served in this study provides a novel approach to the synthesis of a new class of high-order monosaccharide structures. Given that the enzyme is readily available and highly stable as a free or immobilized form,[7a,b,12] NeuAc aldolase appears to be a useful catalyst for the synthesis of a number of uncommon sugars.

(12) Kim, M.-J.; Hennen, W. J.; Sweers, H. M.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 6481.

Observation of Noncovalent Enzyme–Substrate and Enzyme–Product Complexes by Ion-Spray Mass Spectrometry

Bruce Ganem*

Department of Chemistry, Baker Laboratory Cornell University, Ithaca, New York 14853

Yu-Tsyr Li and Jack D. Henion*

Drug Testing and Toxicology, New York State College of Veterinary Medicine, Cornell University 925 Warren Drive, Ithaca, New York 14850

Received June 19, 1991

The newly developed techniques of electrospray[1] and ion-spray[2] (pneumatically assisted electrospray) mass spectrometry (MS) permit the formation of gas-phase macromolecular ions directly from solution at atmospheric pressure via protonation and ion evaporation. Recently we described for the first time the successful detection of noncovalent receptor–ligand complexes using continuous-introduction ion-spray MS.[3] Here we report that noncovalent enzyme–substrate and enzyme–product complexes of hen egg white lysozyme (HEWL) can also be detected as their protonated, multiply charged species. That the observed complexes are active-site directed and not anomalous aggregates resulting from the electrospray ionization process is supported by several lines of evidence.

Figure 1 depicts a typical ion-spray mass spectrum of HEWL (MW 14305) showing the expected distribution of multiply protonated, multiply charged parent ions.[4] Since ion-spray MS does not generally cause molecular fragmentation, the possibility of detecting noncovalent association complexes between HEWL and its substrates or products may be considered.

HEWL hydrolyzes a hexasaccharide of *N*-acetylglucosamine (NAG_6) with principal cleavage between residues 4 and 5 to produce NAG_4 and NAG_2.[5] From an X-ray structure determination of the enzyme–NAG_3 complex, Perkins et al. propose a model for the lysozyme binding site in which six subsites (designated A–F) can accommodate up to a hexasaccharide substrate domain.[6]

The reaction of HEWL with NAG_6 can be monitored at its optimum pH (NH_4OAc buffer, pH 5)[7] by ion-spray MS, as illustrated in Figure 2, parts A–C. Besides the expected multiply protonated molecule ions at m/z 1789 $(HEWL + 8H)^{8+}$ and 2045 $(HEWL + 7H)^{7+}$, a mass spectrum immediately after mixing (Figure 2A) reveals a new protonated ion at m/z 1943 corresponding to $(HEWL + NAG_6)^{8+}$. A weak ion at m/z 1893 is observed in Figure 2A which increases with time (Figure 2, parts B and C) and represents the $(HEWL + NAG_4)^{8+}$ enzyme–product complex. Binding of HEWL to NAG_2 is almost 100 times weaker (Table I);[8] thus $(HEWL + NAG_2)^{8+}$ is not detected. Slow hydrolysis of NAG_4 and NAG_3 and NAG accounts for the appearance of a weak $(HEWL + NAG_3)^{8+}$ ion at m/z 1868 (Figure 2B).

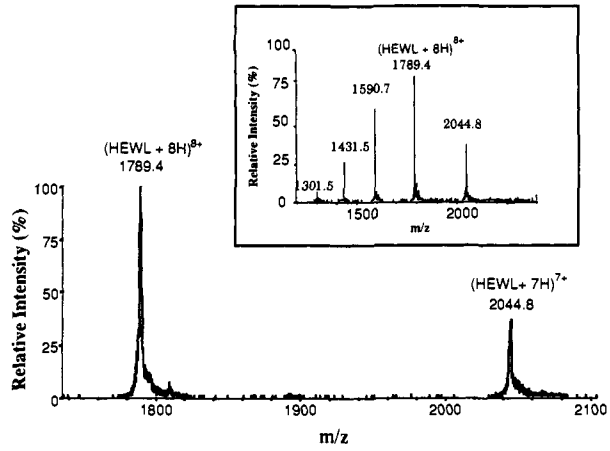

Figure 1. Ion-spray mass spectrum of HEWL recorded on a Sciex TAGA 6000E triple quadrupole mass spectrometer. HEWL was infused (1 μg/μL in 10 mM NH_4OAc buffer, pH 5.0) at 2 μL/min through the ion-spray interface. The instrument was calibrated using the multiply charged ions of HEWL. Unit mass resolution was adjusted to give an approximately 30% valley on the singly charged ions of PEG 2000. The spectrum is an averaged sum of 10 scans from m/z 1200 to 2400 at a scan rate of 2 s/scan. The envelope of multiply protonated, multiply charged ions ranges from the $(M + 7H)^{7+}$ to the $(M + 11H)^{11+}$ charge states of HEWL (figure inset). The mass-to-charge region between the 8+ and 7+ charge states (main figure) is the region of interest for detecting noncovalent enzyme–substrate and enzyme–product complexes described in this work.

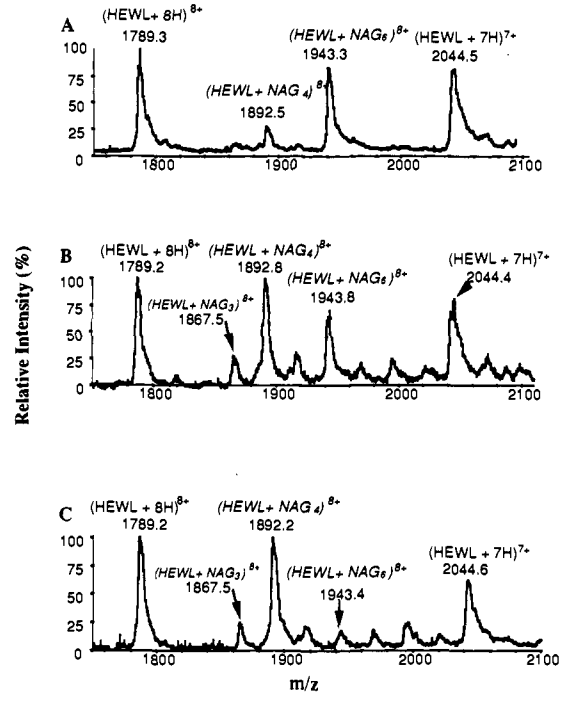

Figure 2. An on-line ion-spray MS time-course study for the hydrolysis of NAG_6 by HEWL at room temperature over 60 min. A mixture of HEWL (3.17×10^{-5} M) and NAG_6 (4.41×10^{-4} M) in buffer (10 mM NH_4OAc, pH 5.0) was infused through the ion-spray interface at 2 μL/min. Mass spectra were obtained as 1 min (panel A), 10 min (panel B), and 60 min (panel C).

(1) (a) Wong, S. F.; Meng, C. K.; Fenn, J. B. *J. Phys. Chem.* **1988**, *92*, 546. (b) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.

(2) (a) Bruins, A. P.; Covey, T. R.; Henion, J. D. *Anal. Chem.* **1987**, *59*, 2642. (b) Covey, T. R.; Bonner, R. F.; Shushan, B. I.; Henion, J. D. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249.

(3) Ganem, B.; Li, Y.-T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 6294.

(4) Mann, M.; Meng, C. K.; Fenn, J. B. *Anal. Chem.* **1989**, *61*, 1702.

(5) Rupley, J. A.; Gates, V. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *57*, 496.

(6) Perkins, S. J.; Johnson, L. N.; Philips, D. C.; Dwek, R. A. *Biochem. J.* **1981**, *193*, 554.

(7) Banerjee, S. K.; Kregar, I.; Turk, V.; Rupley, J. A. *J. Biol. Chem.* **1973**, *248*, 4786.

(8) Schindler, M.; Assaf, Y.; Sharon, N.; Chipman, D. M. *Biochemistry* **1977**, *16*, 423.

0002-7863/91/1513-7818$02.50/0

Table I. Summary of Binding and Hydrolysis Data for Oligosaccharides of *N*-Acetylglucosamine

saccharide	$K_{association}$[a]	relative hydrolysis rate[b]
NAG	0.03 M^{-1}	
NAG_2	3.2×10^3 M^{-1}	0.003
NAG_3	1.1×10^5 M^{-1}	1
NAG_4	1.8×10^5 M^{-1}	8
NAG_6	$\sim NAG_4$[c]	30000

[a] At pH 6.58; data from Schindler, M., et al. (ref 8). [b] Data from Rupley and Gates, 1967 (ref 5). [c] Apparent first-order kinetics of lysozyme-catalyzed hydrolysis at $[NAG_6]$ above K_M indicate essentially identical equilibrium binding of substrate and one product: Banerjee et al. (ref 7).

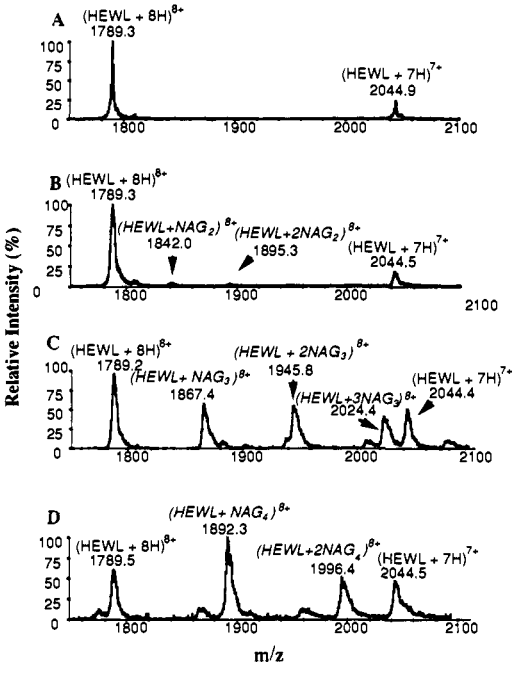

Figure 3. Ion-spray mass spectra of HEWL (3.47×10^{-5} M) with NAG_n (n = 1–4; 2.97×10^{-4} M) at pH 5.0 in NH_4OAc buffer. Each spectrum is an averaged sum of 10 scans recorded 10 min after mixing at room temperature. Panel A: NAG. Panel B: NAG_2. Panel C: NAG_3. Panel D: NAG_4. Only the mass-to-charge region of interest for the most abundant $(M + 8H)^{8+}$ charge state with the NAG_n species is displayed.

Other evidence suggests that the (HEWL + NAG_6) and (HEWL + NAG_4) complexes are active-site directed and not anomalous association complexes. Relative ion intensities of $(HEWL + NAG_n)^{8+}$ complexes (Figure 3, parts A–D) qualitatively parallel the known association constants for (HEWL + NAG_n) in solution (Table I),[8] with $(HEWL + NAG_4)^{8+}$ and $(HEWL + NAG_3)^{8+}$ complexes significantly more abundant than $(HEWL + NAG_2)^{8+}$, which is barely detectable (Figure 3B). No complex is observed between HEWL and NAG (Figure 3A).

Additional ions are observed in parts C and D of Figure 3 corresponding to higher order 1:2 and 1:3 complexes of HEWL with NAG_4 (m/z 1996) and NAG_3 (m/z 1945, 2024) which can be reduced but not eliminated by increasing the declustering voltage energy at the ion-sampling orifice.[9] Such complexes may be artifacts of proton-bound NAG_n adducts complexed to HEWL.[10] Alternatively, higher order HEWL-NAG_3 and HEWL-NAG_4 complexes may result from multiple binding of these smaller substrates to the enzyme's hexasaccharide binding domain. Recent calculations suggest that the active site of HEWL is bifurcated at subsite D,[11] raising the possibility that short NAG

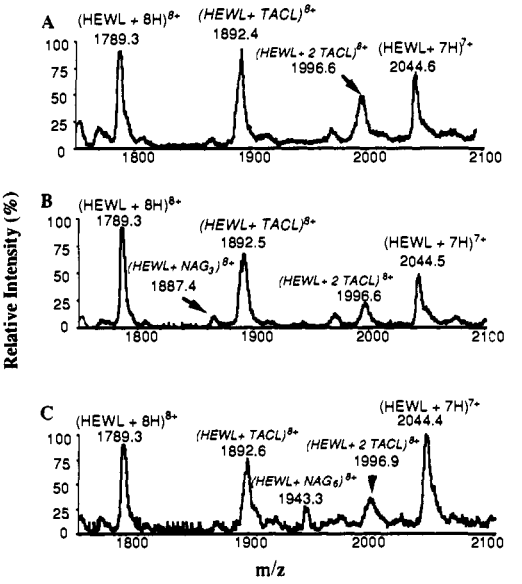

Figure 4. The effect of TACL on HEWL–NAG_n mixtures. Panel A: HEWL (3.93×10^{-5} M) and TACL (2.1×10^{-4} M). Panel B: HEWL, NAG_3 (2.1×10^{-4} M), and TACL. Panel C: HEWL, NAG_6 (2.1×10^{-4} M), and TACL. These mass spectra were recorded on line at room temperature, 1 min after mixing.

oligomers might simultaneously occupy up to three regions of the active site (subsites ABC, EF, and E'F').[12]

Additional mass spectrometric measurements with tetra-*N*-acetylchitotetraose δ-lactone (TACL), a well-known competitive inhibitor of HEWL,[13] are shown in Figure 4. From its association constant at pH 5.0 (3.3×10^6 M^{-1}), TACL is some 20–30 times more strongly bound at the active site than NAG_3, NAG_4, or NAG_6;[13] accordingly the $(HEWL + TACL)^{8+}$ complex at m/z 1892 is readily detected by ion-spray MS (Figure 4A). Some $(HEWL + 2TACL)^{8+}$ complex at m/z 1997 is also observed, as is the proton-bound TACL dimer in the mass spectrum of inhibitor alone.

Parts B and C of Figure 4 illustrate the effect of TACL on the complexation of HEWL with NAG_3 and NAG_6, respectively. For the hexasaccharide substrate, comparison of Figures 4C and 2A clearly shows a diminution in ion intensity for the $(HEWL + NAG_6)^{8+}$ complex at m/z 1943 relative to free HEWL when an equimolar amount of TACL is added. The inhibitor has the same effect on complexation of NAG_3, as is evident from the ability of TACL to suppress the $(HEWL + NAG_3)^{8+}$ ion at m/z 1867 (compare Figure 4B with Figure 3C). Such clear trends between relative ion abundances and both substrate and inhibitor association constants are highly unlikely if the oligosaccharides are randomly aggregating on the exterior surface of the protein.

The experiments described herein suggest that other host–guest complexes, and perhaps even supramolecular protein–protein and protein–nucleic acid complexes, may be detectable under conditions which are compatible with ion-spray MS.[14] If so, the method should prove useful in probing noncovalent binding interactions responsible for molecular recognition and other biological phenomena.

Acknowledgment. We thank Professor Taichi Usui for providing NAG_6 and Professor Gustav Lienhard for providing TACL. This research was supported by a grant from the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office, and the National Science Foundation.

(9) Smith, R. D.; Loo, J. A.; Barinaga, C. J.; Edmonds, C. J.; Udseth, H. P. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 53.

(10) Mass spectra of pure NAG_4 and NAG_3 in the absence of HEWL exhibit weak, concentration-dependent ions corresponding to $(NAG_3)_2$, $(NAG_3)_3$, and $(NAG_4)_2$.

(11) Pincus, M. R.; Scheraga, H. A. *Acc. Chem. Res.* **1981**, *14*, 299.

(12) Smith-Gill, S. J.; Rupley, J. A.; Pincus, M. R.; Carty, R. B.; Scheraga, H. A. *Biochemistry* **1984**, *23*, 993.

(13) Secemski, I. I.; Lehrer, S. S.; Lienhard, G. E. *J. Biol. Chem.* **1972**, *247*, 4740.

(14) Noncovalent polysaccharide–lipid interactions have recently been probed by fast-atom-bombardment MS: Ballou, C. E.; Dell, A. *Carbohydr. Res.* **1985**, *140*, 139.