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Evidence for enzymatic activity in the absence of solvent in gas-phase complexes of lysozyme and oligosaccharides

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Abstract

Hen egg-white lysozyme complexed to substrate and nonsubstrate oligosaccharides are examined by electrospray ionization mass spectrometry. Heated capillary dissociation and collision induced dissociation (CID) are used to characterize the complexes. The relative order of stability obtained in gas phase agrees well with their solution-phase association constants. A proton transfer reaction occurs during the CID of substrate complexes that is not observed with non-substrate complexes. Oligosaccharide fragments are also observed with lysozyme–chitohexaose complex during CID. The possibility of lysozyme activity in the gas phase, or a gas-phase enzymatic activity, is explored. (Int J Mass Spectrom 193 (1999) 103–114) © 1999 Elsevier Science B.V.

Keywords: CID; Lysozyme; Gas phase; Enzyme

1. Introduction

Mass spectrometry has recently been used to investigate the higher-order structures of proteins. McLafferty and co-workers [1] first reported the folding and unfolding of protein in vacuo. Since then several papers on folding and unfolding of proteins in the gas phase, including cytochrome *c* and lysozyme [2–8] have been reported. A key question remains, however, as to whether the gas-phase structures are related to those in solution. Several reports address this question by different means. Smith and co-workers studied peptide and protein dimers by two different electrospray ionization (ESI) interfaces, one with the countercurrent flow of bath gas and the other with heated metal capillary, both coupled with collision induced dissociation (CID) [9,10]. The authors concluded that at least some forms of noncovalent associations found in solution are preserved during the ESI process. Williams and co-workers utilized proton transfer reactivity to investigate isolated charge states of hen egg-white lysozyme [2]. It was found that the 9+ and 10+ charge states have reactivity consistent with their crystal structures. Recently, Loo *et al*. examined the structure of RES-701-1, a hexadecapeptide isolated from *Streptomyces*, and its synthetic analog [11]. The naturally occurring peptide has a unique highly ordered structure, which can not be duplicated synthetically. They reported significant differences in the two peptides in their collisional induced dissociation (CID) and solution-phase H/D exchange. The authors surmised that the natural peptide can withstand the solution-to-gas

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phase transition and preserve its solution-phase structure.

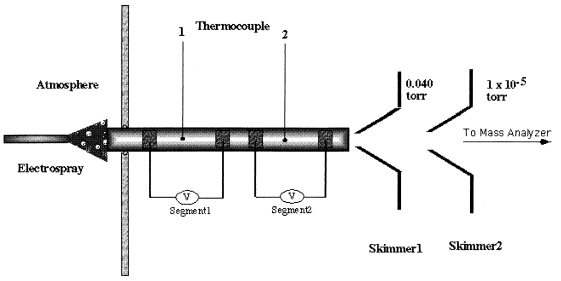
In this article, we use ESI Fourier transform mass spectrometry (FTMS) to investigate enzyme substrate complexes. If enzyme–substrate complexes are found to have enzymatic activity in vacuum, it would be the most direct evidence to indicate whether solution-phase structures are preserved in the gas phase. The lysozyme used in this research, hen egg-white lysozyme, is a single protein chain of 129 residues. It is a compact, highly stable protein with four disulfide bonds. Therefore, it serves as a good candidate for studying the stability of enzyme–substrate complexes and enzymatic activity in the gas phase by electrospray mass spectrometry.

Hen egg-white lysozyme was chosen as the model enzyme to examine gas-phase enzymatic activities. Lysozyme is widely found in living organism and was one of the first enzymes whose crystal structure was determined [12,13]. In solution, it hydrolyzes β(1–4)-glycosidic bonds in bacterial cell wall carbohydrates. Oligosaccharides composed of *N*-acetylglucosamine oligomers (commonly known as chitin) [14] are also natural substrates of lysozyme. It is well accepted that lysozyme contains a grove able to bind up to six contiguous saccharide residues. An essential requirement for the specific binding of oligosaccharides to lysozyme is the *N*-acetyl group on the sugar residue, which interacts strongly with the enzyme by forming two hydrogen bonds. The aspartic acid 52 (Asp 52) and glutamic acid 35 (Glu 35) participate directly in the catalysis [14–17]; the COOH group of Asp 52 donates a H^+ to the glycosidic oxygen to cleave the glycosidic bond through a positively charged *carbonium ion* intermediate. This process is facilitated by the resulting negative charge on the Asp 52, which electrostatically stabilizes the positive charge. The nascent *carbonium ion* intermediate then reacts with H_2O from the solution and produces the hydrolysis products. Henion and co-workers studied lysozyme complexes with *N*-acetylglucosamine oligosaccharides [18]. By electrospraying enzyme substrate solutions, they were able to determine the relative abundance of complex peaks in the mass spectra. A good agreement was found between the complex intensity and their solution-phase binding constants. Furthermore, they investigated the complexation when tetra-*N*-acetylchitotetraose δ-lactone, one of the lysozyme inhibitors, was added to the solution. They concluded that the close correlation observed between relative ion abundance and substrate or inhibitor association constants are unlikely to be a random aggregation of oligosaccharides on the exterior surface of the enzyme. However, the probe of the gas-phase structure of the complexes has not been reported. Nonetheless, this study was important for understanding structures and behavior of noncovalent complexes produced by electrospray ionization.

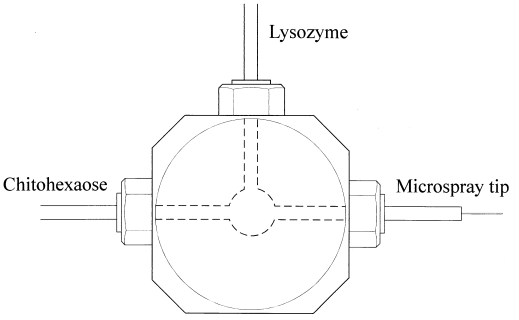
2. Experimental

Hen egg-white lysozyme (L) and all the glucose polymers: maltose (M_2), maltotriose (M_3), maltotetraose (M_4), maltopentaose (M_5), and maltohexaose (M_6) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. All *N*-acetylglucosamine oligosaccharides: chitobiose (O_2), chitotriose (O_3), chitotetraose (O_4), chitopentaose (O_5), and chitohexaose (O_6) were obtained from Seikagaku Corporation (Tokyo, Japan) and used without further purification. The electrospray solutions were prepared by dissolving the protein and oligosaccharides (1:5) in 50:50 water:methanol solvent at a lysozyme concentration of 1.0×10^{-5} M, unless otherwise specified.

The experiments were carried out on a home-built electrospray FTMS described in an earlier publication [19]. The ions produced from the needle travel through a heated capillary and a set of skimmers and lenses. Upon exiting the source, the ions are guided through the fringing field of the 3.0 tesla superconducting magnet by using rf-only quadrupole rods to the ion cyclotron resonance (ICR) cell where the ions are detected. The analyte solution is transported from the syringe pump to the electrospray needle at a flow rate of 5 mL/h in all experiments unless otherwise stated.



Scheme 1. Differentially heated capillary.



Scheme 2. “Tee” configuration.

2.1. Heated capillary dissociation

Heated capillary dissociation (HCD) experiments were carried out on a differentially heated capillary. The apparatus is shown in Scheme 1. The capillary is mounted inside a home-built electrospray source whose operation is described elsewhere [20,21]. Only the relevant features of the newly designed capillary will be described here. The capillary is made of stainless steel with 1.9 mm o.d., 0.5 mm i.d., and 25 cm length. The capillary is divided into two segments of equal length, with the one close to the electrospray needle (front end) designated as segment1, and the one close to the ICR cell (back end) designated as segment2. Both segment1 and segment2 are resistively heated by two dc power supplies. Two copper blocks connect the capillary and the leads on each segment. Each segment has a J-type thermocouple connected to the middle of the segment to monitor the temperature during the experiment. Because of the dynamic nature of the system, a temperature gradient exists in the capillary. The temperature is measured in the middle of each segment to monitor the hottest point. The front end of the capillary is attached to a counter electrode which is charged to 71 V. An O-ring seals the atmosphere from the stage 1 of the differential pumping system. The back end of the capillary points to the hole of skimmer1, which opens to skimmer2 and pumping stage 2.

The temperature response of one segment when the other is heated has been characterized [21]. To ensure that the dissociation is a gas-phase rather than a solution phase process, the HCD experiments are performed by setting the temperature of the first segment to desolvate ions. The temperature on the second segment is then increased to perform the dissociation. We have earlier shown that the dissociation process is a gas-phase process [21]. During the HCD experiments, all other conditions including needle, capillary, lens, and skimmer voltages are held constant, unless specified. The temperature on segment2 is monitored and the temperature at which the complex is no longer observed ($<$1%) is designated as the dissociation temperature (T_d). We have earlier shown that T_d is a relative measure of complex binding strength [20,21].

For the HCD and CID experiments, an intense signal of the intact complex is required. For the oligomers chitobiose, chitotriose, and chitotetraose premixing the lysozyme and the oligosaccharide in the same solution was sufficient. However, chitopentaose and chitohexaose reacted too quickly to produce the intact complex. To obtain stronger signals of intact complexes of chitopentaose and chitohexaose, a “Tee” type connector was used to mix the two solutions immediately before the electrospray, as shown in Scheme 2. The Tee has a 0.75 mm bore (fitted for 1/16 in. tubing) with a length (horizontal) of 23 mm.

The length from the center of the Tee connector to the needle tip is 20 mm, and the inner diameter of the stainless steel needle is 100 μm. With the typical flow rate of 5 mL/h, the mixing period of the two solutions is about 0.5 s. Under these conditions, only a small portion of the oligosaccharides are hydrolyzed before

introduction into the gas phase. With this setup, abundant signals corresponding to the desired complexes of chitopentaose and chitohexaose with lysozyme are observed.

2.2. Collision-induced dissociation

There are basically two types of CID experiments in FTMS: on-resonance and sustained off-resonance irradiation (SORI) [22]. Both were performed on the lysozyme complexes. For both methods, the complex ions are isolated with a series of pulses and bursts produced by an arbitrary waveform generator as part of the IonSpec data system. Ions are excited to increase the translational energy and allowed to collide with gas pulsed to a maximum pressure of 5×10^{-7} Torr. During collisions, translational energies are converted into internal energies resulting in the dissociation of the complex ions. With on-resonance CID, the parent ion was excited by a 0.5 ms rf pulse with frequency corresponding to the cyclotron frequency. Ions were detected 6 s after the excitation to allow sufficient time for collisions. With SORI-CID, the ion was excited by a rf a 800 Hz above the cyclotron frequency for a duration of 1 s. Two nitrogen pulses in 500 ms intervals were applied to maintain a pressure of about 5×10^{-7} Torr during the CID event. SORI-CID results in the sequential activation of ions by multiple, low energy ($<$10 eV) collisions [22]. As a consequence, only small increments of internal energy are deposited into the ion throughout the duration of the event. This mode of activation is analogous to infrared multiphoton dissociation using low power lasers; it can be used as a probe of the lowest decomposition pathway of ions.

3. Results and discussion

3.1. Complex formation

β-1,4 linked oligomers of *N*-acetylglucosamine are natural substrates of lysozyme. Table 1 lists the association constants [23] and the relative hydrolysis rate constants [24] of chitin oligosaccharides when reacted with lysozyme. For chitotetraose (O_4), chitotriose (O_3), and chitobiose (O_2), the hydrolysis rates are very slow. Indeed, after mixing O_4, O_3, and O_2 with lysozyme in 50:50 H_2O/methanol solution, no hydrolysis products are observed even after 70 min. Fig. 1(a) shows the spectrum of lysozyme mixed with chitotetraose with a capillary temperature of 215 °C. The only complex peak observed belongs to the +8 charge state. Complexes of other charge states (+9, +7) are not observed in this spectrum but are observed under conditions of low capillary temperature (125 °C). However, they remain with very low abundances and dissociate readily when the temperature of the capillary is increased. Chitotriose (O_3) and chitobiose (O_2) yield similar results.

Table 1
Association constants K_{assoc} and relative rate constants of hydrolysis of hen egg white lysozyme and *N*-acetylglucosamine oligosaccharides complexes in solution; the deviation in the dissociation temperature is ±0.5 °C

Oligosaccharide	K_{assoc} (M^{-1})[a]	Relative rate of hydrolysis	T_d (°C)
$(GlcNAc)_2$	3.2×10^3	0	262
$(GlcNAc)_3$	1.1×10^5	1	335
$(GlcNAc)_4$	1.8×10^5	8	373
$(GlcNAc)_5$	~$(GlcNAc)_4$	4 000	405
$(GlcNAc)_6$	~$(GlcNAc)_4$	30 000	409
$(GlcNAc)_8$	~$(GlcNAc)_4$	30 000	

[a] See [23].

When lysozyme is mixed with chitopentaose (O_5) and chitohexaose (O_6), hydrolysis products are immediately observed. Fig. 1(b) shows the spectrum of lysozyme and chitohexaose (O_6) dissolved in 50:50 H_2O/methanol at a capillary temperature of 220 °C. The hydrolysis products corresponding to O_3 and O_4 are observed complexed to lysozyme. The O_4 complex is observed primarily in the +8 charge state, while the O_3 complex is observed in the +7 charge state. The intact complexes of chitohexaose $[L{:}O_6 + 7H]^{+7}$ and $[L{:}O_6 + 8H]^{+8}$ have very low intensities. Another product, O_2, is observed complexed to lysozyme at low capillary temperatures (below 90 °C, spectrum not shown).

Fig. 2(a) shows the spectrum of lysozyme and chitohexaose using the Tee arrangement. The intact

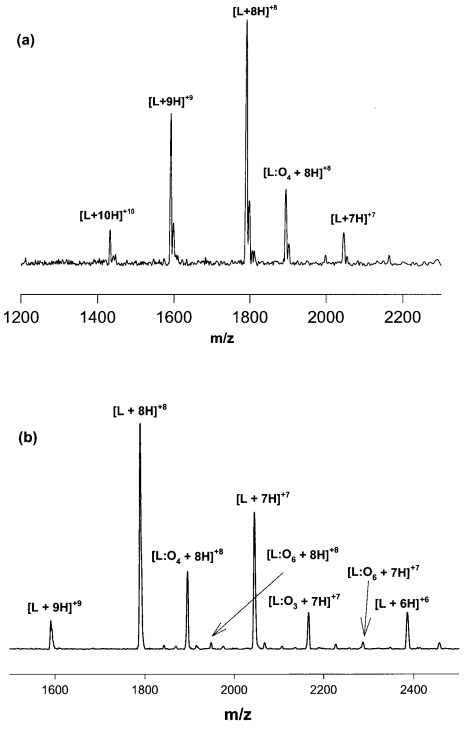


Fig. 1. ESI-FTMS spectra of lysozyme (1×10^{-5} M) in 50:50 H_2O/methanol with (a) chitotetraose (O_4) at capillary temperature of 215 °C and (b) chitohexaose (O_6) at capillary temperature of 220 °C.

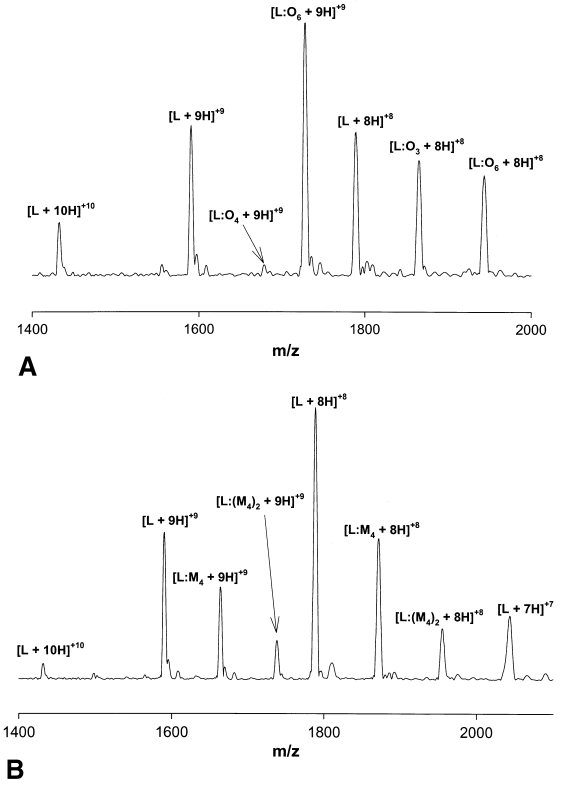


Fig. 2. ESI-FTMS spectra of lysozyme using the Tee (Scheme 2) with (a) chitohexaose (O_6) and (b) maltotetraose (M_4). See text for additional information.

complex peak corresponding to the +9 charge state, $[L:O_6 + 9H]^{+9}$, is the most abundant peak. The +8 charge state of the intact complex is about 40% of the base peak. Also observed are the complexes of hydrolysis products $[L:O_4 + 9H]^{+9}$ and $[L:O_3 + 8H]^{+8}$. The $[L:O_3 + 8H]^{+8}$ is about 50% relative to the base peak, while the intensity of $[L:O_4 + 9H]^{+9}$ is significantly lower. It is clear that using the Tee greatly reduces the hydrolysis before ionization and enhances the signal intensity of the intact complex peaks. In this article, all the HCD and CID results with chitopentaose and chitohexaose were obtained in this manner.

For comparison, nonsubstrate oligosaccharides (glucose and its oligomers, M_n, $n = 2, 3, \ldots, 6$) were examined. It is known that lysozyme does not hydrolyze these glucose oligomers in solution. When they are mixed with lysozyme and electrosprayed into the gas phase, their complexes with lysozyme are also observed. However, no hydrolysis products are observed even with the maltopentaose and maltohexaose (M_5 and M_6). Fig. 2(b) shows the spectrum of lysozyme with maltotetraose (M_4). No hydrolysis products are observed. In addition to single adduct complexes, e.g. $[L:M_4 + 9H]^{+9}$ and $[L:M_4 + 8H]^{+8}$, the double adduct species, $[L:(M_4)_2 + 9H]^{+9}$ and $[L:(M_4)_2 + 8H]^{+8}$ are also observed. Maltotriose and maltose also show similar adducts however with significantly lower intensities.

The typical condition for lysozyme enzymatic activity involves water at a pH 5.0 [12]. Complex formation followed by ESI were also performed under these conditions. In general, all spectra obtained from aqueous solution (pH 5.0) are similar to those obtained from 50:50 H_2O/methanol solution. Abundant products are observed with O_5 and O_6, however, no

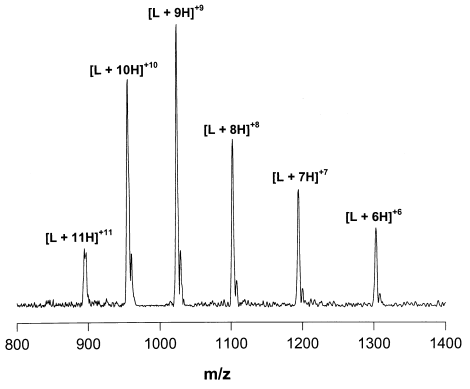


Fig. 3. ESI-FTMS spectrum of denatured hen egg-white lysozyme with chitotetraose (O_4) in H_2O/methanol solution.

hydrolysis products are observed with the rest of oligosaccharides, as before. Using the Tee connector also greatly increases the signal intensities of intact complex peaks of O_5 and O_6.

For comparison, denatured lysozyme was also examined. Lysozyme has four disulfide bonds that keep the protein in a very compact conformation. Reducing the four disulfide bonds unfolds the protein [25]. The denatured lysozyme is prepared by boiling a solution of lysozyme in 0.02 M dithiothreitol for 30 min immediately before it was electrosprayed. A spectrum of denatured lysozyme with chitotetraose is shown in Fig. 3. The charge state distribution of denatured lysozyme in the mass spectrum is shifted to high charge states. The dominant peak is the +14 charge state rather than the +8 charge state (Fig. 2) indicating that the denatured protein has an unfolded conformation. The molecular weight obtained from the ESI mass spectrum is eight mass unit higher, resulting from the reduction of four disulfide bonds. Interestingly, there are no complexes of denatured lysozyme and oligosaccharides observed. Evidently, denatured lysozyme does not form complexes with either type of oligosaccharides.

4.2. Heated capillary dissociation

Fig. 4 shows the result of a typical HCD experiment. A solution of lysozyme was mixed with both O_3 and O_4 (1:2 lysozyme:oligosaccharide) and the spectrum obtained at different temperatures. At a temperature of 202 °C, lysozyme complexes of both oligosaccharides are observed as the +8 charge state [Fig. 4(a)]. Complexes of other charge states such as +7 and +9 were observed at lower temperatures, albeit with very low abundances. They dissociated readily when the temperature is increased. When temperature is increased further to 220 °C, $[L{:}O_3 + 8H]^{8+}$ is almost fully dissociated [Fig. 4(b)]. The lysozyme complex with chitotetraose $[L{:}O_4 + 8H]^{8+}$ fully dissociates at a higher temperature (257 °C) Fig. 4(c). No fragmentation of the individual moieties (protein and oligosaccharides) was observed. It is believed that only the non-covalent complex dissociates under these conditions. For clarity, "fragment" will refer to fragmentation of individual moieties and "dissociate" will refer to the dissociation of the complex into two intact moieties.

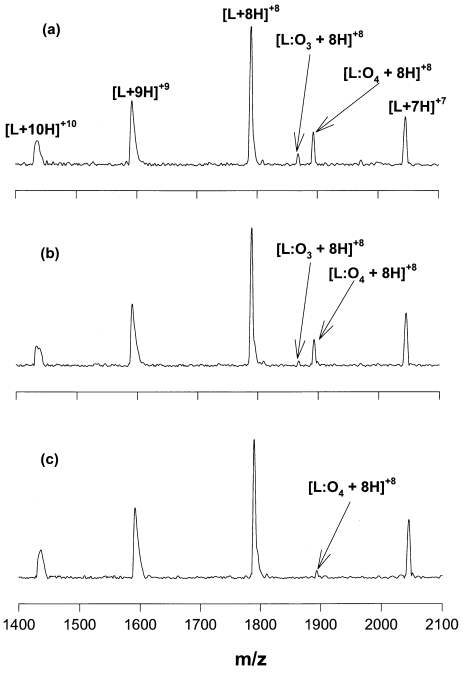


Fig. 4. ESI-FTMS spectra of lysozyme with chitotriose and chitotetraose at different temperatures of segment2. (a) 202, (b) 220, (c) 257 °C.

Note that the $[L{:}O_4 + 8H]^{8+}$ peak is already more intense than $[L{:}O_3 + 8H]^{8+}$ at low temperature [Fig.

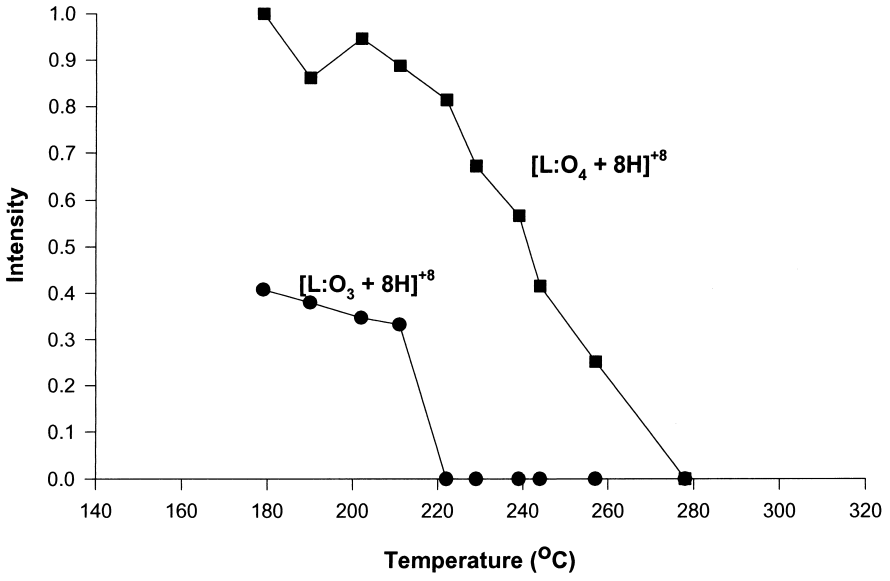


Fig. 5. HCD plot of lysozyme complexed with chitotriose $[L:O_3 + 8H]^{+8}$ and chitotetraose $[L:O_4 + 8H]^{+8}$. The dissociation temperature (T_d) corresponds to 220 °C for O_3 and 278 °C for O_4.

4(a)]. It should be pointed out that the lower intensity of the complex $[L:O_3 + 8H]^{8+}$ does not necessarily give it a lower T_d. We have observed less intense signals that produced higher T_d than more intense ones in the HCD of peptides complexed to cyclodextrins [20,21]. The relative intensities are likely a reflection of the relative solution-phase affinities. It is known that chitotetraose forms a stronger complex than chitotriose with lysozyme in solution (Table 1). It has been suggested that the peak intensities in the mass spectra are related to the relative binding affinity of noncovalent complexes in solution. Attempts to obtain solution-phase association constants by measuring peak intensities have been made [18]. The correlation between solution-phase association constants and the peak intensities further supports this notion.

By plotting the complex peak intensities versus temperatures, the HCD plot is obtained. Fig. 5 shows the HCD behavior of the complex $[L:O_4 + 8H]^{8+}$. This species fully dissociates at about 278 °C. A similar plot constructed for $[L:O_3 + 8H]^{8+}$ produces a lower dissociation temperature of 220 °C. The plots clearly show that $[L:O_{3,4} + 8H]^{8+}$ and $[L:O_4 + 8H]^{8+}$ have distinct dissociation temperatures, T_d. The summary of the results is tabulated in Table 2. The higher T_d of the chitotetraose complex relative to the chitotriose complex is consistent with their relative stability in the solution.

Two intriguing trends are found in Table 2. First,

Table 2
Dissociation temperatures (T_d) of substrate and nonsubstrate sugars complexed with hen white lysozyme, determined by heated capillary dissociation (HCD); the values have an uncertainty of ±5 °C

Sugar	M_2	M_3	M_4	M_5	M_6
T_d (°C)	<200	274	277	327	331
Sugar	O_2	O_3	O_4	O_5	O_6
T_d (°C)	262	335	373	405	409

the dissociation temperatures of complexes with substrate oligosaccharides (O_n) are consistently higher than the corresponding nonsubstrate oligosaccharides (M_n). For instance, the maltohexaose complex dissociates at a temperature of 331 °C, while chitohexaose dissociates at 409 °C. The other oligosaccharides in Table 2 show similar behavior.

Second, the relative order of stability of substrate oligosaccharide agrees with the K_{assoc} (Table 1). The relative gas-phase stability follows the order $O_6 > O_5 > O_4 > O_3 > O_2$, which is the same order as K_{assoc}. The fact that the results obtained in gas phase agree well with those in solution phase might be an indication that lysozyme complexes have similar structures and relative stability in both the gas phase and the solution phase. As Dobson and co-worker [26] reported using ^{1}H nuclear magnetic resonance (NMR) studies, the specific binding are the consequence of interactions including hydrogen bonds to the *N*-acetyl group, rather than to the pyranose rings. They further concluded that the complex formation employs an induced fit mechanism with the main driving force being hydrogen bonding. This may explain the consistency of the relative stability between gas and solution phase. It also suggests that although solvation is one of the major driving forces of complex formation of many noncovalent systems, it may not be as important in the lysozyme complexes.

4.3. Collision-induced dissociation

Both on-resonance and sustained off-resonance irradiation (SORI) CID were performed on the lysozyme complexes. Fig. 6(a) shows an on-resonance CID spectrum of the lysozyme-maltotriose (M_3) complex. The parent peak of $[L{:}M_4 + 8H]^{8+}$ was isolated by a series of sweeps and bursts. An on-resonance excitation pulse with 12.5 V (base to peak) was applied to the parent peak at the same frequency as its natural cyclotron frequency. The only product observed was the $[L + 8H]^{8+}$ species, as shown in:

$$[L{:}M_3 + 8H]^{8+} \xrightarrow{\text{CID}} [L + 8H]^{8+} + M_3 \qquad (1)$$

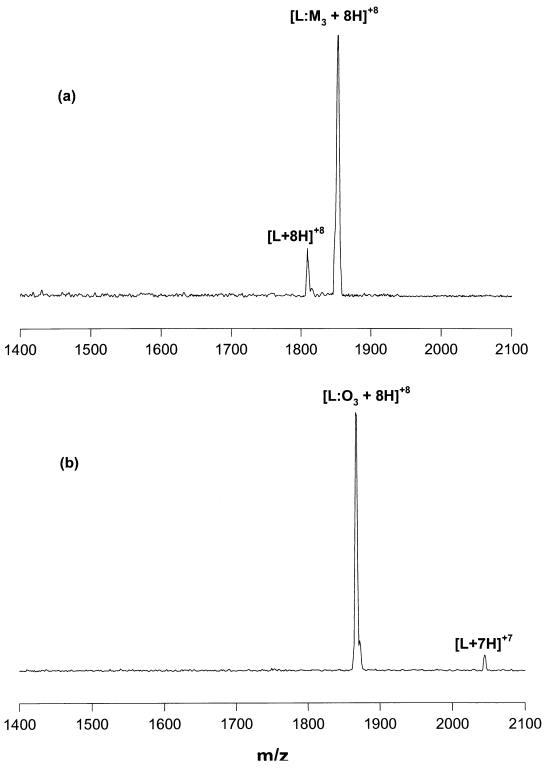


Fig. 6. On-resonance CID spectrum of (a) lysozyme–maltotriose complex with 12.5 V (base-peak) amplitude and (b) lysozyme–chitotetraose complex with 12.5 V (base-peak) amplitude.

Neither fragmentation of the lysozyme nor of the oligosaccharide is observed. Only dissociation occurs with the oligosaccharide leaving as a neutral species, which cannot be detected by mass spectrometry. The same results are obtained with lysozyme complexes of other non-substrate oligosaccharides M_2, M_4, M_5, and M_6.

It is believed that the complexes of lysozyme and nonsubstrate oligosaccharides are not specific. It was suggested by Henion that these nonspecific adducts are simple aggregation of the oligosaccharides on the protein surface [18]. With no specific interaction between the enzyme and oligosaccharide, the nonsubstrate oligosaccharide dissociates from the protein during CID.

When CID is performed on the complexes $[L{:}O_3 + 8H]^{8+}$ as shown in Fig. 6(b), the major product observed was the deprotonated lysozyme

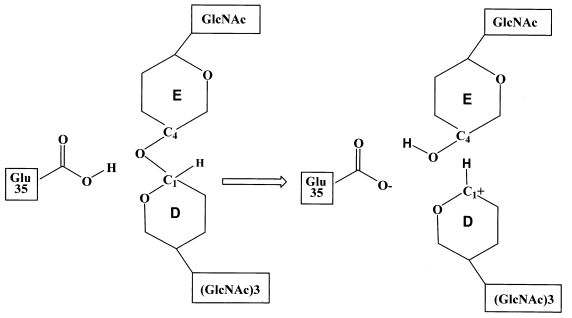
$[L + 7H]^{7+}$. The results correspond to a net charge transfer from the lysozyme to the oligosaccharide during the dissociation:

$$[L{:}O_3 + 8H]^{8+} \xrightarrow{CID} [L + 7H]^{7+} + [O_3 + H]^{+} \quad (2)$$

However, the singly charged chitotriose was not observed in the CID spectrum under the current experimental conditions. There are several reasons for the absence of the protonated oligosaccharide. The experimental conditions optimized for detecting high-mass species may not work as well for low-mass species. Similarly, the multiply charged protein may exert a strong columbic repulsion towards the singly charged oligosaccharide after it dissociates. The lighter fragment then escapes from the ICR cell. Another possible reason is that the singly charged oligosaccharide undergoes proton transfer with trace amount of water or methanol (from electrospray source) in the ICR cell. The charged water and methanol molecules are too small to be detected under current conditions. It is not clear at this point which of these possibilities exists. The other substrate oligosaccharides O_2, O_4, and O_5 yield the same dissociation behavior.

SORI-CID was also performed on the same complexes. The same dissociation behavior was obtained with SORI-CID as with on-resonance CID. The fact that both on-resonance CID and SORI-CID yield the same results suggests that "complex dissociation" is the lowest pathway.

The reason that substrate and nonsubstrate oligosaccharide complexes give different products during CID can be rationalized by the structural differences between specific and nonspecific complexes [26]. In the hydrolysis catalyzed by lysozyme, proton donation is the first step of the process. It happens inside the cavity where the Glu 35 group from the protein donates a proton to the glycosidic bond of the substrate sugar, cleaving the bond as shown in Scheme 3 [24]. This process occurs only when the substrate is complexed to the active site. In the gas phase, this endothermic proton transfer reaction is facilitated by



Scheme 3. Hydrolysis of substrate sugar by lysozyme.

the CID event. Similar endothermic proton transfer reactions on other noncovalent complexes have also been observed in this laboratory [27]. While proton transfer does occur with the smaller oligomers (O_2, O_3, O_4, and O_5), there is no glycosidic bond cleavage observed. With the exception of O_5, the oligomers are too short to be cleaved because cleavage generally occurs between the fourth and fifth residues. These observations are consistent with solution-phase results that show *N*-acetylglucosamine oligosaccharides with less than 5 units have significantly slower hydrolysis rates (Table 1). For both O_5 and O_6, the Glu 35 is positioned close to a glycosidic bond so that bond cleavage occurs immediately after the proton transfer in solution. In the gas phase, O_6 shows cleavage of the oligosaccharide bond (vida supra); however, O_5 does not. In solution, the O_6 oligomer is cleaved at a rate that is an order of magnitude larger than O_5. The differences in the gas phase fragmentation behavior of the two compounds may simply be a reflection of the large differences in the solution kinetics.

4.4. CID fragmentation of lysozyme–chitohexaose (O_6) complex

CID of the lysozyme–chitohexaose complex yields fragments different from O_2, O_3, O_4, and O_5. When O_6 is mixed with lysozyme by using the Tee configuration, two abundant charge states of the intact complex are obtained: $[L{:}O_6 + 9H]^{+9}$ and $[L{:}O_6 + 8H]^{+8}$. Fig. 7 shows the CID results of the $[L{:}O_6 + 9H]^{+9}$ complex. Note that the isolation of the com-

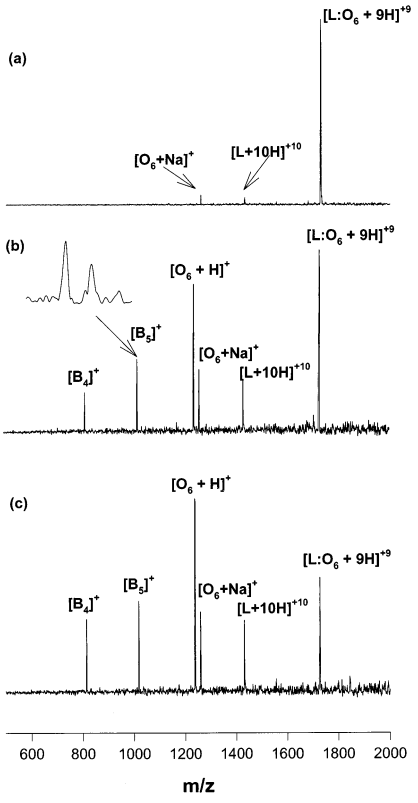


Fig. 7. CID of the lysozyme–chitohexaose complex $[L{:}O_6 + 9H]^{+9}$. (a) Isolation. (b) SORI-CID with 3.8 V amplitude (base-peak). (c) SORI-CID with 4.0 V amplitude (base-peak).

plex peak in Fig. 7(a) is incomplete; the $[O_6 + Na]^+$ and $[L + 10H]^{+10}$ peaks are also present. There were a total of more than ten peaks including different charge states of lysozyme and its complexes present in the original spectrum. Several species have cyclotron frequencies that are close to the desired peak. It was difficult to completely isolate the desired complex peak without attenuating the signal. The isolation spectrum shown in Fig. 7(a) was a compromise between complete isolation and optimal signal intensity. Nevertheless, the unejected ions do not interfere with the CID results. The two peaks $[L + 10H]^{+10}$ and $[O_6 + Na]^+$ could not produce the fragments observed.

Fig. 7(b) and (c) show the CID spectra at excitation amplitudes of 3.8 V (base-peak) and 4.0 V, respectively. Protonated chitohexaose $[O_6 + H]^+$ and two

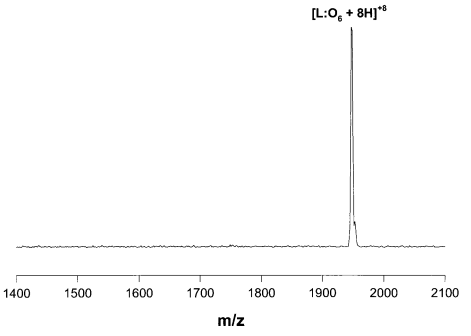


Fig. 8. CID spectrum of lysozyme–chitohexaose complex $[L{:}O_6 + 8H]^{+8}$ with 3.5 V amplitude (base-peak). No fragments are observed. Only the disappearance of the complex is observed with high amplitudes.

fragments B_5 and B_4 are observed. The proton on $[O_6 + H]^+$ comes undoubtfully from the enzyme. The B_5 and B_4 are fragments of the oligosaccharide, corresponding to a five residue and a four residue sequence, respectively. The oligosaccharide fragment products in the gas phase differ from the hydrolysis products in solution because there is no water molecule available. For yet undetermined reasons, the complement $[L + 8H]^{+8}$ product is not observed.

There are two possible pathways for the formation of the B_5 and B_4 fragments. They may fragment directly from the complex after the proton transfer. Alternatively, they may be secondary fragments from the $[O_6 + H]^+$ that has dissociated from the complex. The $[O_6 + H]^+$ species from the complex may have excess energy allowing it to undergo subsequent fragmentation. It is not clear at this point which mechanism is responsible for the B_5 and B_4 fragments.

CID on the +8 charge state of the chitohexaose complex $[L{:}O_6 + 8H]^{+8}$ was also performed. The spectra at an amplitude 3.5 V (base-to-peak) is shown in Fig. 8. Neither dissociation nor fragmentation is observed. Only ion loss is observed when the excitation amplitude is increased. The results suggest that there are some structural differences between the +9 and +8 charge states of the lysozyme–chitohexaose complexes. Williams and co-workers [2] have suggested that the conformer of the +9 ion of lysozyme is consistent with the crystal structure. However, the

+8 conformer has a similar structure to a denatured one. In their studies, the +9 ions were produced directly from the electrospray whereas the +8 ions were produced by charge stripping +9 ions. They further concluded that charge stripping denatures the intermediate charge states. In this article, both the +9 and +8 complexes are formed directly from electrospray. It is possible that the +8 charge state is inherently denatured.

5. Conclusion

The presence of the complex in the mass spectrum with electrospray ionization is not a sufficient criterion for the presence of a specific protein–substrate interaction in solution. The substrate chitose oligomers and the nonsubstrate maltose oligomers both show strong intensities for the lysozyme in the ESI-MS spectra. However, HCD and CID are shown to be useful probes for characterizing gas-phase noncovalent complexes. The dissociation temperature of the substrate oligosaccharides are generally higher than the similarly length nonsubstrate oligosaccharides. In addition, the relative order of stability obtained by HCD agrees well with solution phase association constants for the substrate oligosaccharides suggesting that HCD may be generally useful for predicting the relative strengths of association in solution.

Collision-induced dissociation of the lysozyme substrate complex is also consistent with solution phase results. Nonsubstrate oligosaccharides, presumed to be nonspecifically bound, dissociate during CID. Substrate oligosaccharides, presumed to be specifically bound, dissociate by first transferring a proton from the more basic protein to the less basic oligosaccharide. This endothermic proton transfer reaction has been recently reported in other systems. For example, cyclodextrin–amino acid complexes produce protonated cyclodextrin fragments upon CID. The reaction in the enzyme lysozyme is driven by collision energy but is consistent with the putative mechanism for enzymatic degradation in solution. The reaction of chitohexaose, a known substrate for lysozyme, in the gas phase is consistent with the known mechanism in the solution phase. Fragmentation of the oligosaccharide is observed presumably through the proton transfer and the proton catalyzed fragmentation of the glycosidic bond. The results further suggest that once the complex is formed, the reaction occurs even in the absence of solvent molecules.

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