polysialic Acid by Bacteriophage Endosialidases of Different Properties and Origins

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The cleavage specificities of seven bacteriophage endosialidases degrading the α2–8-linked polysialic acid common to bacterial polysaccharides and to the cell adhesion molecule *N*-CAM were investigated. The bacteriophages studied represented five different phenotypic groups by protein and DNA fragment analysis and two different morphology groups by electron microscopy. Characterization of the fragments arising from the native or chemically modified substrates of different sizes showed that cleavage specificity was influenced by enzyme concentration. At the initial phase of degradation, at concentrations ranging from 20- to 100-fold, the minimum substrate size was an oligomer of eight (in one case, nine) sialic acid units that was preferably cleaved at the same position. Under exhaustive conditions, the oligomers were degraded further, and each enzyme type had its own specificity. The similar initial cleavage of polysialic acid by endosialidases associated with phages of different properties and morphology suggests a conserved mechanism of enzyme-substrate interaction. This mechanism may be conformationally determined and related to the specific properties of polysialic acid in other molecular interactions.

Polysialic acid, a polymer of α2–8-linked *N*-acetylneuraminic acid units, occurs as a developmentally regulated unit in the carbohydrate chains of the neural cell adhesion molecule *N*-CAM (7) and as the capsular polysaccharide of the pathogenic bacteria *Escherichia coli* K1, *Neisseria meningitidis* group B, and *Pasteurella haemolytica* A2 (1, 3, 11, 26). In animal tissues, polysialic acid is involved in cell adhesion of neural and other cells (16, 34, 35). In bacteria, the capsular polysialic acid contributes to bacterial virulence and is often encountered in strains causing meningitis and sepsis (1, 10, 32). A third biological interaction of polysialic acid is the binding and subsequent degradation of the polysialic acid capsule by K1-specific bacteriophages of *E. coli* (12, 19, 21, 37).

Endosialidases of the K1-specific coliphages have been used for the detection and selective splitting of polysialic acid in studies of the structure, metabolism, and function of polysialic acid in various biological systems (8, 34, 40). Polysialic acid interacts with other molecules, such as K1-specific antibodies, in an exceptional way (6, 8, 17, 18). Elucidation of the mechanisms involved in the enzyme-substrate interactions of the K1 bacteriophages is needed for further insight into the role of this unique polymer in biological processes.

Like other capsule-specific bacteriophages, the phages specific for the K1 capsule of *E. coli* carry the capsule-degrading enzyme in their tail spikes (23). Phages PK1A to PK1E differ in ability to infect *E. coli* K1 strains (9, 12). The endosialidase of the bacteriophage ϕ1.2 degrades efficiently only the K1 polysaccharide, whereas that of ϕ92 splits both the K92 (polymer of *N*-acetylneuraminic acid with alternating α2–8 and α2–9 linkages) and the K1 polysaccharides (19, 20).

In this report, we describe the interaction between polysialic acid and different phage endosialidases. At the in vivo enzyme concentration of bacterial phage lysates, all enzymes initially cleave polysialic acid in an almost identical fashion despite the different biochemical and morphological properties of the bacteriophages of different origins. This conserved mechanism of interaction appears to be determined by the substrate conformation and may reflect a common property in the molecular interactions of polysialic acid.

MATERIALS AND METHODS

Abbreviations. Sia_n, Oligomer of *n* α2–8-linked *N*-acetylneuraminic acid units; Sia_n-ol, oligomer of *n* α2–8-linked sialyl units with a ^3H-reduced sialitol reducing end; P-Sia_n, oligomer of *n* α2–8 sialyl units with a periodate-oxidized and ^3H-reduced nonreducing end.

Propagation and purification of bacteriophages. Bacteriophages PK1A to PK1E (12) and the host strain *E. coli* K1 (IH3088) were supplied by P. H. Mäkelä, National Public Health Institute, Helsinki, Finland. Bacteriophages ϕ1.2 and ϕ92 (19, 21) and their host strains K235 (*E. coli* O1:K1) and Bos 12 (*E. coli* O16:K92), respectively, were provided by S. Stirm, Biochemisches Institut, Giessen, Federal Republic of Germany. L broth, L-agar plates, and soft agar (25) were used for cultivation of the organisms.

The phages were propagated by standard methods (24), starting from a single plaque. The phage particles were precipitated by polyethylene glycol (19) and purified by two-step CsCl (ultra pure; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) gradient centrifugation (8), followed by dialysis against 10 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl at 4°C (6, 20). The purified stocks contained 1×10^{12} to 5×10^{12} PFU/ml and 2×10^{15} to 10×10^{15} PFU/g of protein and were stored in equal portions at −70°C.

Characterization of phage morphology, proteins, and DNA. For electron microscopy, 5 μl of phage stock was placed on a carbon-coated specimen grid. The grids were washed in 10

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mM Tris hydrochloride (pH 7.0) containing 10 mM NaN_3 and 1 mM Mg^{2+} and counterstained with aqueous uranyl acetate. Gel electrophoresis of total phage proteins was carried out in 10% polyacrylamide gels as described previously (6, 22). For DNA characterization, phages (10^{11} to 10^{12} PFU) were lysed in 2 ml of 50 mM Tris hydrochloride (pH 8) containing 20 mM EDTA, 0.5% sodium dodecyl sulfate, and 100 μg of pronase per ml (50°C, 1 h), and the DNA was further isolated by phenol-chloroform extraction as described previously (25). Restriction endonuclease digestions (*Bgl*II, *Hin*dIII, and *Pvu*II; 10 U of each) were performed as recommended by the supplier (New England BioLabs, Inc., Beverly, Mass.); the fragments were electrophoresed in agarose gels and visualized by ethidium bromide (25).

Preparation of substrates. To obtain oligomers eight or more sialyl residues long, the oligomers isolated from colominic acid (sodium salt; Sigma Chemical Co., St. Louis, Mo.) by high-performance liquid chromatography (30) and tested for sialic acid content (28) were ^3H radioreduced (30) and analyzed for sialyl chain length by gel electrophoresis (30). The putative lipid moieties of the reducing ends of the oligomers (10) and the lactone linkages (26) were removed by procedures involving mild acid treatment, saponification, neutralization, and desalting (29, 30). After lyophilization, the oligomers were used as the endosialidase substrate. Reduced radiolabeled sialyl oligomer substrate was prepared by NaB^3H_4 reduction (5 Ci/mmol; Amersham International, Amersham, United Kingdom) (30). Sialyl oligomers of defined lengths labeled at the reducing or nonreducing end were prepared as described previously (8).

Endosialidase digestion of sialic acid oligomers. Unlabeled sialyl oligomers (2.5 to 5 μg) were incubated with endosialidase (10^6 to 10^{11} PFU) in 10 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl and 1% (wt/vol) chloroform in a volume of 50 μl at 37°C for 24 h. The samples were desalted on P-2 microcolumns (29) and labeled by reduction with NaB^3H_4 (100 μCi per sample) (30). Control experiments showed that no significant loss of even the smallest oligosaccharides occurred during lyophilization and desalting. Digestions of the ^3H-labeled sialyl oligosaccharides were performed under the same conditions except that 5 μg of the reduced oligomer mixture or 0.75 μg of sialyl oligomers of a single length labeled at the reducing or nonreducing end was used as the substrate.

After incubation, the samples were lyophilized and analyzed by polyacrylamide gel electrophoresis as described previously (30). For fluorographic detection of the digestion fragments, the gels were treated with 2,5-diphenyloxazole (36), and the films were exposed at −70°C for 2 to 5 days. The polysialic acid standard was isolated from *E. coli* K1, hydrolyzed at pH 2 at 80°C for 10 min, and radioreduced by borotritide (30). The electrophoretic mobility of the tracking dye phenol red matched that of tetrasialic acid.

RESULTS

Purification and properties of endosialidase-containing bacteriophages. Preliminary attempts to purify PK1A endosialidase indicated that the soluble form of the enzyme rapidly lost activity in its more purified forms, a finding similar to that observed for the acetyltransferase specific for polysialic acid (14). In contrast, the endosialidase associated with the bacteriophages purified by the cesium chloride method was stable during prolonged storage at 4 or −20°C. Thus, purification of the bacteriophages was considered the method of choice to obtain endosialidase in a stable form. Studies of the endosialidase of phage K1F have shown that the purified, free enzyme acts similarly to the phage-associated enzyme (13).

The infective activities of the different purified phage preparations were similar (2×10^{15} to 10×10^{15} PFU/g of protein). Morphological findings (Fig. 1) indicated that the phage particles had been purified in their intact forms. The morphology of the K1 capsule-specific phages PK1A to PK1E and φ1.2 was typical of Bradley group C, whereas the K92 capsule-specific φ92 belonged to Bradley group A (Fig. 1) (4).

The general protein band patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PK1A to PK1E and φ1.2 were similar to each other, but the phages differed slightly but reproducibly in the electrophoretic mobilities of some of the bands (Fig. 2). Phage φ92, on the other hand, was totally different from the other phages. Within the PK1 group of phages, the pair PK1B and PK1C as well as the pair PK1D and PK1E gave identical band patterns. It is of interest that the bands of different mobilities included the bands at molecular weights of 74,000 and 38,500 (Fig. 2, lane E) that have been identified for PK1E to represent the endosialidase molecule (38).

The phage DNA fragments digested by using *Pvu*II-*Hin*dIII and *Pvu*II-*Bgl*II double digestions were in accordance with the protein band patterns; each phage gave its own band pattern except the pairs PK1B-PK1C and PK1D-PK1E, whose patterns were identical (Fig. 3; data for φ1.2 and φ92 not shown).

Degradation of polysialic acid by endosialidases in two phases. The cleavage of different polysialic acid substrates by the endosialidases was analyzed by a gel electrophoretic method that allowed the separation of sialic acid oligomers down to a monomer (30). In accordance with previous findings (8), there was no further degradation of the ^3H-reduced polysialic acid substrate during prolonged incubation for 1 to 2 days with PK1A endosialidase, using a high amount (10^9 PFU) of enzyme (Fig. 4). However, after 3 days of incubation some further degradation of the sialyl oligomers became evident, especially with φ92 endosialidase.

To study the effect of enzyme concentration, the ^3H-reduced polysialic acid substrate was incubated in the presence of PK1A or φ92 endosialidase at concentrations ranging over 10^5-fold. Two phases of enzymatic cleavage were observed (Fig. 5). At the lowest concentrations, essentially no cleavage was observed. With increasing concentrations, the cleavage seemed to stop essentially at the same level at concentrations ranging from 20- to 100-fold (10^7 to 10^9 PFU). The major fragments produced from the reducing end were the reduced oligomers containing five to seven sialyl residues (Sia_5-ol to Sia_7-ol). This result is in accordance with the cleavage specificity of the PK1A enzyme characterized previously (8). At extreme concentrations, however, degradation proceeded further, yielding the disialyl fragment Sia_2-ol as the main product. The substrate was degraded in two phases also by the other endosialidases except φ1.2, which did not degrade the oligomers further even at the extreme concentrations (Fig. 6). The endosialidases differed from each other in the relative amounts of Sia_2-ol produced except for the pairs PK1B-PK1C and PK1D-PK1E, which gave almost identical results.

When the digestion process was examined more closely near the transition between the two phases of degradation, all enzymes except φ1.2 behaved similarly and cleaved the ^3H-reduced substrate to the level of Sia_5-ol to Sia_7-ol (results not shown). This result indicated that Sia_8-ol was the small-

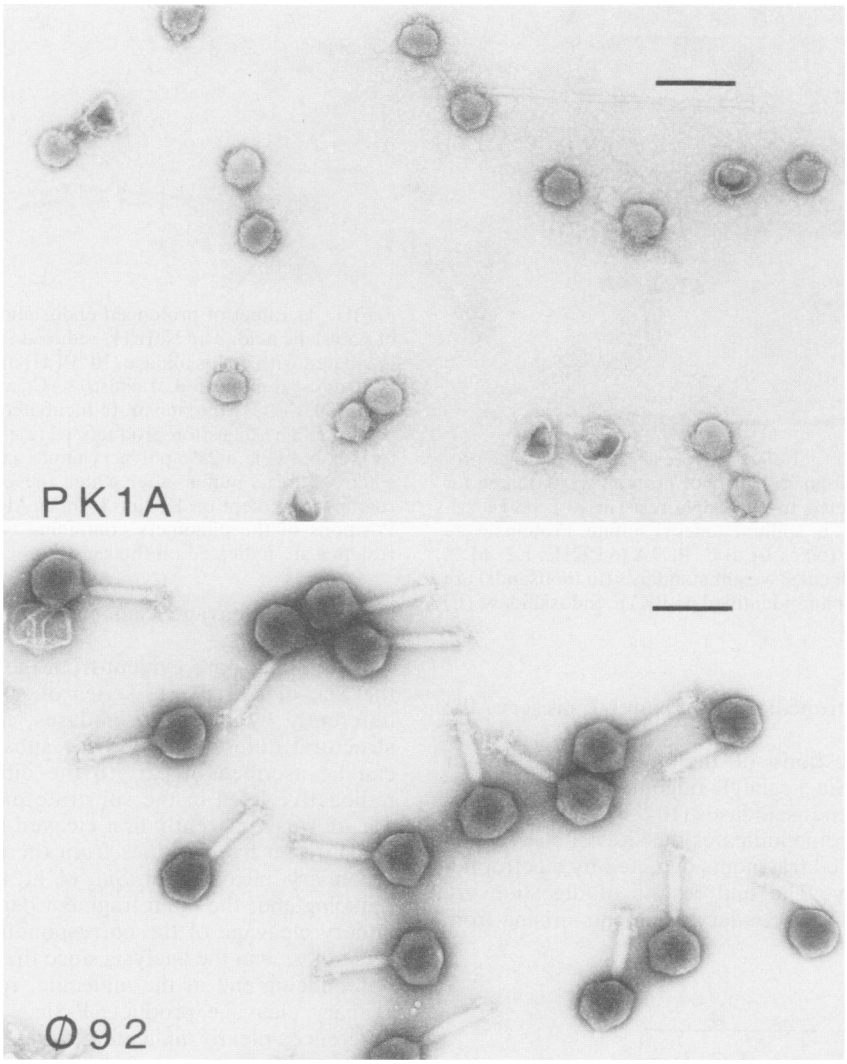

FIG. 1. Morphology of endosialidase-containing bacteriophages. The phages were processed for electron microscopy by using negative staining. The morphology of PK1A corresponds to Bradley group C, and that of ϕ92 corresponds to Bradley group A. Bar, 50 nm.

est degradable molecule. The ϕ1.2 endosialidase products were Sia_5-ol to Sia_8-ol, the smallest degradable molecule being Sia_9-ol. In the second phase of degradation (5×10^9 PFU), the endosialidases differed from each other markedly. PK1A and ϕ92 yielded the highest amount of the fragment Sia_2-ol. In contrast, ϕ1.2 did not degrade the substrate further, in accordance with the result shown in Fig. 6.

These results suggested that the endosialidases cleaved the polysialic acid substrates up to the transition point relatively similarly, whereas differences became evident at the extreme concentrations.

Digestion of native polysialic acid substrate. The results described above were obtained with the substrate labeled at its reducing end; therefore, only the products originating from the reducing end of the molecule were detected by subsequent gel electrophoresis and fluorography. To reveal the fragments arising from the nonreducing end of the oligomers and to investigate whether the differences observed at the extreme enzyme concentration also applied to native sialic acid oligomers, incubations were carried out with the unreduced substrate. The products were labeled after digestion by ^3H reduction. The fragments produced from the native sialic acid substrate after exhaustive digestion (10^{10} PFU) were Sia_3 to Sia_5 for all of the enzymes except ϕ1.2, which yielded Sia_3 to Sia_7 (Fig. 7). As with the reduced substrate, the proportions of the products varied among the enzymes. The main product of PK1A was Sia_4, and that of ϕ92 was Sia_3. The main product of PK1B to PK1E was Sia_5, but interestingly, the pairs PK1B-PK1C and PK1D-PK1E yielded slightly different proportions of products. The smallest fragment obtained after digestion of the unreduced substrate with these phages was a sialyl trimer.

Probing of active sites by using modified oligomers of defined length. To study the different splitting patterns observed at the extreme enzyme concentrations, we used sialic acid oligomers of defined length as the substrate. The oligomers chemically modified at the reducing or nonreducing end were used as probes to examine the differences in subsites of the active centers of the enzymes (see Discussion). In particular, conversion of the reducing-end residue of the sialyl oligomers to sialitol (Sia_n-ol series) induces a significant change in the substrate by opening the ring structure. The oligomers labeled at the nonreducing end by periodate oxidation (P-Sia_n-series) and reduction, on the

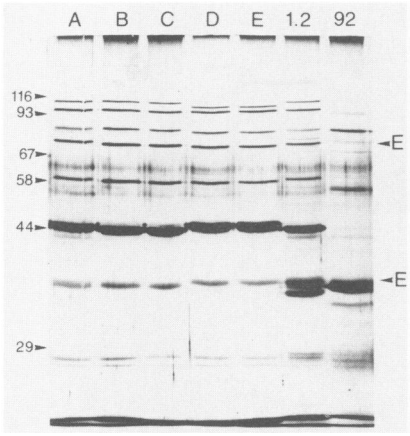

FIG. 2. Polyacrylamide gel electrophoresis of total phage proteins. The purified bacteriophages (1 μg of protein) were treated for 5 min at 95°C and subjected to electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. Proteins were visualized by silver stain. Lanes: A to E, PK1A to PK1E; 1.2, ϕ1.2; 92, ϕ92. Mobilities of molecular weight standards (in thousands) are indicated on the left; the band identified as PK1E endosialidase (E) (30) is shown on the right.

other hand, contain a truncated side chain but preserve the ring structure (39).

The results of digestions of the reduced (Sia_n-ol) or periodate-labeled (P-Sia_n) sialyl oligomers with PK1A, PK1B, ϕ1.2, and ϕ92 endosialidases (10^{10} PFU) are summarized in Fig. 8. The scheme indicates the sites of cleavage as deduced from the labeled fragments detected by electrophoresis and fluorography. The end results of digestion are presented; therefore, the secondary fragments arising from

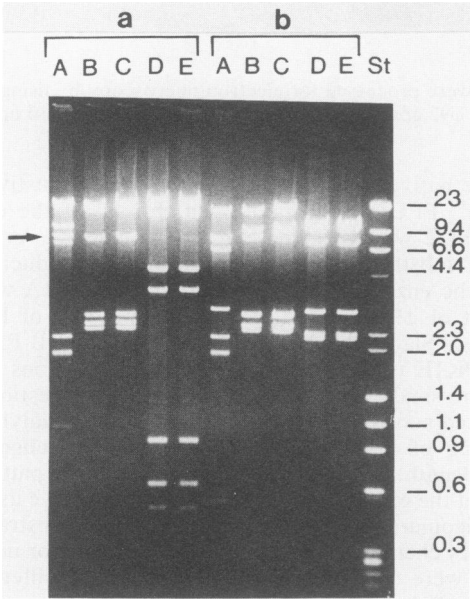

FIG. 3. Restriction enzyme analysis of phage DNA. Phage DNA was double digested with *Pvu*II-*Hin*dIII (a) and *Pvu*II-*Bgl*II (b), and the fragments were electrophoresed in a 1.0% agarose gel. Lanes: A to E, PK1A to PK1E; St, molecular weight marker. Sizes of the standards (in kilobases) are indicated on the right. The arrow points to the band of a partially digested fragment.

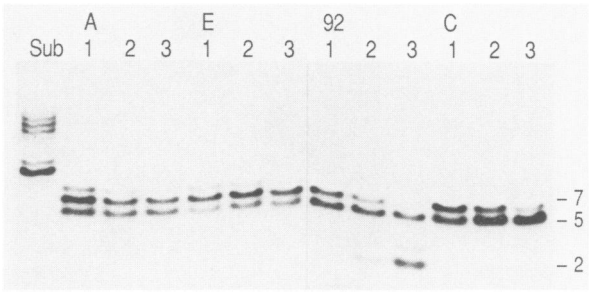

FIG. 4. Effect of prolonged endosialidase digestion on cleavage of polysialic acid. The NaB^3H_4-reduced sialyl oligomers (5 μg) were incubated with endosialidase (10^9 PFU) in a volume of 50 μl for 1, 2, or 3 days as indicated. Lanes: A, C, and E, PK1A, PK1C, and PK1E; 92, ϕ92; Sub, substrate incubated for 3 days without endosialidase. The digestion products were electrophoresed at 35 V/cm for 3 h at 4°C in a 25% polyacrylamide gel in glycine-borate-EDTA electrophoresis buffer, after which the gel was processed for fluorography and kept on Kodax X-Omat AR film for 3 days at −70°C. Positions of the oligomers containing two, five, and seven sialyl residues are indicated on the right.

the primary cleavage fragments of the longer substrates are also seen.

Two findings are evident from the results in Fig. 8. First, the Sia_n-ol and P-Sia_n series of substrates were digested differently by the endosialidases, in accordance with the structural differences of these substrates. The differences can be ascribed in part to the different locations of the radioactive label in the substrate molecules. For example, Sia_8-ol was apparently first cleaved by ϕ92 endosialidase at the position five residues from the reducing end, but after secondary cleavage of Sia_5-ol at two residues from the reducing end, the main fragment detected was Sia_2-ol. Secondary cleavage of the corresponding product from P-Sia_8 was not seen in the analysis since the label was located at the nonreducing end of the molecule, represented by the main primary cleavage product, P-Sia_3. However, most of the differences clearly indicate differential cleavage of the two series of substrates.

Second, the endosialidases seemed to differ from each other in the cleavage patterns of the modified substrates. This finding is in contrast to those obtained at the first stage of digestion, when most enzymes digested both the native and the reduced substrates in remarkably similar fashions. The results, therefore, indicate that differences in the active sites of the enzymes were revealed only by the use of the modified substrates at the extreme enzyme concentrations.

DISCUSSION

Interaction of the endosialidases with sialic acid oligomers. The differential cleavage of the sialyl oligomers at exhaustive conditions of digestion indicates differences in the active centers of the endosialidases. Models proposed to explain the action of enzymes on polymeric substrates involve multiple subsites binding the individual residues of the substrate (2, 5, 15). A similar model can be implicated in the interaction of the endosialidases with the polysialic acid substrate. An alternative model, without subsites and with the preferential positions of cleavage being controlled only by the conformation of the substrate, can be ruled out by this study, in which different enzymes were shown to display different cleavage patterns for the same substrates.

The mode of interaction between the endosialidases and the substrate is discussed with reference to the multiple

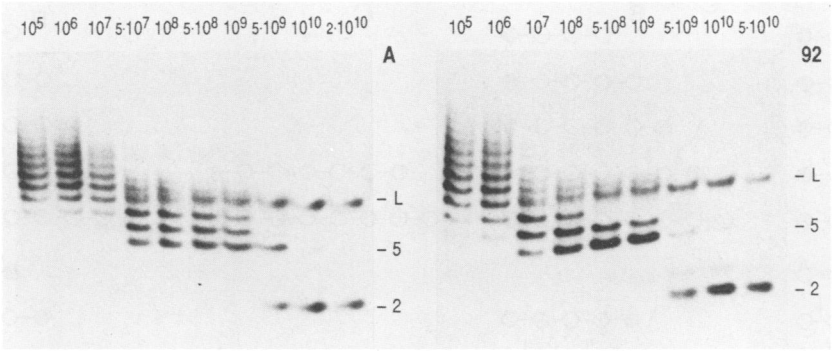

FIG. 5. Effect of endosialidase concentration on cleavage of polysialic acid. The NaB^3H_4-reduced sialyl oligomers (5 μg) were incubated with increasing amounts (1×10^5 to 5×10^{10} PFU) of endosialidase (A, PK1A; 92, ϕ92) for 24 h and subjected to gel electrophoresis as described for Fig. 4. Band L should be ignored in interpretation of the results, since it arose from a subfraction of colominic acid that contained a lipid moiety attached to the nonreducing end of the oligosaccharides (10). The identity of the band was confirmed by control digestion with *Vibrio cholerae* neuraminidase and by mild acid hydrolysis (30) (data not shown).

subsite model and PK1A and PK1B as examples (Fig. 9). At the first stage of digestion, the smallest degradable substrate is the oligomer containing eight sialyl residues. The active centers of both enzymes bind to this substrate similarly, so that three subsites at the nonreducing end (subsites N_1 to N_3) and five at the reducing end (subsites R_1 to R_5) are occupied (Fig. 9, top schemes). At the highest concentration of enzyme, differences become evident. This model is analogous to the recently described cleavage mechanism of polyphosphate glucokinase (31). The preferential cleavage of Sia_5-ol and Sia_6-ol at two residues from the reducing end by PK1A suggests that subsite R_2 preferentially binds the reduced sialitol residue. The possible presence of an additional subsite, N_4, is suggested by the better cleavage of Sia_6-ol than Sia_5-ol (Fig. 8). The periodate-modified nonreducing-end residue of the P-Sia oligomers does not seem to influence the binding as strongly as the sialitol residue, which is indicated by the multiple possible cleavage sites (Fig. 8). The cleavage of P-Sia_5 and P-Sia_6 at four residues from the nonreducing end indicates a stronger interaction of PK1A with the nonreducing-end subsites (N sites) than with the reducing-end subsites (R sites).

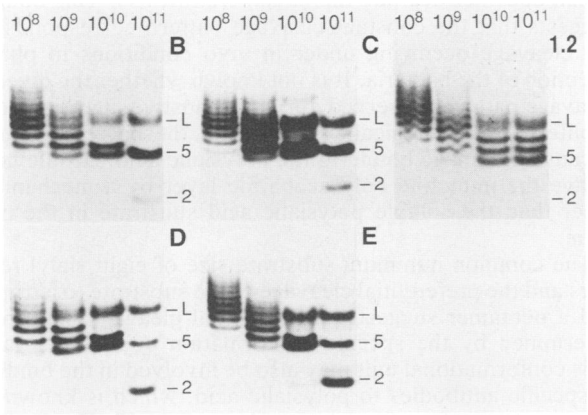

FIG. 6. Effect of endosialidase concentration on cleavage of polysialic acid. The NaB^3H_4-reduced sialyl oligomer substrates (5 μg) were incubated with increasing amounts (10^8 to 10^{11} PFU) of endosialidase (B to E, PK1B to PK1E; 1.2, ϕ1.2) for 24 h. The products were analyzed by gel electrophoresis as described for Fig. 4 and 5.

PK1B cleaves the reduced substrates preferably at five residues from the reducing end, indicating the presence of a preferentially sialitol-binding subsite, R_5, analogous to site R_2 of PK1A. In contrast to PK1A, PK1B seems to bind the periodate-modified substrates without preference for certain N or R sites. This is seen most clearly in the cleavage of P-Sia_6, which is cleaved equally well at one, two, three, or four residues from the reducing end by PK1B but only at two residues from the reducing end by PK1A (Fig. 8 and 9).

Each of the enzymes studied for the pairs PK1B-PK1C and PK1D-PK1E displayed its own cleavage pattern at the extreme enzyme concentration, which indicates differences in the active centers of the enzymes. This finding may also be extended to the recently described endosialidase of coliphage K1F (13), which differs from the PK1A and PK1B enzymes, among others, in having a sialitol-binding site at position R_3 instead of at R_2 and R_5, respectively.

The ϕ1.2 endosialidase behaved exceptionally in that (i)

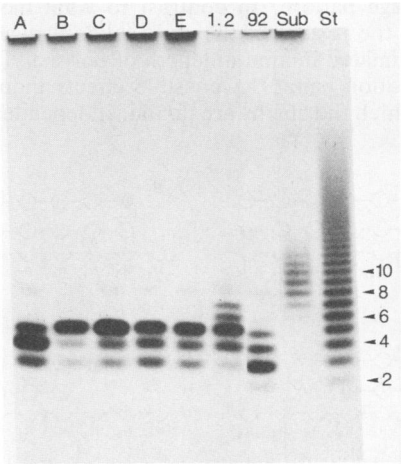

FIG. 7. Endosialidase digestion of native sialyl oligomer. The native unlabeled sialyl oligomer substrate (Sub) was digested for 24 h with endosialidases PK1A to PK1E (A to E), ϕ1.2 (1.2), and ϕ92 (92) (10^{10} PFU each), after which the products were labeled by reduction with NaB^3H_4. The products as well as undigested substrate (Sub) and a reference sialyl polymer preparation (St) were subjected to gel electrophoresis as described for Fig. 4. The numbers of sialyl residues in the individual bands are indicated on the right.

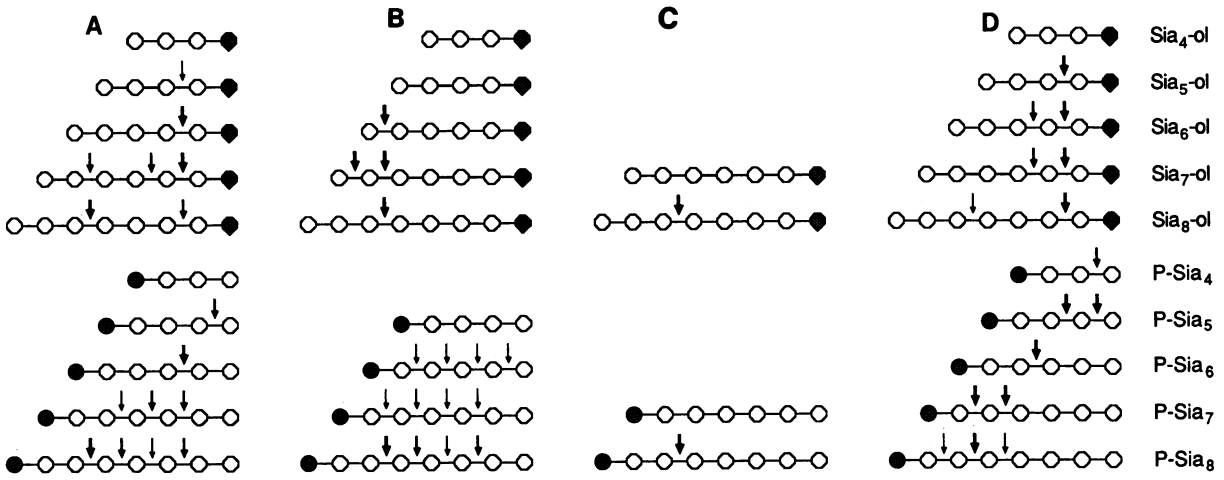

FIG. 8. Cleavage of sialyl oligomers of defined length with endosialidases PK1A (A), PK1B (B), ϕ1.2 (C), and ϕ92 (D). Individual sialyl oligomers radiolabeled at the reducing end by NaB^3H_4 reduction (Sia_n-ol) or at the nonreducing end by periodate oxidation and NaB^3H_4 reduction (P-Sia_n) were incubated with the endosialidases (10^{10} PFU), and the digestion products were analyzed by gel electrophoresis. Positions of cleavage (↓) were deduced from the digestion products. The thickness of each arrow corresponds to the relative proportion of fragments observed (thick arrow, major fragment; medium arrow, medium fragment; thin arrow, minor fragment). Only the products carrying the labeled (hatched) end residues were detected in the fluorographs. Since the fragments observed represent the end products of digestion, the sites of cleavage include those arising from the primary cleavage products after secondary cleavage.

the sialyl fragments were not digested further at increasing enzyme concentrations and (ii) the chemical modification of the substrate did not influence the cleavage pattern. The same results were obtained with separately purified preparations, which indicated that these properties were not caused by, for example, an occasional lower activity of the ϕ1.2 endosialidase preparation studied.

Use of the bacteriophage endosialidases as reagents. PK1A endosialidase has been used for the study of the polysialic acid moiety of the cell adhesion molecule N-CAM (6, 8, 33). The presence of long polysialic acid chains containing at least 12 residues in these glycans was concluded from these studies, using the enzyme concentration that gave the constant cleavage pattern. In contrast to what has been proposed (13), the results of the endosialidase digestions support the estimated minimum length of polysialyl chains, the only reservation being the possible effects induced by the glycan to which the chains are bound. Independent evidence for the long sialic acid chains has recently been obtained from immunochemical data as well (6).

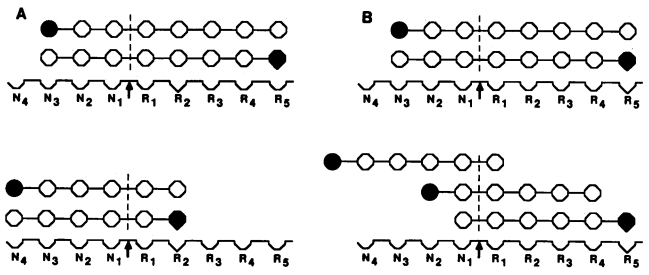

FIG. 9. Model for the binding and cleavage of sialic acid oligomers by PK1A (A) and PK1B (B) endosialidases. The active site is postulated to consist of a series of subsites at the nonreducing end (N_n) and reducing end (R_n) of the site of cleavage (↑). The upper schemes indicate the preferential mode of interaction for the substrates containing eight sialyl residues (minimum substrate size at first stage of digestion); the lower schemes represent the mode of interaction for the substrates containing six sialyl residues (cleavage under exhaustive digestion conditions). See the legend to Fig. 8 for identification of labeled substrates and the text for further details.

The use of exhaustive conditions of digestion is not practical for selective splitting of polysialyl moieties, since considerable amounts of bacteriophage are required. Moreover, because the cleavage would be critically influenced by the exact structure of the substrate, the cleavage pattern would be difficult to predict. In contrast, cleavage at the first stage of digestion appears to occur in a constant manner for different substrates and to give reliable results, provided the conditions of digestion have been controlled.

Implications of the similar initial cleavage by the endosialidases. Bacterial K1 phage lysates contain about 10^9 to 10^{10} PFU/ml, which corresponds to the concentration of endosialidase giving the constant cleavage pattern. Interestingly, the switch to the second phase of digestion occurs at about the same concentration with all of the enzymes (except ϕ1.2, which does not degrade the oligomers further). This finding suggests that the constant cleavage pattern corresponds to the cleavage occurring under in vivo conditions in phage infection of the bacteria. It is not known whether the diverse cleavage patterns observed during exhaustive digestion contribute to the biological properties of the bacteriophages, because the phage bound to the polysialic acid capsule may cleave the immobile polysaccharide layer by a mechanism other than the soluble polysialic acid substrate in the medium.

The common minimum substrate size of eight sialyl residues and the preferential cleavage of the substrate to a trimer and a pentamer suggest that the initial cleavage pattern is determined by the specific conformation of the substrate. This conformational unit may also be involved in the binding of specific antibodies to polysialic acid, which is known to require at least 8 to 10 sialyl residues (6, 8, 17, 18). Recent nuclear magnetic resonance data on the conformation of sialyl oligosaccharides indicate that the interresidue linkage conformations of the terminally located two sialic acid residues differ from those of the inner residues (27). Binding of the antibodies only to the internal residues of the sialic

TABLE 1. Morphology, protein and DNA band patterns, and minimum substrate sizes and cleavage patterns of seven bacteriophages cleaving α2–8-linked polysialic acid[a]

Endosialidase	Bradley group[b]	Protein and DNA band pattern[c]	Amt of enzyme (PFU)				Sia[6]-ol cleavage[d]
			≤ 1 × 10[9]		≥ 5 × 10[9]		
			Minimum unmodified substrate	Reducing-end fragment[e]	Minimum unmodified substrate	Reducing-end fragments	
PK1A	C	A	Sia_8	Sia_{5-7}-ol	Sia_6	Sia_2-ol	4 + 2
PK1B	C	B-C	Sia_8	Sia_{5-7}-ol	Sia_6	Sia_5-ol	1 + 5
PK1C	C	B-C	Sia_8	Sia_{5-7}-ol	Sia_6	Sia_5-ol	1 + 5
PK1D	C	D-E	Sia_8	Sia_{5-7}-ol	Sia_6	Sia_2-ol, Sia_5-ol	4 + 2
PK1E	C	D-E	Sia_8	Sia_{5-7}-ol	Sia_6	Sia_2-ol, Sia_5-ol	4 + 2
ϕ1.2	C	1.2	ND	Sia_{5-8}-ol	Sia_8	Sia_5-ol	
ϕ92	A	92	ND	Sia_{5-7}-ol	Sia_6	Sia_2-ol	4 + 2, 3 + 3

[a] Data are compiled from Fig. 1 to 3 and 5 to 8 and from previous results (8). For simplicity, only the major cleavage patterns of the substrates are presented.
[b] Morphology of the bacteriophages; see Fig. 1.
[c] Band patterns obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2) and restriction enzyme analysis (Fig. 3) for PK1A (A), PK1B and PK1C (B-C), PK1D and PK1E (D-E), ϕ1.2 (1.2), and ϕ92 (92).
[d] Lengths (in sialyl residues) of the nonreducing- and reducing-end fragments arising from digestion of Sia_6-ol (see legend to Fig. 8) are indicated by the first and second numbers, respectively.
[e] Labeled fragment arising from digestion of the NaB^3H_4-reduced polysialic acid substrate. ND, Not determined.

acid polymer, as described recently (18), could explain the requirement for the exceptionally long oligosaccharide determinant. Similarly, the phage endosialidases appear to degrade the substrate readily as long as the interresidue linkages of the internal sialyl residues are cleaved. The reason for this, and for the requirement of as many as eight residues for initial cleavage, is, however, not clear. The endosialidases of phages of different origins and morphologies seem to function in a highly conserved manner, which may reflect a specific property of the polysialic acid substrate.

The properties of the seven endosialidases studied are summarized in Table 1. Taking into account the similarity of the pairs PK1B-PK1C and PK1D-PK1E, five different types of endosialidases can be discerned by differences in the proteins and DNAs of the phages as well as by differences in the cleavage patterns at exhaustive digestion conditions. Moreover, one of the phages (ϕ92) also differed from the others in morphology. The properties of K1F endosialidase recently reported (13) appear different from the properties of any of the enzymes described in this report. Therefore, the properties of one particular endosialidase (13) should not be directly compared with those of other enzymes studied at different conditions.

It is striking that despite the differences observed in phage protein, DNA, enzymatic function, and morphology, all of the endosialidases appear to cleave the polysialic acid substrate in a similar manner at the initial phase of digestion. This enzymatic property seems highly conserved and therefore critical for the biological function of the endosialidases. In the case of ϕ92 bacteriophage, which is of totally different morphology and origin, the possibility that this property may even have arisen by convergent evolution must be considered.

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