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Volumetric Characterization of Tri-*N*-acetylglucosamine Binding to Lysozyme

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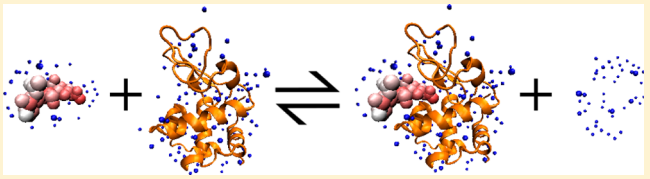
ABSTRACT: Volumetric characteristics of protein recognition events determine the direction of pressure-induced shifts in the recognition reaction, while also providing insights into the structural, dynamic, and hydration changes. We report changes in volume, ΔV, and adiabatic compressibility, ΔK_S, accompanying the binding of tri-*N*-acetylglucosamine [$(GlcNAc)_3$] to lysozyme at 25 °C in a pH 5.5 sodium acetate buffer. We interpret our measured changes in volume and compressibility in terms of changes in hydration and dynamic properties of the protein. On the basis of our ΔV data, we find that 79 ± 44 water molecules are released to the bulk from the hydration shells of the protein and the ligand. Our ΔK_S data suggest a 4 ± 2% decrease in the mean-square fluctuations of the intrinsic volume of the protein, $\langle \delta V_M^2 \rangle$ (or a 2% decrease in δV_M). Thus, the trisaccharide-bound state of the enzyme is less hydrated, more rigid, and less dynamic compared to the unbound state. In general, we discuss the importance of volumetric insights into the molecular origins of protein recognition events.



A detailed understanding of the thermodynamic forces participating in the control of ligand−protein recognition events is required for characterization and, even to a larger degree, prediction of the affinity and specificity of such events.[1−6] Among these forces, hydration/dehydration of interacting surfaces and, possibly, more distant loci of the protein occupy a special place both due to the sizable contribution of hydration to the binding affinity and specificity and the difficulties involved in quantifying changes in hydration and evaluating its net thermodynamic effects.[2−4,7−15] Changes in hydration and the related thermodynamics have been implicated as a major factor contributing to the ubiquitous phenomenon of enthalpy−entropy compensation in protein recognition.[4,16,17]

The free energies of waters solvating protein hydration sites may be higher or lower relative to waters in the bulk phase.[12,14] This notion is consistent with a picture in which the ligand-induced release of water molecules from the protein hydration shell to the bulk may contribute positively or negatively to the affinity of the binding reaction. In fact, it has been proposed that "druggable" domains on the protein surface are all characterized by high-energy hydration sites which drives the binding affinity.[14]

Characterization of the role of hydration in protein−ligand association requires the use of physical parameters that can discriminate between waters solvating various protein and ligand sites and waters in the bulk. Volumetric observables such as volume, compressibility, and expansibility offer one way for tackling the problem of hydration in protein recognition.[18−21] These parameters are sensitive to solute-induced changes in the properties of waters of hydration. Significantly, they sense the entire population of thermodynamically altered water molecules and not restricted just to highly localized or immobilized waters as is the case for X-ray and NMR. In addition, the volumetric properties of water molecules interacting with charged, polar, and nonpolar groups are distinct in both their absolute magnitude and temperature dependences, a feature that, potentially, can be used for discriminating between the types of hydration.[19,20]

A survey of the literature reveals only a few volumetric studies of protein binding events.[22−27] The dearth of volumetric data is unfortunate. Volumetric data are complementary to more abundant calorimetric data, and furthermore, their combination may produce additional insights into the thermodynamic forces driving protein recognition. To implement to the full extent the possibilities provided by such a combination, larger volumetric databases on protein−ligand association are required.

The complexation between tri-*N*-acetylglucosamine [$(GlcNAc)_3$] and lysozyme has been well characterized by structural and thermodynamic means. This makes the lysozyme-$(GlcNAc)_3$ an attractive target for a volumetric characterization. The thermodynamics of the binding of $(GlcNAc)_3$ to hen egg-white lysozyme has been studied in a number of publications.[28−30] This is an enthalpy-driven process with an unfavorable change in entropy.[30]

The structures of various avian lysozymes associated with *N*-acetylglucosamines have been investigated by X-ray crystallographic, powder diffraction, and NMR spectroscopic techniques.[31−35] The three sugar rings of $(GlcNAc)_3$ become buried within the binding cleft of hen-egg lysozyme to a different degree.[31] The specificity of the binding results mainly from an

Received: May 28, 2012
Revised: June 22, 2012
Published: June 25, 2012

extensive network of hydrogen bonds between the protein and the inhibitor as well as the five bound water molecules.[31] The major $(GlcNAc)_3$-induced conformational change involves the amino acid residues in the vicinity of subsite C of the active site of the enzyme.[31−33] However, changes on a smaller scale occur in regions remote from the active site.[33] Importantly, judging by the average side-chain B values of the protein, the binding of $(GlcNAc)_3$ causes a stiffening of the atoms of the amino acid residues directly interacting with the bound inhibitor.[31] For example, in the native state, the temperature factors of the five- and six-membered rings of Trp62 are 39 (or 32 Å^2), respectively, while decreasing to 22 (or 14 Å^2), respectively, in the trisaccharide-bound state.[31]

In this work, we report changes in volume and adiabatic compressibility accompanying the binding of $(GlcNAc)_3$ to hen egg-white lysozyme. We interpret our volumetric results in conjunction with X-ray crystallographic data on the unligated enzyme and its complex with the inhibitor to estimate the binding-induced changes in hydration and internal dynamics of the protein. We also discuss implications of our results for understanding the various components of thermodynamic forces driving protein−ligand complexation events.

MATERIALS AND METHODS

Materials. Lysozyme from chicken egg white was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada) and was exhaustively dialyzed against buffer. Tri-*N*-acetylglucosamine [tri-*N*-acetylchitotriose] was acquired from V-laboratories (Covington, LA) and used without further purification. All measurements were performed in a pH 5.5 buffer consisting of 10 mM sodium acetate/acetic acid and 10 mM NaCl. The concentration of lysozyme was determined from the absorbance measured at 25 °C with a Cary 300 Bio spectrophotometer (Varian Canada, Inc., Mississauga, ON, Canada) using a molar extinction coefficient ε_{280} = 37 900 M^{-1} cm^{-1}.[36] For the fluorescence measurements, the protein concentration was ∼35 μM. For the densimetric and ultrasonic velocimetric experiments, lysozyme concentration was ∼100 μM.

Fluorescence. Fluorescence intensity measurements were performed at 25 °C using an Aviv model ATF 105 spectrofluorometer (Aviv Associates, Lakewood, NJ). Fluorescence titration profiles were measured by the incremental addition of aliquots of $(GlcNAc)_3$ to a 10 mm path length cell containing a known amount of lysozyme. The protein samples were excited at 296 nm, and the intensity of emission light was recorded through a monochoromator at 345 nm. When calculating the relative fluorescence intensity of lysozyme, we have taken into account the change in the concentration of the protein upon each addition of the titrant $[(GlcNAc)_3]$.

Volumetric and Densimetric Measurements. All densimetric and ultrasonic velocimetric investigations reported here were conducted at 25 °C. Densities were measured with a precision of ±1.5 × 10^{-6} g cm^{-3} using a vibrating tube densimeter (DMA-5000, Anton Paar, Graz, Austria). The partial molar volume, $V°$, of the protein was calculated from density values using the relationship

$$V° = M/\rho_0 - (\rho - \rho_0)/(\rho_0 C) \quad (1)$$

where ρ and ρ_0 are the densities of the protein solution and the solvent, respectively, C is the molar concentration of the protein, and M is the protein's molecular weight.

Solution sound velocity measurements were carried out at ∼7.2 MHz by analyzing the amplitude-frequency characteristics of an ultrasonic resonator as described previously.[37−39] The analysis of the frequency characteristics of the resonator was performed by a Hewlett-Packard model E5100A network/spectrum analyzer (Mississauga, Ontario, Canada).

The key characteristic of a solute directly derived from ultrasonic velocimetric measurements is the relative molar sound velocity increment, $[U] = (U - U_0)/(U_0C)$, where U and U_0 are the sound velocities in the protein solution and the neat solvent, respectively.

Values of the relative molar sound velocity increment, $[U]$, were used in conjunction with the measured partial molar volume data, $V°$, to calculate the partial molar adiabatic compressibility, $K°_S$, of the protein using the relationship[18,40,41]

$$K°_S = \beta_{S0}(2V° - 2[U] - M/\rho_0) \quad (2)$$

where β_{S0} is the coefficient of adiabatic compressibility of the solvent. The volumetric and ultrasonic velocimetric experiments have been performed at least three times with the average values of $[U]$ and $V°$ being used for $K°_S$ determination.

Densimetric and ultrasonic titrations were performed at 25 °C by adding aliquots of the ligand $[(GlcNAc)_3]$ to lysozyme solution following previously described experimental protocols.[42,43]

Determination of Intrinsic Volumes and Solvent-Accessible Surface Areas. The atomic coordinates of unligated lysozyme and the $(GlcNAc)_3$−lysozyme complex needed for calculating intrinsic volumes and solvent-accessible surface areas were obtained from the RSCB Protein Data Bank. For unligated lysozyme, we used the 4LYZ, 1AK1, 1VDQ, and 2YVB PDB entries as well as the protein domain extracted from the 1HEW entry. For the $(GlcNAc)_3$−lysozyme complex, we used the 1HEW entry. We calculated the solvent-accessible surface area, S_A, for each structure as the sum of the accessible surface areas of all atoms in the structure. The intrinsic volumes, V_M, of unligated lysozyme, free ligand, and the ligand−protein complex were calculated as molecular volumes as described by Richards.[44,45]

Each PDB file was stripped of water molecules and cleaned using VMD (version 1.9.1) on a Linux platform. The program MSP (Molecular Surface Package) Version 3.9.3 was obtained from Dr. Michael Connolly at www.biohedron.com and used to calculate the solvent-accessible surface area and molecular volume for each structure, using a 1.4 Å probe radius on a Linux platform.

RESULTS

Figure 1 presents the dependence of the relative fluorescence intensity of lysozyme on the ligand-to-protein binding ratio, $r = [L_T]/[P_T]$, where $[L_T]$ is the total concentration of $(GlcNAc)_3$ and $[P_T]$ is the total concentration of lysozyme. The experimental points presented in Figure 1 were approximated by an analytical function representing a one-to-one stoichiometric binding:

$$X = X_0 + \alpha\Delta X \quad (3)$$

where X is a binding-dependent observable (in this case, X is the relative fluorescence intensity), X_0 is the initial value of X in the absence of the ligand, ΔX is the maximum change in X when the protein is saturated with the ligand, and α is the fraction of protein molecules associated with the ligand. The

dx.doi.org/10.1021/bi3006994 | Biochemistry 2012, 51, 5784−5790

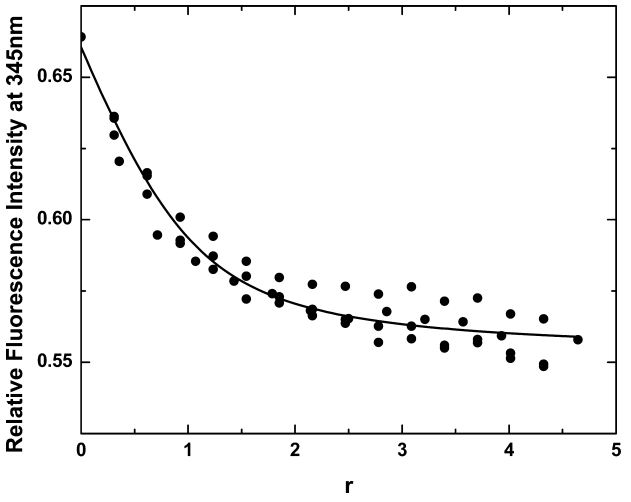


Figure 1. Relative fluorescence intensity of a solution containing lysozyme plotted against the $(GlcNAc)_3$-to-lysozyme molar ratio, *r*. The excitation and emission wavelengths are 296 and 345 nm, respectively. The initial concentration of lysozyme is 35 μM. The experimental points are fitted using eq 3 (solid lines).

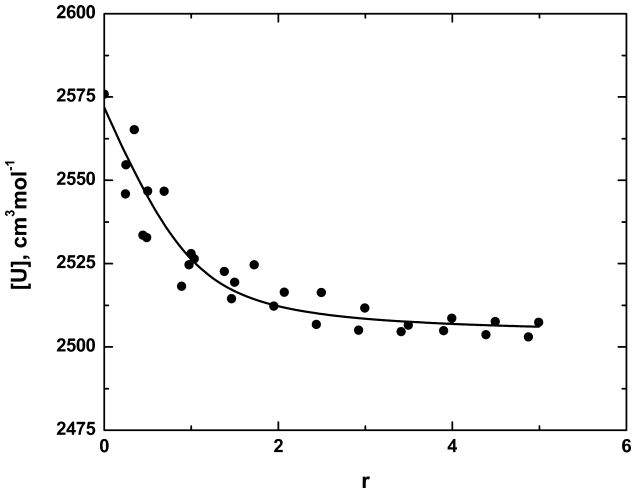


Figure 2. Change in the relative molar sound velocity increment of lysozyme plotted against the $(GlcNAc)_3$-to-lysozyme molar ratio, *r*. The initial concentration of lysozyme is 90 μM. The experimental points are fitted using eq 3 (solid lines).

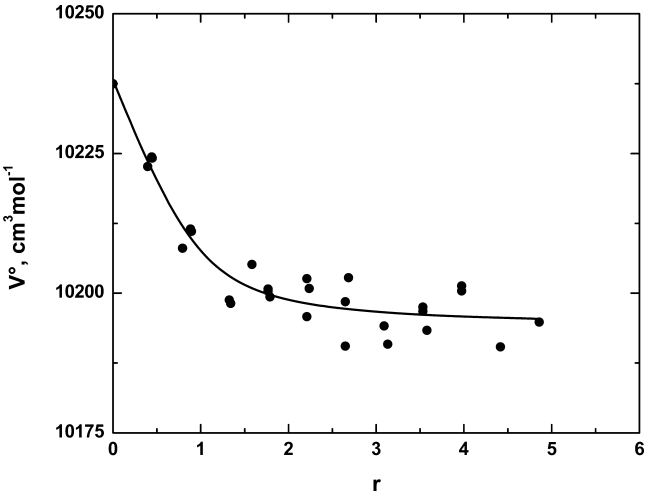


Figure 3. Change in the partial molar volume of lysozyme plotted against the $(GlcNAc)_3$-to-lysozyme molar ratio, *r*. The initial concentration of lysozyme is 90 μM. The experimental points are fitted using eq 3 (solid lines).

fraction of ligated protein, $\alpha = [PL]/[P_T]$, is given by the relationship

$$\alpha = 0.5(r + 1) + Y^{-1} - [0.25(r - 1)^2 + (r + 1)/Y + Y^{-2}]^{1/2} \quad (3a)$$

where $Y = 2K_b[P_T]$; $K_b = [PL]/([P][L])$ is the binding constant, [PL] is the concentration of the $(GlcNAc)_3$–lysozyme complex, [P] is the concentration of the unligated protein, and [L] is the concentration of the free ligand.

Equation 3a can be derived for a one-to-one stoichiometric reaction by presenting the binding constant as $K_b = [PL]/([P][L]) = [PL]/\{([P_T] - [PL])([L_T] - [PL])\}$. This relationship can be easily transformed into the quadratic equation $Y\alpha^2 - (Y + Yr + 2)\alpha + Yr = 0$. Solving the latter with respect to the fraction of ligated protein, α, yields eq 3a.

By fitting the binding profile shown in Figure 1 by eq 3, we calculated a binding constant, K_b, of $(1.2 \pm 0.2) \times 10^5$ M^{-1} for $(GlcNAc)_3$ association with lysozyme. Our measured binding constant is in good agreement with previous estimates of 1.3×10^5 M^{-1} [29] and 1.2×10^5 M^{-1}.[30] The observed agreement between our and reported binding constants lends credence to our experimental and data fitting protocols, although our fluorescence measurements were performed at somewhat high protein concentrations (~35 μM). Using $\Delta G° = -RT \ln K_b$, we calculated a binding free energy, $\Delta G°$, of -6.9 ± 0.1 kcal mol^{-1}.

Figures 2 and 3 show respectively the changes in relative molar sound velocity increment, $\Delta[U]$, and partial molar volume, ΔV, of lysozyme in the absence and presence of the inhibitor at various $(GlcNAc)_3$-to-lysozyme binding ratios, *r*. We used eq 3 to fit the binding profiles presented in Figures 2 and 3 and determine changes in relative molar sound velocity increment, $\Delta[U]_b$, and volume, ΔV_b, accompanying saturation of lysozyme with $(GlcNAc)_3$. It should be noted that the binding constants, K_b, determined from fitting sound velocity (Figure 2) and volume (Figure 3) data are in good agreement with our fluorescene intensity-based evaluation (Figure 1). A change in adiabatic compressibility, ΔK_{Sb}, accompanying ligand–protein association can be calculated from the values of $\Delta[U]_b$ and ΔV_b by modifying eq 2 to the form $\Delta K_{Sb} = 2\beta_{S0}(\Delta V_b - \Delta[U]_b)$. Our determined values of $\Delta[U]_b$, ΔV_b, and ΔK_{Sb} are -69 ± 4 cm^3 mol^{-1}, -44 ± 2 cm^3 mol^{-1}, and $(22 \pm 4) \times 10^{-4}$ cm^3 mol^{-1} bar^{-1}, respectively. The reported uncertainties reflect the scattering of measurement data obtained from repetitive experiments and the standard deviation of data fitting by eq 3.

Table 1 lists the intrinsic volumes, V_M, and solvent-accessible surface areas, S_A, of unligated lysozyme, $(GlcNAc)_3$–lysozyme complex, free $(GlcNAc)_3$, and lysozyme in the complex. The listed values of V_M and S_A for unligated lysozyme are the averages of the calculations performed on each PDB entry used in our analysis as explained in Materials and Methods. The uncertainties of the V_M and S_A values for the unligated protein are the standard deviations for the calculations performed for the four PDB entries. Since there is only a single PDB entry for the ligand–protein complex, it is difficult to estimate the error of our calculated V_M and S_A values for the complex, the ligand, and lysozyme in the complex. However, the uncertainties

dx.doi.org/10.1021/bi3006994 | *Biochemistry* 2012, 51, 5784–5790

Table 1. Molecular Volumes, V_M, and Solvent Accessible Surface Areas, S_A, of Unligated Lysozyme, Lysozyme−$(GlcNAc)_3$ Complex, Lysozyme in the Complex, and Free $(GlcNAc)_3$

protein	S_A, Å^2	V_M, cm^3 mol^{-1}
lysozyme	6588 ± 113	9746 ± 58
complex	6777	10231
lysozyme in the complex	6773	9774
$(GlcNAc)_3$	875	341

should be of the same order as those estimated for unligated lysozyme (second row in Table 1).

DISCUSSION

Evaluation of the Binding-Induced Changes in Hydration. The partial molar volume, $V°$, of a solute is the sum of the following contributions:[19,46,47]

$$V° = V_M + V_T + V_I + \beta_{T0}RT \quad (4)$$

where V_M is the intrinsic volume of a solute, that is, the volume inaccessible to any part of a spherical probe with a radius of 1.4 Å that is rolled over the surface of a solute; V_T is the thermal volume, that is, the volume of the effective void created around the solute due to thermally induced mutual vibrational motions of solute and solvent molecules as well as steric and structural effects; V_I is the interaction volume, that is, the volume reduction due to solute−solvent interactions; β_{T0} is the coefficient of isothermal compressibility of the solvent; R is the universal gas constant; and T is the absolute temperature. The ideal term $\beta_{T0}RT$ reflects the volume effect arising from the translational degrees of freedom available to the solute molecule.

Based on eq 4, the change in volume accompanying a ligand−protein association event can be viewed as the sum of changes in the intrinsic, ΔV_M, thermal, ΔV_T, and interaction, ΔV_I, contributions:[47]

$$\Delta V = \Delta V_M + \Delta V_T + \Delta V_I \quad (5)$$

To rationalize the observed volumetric changes in terms of hydration, we need to use the structural data on solvent accessible surface area, S_A, and intrinsic volume, V_M, presented in Table 1. A change in the intrinsic contribution, ΔV_M, is 144 ± 58 cm^3 mol^{-1} (10231 − 9746 − 341) as can be calculated from the data on the molecular volumes of the complex, the protein, and the ligand presented in Table 1. A change in thermal volume, ΔV_T, correlates with a change in solvent accessible surface area, ΔS_A. In general, the thermal contribution, V_T, to the partial molar volume of a solute is proportional to its solvent accessible surface area, S_A, with the proportionality coefficient equal to the effective thickness, δ, of the thermal volume: $V_T = \delta S_A$.[46−48] The value of δ appears to depend on the size of the solute molecule; it increases sigmoidally from ∼0.6 Å for small molecules to ∼1.0 Å for large molecules of the size corresponding to proteins.[48]

A change in thermal volume, ΔV_T, is, thus, given by the relationship

$$\Delta V_T = \delta_L \Delta S_{AL} + \delta_P \Delta S_{AP} \quad (6)$$

where δ_L and δ_P are the values of δ for the ligand and the protein, respectively, and ΔS_{AL} and ΔS_{AP} are the changes in the solvent accessible surface area of the ligand and the protein, respectively (note that $\Delta S_A = \Delta S_{AL} + \Delta S_{AP}$).

In our estimates, we take δ_L = 0.6 Å (as for small molecules) and δ_P = 1.0 Å (as for proteins). We further assume that $\Delta S_{AL} = \Delta S_{AP} = 0.5\Delta S_A$. With these assumptions, a change in thermal volume, ΔV_T, is given by $\Delta V_T = 0.5(\delta_L + \delta_P)\Delta S_A = 0.8\Delta S_A$. A change in solvent-accessible surface area, ΔS_A, equals −686 ± 113 Å^2 (6777 − 6588 − 875) as can be calculated from the data on the solvent-accessible surface areas of the complex, unligated lysozyme, and the trisaccharide given in Table 1. Thus, a change in thermal volume, ΔV_T, accompanying the binding of $(GlcNAc)_3$ to lysozyme is equal to −549 ± 90 Å^3 (−0.8 × 718) or −330 ± 54 cm^3 mol^{-1}. With our estimates of ΔV_M and ΔV_T, a change in interaction volume, ΔV_I, can be determined from eq 5 to be 143 ± 79 cm^3 mol^{-1} (−44 − 144 + 331).

The change in interaction volume, ΔV_I, is the only component of ΔV that reflects redistribution of water molecules between the bulk and hydration phases. The value of ΔV_I is given by the sum

$$\Delta V_I = \sum_i \Delta n_{hi}(V_{hi} - V_0) \quad (7)$$

where Δn_{hi} is the number of water molecules taken up by the ith domain of the ligand or the protein upon their association and V_{hi} and V_0 are the partial molar volumes of water of hydration of the ith solute domain and bulk water, respectively. Under the assumption of the uniformity of the hydration shells of the ligand and the protein, eq 7 simplifies to the form $\Delta V_I = \Delta n_h(V_h - V_0)$, from which $\Delta n_h = \Delta V_I/(V_h - V_0)$. The partial molar volume of water solvating proteins and simple sugars, V_h, is roughly 10% smaller than that of bulk water.[49−51] Assuming $(V_h - V_0)$ = −1.8 cm^3 mol^{-1}, the value of Δn_h is −79 ± 44 (−143/1.8). Thus, 79 ± 44 water molecules become released to the bulk from the hydration shells of $(GlcNAc)_3$ and lysozyme following their association.

Our determined number of water molecules released to the bulk, 79 ± 44, is similar to 76, the number of water molecules in direct contact with the associating surfaces. The latter can be evaluated by dividing the net change in solvent-accessible surface area, ΔS_A, of 686 Å^2 by 9 Å^2, the effective cross section of a water molecule. Thus, we conclude that only waters contained within the first coordination spheres of the enzyme and the inhibitor predominantly contribute to the binding.

Dehydration of the interacting surfaces has a pronounced effect on the thermodynamic and kinetic properties of a binding reaction. The release of water molecules from the hydration shell of a protein may be fast or slow depending on the free energy barrier separating a specific water of hydration from the bulk water.[52] The slow exchange kinetics is predominantly displayed by water molecules which are doubly hydrogen bonded with protein groups.[52] Although the number of such water molecules is only 5−10% of the total number of waters of hydration, they play a prominent kinetic and thermodynamic role in controlling the conformational stability and recognition events of proteins.[52]

It is difficult to estimate the thermodynamic impact of the collective release of 79 water molecules. Although the thermodynamic contribution of water molecules involved in protein binding and folding events is known to be on the order of or even exceed the net thermodynamics of the binding or folding reactions, the ligand-induced release of individual water molecules may contribute favorably or unfavorably to the net binding energetics depending on the location of the hydration site.[12−14,53−56] Waters of hydration are highly heterogeneous with respect to their structural, dynamic, and thermodynamic

dx.doi.org/10.1021/bi3006994 | Biochemistry 2012, 51, 5784−5790

properties.[12,14,52,57] In particular, waters near a protein surface which form hydrogen bonds with 0, 1, 2, 3, or 4 functional groups are vastly different with respect to their enthalpic, entropic, and free energy contributions, while all of them are loosely defined as waters of protein hydration.[7,14,58,59] It should be noted that, in globular proteins, the majority of waters of hydration form one or zero hydrogen bonds with protein groups.[14] In lysozyme, there are 195 water molecules which form a single hydrogen bond with protein groups and only 38 waters forming two or more hydrogen bonds.[57] Further studies preferably combining theoretical and experimental approaches are needed to evaluate the thermodynamic role of the 79 water molecules released to the bulk. Our current results represent the first quantitative step in that direction.

Evaluation of the Binding-Induced Change in the Intrinsic Compressibility of Lysozyme. With Δn_h estimated, we now proceed to evaluate a change in the intrinsic compressibility of lysozyme accompanying its association with $(GlcNAc)_3$. Note that the intrinsic compressibility, $K_M = \beta_M V_M$, of a protein is a linear function of its volume fluctuations.[60,61] The mean-square fluctuations of the intrinsic volume, $\langle \delta V_M^2 \rangle$, is related to the intrinsic coefficient of isothermal compressibility, β_{TM}, of the protein molecule:[60,61]

$$\langle \delta V_M^2 \rangle = k_B T V_M \beta_M \quad (8)$$

where k_B is Boltzmann's constant and T is the absolute temperature.

The mean-square fluctuations of the intrinsic volume, $\langle \delta V_M^2 \rangle$, represents an effective measure of protein dynamics. Thus, the value of V_M can be used in conjunction with β_M for quantitative characterization of the binding-induced change in the conformational dynamics of lysozyme as reflected in $\langle \delta V_M^2 \rangle$. A change in adiabatic compressibility, ΔK_S, accompanying ligand−protein binding is given by the sum of the intrinsic, ΔK_M, and hydration, ΔK_h, contributions:

$$\Delta K_S = \Delta K_M + \Delta K_h \quad (9)$$

Analogous to the change in interaction volume, a change in the hydration component, ΔK_h, can be presented as the sum

$$\Delta K_h = \sum_i \Delta n_{hi}(K_{hi} - K_0) \quad (10)$$

where K_{hi} and K_0 are the partial molar adiabatic compressibilities of water of hydration of the ith solute domain and bulk water, respectively.

By assuming uniformity of the hydration shells of the ligand and the protein, eq 10 simplifies to the form $\Delta K_h = \Delta n_h(K_h - K_0)$. The partial molar adiabatic compressibility of water solvating proteins and simple sugars, K_h, is roughly 20% smaller than that of bulk water.[51,62] Assuming $(K_h - K_0) = -1.3 \times 10^{-4}$ cm^3 mol^{-1} bar^{-1} and given Δn_h of -79 ± 44, the value of ΔK_h is $(103 \pm 57) \times 10^{-4}$ cm^3 mol^{-1} bar^{-1} ($79 \times 1.3 \times 10^{-4}$). A change in the intrinsic compressibility of lysozyme, ΔK_M, can be estimated from eq 9 to be $-(81 \pm 57) \times 10^{-4}$ cm^3 mol^{-1} bar^{-1} ($22 \times 10^{-4} - 103 \times 10^{-4}$). Since $\Delta K_M = \beta_M \Delta V_M + V_M \Delta \beta_M$, a change in the intrinsic compressibility of lysozyme accompanying its association with the trisaccharide is given by $\Delta \beta_M = (\Delta K_M - \beta_M \Delta V_M)/V_M$. With the average intrinsic compressibility, β_M, of a globular protein of 25×10^{-6} bar^{-1} [19,63,64] and the values of $V_M = 9700$ cm^3 mol^{-1} and $\Delta V_M = 74$ cm^3 mol^{-1} (9774 − 9700) from Table 1, we calculate $\Delta \beta_M$ of $-(1.0 \pm 0.6) \times 10^{-6}$ bar^{-1}. Thus, the binding of lysozyme to $(GlcNAc)_3$ renders the former $4 \pm 2\%$ ($1.0 \times 10^{-6}/25 \times 10^{-6}$) less compressible compared to the unligated enzyme. A similar decrease of ~1% has been observed for the intrinsic compressibility of hexokinase upon its association with glucose.[26]

According to eq 8, the observed 4% decrease in β_M reflects a similar decrease in the mean-square fluctuations of the intrinsic volume, $\langle \delta V_M^2 \rangle$ (or 2% decrease in δV_M). The ligand-bound state of lysozyme is, thus, more rigid and less dynamic compared to its unbound state. The observed decrease in conformational dynamics as reflected in $\langle \delta V_M^2 \rangle$ is in agreement with the results of crystallographic and MD simulation studies.[31,65] These studies have revealed a significant decrease in the temperature B factors of the residues directly interacting with the bound inhibitor, in particular, Trp62.[31,65] It should be noted that the observed decrease in $\langle \delta V_M^2 \rangle$ may reflect and correlate with a decrease in the configurational entropy of the protein accompanying its association with $(GlcNAc)_3$. In this respect, recall that the binding of $(GlcNAc)_3$ to lysozyme is an enthalpy-driven process with an unfavorable change in entropy.[30] Thus, the decrease in configurational entropy of lysozyme may be one of the contributors to the overall unfavorable change in entropy accompanying the process.

CONCLUDING REMARKS

We applied high precision densimetric and ultrasonic velocimetric measurements to characterize the binding of $(GlcNAc)_3$ to lysozyme at 25 °C in a pH 5.5 sodium acetate buffer. This enzyme−inhibitor association event causes changes in volume, ΔV, and adiabatic compressibility, ΔK_S, of -44 ± 2 cm^3 mol^{-1} and $(22 \pm 4) \times 10^{-4}$ cm^3 mol^{-1} bar^{-1}, respectively. We interpreted these results in conjunction with X-ray crystallographic data in terms of changes in hydration of the ligand and the protein and the dynamic properties of the latter. On the basis of our ΔV data, we estimate that 79 ± 44 water molecules are released to the bulk from the hydration shells of the protein and the ligand. Our ΔK_S data suggest a $4 \pm 2\%$ decrease in the mean-square fluctuations of the intrinsic volume of the protein, $\langle \delta V_M^2 \rangle$ (or 2% decrease in δV_M). Thus, ligand binding stiffens the enzyme and renders it less dynamic compared to the unbound state. In general, we discuss the importance of volumetric insights into the molecular origins of protein recognition events.

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Funding

This work was supported by grant RGPIN 203816 from NSERC to T.V.C. Y.L.S. acknowledges his graduate support from the CIHR Protein Folding Training Program.

Notes

The authors declare no competing financial interest.

ABBREVIATION

$(GlcNAc)_3$, tri-*N*-acetylglucosamine.

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