Gas Phase Noncovalent Protein Complexes that Retain Solution Binding Properties: Binding of Xylobiose Inhibitors to the β -1, 4 Exoglucanase from *Cellulomonas fimi*

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Tandem mass spectrometry has been used to compare gas-phase and solution binding of three small-molecule inhibitors to the wild type and three mutant forms of the catalytic domain of Cex, an enzyme that hydrolyses xylan and xylo-oligosaccharides. The inhibitors, xylobiosyl-deoxynojirimycin, xylobiosyl-isofagomine lactam, and xylobiosyl-isofagomine consist of a common distal xylose linked to different proximal aza-sugars. The three mutant forms of the enzyme contain the substitutions Asn44Ala, Gln87Met, and Gln87Tyr that alter the binding interactions between Cex and the distal sugar of each inhibitor. An electrospray ionization (ESI) triple quadrupole MS/MS system is used to measure the internal energies, ΔE_{int} , that must be added to gas-phase ions to cause dissociation of the noncovalent enzyme-inhibitor complexes. Collision cross sections of ions of the apo-enzyme and enzyme-inhibitor complexes, which are required for the calculations of ΔE_{int} , have also been measured. The results show that, in the gas phase, enzyme-inhibitor complexes have more compact, folded conformations than the corresponding apo-enzyme ions. With the mutant enzymes, the effects of substituting a single residue can be detected. The energies required to dissociate the gas-phase complexes follow the same trend as the values of ΔG^0 for dissociation of the complexes in solution. This trend is observed both with different inhibitors, which probe binding to the proximal sugar, and with mutants of Cex, which probe binding to the distal sugar. Thus the gas-phase complexes appear to retain much of their solution binding characteristics. (J Am Soc Mass Spectrom 2007, 18, 64–73) © 2007 American Society for Mass Spectrometry

The discovery that electrospray ionization (ESI) can produce gas-phase ions of noncovalently bound solution complexes [1–3] led to the question of how the binding in the gas-phase complexes compares with the initial solution binding [4, 5]. Many of the noncovalent complexes reported to date involve proteins. Because the gas-phase ions are completely free of solvent, the ensemble of conformations adopted by a gas-phase protein may differ from that of the same protein in solution [6] leading to differences in the structural and energetic bases for protein-ligand interactions. In some cases, water molecules are directly involved in the solution binding interactions [7] and, therefore, in a gas-phase complex free of water, additional differences in binding may occur. Nevertheless, there have been several reports of complexes that retain at least some of their solution binding characteristics [8–17].

An experimentally tractable method to investigate the details of binding in gas-phase complexes is to make systematic changes to the binding partners. This method has been used to investigate the effects of individual hydrogen bonds between heme and myoglobin and cytochrome b_5 [8], binding in double stranded DNA [9, 10], and to investigate binding of inhibitors to aldose reductase [11], peptides to vancomycin [12], and avoparcin [13], small molecules to carbonic anhydrase [14], peptides to OppA [15], an antibody single chain fragment to a series of structurally related trisaccharides [7, 16], and a series of trisaccharides to an antibody single chain fragment [17].

In this study, tandem mass spectrometry is used to compare, in detail, the binding energetics of noncovalent complexes of the catalytic domain of the wild type (WT) and three mutant forms (Asn44Ala, Gln87Met, and Gln87Tyr) of the catalytic domain of the β -1,4 glucanase Cex, with three small-molecule inhibitors in solution and in the gas phase. Cex is a family 10 glycosyl hydrolase, found in the microorganism *Cellulomonas fimi*. It catalyzes the hydrolytic cleavage of β -1,4-linked polymers of xylose [18]. Five high-affinity

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Table 1. Inhibition constants, K_i , at pH 7 and 37°C in 10 μ M ammonium acetate

	K_i (μ M)		
	X ₂ DNJ	X ₂ IL	X ₂ IF
WT*	5.8	0.34	0.13
Gln87Met	18	2.1	0.39
Gln87Tyr	62	11	1.2
Asn44Ala	180	24	7.1

*Values of K_i for the WT protein are from [19a]; for the mutants, [19b].

xylobiose-derived aza-sugars that inhibit the catalytic activity of Cex have been described: imidazole, lactam oxime, deoxynojirimycin, isofagomine lactam, and isofagomine [19]. These were designed as possible transition-state analogues and all exhibit competitive inhibition, binding noncovalently at the active site. Three-dimensional structures of complexes of each inhibitor with Cex have been determined [20].

Solution binding affinities have been determined as inhibition constants, K_i , which represent dissociation constants for the following equilibrium [19]:

with inhibition constant

$$K_i = \frac{[E][I]}{[EI]} \quad (2)$$

where E represents the enzyme, I represents an inhibitor and EI the complex. Values of K_i for the complexes studied here are shown in Table 1. Figure 1 shows the structures of the three inhibitors used here, with binding affinity increasing in the order xylobiosyl-deoxynojirimycin (X₂DNJ), xylobiosyl-isofagomine lactam (X₂IL), and xylobiosyl-isofagomine (X₂IF). All inhibitors consist of two sugars: a common distal xylose which binds to the –2 sub-site of Cex, and a distinct proximal aza-sugar which binds to the –1 sub-site [18, 20]. Cex binds to the proximal and distal sugars through van der Waals interactions, electrostatic interactions, and a series of hydrogen bonds to amino acid side chains. Changes in the inhibitor structures probe binding of a given form of Cex to the proximal sugar. The mutant proteins used here probe binding to the distal xylose.

The X-ray crystal structures of the complexes provide insights into details of binding of the complexes [19, 20]. Figure 2 shows, for example, the interactions between the active site of WT Cex and X₂DNJ [20]. In comparison, when WT Cex binds X₂IF [20], the interactions with the distal xylose are largely preserved, although the hydrogen bonds may have different lengths. With the proximal sugar, only the bond between His80 and OH-3 is preserved, but with a different length. Two new bonds are formed with the “anomeric” nitrogen N-1: one with the nucleophile Glu233 and a second with

Gln203. When WT Cex binds X₂IL [19a, 19b] instead of X₂DNJ, again the hydrogen bonds with the distal xylose are largely preserved. At the proximal sugar, only the interaction between His80 and OH-3 is preserved. There are new interactions of O-2 with His80, Asn126, and the catalytic nucleophile Glu233, and of nitrogen N-1 with the acid-base catalyst Glu127 and the catalytic nucleophile Gln203.

Based upon the crystal structures of WT Cex with each of the inhibitors [20], as well as structures of some of these mutants with other inhibitors [21], it is possible to predict the consequences of these mutations upon ligand binding. In general, the interactions with the proximal sugar will be preserved as they are in WT Cex [21]. With the Asn44Ala mutant, the interaction between Asn44 and OH-5 of the distal xylose is eliminated for all three inhibitors. Likewise, with the Gln87Met mutant, the hydrogen bond between Gln87 and O-2 of the distal xylose is removed for all three inhibitors. However, with the Gln87Tyr mutant, while the bond between Gln87 and O-2 of the distal xylose is removed, a new bond is formed with OH-4.

Tandem mass spectrometry was used to determine the internal energies, ΔE_{int} , that must be added to gas-phase ions of the complexes to cause dissociation. Collision cross sections, which are required to calculate ΔE_{int} , were measured by ion energy loss experiments. Different dissociation mechanisms are observed for different complexes. For wild type Cex, the complex with the strongest inhibitor, X₂IF, exhibits only neutral

Figure 1. Structures of xylobiose-derived inhibitors of Cex. The distal xylose is on the left and the proximal sugar is on the right.

Figure 2. Interaction map showing the binding between the active site of wild type Cex and the proximal sugar and distal xylose of X₂DNJ (PDB Code:1FH7).

inhibitor loss, while the complexes with the weaker inhibitors X₂DNJ and X₂IL show both neutral and some (<20%) charged inhibitor loss. The ratio of charged-to-neutral inhibitor loss is largely independent of collision energy. All mutant proteins showed only neutral inhibitor losses. In the gas-phase ions, binding of the inhibitors appears to contribute significantly to stabilization of the protein fold, since complexes that are more strongly bound have lower collision cross sections. Values of ΔE_{int} were compared with ΔG^0 for dissociation in solution. The results show that the complexes that are more strongly bound in solution require higher internal energies to cause dissociation in the gas phase. This trend was observed both with different inhibitors and with the mutant proteins. Thus binding of the enzyme to both the proximal and distal sugars in the gas phase seems to retain many of its solution properties.

Experimental

Protein Expression and Purification

All experiments used the catalytic domain of Cex and its mutants, expressed and purified according to methods adapted from earlier procedures [21–23]. Details are given in the Appendix. The final stock solution concentrations of all the proteins were about 1–4 mg/mL.

Reagents and Materials

Solutions of purified Cex or its mutants were 10 μ M. Solutions of the complexes were prepared simply by mixing the enzyme and the inhibitors in ratios calculated to produce nearly fully complexed enzyme in solution, e.g., for the WT-X₂DNJ complex, 17 μ L of a 2.4 mg/mL (69 μ M) stock solution of WT-Cex was mixed with 2 μ L of a 4.0 mM stock solution of X₂DNJ in a total

volume of 240 μ L. The resulting equilibrium concentrations of the enzyme, inhibitor, and complex, calculated from the K_i value, are about 1, 29, and 4 μ M, respectively. Solutions contained 10% methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ), and 10 mM ammonium acetate (Fisher Scientific) buffer, to minimize pH changes in electrospray. The addition of 10% alcohol to a protein solution does not generally lead to conformation changes, unless the protein is otherwise near to a folding transition [24]. Solution pH was measured with an Accumet pH meter (Fisher Scientific, model 15). Solutions of the complexes with mutants were prepared in the same manner.

Mass Spectrometry

Experiments were performed with an ESI-triple quadrupole mass spectrometer system, described previously [8, 25, 26]. The sprayer consisted of a 2 cm length of fused silica capillary (Polymicro Technologies, Phoenix, AZ), with i.d. and o.d. of 76 and 150 μ m, respectively, connected to a 3 cm long stainless steel tube (Small Parts Inc., Miami Lakes, FL) with i.d. and o.d. of 0.02 and 0.04 cm. Samples were infused to the stainless steel tube at 1 μ L/min. High voltage (4000 V) was applied to the stainless steel tube. Ions pass through a nitrogen curtain gas (manufacturer's stated purity 99.999%, Praxair, Mississauga, Ontario, Canada), a 0.25 mm-diameter sampling orifice into a region with a background pressure of 1.3 torr, then through a skimmer with a 0.75 mm diameter orifice, into an RF only quadrupole ion guide, Q0, operated at 4×10^{-3} torr. Ions then pass into the main chamber containing quadrupoles Q1, Q2, and Q3 (background pressure 8×10^{-6} torr). Ion counting was used for detection.

The potential difference between the orifice and the skimmer was 100 V in all experiments. The curtain plate

was held at 1400 V. The voltages on the lenses between the quadrupoles, Q0/Q1, Q1/Q2, Q2/Q3, and on the short RF only rods before Q1 were 100, 103, 85, 103 V, respectively. The aperture at the exit of Q3 was 0 V. For MS experiments the rod offsets on the quadrupoles Q0, Q1, Q2 and Q3 were 119, 110, 100 and 90 V, respectively. For MS/MS experiments, the Q2 and Q3 rod offsets were varied, as described below. The frequency of the quadrupole RF was 0.768 MHz, giving a mass range of up to $m/z = 6500$ (quadrupole field radius, 4.16 mm). In all experiments, the collision gas was argon (Praxair, manufacturer's stated purity 99.9995%). The pressure in the collision cell (length 20.6 cm) was varied in the experiments and monitored by a capacitance manometer (MKS Instruments, Baratron model 120AA, Boulder, CO). All experiments were repeated four times. Uncertainties are the standard deviations of four repeated experiments.

Collision Cross Section Measurements

Ions are collisionally cooled to kinetic energy spreads of 1 eV or less at the exit of Q0 [26,27]. For collision cross section experiments, Q1 was operated as an ion guide in RF only mode. Kinetic energies of ions leaving Q2 after collisions with argon were determined by increasing the rod offset voltage of Q3 until the ion signal was decreased by several orders of magnitude, to generate stopping curves. The pressure of the collision gas was varied and stopping curves were obtained for each pressure. An aerodynamic drag model [26] was used to calculate the protein cross sections from

$$\frac{E}{E^0} = \exp\left(-\frac{C_D n m_2 \sigma l}{m_1}\right) \quad (3)$$

where E^0 is the energy of an ion at the cell entrance, E is the energy of an ion at the cell exit, C_D is a drag coefficient, n is the gas number density in the cell, σ is the collision cross section, l is the cell length, m_1 is the ion mass, and m_2 is the collision gas mass. Energies E were taken as the energy at which the ion intensity was decreased to one-tenth of the initial value for each pressure. The collision cross section for each charge state was determined by plotting $-\ln \frac{E}{E^0}$ versus $\frac{C_D n m_2 l}{m_1}$. Drag coefficients for diffuse scattering were used [26].

Tandem Mass Spectrometry

In MS/MS experiments, the internal energy that must be added to ions of the complexes to induce dissociation is determined. The ion of interest was mass selected in Q1 and injected into the collision cell, where multiple collisions with argon caused dissociation. Fragment ions were mass analyzed in Q3. The Q0-Q2 rod offset difference was systematically increased to determine the voltage difference required to cause complexes to

dissociate. The Q3 rod offset was set equal to the Q2 rod offset. For most complexes, where there is only loss of a neutral inhibitor, the dissociation voltage is taken as the Q0-Q2 voltage difference giving a 50% loss of the inhibitor from the complex. The internal energy added to cause dissociation, ΔE_{int} is calculated from

$$\Delta E_{int} = \Phi \frac{m_2}{M} E^0 \frac{m_1}{m_2 C_D} \left[1 - e^{-\frac{C_D n m_2 \sigma l}{m_1}}\right] \quad (4)$$

where Φ is the average fraction of the centre of mass kinetic energy transferred to internal energy in a collision (taken as $\Phi = 1.0$), E^0 is the initial kinetic energy at the entrance of the collision cell under conditions where there is a 50% fragment yield, m_1 is the complex ion mass, and $M = m_1 + m_2$. This model corrects for the different numbers of collisions of ions that have different collision cross sections and also for the energy losses of ions as they move through the cell [25].

Internal energies needed for dissociation were calculated for all complexes at different pressures of the collision gas, ranging from 0.5 to 2.74 mTorr. The times available for reaction to occur were calculated as in [25], since the internal energies needed to induce dissociation, are best compared for equal reaction times. The added internal energies, ΔE_{int} , which cause 50% dissociation in 25 μ s are reported below.

Results and Discussion

Mass Spectra

The production of the catalytic domain of Cex involves the in vivo post-translational removal of the Cex N-terminal leader peptide, as well as the in vitro cleavage and removal of a C-terminal linker and cellulose-binding region. In comparison with the published sequence from which the crystal structure was derived [28], the catalytic domain of Cex produced and used for these experiments contains the additional amino acids GAS at the C-terminus, and contains the residues VVLPAQA at the N-terminus to give a calculated molecular weight of 34,849 Da. The mass spectrum of denatured Cex gave a mass of $34,866 \pm 44$ Da in reasonable agreement with the calculated mass.

The optimum pH range for the catalytic activity of xylanases is typically 4 to 7 [29]. Figure 3a shows a mass spectrum of a solution of wild-type Cex (WT) and X_2 IF at pH 5.8. Peaks from enzyme (E) and complex (EI) are seen. A narrow distribution of charge states from +9 to +12, with +10 being the most intense, is formed as expected for the protein in its native conformation [25, 30]. The peaks of the complex (EI) are 30 to 40% as intense as those of the enzyme (E) for the same charge state, even though, from the solution K_i , it is calculated that almost all the enzyme was in the complexed form. The gas-phase abundances do not reflect the solution

Figure 3. (a) Mass spectrum of a solution of wild type Cex with X₂IF in 10/90% methanol/water at pH 5.8. Calculated solution concentrations [EI] $\approx$ 4.9 μ M, [E] $\approx$ 0.01 μ M. (b) Relative abundances of EI⁺¹⁰ and E⁺¹⁰ ions in MS/MS spectra of WT-X₂IF ions versus Q0-Q2 rod offset voltage difference at a collision cell pressure of 1.5 mTorr of Ar.

equilibria. The lower abundance of ions of the complex may be the result of the addition of 10% methanol to the solution, some dissociation of the complex in the orifice-skimmer region, or a shift to lower pH during the spray process. A small third peak for each charge state appears in the spectrum corresponding to the mass of E + 2I. Since neither Cex nor its mutants have more than one binding site, this peak is attributed to non-specific binding.

With all the forms of Cex, spectra showed narrow charge state distributions with charges +9 to +12. Spectra of the complexes with X₂DNJ and X₂IL always had +11 as the most intense peak, while in the spectra of the complexes with X₂IF, the most intense was +10. Spectra of the mutants were slightly shifted in mass compared to the WT, corresponding to the difference in mass of the native and altered amino acid residues. The charge states of the complexes with mutants were the same as of the WT for a given inhibitor, suggesting that the charge state distribution depends somewhat on the inhibitor.

Collision Cross Sections

Collision cross sections were measured for E and EI ions from solutions of all the complexes, and for the E ions from solutions in which only protein was present. Table 2 lists all the cross sections obtained. Uncertainties are standard deviations of four replicate measurements. There is a small increase in the cross sections with the charge state of the ions, although in some cases this increase is less than the combined uncertainties in the cross sections. This is likely the result of Coulomb repulsion within the ions, causing them to unfold. Free protein ions formed from solutions in which only enzyme is present have slightly, but consistently, higher cross sections for a given charge than free protein ions from solutions of the complexes of the same protein, and this difference is most significant for the WT. Possibly in the presence of the solution complex, some protein ions are formed by dissociation of the complex in the orifice-skimmer region. If protein ions retain the same conformation as in the complex on the time scale of the experiment, they may retain more compact configurations. Ions of the complexes may be dissociated in the orifice-skimmer region by energetic collisions that “heat” the complexes. If energy that would otherwise cause the protein ions to unfold somewhat is used to dissociate the complexes, less energy is available to cause the protein ions to unfold, leaving them with slightly more compact structures. Ions of the complexes consistently have slightly lower cross sections than protein ions of the same charge formed from solutions of protein only, suggesting that binding the inhibitors helps to stabilize the protein in more compact structures. This is discussed further below.

Tandem Mass Spectra

Most complexes were found to dissociate simply via loss of a neutral inhibitor. As the collision energy is increased, the intensity of the EI⁺ⁿ peak in the MS/MS spectrum decreases, accompanied by an increase in the intensity of the E⁺ⁿ peak. Relative intensities of the precursor (complex) and fragment (enzyme) ions were calculated from:

$$I_i(\%) = \frac{I_i}{I_{\text{precursor}} + I_{\text{fragment}}} \cdot 100\% \quad (5)$$

where $I_{\text{precursor}}$ is the intensity of the precursor, I_{fragment} is the intensity of a fragment (complex minus neutral inhibitor) and i = precursor or fragment. Dissociation curves, plots of relative intensities of precursor (EI⁺¹⁰) and fragment (E⁺¹⁰) ions versus the Q0-Q2 voltage difference, for the +10 charge state of the complex WT-X₂IF at a cell pressure of 1.5 mTorr, are shown in Figure 3b. Similar curves were obtained for all the complexes with mutant proteins.

The two complexes, WT-X₂DNJ and WT-X₂IL, showed different behavior, with small amounts (<20%)

Table 2.

(a) Collision Cross Sections ($\text{\AA}^2$) of wild type Cex and WT complexes							
	WT	WT-X ₂ DNJ		WT-X ₂ IL		WT-X ₂ IF	
Charge	E	E	EI	E	EI	E	EI
+9	1727 ± 31	1320 ± 91	1398 ± 44	1348 ± 69	1428 ± 36	1546 ± 37	1218 ± 44
+10	1790 ± 49	1362 ± 61	1378 ± 15	1444 ± 10	1448 ± 29	1636 ± 55	1257 ± 26
+11	1803 ± 44	1591 ± 68	1552 ± 38	1578 ± 73	1526 ± 36	1637 ± 33	1318 ± 65
(b) Collision Cross Sections ($\text{\AA}^2$) of Gln87Met Cex and Gln87Met complexes							
	Gln87Met	Gln87Met -X ₂ DNJ		Gln87Met -X ₂ IL		Gln87Met -X ₂ IF	
Charge	E	E	EI	E	EI	E	EI
+9	1620 ± 64	1529 ± 34	1412 ± 53	1548 ± 32	1403 ± 21	1432 ± 56	1262 ± 22
+10	1720 ± 72	1548 ± 56	1486 ± 48	1590 ± 36	1526 ± 46	1476 ± 28	1354 ± 46
+11	1780 ± 46	1620 ± 23	1595 ± 81	1635 ± 74	1582 ± 36	1498 ± 43	1426 ± 33
(c) Collision Cross Sections ($\text{\AA}^2$) of Asn44Ala Cex and Asn44Ala complexes							
	Asn44Ala	Asn44Ala -X ₂ DNJ		Asn44Ala -X ₂ IL		Asn44Ala -X ₂ IF	
Charge	E	E	EI	E	EI	E	EI
+9	1605 ± 44	1591 ± 32	1581 ± 22	1598 ± 33	1518 ± 18	1522 ± 72	1422 ± 44
+10	1638 ± 31	1636 ± 98	1612 ± 69	1655 ± 50	1618 ± 58	1562 ± 27	1486 ± 70
+11	1730 ± 12	1701 ± 33	1685 ± 74	1699 ± 39	1605 ± 46	1582 ± 43	1482 ± 62
(d) Collision Cross Sections ($\text{\AA}^2$) of Gln87Tyr Cex and Gln87Tyr complexes							
	Gln87Tyr	Gln87Tyr -X ₂ DNJ		Gln87Tyr -X ₂ IL		Gln87Tyr -X ₂ IF	
Charge	E	E	EI	E	EI	E	EI
+9	1642 ± 46	1573 ± 32	1562 ± 36	1554 ± 33	1554 ± 87	1465 ± 85	1437 ± 48
+10	1670 ± 42	1638 ± 57	1595 ± 51	1654 ± 100	1601 ± 52	1450 ± 72	1408 ± 58
+11	1762 ± 50	1630 ± 43	1606 ± 51	1722 ± 83	1599 ± 44	1518 ± 44	1475 ± 49

of charged inhibitor loss. The ratio of charged-to-neutral inhibitor loss was largely independent of collision energy, indicating that similar energies are required for dissociation by either channel. The data for these two complexes were analyzed in two ways. First, only the neutral loss channel was included, and the dissociation voltage was determined as above. Second, the sum of charged and neutral losses was plotted versus energy and the voltage where the EI^{+n} intensity equalled the sum of E^{+n} and $E^{+(n-1)}$ intensities was taken as the dissociation voltage. This second method produces dissociation voltages that are 2 to 11% lower than the first. Values of ΔE_{int} calculated from the second method are used here.

Internal Energies

Dissociation voltages were determined for the +10 and +11 charge states of all the complexes at collision cell pressures ranging from 0.50 to 2.74 mTorr. At lower pressures, higher dissociation voltages are required for dissociation because ions have fewer collisions and therefore more energy per collision is required. Energy

losses of ions moving through the cell must be considered and losses are lower at lower pressures. In addition, at lower pressures, ions travel faster through the cell, have less time available for reaction, and therefore need more internal energy to give a 50% fragmentation yield than at higher pressures.

Under conditions that give 50% fragment yield, the reaction occurs over a length, l' , of about 5 cm near the cell exit, about 18 cm from the entrance [25]. The ion energy and hence the ion speed in this region can be calculated from the translational energy using eq 3 with $l = 18$ cm. This gives the expression for the reaction time as follows:

$$t = \frac{l'}{\left(\frac{2E^0}{m_1} \exp \left(-\frac{C_D n m_2 l \sigma}{m_1} \right) \right)^{1/2}} \quad (6)$$

with $l' = 5$ cm.

Figure 4 shows a plot of ΔE_{int} versus reaction time for complexes of WT Cex. It can be seen that within the uncertainties, similar energies are required to dissociate

Figure 4. Added internal energies to cause dissociation, ΔE_{int} versus the time available for reaction for the +10 and +11 charge states of complexes of wild type Cex with three inhibitors: X_2DNJ , X_2IL , and X_2IF .

the +10 and +11 charge states for each complex. The values of ΔE_{int} for a given reaction time follow the order $WT-X_2IF > WT-X_2IL > WT-X_2DNJ$.

The curves of ΔE_{int} versus reaction time for the complexes with mutants are similar to the curves for the complexes with WT. The mutants show the same trend as WT. The greatest internal energy is needed for the dissociation of complexes with X_2IF , and the lowest for complexes with X_2DNJ . The internal energies needed for the dissociation of all complexes with mutants are lower than for the corresponding complexes for WT. Among the mutants, Gln87Met requires the highest, Gln87Tyr less, and Asn44Ala the lowest energy for dissociation. Values of ΔE_{int} used below were taken from plots such as those in Figure 4 for equal reaction times of 25 μs . Values of ΔE_{int} for dissociation of +10 and +11 ions of all the complexes are shown in Table 3. Also shown are values of the free-energy change for dissociation of the complexes in solution at 37 °C, $\Delta G^0_{dissociation}$, calculated from $\Delta G^0_{dissociation} = -k_B T \ln K_d$, where k_B is Boltzmann's constant ($k_B = 8.617 \times 10^{-5}$ eV K⁻¹).

The complexes with two bound inhibitors were not studied in detail. A preliminary experiment with +11 $WT-X_2DNJ_2$ ions showed that this complex dissociates by roughly equal losses of charged and neutral inhibitor. The internal energy required to reduce the precursor intensity to one half of its initial value was found to be $\Delta E_{int} = 45$ eV (0.8 mTorr cell pressure, 30 μs reaction time). This energy is about one half of that required for dissociation of +11 $WT-X_2DNJ$ complex ions in about the same reaction time (95 eV, Table 3, and Figure 4), suggesting weaker binding of the non-specific complex. This contrasts to the findings of Wang et al. where non-specific binding was found to be stronger than specific binding in a protein-carbohydrate complex [31].

Collision Cross Sections and Gas Phase Binding

The cross sections and ΔE_{int} values show that binding of inhibitors in the gas-phase complex causes the protein to adopt more compact conformations. Figure 5a and b show cross sections for the complexes versus the ΔE_{int} values for dissociation of the complexes for the +10 and +11 ions respectively. Also shown in Figure 5, by dashed lines, are the cross sections of the protein ions produced from solutions of protein only. All complexes have cross sections lower than those of the protein ions formed from solutions of protein only. The complexes and protein ions in these low charge states retain considerable folded structures; apo-protein ions with charge states +22 to +35 formed from Cex denatured in solution have considerably greater cross sections of 3300 to 4000 Å² (data not shown). There is a strong correlation between the cross sections and ΔE_{int} values, with the more strongly bound complexes having smaller cross sections. The lines in Figure 5 are second-order fits, simply to illustrate the trend. Binding of the inhibitor apparently helps keep the protein folded, most likely around the binding pocket, with stronger binding giving more tightly folded conformations. A related effect was seen with gas-phase holomyoglobin ions [32].

The change in average conformation with inhibitor binding is readily detected in the gas-phase ions. In

Table 3. Free energy changes for the dissociation of complexes in solution and internal energies required for the dissociation of gas phase complexes in 25 μs

Complex	$\Delta G^0_{dissociation}$ (eV)	+ 10 ions ΔE_{int} (eV)	+ 11 ions ΔE_{int} (eV)
WT- X_2DNJ	0.318	86 ± 3	95 ± 3
WT- X_2IL	0.393	113 ± 6	105 ± 3
WT- X_2IF	0.419	154 ± 5	147 ± 5
Gln87Met- X_2DNJ	0.288	69 ± 4	79 ± 4
Gln87Met- X_2IL	0.345	102 ± 3	92 ± 2
Gln87Met- X_2IF	0.390	131 ± 3	139 ± 2
Gln87Tyr- X_2DNJ	0.256	68 ± 4	53 ± 2
Gln87Tyr- X_2IL	0.302	81 ± 3	73 ± 4
Gln87Tyr- X_2IF	0.360	122 ± 4	125 ± 6
Asn44Ala- X_2DNJ	0.228	45 ± 3	48 ± 3
Asn44Ala- X_2IL	0.281	53 ± 4	53 ± 3
Asn44Ala- X_2IF	0.313	95 ± 4	93 ± 4

Figure 5. Collision cross sections of the complexes versus the added internal energy to cause dissociation, ΔE_{int} , for (a) +10 ions and (b) +11 ions.

contrast, the crystal structures show no significant structural changes of the protein upon binding of the inhibitors [19, 20]. However, proteolysis and thermal denaturation studies show that the formation of a covalent glycosyl-enzyme intermediate stabilizes Cex against unfolding [33]. These observations suggest that, rather than a distinct different conformation being stabilized, the range of conformational space occupied is reduced in the presence of the inhibitor.

Comparisons of Solution and Gas Phase Binding

Figure 6 shows values of ΔE_{int} plotted against the values of ΔG^0 for dissociation of the complexes in solution. In solution, the inhibition strength increases in the order: X₂DNJ < X₂IL < X₂IF for all the proteins. As can be seen from Table 3 and Figure 6, the ΔE_{int} values for all the ions of the protein complexes follow the same trends.

From Figure 6, it is evident that for each inhibitor the internal energy needed for dissociation in the gas phase increases in the order Asn44Ala < Gln87Tyr < Gln87Met < WT, for both charge states, the same as the order of increase of ΔG^0 for dissociation in solution. These same changes in binding are seen irrespective of the identity

of the proximal aza-sugar. Thus, binding to the proximal sugar in the gas-phase complex appears to be similar to the binding in solution. Mutations that remove or alter individual hydrogen bonds to the distal xylose to reduce the solution binding strength, also reduce the energy required to cause dissociation in the gas-phase ions. Therefore individual hydrogen bonds involved in interactions with Gln87 and Asn44 in solution probably remain in the gas phase. Individual hydrogen bonds between heme and myoglobin were previously shown to persist in the gas-phase holoprotein [8]. Likely, the solution structures are partially preserved in the gas phase in these protein small-molecule complexes in part because inhibitor binding stabilizes the complex, holding the binding pocket together.

Kitova et al. reported activation energies for dissociation of trisaccharides from an antibody single chain fragment measured in blackbody infrared dissociation (BIRD) experiments [7], and found that activation energies did not correlate with ΔH^0 for association of the

Figure 6. Internal energies needed for dissociation of ions of the complexes of WT, Gln87Met, Gln87Tyr, and Asn44Ala with inhibitors X₂DNJ, X₂IL, and X₂IF in the gas phase versus ΔG^0 for dissociation of the complexes in solution for charges (a) +10 and (b) +11.

complexes in solution. This was attributed to structural differences between the solution and gas-phase proteins. If such differences exist in the Cex complexes, they do not alter the order of the binding energies in the gas phase. It is possible the difference in time scales between these tandem MS/MS experiments and the BIRD experiments contributes to these different results. Ions are formed and dissociate in tens of microseconds in the triple quadrupole experiments. In the BIRD experiments, the ions are trapped for times of a few to more than a hundred seconds. On this longer time scale the ions may undergo unfolding or refolding transitions to structures that differ markedly from the solution structures [34].

Summary

The results presented here represent a detailed study of binding in a gas-phase protein small-molecule complex. The correlation between cross section and dissociation energies suggests that binding of the small molecule helps stabilize the complex in the gas phase. Mutant versions of the protein can be used to probe the effects of removal of individual sets of interactions in the complex. The strong correlation between gas-phase dissociation energies and ΔG^0 for dissociation in solution suggests that much of the solution binding pocket is retained in the gas-phase ion. The method used here, combining tandem mass spectrometry with systematic alterations to the binding partners, should be applicable to a broad range of noncovalent complexes.

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Appendix

Protein Production and Purification

The plasmid, pUC12-1.1Cex(PTIS) [35] codes for Cex with an endogenous secretory sequence that is cleaved upon transport to the periplasm. The plasmids used, coding for Cex Asn44Ala, Gln87Met, and Gln87Tyr mutants, were all engineered based on the pUC12-1.1Cex(PTIS) plasmid. The plasmids were transformed into *E. coli* BL21(λ DE3) cells and cultured on agar media containing ampicillin with the resultant colonies selected for growth in liquid LB media and stored at -80°C . Wild-type Cex and its mutants were identically produced starting with a 20 mL overnight culture in a synthetic medium [23] containing ampicillin. A 10 mL sample of the resultant culture was used to inoculate 1 L of synthetic medium, grown to an OD600 of ~ 0.5 , induced with 0.1 mM IPTG, and harvested 48 h after induction. The temperature was 30°C throughout the entire growth and expression period.

The cells were harvested by centrifugation (6000 rpm, 20 min), resuspended in ~ 25 mL of 50 mM potassium phosphate, 0.02% NaN_3 at pH 7.2 in H_2O (K-P-7 buffer), and lysed by passage twice through a French-press cell at 10,000 psi. After addition of Complete protease inhibitor cocktail (Roche, Mannheim, Germany), cell debris was removed by centrifugation (15,000 rpm, 30 min), and the supernatant, along with the original media supernatant, were stirred overnight at 4°C with ~ 20 g of degassed long fibrous cellulose (Sigma, St. Louis, MO) suspended in K-P-7 buffer. The cellulose was packed into a ~ 150 mL FPLC column, washed with ~ 270 mL of 1 M NaCl in K-P-7 buffer, and followed by ~ 200 mL of K-P-7 buffer. The protein was eluted with H_2O at 1 mL/min while collecting 10 mL fractions. The appropriate fractions were pooled and concentrated to ~ 10 mL using a 10 kDa MWCO stirred ultrafiltration cell (Amicon Corp., Danvers, MA), and exchanged into a buffer containing 20 mM sodium phosphate, 10 mM EDTA, and 20 mM cysteine hydrochloride at pH 7. Washed agarose-immobilized papain (150 μL) (Pierce, Rockford, IL) was then added to cleave Cex at multiple sites within the linker region between its catalytic and cellulose binding domains. After incubation overnight at 37°C on a tube roller, the papain was removed by centrifugation at 5000 rpm. The agarose beads were rinsed three times with K-P-7 buffer, and all the supernatants were combined and incubated overnight at 4°C on a tube roller with 2 g of washed avicel (Fluka Biochemika, Steinheim, Switzerland). The Avicel, with bound uncleaved Cex and the isolated cellulose binding domain, was removed by centrifugation at 5000 rpm. The supernatant, containing the catalytic domain of Cex, was concentrated by ultrafiltration using a 3 kDa MWCO membrane and exchanged into 20 mM potassium phosphate (K-P-8) buffer (pH 8), loaded onto a Hi-Trap Q HP column (GE Healthcare, Piscataway, NJ), and washed with 10 mL of 20 mM K-P-8 buffer (pH8). The various mutants were eluted from the column using the above washing buffer supplemented with 1.0 M NaCl at a flow rate of 0.5 mL/min. Proteins can be detected from 0.6 M to 1.0 M NaCl, and the appropriate fractions were collected, buffer-exchanged into distilled H_2O , and concentrated to a final volume of ~ 1 mL. The final enzyme concentrations were determined spectrophotometrically using a predicted value of $\epsilon_{280\text{nm}} = 52,870\text{M}^{-1}\text{cm}^{-1}$ [36].

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