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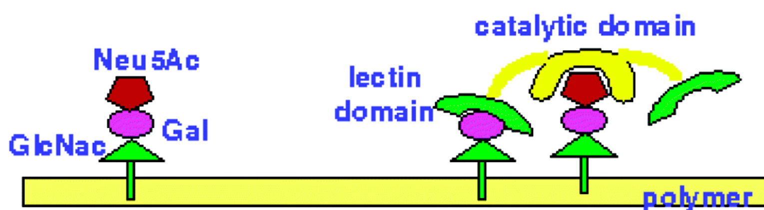

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Multivalency and the Mode of Action of Bacterial Sialidases

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Multivalent binding events, in which multiple ligands on one entity interact simultaneously with multiple receptors on a complementary entity, are widespread in nature.[1−3] This type of interaction has been demonstrated to be mechanically and functionally distinct from its monovalent alternative and is relatively commonplace in carbohydrate-mediated biological events. The best-studied manifestations of multivalency include dramatically increased functional affinities,[1] enhanced or altered selectivities,[2] and initiation of cell signaling events.[3]

We report here a new manifestation of multivalency and demonstrate for the first time that bacterial sialidases, which contain a catalytic domain together with one or more carbohydrate-binding domains, are able to hydrolyze polyvalent substrates with much greater catalytic efficiency than their monovalent counterparts. The striking difference in enzymatic activity displayed by these enzymes is explained by invoking a model wherein the catalytic and lectin domains interact simultaneously with the polyvalent substrate. These findings have been exploited in the design of a novel polyvalent inhibitor of the sialidase of *Vibrio cholerae* that targets the lectin domain. This inhibitor is the first of its type in that it is not based on a sialic acid related scaffold and demonstrates a simple way of engineering exquisite selectivity for inhibitors of modular enzymes that possess a catalytic domain *together* with one or more binding domains.

Monovalent trisaccharide **1** and glycopolymer **2** (Figure 1) were prepared to study the effect of substrate clustering on steady-state kinetic parameters of hydrolysis by the large sialidases of *Clostridium perfringens* and *V. cholerae* and the small sialidase of *Salmonella typhimurium*. The first two enzymes are typical modular enzymes comprising a catalytic domain flanked by one or more lectin domains.[4] The small sialidase of *S. typhimurium*, however, possesses only a catalytic domain.[5] Thus, it was envisaged that the role of the lectin domains in catalysis could be determined by correlating apparent kinetic parameters of hydrolysis of the mono- and polyvalent substrates by the small and large sialidases.

The enzymatic hydrolysis was carried out at varying substrate concentrations based on equivalents of trisaccharide (valency corrected). The initial rates were determined by quantifying the amounts of free sialic acid using a newly developed HPLC-based method. Michaelis constants (K_m) and the maximum velocities (V_{max}) were determined from nonlinear fittings of the Michaelis−Menten equation. The relative V_{max} (rel V_{max}) of monovalent compound **1** was arbitrarily set at 1.

The hydrolysis of polyvalent 3′-siayl *N*-acetyllactosamine **2** by the two large sialidases is seen to display apparent kinetic parameters that are markedly different from those of the monovalent derivative **1**. For both of these enzymes, the K_m for the polyvalent substrate is in the order of 100-fold smaller than that of the monovalent derivative, whereas the rel V_{max} for both is slightly diminished (Table 1). It follows that the catalytic efficiency (V_{max}/K_m) for the hydrolysis of the polyvalent substrate is significantly enhanced due to a dramatically smaller K_m. In striking contrast,

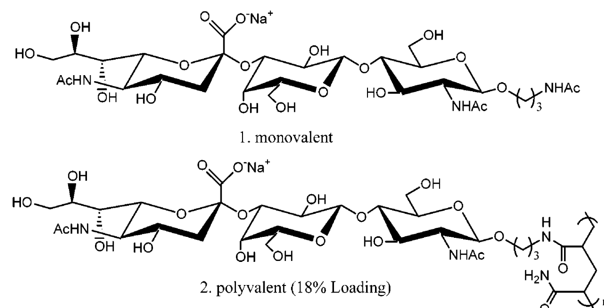

Figure 1. Mono- and polyvalent 3′-sialyl *N*-acetyllactosamine.

Table 1. Apparent Kinetic Parameters of Hydrolysis of Monovalent **1** and Polyvalent **2** by Sialidases

sialidase	compd	K_m (mmol)	rel V_{max}	V_{max}/K_m
Clostridium perfringens	**1**	2.2 ± 0.3	1	0.45
	2	0.04 ± 0.02	0.85	21
Vibrio cholerae	**1**	5.7 ± 0.4	1	0.18
	2	0.04 ± 0.01	0.23	5.8
Salmonella typhimurium	**1**	2.1 ± 0.5	1	0.48
	2	2.9 ± 0.3	1.4	0.48

the small enzyme of *S. typhimurium* displays very similar apparent kinetic parameters for both the mono- and polyvalent substrates, strongly suggesting that the observed increased catalytic efficiencies of the large enzymes are a consequence of their lectin domains.

The greatly enhanced catalytic efficiency of desialylation of polyvalent **2** by large sialidases can be rationalized by a model whereby the catalytic and lectin domain bind simultaneously with the polyvalent substrate, leading to enhancement of affinity.[1] We reasoned that further evidence for this mechanism could be obtained by simply nullifying the effects of the lectin domains by introducing a high concentration of ligand for the lectin that could effectively compete with the polymeric substrate. Under these conditions, apparent kinetic parameters for hydrolysis of the polymer would be expected to be comparable to those for the hydrolysis of the monovalent substrate. We conjectured that D-galactose might serve as an appropriate ligand for the lectin: terminal galactosyl residues are often exposed by the action of sialidases on their oligosaccharide substrates, and moreover, an X-ray crystal structure of the large sialidase of *M. viridifaciens* shows that D-galactose can, indeed, complex to its lectin domain.[6] As expected, when the hydrolysis of the polyvalent sialoside **2** by the sialidase of *V. cholerae* was performed in the presence of increasing concentrations of D-galactose, values for both K_m and rel V_{max} were seen to mount, which resulted in a gradual decrease in catalytic efficiency (Table 2).

To rule out that the observed inhibition was an artifact due to trans-sialylation (transfer of sialic acid from the substrate to galactose),[7] the inhibition assay was repeated using D-lactose in place of D-galactose, and the resulting hydrolysis mixture was examined by Dionex HPLC using sialyllactose as a standard.

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Table 2. Apparent Kinetic Parameters of *Vibrio Cholerae* Sialidase in the Presence of Mono- and Polyvalent Inhibitors

compd	inhibitor amt (mmol)	K_m (mmol)	rel V_{max}	V_{max}/K_m
Inhibitor: Galactose				
2	0	0.04 ± 0.01	0.23	5.8
2	5	0.09 ± 0.02	0.26	2.9
2	10	0.20 ± 0.06	0.33	1.6
2	15	0.5 ± 0.1	0.43	0.86
1	0	5.7 ± 0.4	1	0.18
1	5	5.3 ± 0.4	0.8	0.15
Inhibitor: Polyvalent Melibiose				
2	0.10	0.25 ± 0.04	0.48	1.9
2	0.25	0.54 ± 0.1	0.81	1.5
2	0.50	1.6 ± 0.3	1.1	0.69

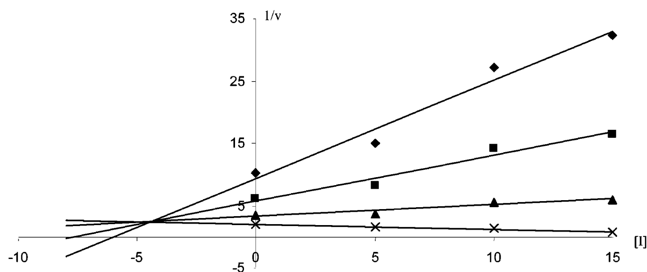

Figure 2. Dixon plot of hydrolysis of sialoside **2** by sialidase of *V. cholerae* in the presence of different concentrations of D-galactose. ◆, 0.01; ■, 0.02; ▲, 0.06; and ×, 4.0 mmol (valency-corrected concentrations of substrate).

Approximately 5% of the trans-sialylation product, sialyllactose, was determined relative to free sialic acid, and correction for this resulted in only a very slight increase in V_{max}. Furthermore, addition of D-galactose had only a marginal effect on hydrolysis of the monomeric trisaccharide substrate **1**, indicating that interaction of D-galactose with the catalytic domain does not play any part in the observed effects on catalysis.

The inhibition data were further analyzed by a Dixon plot (Figure 2), which showed that, at relatively low substrate concentrations, D-galactose competitively inhibits the hydrolysis of the polyvalent substrate, with $K_i = 5$ mmol. On the other hand, at a very high substrate concentration, increasing concentrations of D-galactose led to small increases in velocity, indicating that, under these conditions, D-galactose acts as a weak activator. This observation is, however, in agreement with the data presented in Table 1. Hydrolysis of the polyvalent substrate by the large sialidases led to a small reduction of V_{max}; therefore, at high substrate concentrations, the involvement of the lectin domains will lead to slightly decreased velocities. Thus, inhibition of the lectin domains under these conditions might be expected to lead to activation of hydrolysis, which is observed in the Dixon plot.

Under physiological conditions, concentrations of sialosides are generally very low; therefore, galactosides provide intriguing possibilities for the design and synthesis of more potent inhibitors. We postulated that a polyvalent galactoside may simultaneously interact with the two lectin domains and the catalytic fold. This cluster of weak-binding interactions would result in an enhancement of association over and above that expected on the basis of valency. Thus, aminopropyl melibiose [Gal(1−6)Gal1-$(CH_2)_3NH_2$] appended to a poly[*n*-acrylamide] backbone was synthesized, and varying concentrations of the resulting polyvalent galactoside were employed to inhibit desialylation of **2**.

Increasing the concentration of the polyvalent inhibitor did, indeed, result in gradually magnified values for both K_m and rel V_{max} (Table 2). A value of $K_i = 50$ μmol (valency corrected) was determined from a Dixon plot for the galactoside polymer, which is seen to be a 100-fold better inhibitor than monovalent D-galactose. This finding is significant since it has been difficult to develop potent inhibitors of bacterial sialidases.[8] Furthermore, an important property of the new inhibitor is that it displays an exquisite selectivity for sialidases that have a lectin domain. The latter was born out by the observation that the small sialidase of *S. typhimurium* was not inhibited by polyvalent galactoside (data not shown).

Although modular enzymes are encountered in nature, the possible consequences of this modularity on their mode of catalysis have not, thus far, been well-studied or rationalized.[9] We have successfully been able to unravel important aspects of the mode of action of modular bacterial sialidases. These enzymes, which are often secreted, are involved in pathogenesis of a range of diseases, such as gas gangrene, septicemia, pneumonia, peritonitis, meningitis, and cholera.[10] In the extracellular environment, concentrations of sialosides are generally very low, which, in combination with typical Michaelis constants of sialidases in the millimole range, would lead to negligible activities. However, in biological systems, which are not homogeneous, high local concentrations of sialosides commonly exist on cell surfaces. The results of the present study suggest that certain bacterial sialidases have evolved in such a way that they can efficiently desialylate multivalent entities, such as those on cell surfaces. It seems that these pathogenic bacteria have been able to evolve this advantage by simply "hijacking" lectin domains and affixing these modules to an existing highly developed catalytic domain, thereby creating a modular enzyme that has a greatly enhanced effectiveness for polyvalent entities. It is to be expected that other enzymes, especially those that are secreted and implicated in invasion, may also have developed a mode of action akin to that we have postulated for bacterial sialidases.[11]

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Supporting Information Available: Synthetic procedures and analytical data for **1** and **2**, enzyme assay methodology, and Michaelis−Menten, Lineweaver−Burk, and Dixon plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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