Microcalorimetric Study of Wheat Germ Agglutinin Binding to *N*-Acetylglucosamine and Its Oligomers†

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ABSTRACT: The energetics of association of wheat germ agglutinin (WGA) with *N*-acetylglucosamine (GlcNAc) and its $\beta(1,4)$ oligomers have been measured using isothermal titration calorimetry. Association constants of 0.4, 5.3, 11.1, 12.3, and 19.1 mM^{-1} and enthalpies of binding of −6.1, −15.6, −19.4, −19.3, and −18.2 kcal mol^{-1} were obtained at 26 °C for the titration of WGA with GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$, respectively. The term $T\Delta S$ was always of negative value, indicating that the binding process is enthalpically driven. Titrations of WGA performed at pH 4.5 did not differ significantly from those performed at pH 7.0, suggesting that no groups with a pK_a in this range are directly involved in the binding event. Also, performing the titration in a buffer system with a higher enthalpy of protonation did not change the enthalpy of binding confirming that there is no net protonation or deprotonation when WGA binds GlcNAc residues at pH 7. A model of four independent binding sites was found to adequately describe the binding curves, except in the case of $(GlcNAc)_4$ which exhibited positive cooperativity. The energetic values are discussed within the context of the structure of the WGA–$(GlcNAc)_2$ complex.

Lectins are carbohydrate-binding proteins originally found in plants. In recent years, lectins of animal origin have gained much prominence due to their participation in diverse phenomena of biological significance. These include, to name a few, viral and microbial infection, routing of lysosomal enzymes, phagocytosis by macrophages, and lectin-mediated specific interactions between various types of leukocytes and endothelial cells [for review, see Rademacher et al. (1988) and Sharon and Lis (1989)].

Wheat germ agglutinin (WGA)[1] is a lectin composed of two identical 21.6-kDa subunits at pH 7 [for review, see Goldstein and Poretz (1986) and references within]. Each subunit is comprised of four homologous, but nonidentical, domains. Although the stoichiometry and specificity of GlcNAc-containing ligands for WGA have been extensively studied using equilibrium dialysis (Nagata & Burger 1974), fluorescence (Lotan & Sharon, 1973; Privat et al., 1974a), NMR (Jordan et al., 1977), and X-ray diffraction techniques (Wright, 1984), direct calorimetric measurements of the binding energetics have not been reported. Among the simple sugars, WGA interacts only with GlcNAc and *N*-acetylneuraminic acid. To date, the strongest ligands are the β-(1,4)-linked GlcNAc oligomers. Studies on such homologous GlcNAc oligomers have shown that the binding affinity increases geometrically for ligands containing from one to three GlcNAc units. This observation led Allen et al. (1973) to propose a model which depicts each binding site as being comprised of three subsites.

With the recent advent of sensitive and reliable instrumentation, a direct determination of energetics of biological interactions by titration microcalorimetry has become feasible (Schon & Freire, 1989; Freire et al., 1990). In this paper, we have measured the interaction of WGA with a series of β-(1,4)-linked oligomers of GlcNAc [$(GlcNAc)_n$ where n = 1–5] using high-sensitivity isothermal titration calorimetry. By performing these experiments as titrations, both the affinity of the ligands for WGA and the enthalpy of binding of these ligands were obtained simultaneously, from which the free energy and entropy terms were calculated. In addition, the pH and buffer conditions of the titration were varied in order to investigate the groups which may participate in the binding process.

MATERIALS AND METHODS

WGA was obtained from E–Y Labs, Inc. (San Mateo, CA). GlcNAc and the $\beta(1,4)$-linked oligosaccharides of GlcNAc (from dimer to pentamer) were purchased from SEIKAGAKU America, Inc. (Rockville, MD) and were of greater than 99% purity. WGA was suspended in buffer, sonicated briefly, and then stored at 4 °C overnight. Buffers were 0.1 M sodium acetate at pH 4.5 and 50 mM sodium phosphate/50 mM KCl or 50 mM Tris-HCl/50 mM KCl at pH 7.0. Concentrations of WGA were determined from absorbance at 280 nm using an absorption coefficient of 1.5 mg^{-1} cm^2 (Privat et al., 1974b) and ranged from 10 to 40 μM in all experiments.

Isothermal titration calorimetry was performed using an Omega instrument from Microcal coupled with a Keithley 181 nanovoltmeter used as a preamplifier (Wiseman et al., 1989). All experiments were done at 26 °C. Briefly, saccharide was injected in 10-μL increments into a solution of WGA (cell volume = 1.4 mL) dissolved in the same buffer as the saccharide. The amount of power required to maintain the cell at a constant temperature with each injection was monitored as a function of time. Injections were made until all of the available sites on WGA were saturated, or until no more heat was observed with additional injections. Control experiments were performed by making identical injections

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[1] Abbreviations: WGA, wheat germ agglutinin; $(GlcNAc)_2$, GlcNAcβ4GlcNAc; $(GlcNAc)_3$, GlcNAcβ4GlcNAcβ4GlcNAc; $(GlcNAc)_4$, GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAc; $(GlcNAc)_5$, GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAc; MurNAc, *N*-acetylmuramic acid.

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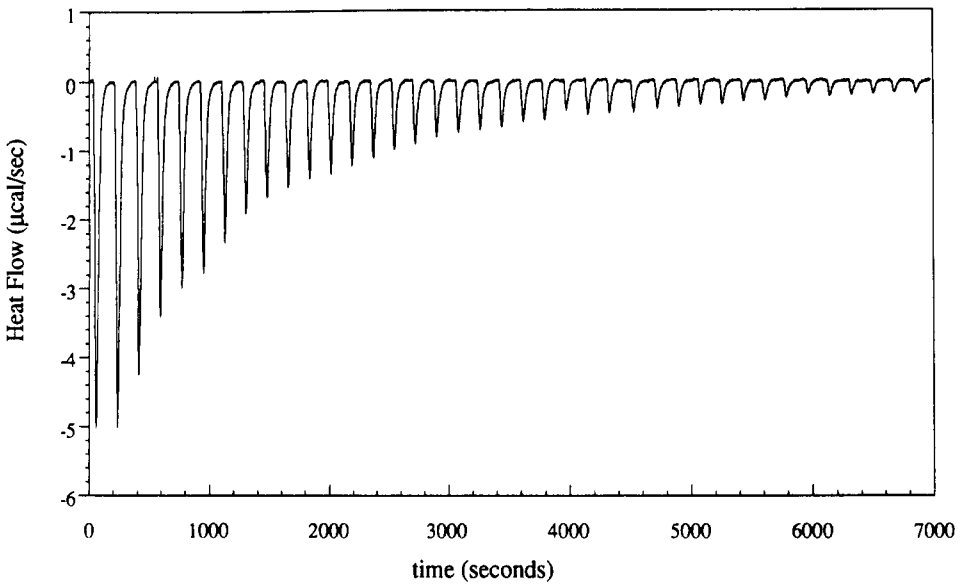

FIGURE 1: Isothermal titration of WGA with GlcNAcβ4GlcNAc at pH 7. The saccharide solution (4.3 mM) was injected in small increments into the protein solution in the cell (29.6 µM) until no more measurable peaks are produced. Each peak represents the thermal power as a function of time. The area under each peak is equal to the amount of heat released by the binding event at a given degree of saturation.

of saccharide into a cell containing buffer with no protein. In all experiments, values for the heats of dilution were less than 3% of the binding enthalpies and have been subtracted from the reported enthalpies.

The data were analyzed with software developed at the Biocalorimetry Center by determination of the area underneath the peak induced by each injection of saccharide into WGA. The resulting heats were plotted as a function of total ligand injected into the cell, and this binding curve was fit for the enthalpy ΔH and the affinity K_a, using a nonlinear least-squares analysis.

RESULTS

Calorimetric Titrations. Figure 1 shows a typical calorimetric titration of WGA with $(GlcNAc)_2$ at pH 7. As shown in the figure, after each injection of the saccharide an exothermic heat effect is observed. The magnitude of this heat effect decreases progressively with each new injection until complete saturation is achieved. The calorimetric binding curve is obtained by determining the area under each peak and plotting the resulting values as a function of the ligand concentration. The binding curve and fit obtained through this analysis for the binding of $(GlcNAc)_2$ to WGA are shown in Figure 2. A model of four identical and independent binding sites was found to adequately describe the binding event in all cases except that of the tetrasaccharide, $(GlcNAc)_4$. The binding curve of $(GlcNAc)_4$ exhibits sigmoidal behavior and yields particularly high residuals upon fitting to this model or to a model with two sets of two different binding sites. Analysis of the tetrasaccharide data in terms of a model exhibiting positive cooperativity improved the goodness of fit by a factor of 10. The cooperative model used for this fit assumed two independent pairs of interacting sites and suggested a cooperative free energy of about −0.5 to −1 kcal·mol^{-1} between interacting sites.

Thermodynamic Analysis. The thermodynamic parameters for the interaction of WGA with GlcNAc and its β(1,4) oligomers at pH 4.5 and pH 7 are summarized in Table I. This data suggest that the interaction between lectin and oligosaccharide is independent of pH in this range, and thus is not coupled to protonation or deprotonation of groups with a pK_a between 4.5 and 7. In addition, a comparison of the

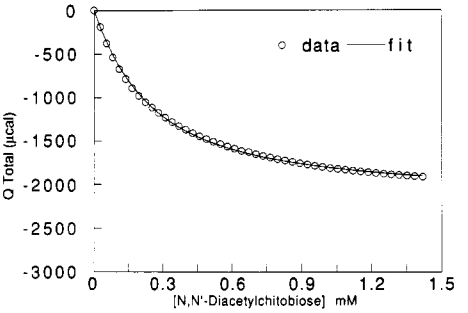

FIGURE 2: Dependence of the cumulative heat (Q) on the total ligand concentration. The experimental data are fitted using a nonlinear least-squares method in order to obtain values for K_a and ΔH, with a model of the protein containing four independent and equivalent sites for sugar binding. Open circles (O) represent points; the solid line represents the fit. At the end of the titration curve the degree of saturation is approximately 90%.

results obtained in the phosphate buffer system with those obtained in the Tris buffer system shows no significant difference in the enthalpy of binding. If a net protonation/deprotonation reaction were occurring during the binding event, the enthalpy of ionization of the buffer system (11.28 kcal·mol^{-1} for Tris and 1.22 kcal·mol^{-1} for phosphate) would either contribute to or subtract from the enthalpy of binding. The absence of a pH effect in this range agrees with the results of others (Privat et al., 1974a). This is, however, a surprising result in light of the existence of a solvent-accessible histidine[2] and a glutamate in two of the four binding sites. According to the crystallographic data (Wright, 1984), this histidine ring lies within van der Waals contact of the nonreducing GlcNAc ring of the WGAII–$(GlcNAc)_2$ complex. Apparently, the ionization of the amino acid groups does not change with the binding of the saccharide molecule.

The observed enthalpy, entropy, and free energy changes for this system are similar to those reported for many other carbohydrate–lectin interactions in that, at 26 °C, the reactions

[2] WGA is a mixture of two main isolectins (WGAI and WGAII) with similar binding affinities which differ in amino acid substitutions at 5 residues. One of the isolectins, WGAI, has a tyrosine instead of this histidine. The fraction of WGAII in affinity-purified WGA is close to 65% (Rice & Etzler, 1975).

Table I: Thermodynamic Parameters Derived from the Titration of WGA with β(1,4)-Linked GlcNAc Oligomers

GlcNAc oligomer	ΔH (kcal·mol^{-1})	$T\Delta S$ (kcal·mol^{-1})	ΔG (kcal·mol^{-1})	K_a[c] ($M^{-1} \times 10^{-3}$)	ΔH per additional saccharide (kcal·mol^{-1})	$T\Delta S$ per additional saccharide (kcal·mol^{-1})	ΔG per additional saccharide (kcal·mol^{-1})	SD[b] (μcal)
				pH 4.5				
GlcNAc	−7.0	−3.5	−3.5	0.39	−7.0	−3.5	−3.5	8.2
$(GlcNAc)_2$	−13.5	−8.6	−4.9	3.9	−6.5	−5.1	−1.4	9.9
$(GlcNAc)_3$	−16.0	−10.4	−5.6	12.2	−2.5	−1.8	−0.7	12.0
				pH 7.0				
GlcNAc	−6.1	−2.4	−3.7	0.41	−6.1	−2.4	−3.7	
$(GlcNAc)_2$	−15.6	−10.5	−5.1	5.3	−9.5	−8.1	−1.4	10.9
$(GlcNAc)_2$[a]	−14.6	−9.7	−4.9	3.9	−8.5	−7.3	−1.2	10.7
$(GlcNAc)_3$	−19.4	−13.9	−5.5	11.1	−3.8	−3.4	−0.4	8.7
$(GlcNAc)_4$	−19.2	−13.6	−5.6	12.3	+0.2	+0.3	−0.1	32.1
$(GlcNAc)_5$	−18.2	−12.4	−5.8	19.1	+1.0	+1.2	−0.2	8.8

[a] This experiment was performed in a Tris buffer system instead of a phosphate buffer system. [b] These values represent the standard deviation between the experimental binding curve and the theoretical curve obtained with the fitted thermodynamic parameters. [c] The errors in K_a are primarily those associated with concentration determinations of the reactants. If we assume a maximal uncertainty of ±5% for both the saccharide and the protein concentration determination, then the maximum error estimate is ±6% of the quoted value in each case.

have a favorable enthalpic component and an unfavorable entropic component [e.g., Lemieux (1989); Sastry et al. (1986), and Munske et al. (1984)]. An analysis of the thermodynamic reaction parameters as a function of the number of linked GlcNAc residues in the binding reaction demonstrates that both the magnitude of the enthalpy change and the free energy change increase as the number of GlcNAc residues increases in the oligosaccharide for up to three residues. However, there is little further increase in these thermodynamic parameters upon linkage of a fourth GlcNAc residue to the trisaccharide. These results agree with the "three subsite" structural binding model proposed by Allen et al. (1973), although there appears to be a small contribution to the overall binding thermodynamics from the fifth GlcNAc residue in our experiments.

A plot of ΔH vs ΔS for the data at pH 4.5 and pH 7.0 yields a straight line with a regression coefficient better than 0.99, indicating that the enthalpy and entropy changes partially compensate each other [see, for example, Lumry and Rajender (1970)]. The slope of this plot defines the "compensation temperature" which for these reactions is 357 K.

DISCUSSION

The WGA monomer consists of four structurally homologous, but spatially distinct, domains (A, B, C, and D domain from the N-terminus) connected in tandem. X-ray crystallographic studies by Wright (1980) indicate that the interaction of two monomers of WGA to form a dimer produces four contact areas. If the two protomers in the dimer are designated I and II, then the four contact areas may be described as occurring between the domains A_I and D_{II}, A_{II} and D_I, B_I and C_{II}, and B_{II} and C_I. The sugar-binding sites are located in these contact areas: i.e., two primary binding sites in the B/C contact regions (B_I/C_{II} and B_{II}/C_I), and two secondary binding sites in the A/D contact regions (A_I/D_{II} and A_{II}/D_I). These two types of sites possess extensive homology both in sequence and structure (rms deviation = 1.39 Å (Wright, 1984)), and various binding studies have not been able to discriminate between them. Also, in our study, we did not observe significant deviations from the expected curves corresponding to four identical binding sites. Except for the tetrasaccharide $(GlcNAc)_4$ which exhibited positive cooperativity, no evidence of site–site interactions could be detected either. This lack of cooperativity is noteworthy, as all the binding sites occur at the interfacial region of the WGA dimer.

Table II: Comparison of WGA–GlcNAc Oligomer Association Constants Obtained through Different Methods[a]

	$K_a \times 10^{-3}$ (M^{-1})					
GlcNAc oligomer	this paper	Lotan and Sharon (1973)	Nagata and Burger (1974)	Privat et al. (1974a)	Jordan et al. (1977)	Matsumoto et al. (1980)
GlcNAc	0.4		1.3	0.7	0.2	
$(GlcNAc)_2$	5.0	13	20	4.5		10
$(GlcNAc)_3$	12	22	83	20		
$(GlcNAc)_4$	12.1	36		23		
$(GlcNAc)_5$	19					

[a] Methods used: fluorometry (Lotan & Sharon, 1973; Privat et al., 1974a); equilibrium dialysis (Nagata & Burger, 1974); ^1H NMR (Jordan et al., 1977); difference UV spectroscopy (Matsumoto et al., 1980).

The association constants of interactions between WGA and GlcNAc oligomers obtained by other groups using different techniques are shown in Table II. Unlike our calorimetric study, the enthalpic and entropic contributions to the association free energies are not available. With respect to association constants, our results agree best with those of Privat et al. (1974a), who determined association constants using the fluorescence enhancement of a tryptophan residue thought to be near the binding site. The major difference between our results and those of Privat is observed for $(GlcNAc)_4$ in which the binding constant differs by a factor of 2. The reasons for this difference are not clear, although the presence of a cooperative binding effect might preclude a simple direct comparison, especially if data were not collected with the same frequency or for a similar range of saturation values.

To date, X-ray crystallographic studies have been performed on several carbohydrate–protein complexes, and an exhaustive comparison has been made of the binding sites (Quiocho, 1986). Although these proteins can differ remarkably in overall tertiary structure, the amino acids that directly interact with carbohydrates are similar in that residues possessing both hydrophobic and hydrophilic character are involved at a high frequency (His, Asp, Glu, Tyr, Arg, and Asn). This class of residues could serve a dual purpose—ensuring that adequate solvent is available for the saccharide in the binding pocket and providing hydrogen bond donors and acceptors for the carbohydrate hydroxyls. The enhanced hydrogen bonding capacity of saccharides is likely to influence protein–carbohydrate interactions and define solvation effects different from those found in other types of protein–ligand interactions.

In the case of the WGAII–$(GlcNAc)_2$ complex (Wright, 1987), four hydrogen bonds appear to be formed in the primary binding site—two between the acetamido group of the nonreducing GlcNAc and a glutamic acid residue, one between the backbone carbonyl of a serine and the same acetamido group, and the fourth between a tyrosine and the C3 hydroxyl of the nonreducing sugar ring. For the secondary site, the same groups in the $(GlcNAc)_2$ molecule participate in hydrogen bonding; however, the role of the glutamic acid is served by an aspartic acid in this site. The roles of the serine and tyrosine residues are conserved between the two types of sites. In both sites, the second residue of the ligand appears to interact only marginally with the protein. While the second residue does not appear to interact with the protein through hydrogen bonding, the sugar ring is positioned within 4 Å of a tyrosine ring, suggesting that van der Waals interactions occur between these two rings at the primary site. This interaction apparently does not occur at the secondary site.

Recently, it has been shown that for those proteins for which both thermodynamic and structural data are available, the enthalpy and heat capacity changes associated with folding/unfolding transitions are related to changes in accessible polar and apolar surface areas through a set of elementary thermodynamic parameters (Murphy et al., 1992a). In addition, the same elementary parameter values derived from protein folding studies have been shown to successfully account for the enthalpy and heat capacity changes associated with the binding of the small peptide angiotensin II to an angiotensin II-reactive antibody (Murphy et al., 1992b). In order to assess how well these protein-derived parameters are able to account for saccharide binding, structural thermodynamic calculations were performed for WGAII and lysozyme, another protein which binds oligomers of GlcNAc and MurNAc.

The structure of the bound $(GlcNAc)_2$ residue was provided to us by C. Wright. These coordinates were used in conjunction with the coordinates of WGAII (Wright, 1987) to estimate the changes in accessible polar and apolar surface area associated with the saccharide binding at the primary binding site. The structure of lysozyme–(MurNAc-GlcNAc-MurNAc) has been solved at 2.5-Å resolution (Kelly et al., 1979). The binding thermodynamics have been obtained by a van't Hoff analysis by Patt et al. (1978). In addition, the binding thermodynamics of lysozyme to $(GlcNAc)_3$ have been measured calorimetrically (Bjurulf & Wadso, 1972).

Structural thermodynamic calculations were performed as described by Murphy et al. (1992a). All calculations were made under the assumption that the binding event is not coupled to a conformational change in the protein. The structures of the saccharide free in solution were obtained by energy minimization of the saccharide using CHARMM (Brooks et al., 1983). Accessible surface areas were calculated using the algorithm of Lee and Richards modified by S. Presnell at UCSF as described in Murphy et al. (1992a). Briefly, the heat capacity change is the parameter with the most straightforward structural relationship. It is directly proportional to the change in polar and apolar solvent-accessible surface area associated with the process:

$$\Delta C_p = \Delta A_{ap}\Delta C_{p,ap}^\circ + \Delta A_{pol}\Delta C_{p,pol}^\circ \quad (1)$$

where ΔA_{ap} and ΔA_{pol} represent the change in accessible apolar and polar surface area, respectively, and $\Delta C_{p,ap}^\circ$ and $\Delta C_{p,pol}^\circ$ represent the elementary ΔC_p contributions per mole-Å^2. $\Delta C_{p,ap}^\circ$ and $\Delta C_{p,pol}^\circ$ have been estimated as 0.45 ± 0.02 cal·K^{-1}·(mol-Å^2)$^{-1}$ and -0.26 ± 0.03 cal·K^{-1}·(mol-Å^2)$^{-1}$ from solid model peptide dissolution studies (Murphy & Gill, 1990) and from an analysis of the protein thermodynamic database (Murphy et al., 1992a).

The intrinsic enthalpy change after correction from protonation effects can be written as:

$$\Delta H = \Delta H^* + \Delta C_p(T - T_H^*) \quad (2)$$

where ΔH^* is the enthalpy change at the reference temperature, T_H^*. For proteins, it has been shown that at this temperature (100 °C) ΔH^* scales linearly with the buried polar surface area and is equal to 35 ± 1 cal·(mol-Å^2)$^{-1}$ of buried polar surface that becomes exposed during the process (Murphy & Freire, 1991; Murphy et al., 1992a).

From the crystallographic structure of WGA–$(GlcNAc)_2$ and its isolated components, we calculate that upon binding 277 Å^2 of polar surface and 237 Å^2 of apolar surface become buried from the solvent. These figures are consistent with a ΔC_p of -35 ± 10 cal·K^{-1}·mol^{-1} and an enthalpy change (at 26 °C) of -7 ± 1.5 kcal·mol^{-1}. The value obtained experimentally for the enthalpy change is -13.5 ± 0.8 kcal·mol^{-1}. These two values are close in absolute magnitude; however, the magnitude of the predicted value is somewhat smaller than the value obtained experimentally. This difference could be explained by a small conformational change not accounted for in the structural data or other saccharide-specific effects not considered in the calculations. Additionally, it should be noted that the crystallographic structure of the $(GlcNAc)_2$–WGAII complex has not been refined, and therefore there may be errors associated with the solvent accessibility calculations.

From the crystallographic structure of the lysozyme complex and that of lysozyme and free saccharide, we calculate that, upon binding, 415 Å^2 of polar surface and 439 Å^2 of apolar surface become buried from the solvent. These values yield a ΔC_p for binding of -90 ± 20 cal·K^{-1} mol^{-1} and a ΔH of binding of -8 ± 2 kcal·mol^{-1} at 25 °C. The experimental enthalpy value obtained noncalorimetrically is -8 kcal·mol^{-1} (Patt et al., 1978). In this system, the calculated value agrees well with the experimental value.

Unfortunately, ΔC_p values for the lysozyme–(MurNAc-GlcNAc-MurNAc) are not available. However, direct calorimetric measurements are available for GlcNAc and $(GlcNAc)_3$ binding to lysozyme, although the coordinates for this complex are still pending (Cheetham et al., 1992). The experimental estimate of ΔC_p for GlcNAc is -52 ± 38 cal·K^{-1}·mol^{-1} and for $(GlcNAc)_3$ is 40 ± 36 cal·K^{-1}·mol^{-1} (Bjurulf & Wadso, 1972). These ΔC_p values are extremely small and are consistent with the absence of a significant conformational change upon binding. For $(GlcNAc)_3$ binding to lysozyme, the experimental value for ΔH at 25 °C and pH 5 is -13.6 kcal·mol^{-1}. This value is also close to the calculated and experimental value for $(GlcNAc)_2$ binding to WGA. It will be interesting to determine whether the calculated value agrees with the experimental value when the structure of $(GlcNAc)_3$–lysozyme becomes available.

The binding of carbohydrates to a number of proteins [see also Dani et al. (1981), Munske et al. (1984)] is characterized by small enthalpy and heat capacity changes. In many cases, the association of the sugar is coupled to changes in solvent accessibilities that result in a negative, albeit small, ΔC_p. This ΔC_p defines the solvation/desolvation contributions to the overall energetics. A negative ΔC_p gives rise to both positive enthalpy and entropy contributions to binding at 25 °C. For WGA–$(GlcNAc)_2$, these contributions amount to 2.5 kcal·mol^{-1} and 8.5 cal·K^{-1}·mol^{-1}, i.e., a destabilizing enthalpy contribution and a stabilizing entropy contribution. The main stabilizing contribution to the total enthalpy of binding must

therefore arise from polar van der Waals interactions and hydrogen bonding. On the other hand, solvation effects partially compensate for the negative entropy change associated with the reduced number of degrees of freedom of the sugar molecule and amino acid residues at the site upon binding. The magnitudes of the overall enthalpy changes, however, are small, and as such they can be significantly affected by the coupling of the binding reaction to a second process, e.g., a protonation/deprotonation reaction. For example, the enthalpy of protonation of one histidine residue is -7 kcal·mol^{-1}, a figure close to the overall enthalpy of binding for many carbohydrate–protein systems. Although protonation apparently does not occur in WGAII binding to $(GlcNAc)_2$, it may be important in other types of reactions and must be considered.

The relative agreement between the experimental and calculated ΔH values is encouraging, especially considering that the fundamental thermodynamic parameters used for the calculations were obtained from the analysis of peptide and protein systems only. These results point to the feasibility of performing accurate structural thermodynamic predictions in the near future. A refined set of parameters for protein–carbohydate interactions should become available as more structural and thermodynamic information for these systems appears in the literature.

ADDED IN PROOF

Structural thermodynamic calculations based upon the newly available crystallographic structure of the lysozyme–$(GlcNAc)_3$ complex (Cheetham et al., 1992) yield a ΔH of -9 ± 2 kcal mol^{-1} at 25 °C and a ΔC_p of -75 ± 20 cal K^{-1} mol^{-1}. The calorimetrically determined values at pH 4.5 and 25 °C are -12 ± 0.5 kcal mol^{-1} and -40 ± 20 cal K^{-1} mol^{-1} (unpublished results from this laboratory).

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