Thermodynamic Analysis of Allosamidin Binding to a Family 18 Chitinase†

F. Henning Cederkvist,‡ Silje F. Saua,‡ Vigdis Karlsen,‡ Shohei Sakuda,§ Vincent G. H. Eijsink,‡ and Morten Sørlie*,‡

Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N - 1432 Ås, Norway, and Department of Applied Biological Chemistry, University of Tokyo, Bunkyo-Ku, Tokyo 113, Japan

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ABSTRACT: Inhibition of family 18 chitinases is emerging as a target for pest and fungal control as well as asthma and inflammatory therapy. One of the best known inhibitors for these enzymes is allosamidin, a natural product. While interactions of this compound with family 18 chitinases have been studied in much detail by X-ray crystallography and standard enzymology, details of the driving forces behind its tight binding remain unknown. We have studied the thermodynamics of allosamidin binding to chitinase B (ChiB), a family 18 chitinase from *Serratia marcescens*, using isothermal titration calorimetry. At pH 6.0, K_d is 0.16 ± 0.04 μM, and the binding reaction is entropically driven ($\Delta S_r = 44$ cal/K mol) with an enthalpic penalty ($\Delta H_r = 3.8 \pm 0.2$ kcal/mol). Dissection of the entropic term shows that a favorable conformational change in the allosamidin−ChiB complex ($\Delta S_{conf} = 37$ cal/K mol) is the main contributor to the reaction. At pH 8.5, K_d decreases to 0.03 μM and the binding reaction is less entropically favorable ($\Delta S_r = 30$ cal/K mol). While the solvation entropy change (ΔS_{solv}) increases from 15 cal/K mol at pH 6.0 to 46 cal/K mol at pH 8.5, ΔS_{conf} becomes small and negative (−8 cal/K mol) because of an enthalpy−entropy compensation. Analyses of proton transfer showed that at pH 6.0 binding of allosamidin requires deprotonation of the Asp142−Glu144 catalytic diad. At pH 8.5, the 142−144 diad is ionized in the native enzyme, relieving the deprotonation penalty of binding and explaining why binding becomes enthalpically favorable ($\Delta H_r = -1.2 \pm 0.2$ kcal/mol).

Chitinases break down chitin, an insoluble linear polymer of β-1,4-linked *N*-acetyl-D-glucosamine. These enzymes belong to the glycoside hydrolase families 18 and 19 (*1*). Whereas family 19 chitinases are primarily found in plants and actinomycetes, family 18 chitinases occur in many different organisms, including humans. Chitin metabolism, including hydrolysis by family 18 chitinases, is an important process in many plague organisms and parasites. For instance, the avian malaria parasite *Plasmodium gallinaceum* is dependent on chitin hydrolysis to penetrate the chitin-containing peritrophic matrix surrounding the blood meal in the mosquito midgut, and the presence of the family 18 chitinase inhibitor allosamidin prevents the parasite from entering the mosquito (*2*). Thus, chitin metabolism is an interesting target area for the development of drugs and pesticides, especially since humans do not possess chitin. In lower organisms, such as plants and fungi, the production of chitinases is associated with defense responses to chitin-containing organisms (*3*−*5*). Whereas humans do not possess chitin, they do possess family 18 chitinases that perhaps are involved in similar defense reactions (*6*). The recent discovery of the expression of human chitinases during TH2 inflammation through an interleukin (IL)-13-dependent mechanism led to the theory that chitinases play a role in the pathogenesis of TH2 inflammation and IL-13 effector pathway activation. It was suggested that chitinase expression in some cases may reflect a parasite-independent antiparasite response that can lead to inflammation such as asthma (*7*−*10*). As a consequence of the known and putative biological roles of family 18 chitinases, inhibition of these enzymes is a target for the development of pesticides (*11*−*13*), fungicides (*14*, *15*), medicines for allergic and inflammatory disorders (*7*−*10*), and anti-malarials (*2*, *16*, *17*).

The best known inhibitor of family 18 chitinases is allosamidin, a natural product isolated from *Streptomyces* sp. (*12*, *18*, *18*−*23*). Allosamidin is a pseudotrisaccharide that binds to the −3 to −1 glycon-binding subsites of family 18 chitinases with affinities in the low and sub-micromolar range (*12*, *23*, *24*). Its allosamizoline group resembles the oxazolinium ion reaction intermediate that emerges in the −1 subsite during the catalytic cycle (Figure 1) (*23*, *25*−*27*). The crystal structures of several family 18 chitinases in complex with allosamidin (*21*, *23*, *25*, *27*) or its derivatives (*24*) have revealed structural details of the enzyme−ligand interaction. This interaction is dominated by residues comprising the −1 subsite, which are conserved throughout the family 18 chitinase family (see below).

Both IC_{50} and K_i values for the allosamidin interaction with various family 18 chitinases have been determined (*12*, *20*, *23*, *28*). An especially interesting feature of the binding reaction is its pH dependency. Several studies report a 5-fold up to a 10-fold reduction in K_i upon increasing the pH (*12*,

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* To whom correspondence should be addressed. Tel: + 47 64 96 59 02. Fax: +47 64 96 59 01. E-mail: morten.sorlie@umb.no.

‡ Norwegian University of Life Sciences.

§ University of Tokyo.

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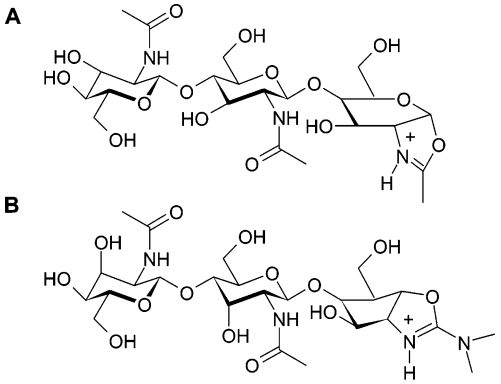

FIGURE 1: Oxazolinium ion and allosamidin. (A) The proposed positively charged reaction intermediate (oxazolinium ion), exemplified by a trisaccharide bound to subsites −3 (left) to −1 (right). (B) Allosamidin.

22, *23*). IC_{50} and K_i values yield no information on the enthalpic and entropic contribution to the enzyme−ligand interaction, whereas such information is fundamental for understanding this interaction and, consequently, inhibitor design. The reaction enthalpy change (ΔH_r) of binding between an inhibitor and an enzyme reflects the change in weak interactions, such as electrostatic−electrostatic interactions, hydrogen bonding, and dipole−dipole interactions, for the inhibitor and the enzyme in going from two solvated free species to a solvated complex. The reaction entropy change (ΔS_r) is normally divided into three contributions: the loss in translational entropy that occurs when two species combine, the change in solvation entropy, and the change in conformational entropy. Typically, ligand binding leads to desolvation, resulting in a gain in entropy that is thought to be of particular importance if hydrophobic groups are involved in the binding (*29*−*35*). The change in conformational entropy originates from changes in conformational freedom for both the ligand and the protein.

Studies of the binding reactions with isothermal titration calorimetry (ITC[1]) enable the direct determination of the equilibrium binding association constant (K_a) and ΔH_r in one single experiment. The reaction-free energy change (ΔG_r), ΔS_r, and the equilibrium binding dissociation constant (K_d) follow from the relationship described in eq 1.

$$\Delta G_r = -RT \ln K_a = RT \ln K_d = \Delta H_r - T\Delta S_r \quad (1)$$

In addition, the temperature dependence of the enthalpy change yields the change in heat capacity (ΔC_p) as described by eq 2:

$$\Delta C_p = \left(\frac{\partial \Delta H_r}{\partial T}\right) \quad (2)$$

The most important quantity in the thermodynamic description of binding is ΔG_r since this determines the binding affinity of a ligand, that is, substrate or inhibitor, to an enzyme. Detailed information of the binding affinity can be obtained by investigating the enthalpic and entropic terms. When examining the entropic term, any entropic change at a given temperature can be calculated once ΔC_p has been obtained and the entropy change has been determined at a reference temperature (T_R) because entropy changes are temperature dependent (eq 3):

$$\Delta S_T = \Delta S_{TR} + \int_{T_R}^{T} \Delta C_p d \ln T \quad (3)$$

For entropy changes, the reference temperature normally corresponds to temperatures at which hydration is zero. This temperature has been estimated to be 385 K. There are several experiments that allude to this temperature. The entropy of transfer of six liquid hydrocarbons, as a model for hydrophobic interaction in protein folding, reaches zero at 385.5 ± 2.2 K (*29*). Also, plotting entropy changes versus heat capacity changes for denaturation of 11 proteins, apolar gases, saturated hydrocarbon gases, and solid cyclic dipeptides yield linear plots, and the temperature of 385 K comes from the slopes after least-squares fits of the data (*31*). A similar result was obtained when 8 different alcohols were investigated (*36*). Inserting $T_R = 385$ K and $\Delta S_{TR} = 0$ into eq 3 and rearranging, the solvation entropy at any given temperature (e.g., 298 K) can be estimated when ΔC_p is known (eq 4).

$$\Delta S_{solv,\ 298\ K} = \Delta C_p \ln\left(\frac{298\ K}{385\ K}\right) \quad (4)$$

Moreover, the loss in translational entropy (ΔS_{mix}) of the binding reaction can be calculated as a statistical correction that reflects the mixing of solute and solvent molecules that effectively accounts for the entropy change due to changes in translational/rotational degrees of freedom (eq 5) (*37*−*39*).

$$\Delta S_{mix} = R \ln\left(\frac{1}{55.5}\right) \quad (5)$$

Since the change in reaction entropy can be derived from the ITC experiment, and as ΔS_{solv} and ΔS_{mix} can be calculated at any temperature and pH, ΔS_{conf} can be derived using eq 6.

$$\Delta S_r = \Delta S_{solv} + \Delta S_{conf} + \Delta S_{mix} \quad (6)$$

Using this relationship, it is possible to determine the extent of conformational changes induced by inhibitor binding. When this approach has been used, experimental data from ITC experiments compare well with structure-based predictions for the binding of hydroxamic acid and carboxylic acid inhibitors to the stromelysin-1 catalytic domain (*40*), 6-methyltetrahydropterin to phenylalanine hydroxylase (*41*), and ovomucoid third domain to elastase (*37*).

Here, we have studied the binding of allosamidin to ChiB, one of three family 18 chitinases produced by the soil bacterium *Serratia marcescens* (*42*). The structure and the catalytic mechanism of ChiB have been studied in much detail. Available data include crystal structures of wild-type and mutant enzymes with and without allosamidin, kinetic parameters for various substrates, and structural and binding data for interactions with a variety of inhibitors (*20*, *23*, *27*, *43*−*48*). Thus, ChiB may serve as a model enzyme for family18 chitinases.

MATERIALS AND METHODS

Isolation of ChiB and Allosamidin. Wild-type ChiB from *Serratia marcescens* was overexpressed in *Escherichia coli*

[1] Abbreviations: ITC, Isothermal titration calorimetry; ChiB, chitinase B of *Serratia marcescens*.

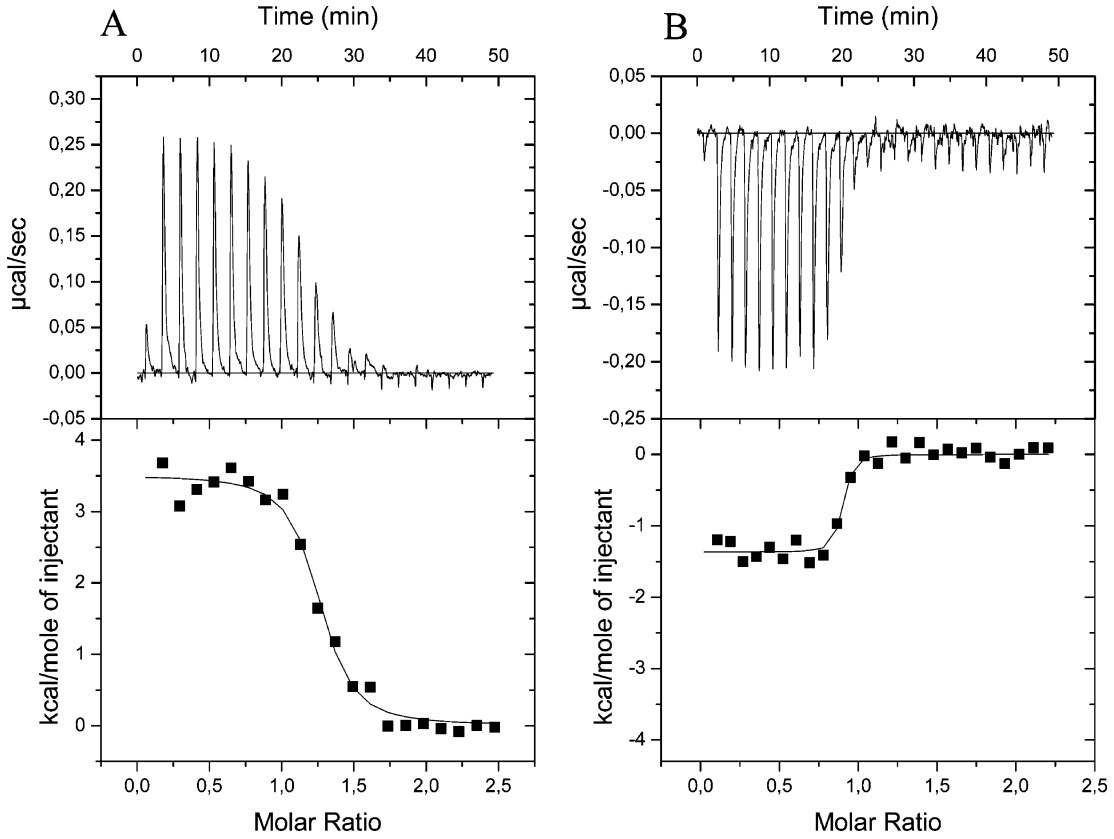

FIGURE 2: Thermograms (upper panels) and binding isotherms with theoretical fits (lower panels) obtained for the binding of allosamidin to ChiB at pH 6.0 (A) and pH 8.5 (B) at 30 °C in 20 mM potassium phosphate. Allosamidin (500 μM, injections of 4 μL) was titrated into the reaction cell containing ChiB (16 μM, 1.4214 mL).

and purified as described elsewhere (*49*). Allosamidin was isolated from *Streptomyces* sp., and the purity was controlled by ^{1}H NMR as described elsewhere (*18*). Previously, the structure of allosamidin had been verified by both NMR and crystallography (*19*).

Isothermal Titration Calorimetry Experiments. ITC experiments were performed with a VP-ITC system from Microcal, Inc (Northampton, MA) (*50*). Solutions were thoroughly degassed prior to experiments to avoid air bubbles in the calorimeter. Standard ITC conditions were 500 μM of allosamidin in the syringe and 16 μM of ChiB in the reaction cell in 20 mM potassium phosphate buffer of pH between 6.0 and 8.5. The concentration of other buffers, PIPES, imidazole, HEPES, tricine, and TRIS, was also 20 mM. The heats of ionization of these buffers are as follows: potassium phosphate, 1.22 kcal/mol (*51*); PIPES, 2.74 kcal/mol (*51*); imidazole, 8.75 kcal/mol (*51*); HEPES, 5.02 kcal/mol (*51*); Tricine, 7.64 kcal/mol (*51*); and TRIS, 11.2 kcal/mol (*52*). For a typical titration of ChiB with allosamidin, 16 μM ChiB was placed in the reaction cell with a volume of 1.4214 mL, and 500 μM of allosamidin was placed in the ITC syringe. Aliquots of 5 μL were injected into the reaction cell at 140 s intervals with a stirring speed of 260 rpm. The titrations were normally completed after 25 injections.

Analysis of Calorimetric Data. ITC data were collected automatically using the Microcal Origin v.7.0 software accompanying the VP-ITC system (*50*). All data were corrected for heat of dilution by subtracting the heat remaining after the saturation of binding sites on the enzyme prior to further data analysis. Data were fitted using a nonlinear least-squares algorithm using a single-site binding model employed by the Origin software that accompanies the VP-ITC system. All data from the binding reactions fitted well with the single-site binding model, yielding the stoichiometry (n), equilibrium binding association constant (K_a), and the enthalpy change (ΔH_r) of the reaction. The value of n was found to be between 0.9 and 1.1 for all reactions. The changes in reaction free energy (ΔG_r) and entropy (ΔS_r) as well as the dissociation constant (K_d) were calculated using eq 1.

RESULTS

Binding of Allosamidin to ChiB. The binding of allosamidin to ChiB in 20 mM potassium phosphate at different temperatures (20−37 °C) and pH (6.0−8.5) was studied by ITC. Figure 2 shows typical ITC thermograms and theoretical fits to the experimental data for two different pH values (pH 6.0, Figure 1A, and pH 8.5, Figure 1B).

At t = 30 °C, K_d is in the submicromolar area (0.16 ± 0.04 μM, pH 6.0) and decreases to 0.03/0.04 μM at pH 7.0 and higher values (Table 1). At pH 6.0, the binding of allosamidin is clearly entropically driven, with an enthalpic penalty: ΔS_r = 44 cal/K mol and ΔH_r = 3.8 ± 0.2 kcal/mol. At higher pH, the binding reaction becomes exothermic, and the entropic term becomes smaller. At the highest pH tested, pH 8.5, ΔH_r = −1.2 ± 0.2 kcal/mol and ΔS_r = 30 cal/K mol. Measurements of the temperature dependency of ΔH_r yielded ΔC_p values (Figure 3). Interestingly, the change of heat capacity of the binding reactions also showed a clear pH dependency (Table 1), with values decreasing from −63 ± 4 cal/K mol at pH 6.0 to −190 ± 13 cal/K mol at pH 8.5.

Table 1: Thermodynamic Parameters Obtained for the Binding of Allosamidin to ChiB at $t = 30$ °C in 20 mM Potassium Phosphate

pH	K_d (μM)	ΔG_r (kcal/mol)	ΔH_r (kcal/mol)	ΔS_r (cal/K mol)	ΔC_p (cal/K mol)[a]
6.0	0.16 ± 0.04	-9.4 ± 0.1	3.8 ± 0.2	44 ± 1	-63 ± 4
6.5	0.13 ± 0.01	-9.5 ± 0.1	3.5 ± 0.1	43 ± 1	-60 ± 4
7.0	0.03 ± 0.01	-10.4 ± 0.2	2.6 ± 0.1	43 ± 1	-120 ± 9
7.5	0.04[b]	−10.3[b]	0.3 ± 0.1	35	-131 ± 8
8.0	0.04[b]	−10.3[b]	0.1 ± 0.2	34	-193 ± 9
8.5	0.033 ± 0.004	-10.4 ± 0.1	-1.2 ± 0.2	30 ± 1	-190 ± 13

[a] These data are derived from the temperature dependence of ΔH_r; see Figure 3 and text. [b] Interpolated from K_d and ΔG data at $t = 20$, 25, and 37 °C.

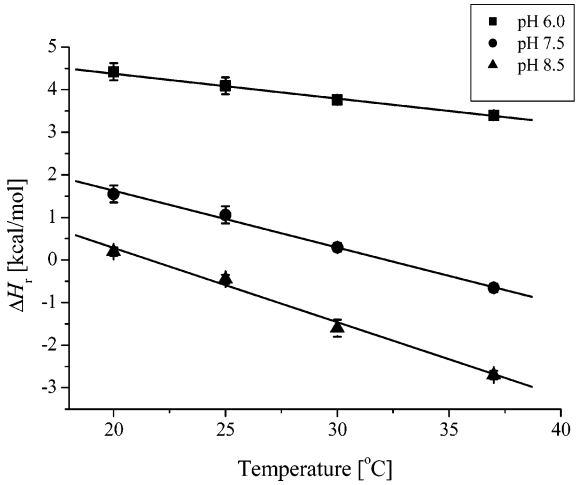

FIGURE 3: Temperature dependence of the enthalpy change for the binding of allosamidin to ChiB in 20 mM potassium phosphate at different pH values, as measured by ITC. The results shown are the mean values from three independent measurements at each pH and temperature. The slope of the linear regression of ΔH_r vs temperature yields a $\Delta C_p = -63 \pm 4$ cal/K mol for pH 6.0 (■), -131 ± 8 cal/K mol for pH 7.5 (●), and -190 ± 13 cal/K mol for pH 8.5 (▲). The plots for pH 6.5 ($\Delta C_p = -60 \pm 4$ cal/K mol), pH 7.0 ($\Delta C_p = -120 \pm 9$ cal/K mol), and pH 8.0 ($\Delta C_p = -193 \pm 9$ cal/K mol) were omitted for clarity.

Table 2: Division of the Entropic Term of Allosamidin Binding in 20 mM Potassium Phosphate Buffers

pH	ΔS_r (cal/K mol)	ΔS_{mix} (cal/K mol)[a]	ΔS_{solv} (cal/K mol)[b]	ΔS_{conf} (cal/K mol)[c]
6.0	44	−8	15	37
6.5	43	−8	14	37
7.0	43	−8	20	31
7.5	35	−8	22	21
8.0	34	−8	46	−4
8.5	30	−8	46	−8

[a] $\Delta S_{mix} = R \ln(1/55.5) = -8$ cal/K mol ("cratic" term). [b] $\Delta S_{solv,calc} = \Delta C_p \ln(T_{298\ K}/T_{385\ K})$. [c] $\Delta S_r = \Delta S_{solv} + \Delta S_{mix} + \Delta S_{conf}$.

Parametrization of the Entropic Term. The entropic term, ΔS_r, can be split into three terms, as shown in eq 5: ΔS_{solv}, ΔS_{mix}, and ΔS_{conf}. The availability of ΔC_p values for the binding of allosamidin to ChiB at selected pH enables the estimation of ΔS_{solv} using the relationship in eq 3. Table 2 shows that ΔS_{solv} increases with increasing pH, from 15 cal/K mol at pH 6.0 to 46 cal/K mol at pH 8.5.

Because the loss of translational entropy, ΔS_{mix}, is independent of pH, it is possible to calculate the change in entropy with respect to conformational changes, ΔS_{conf}, may be calculated from ΔS_r and ΔS_{solv}, using eq 6. Table 2 shows

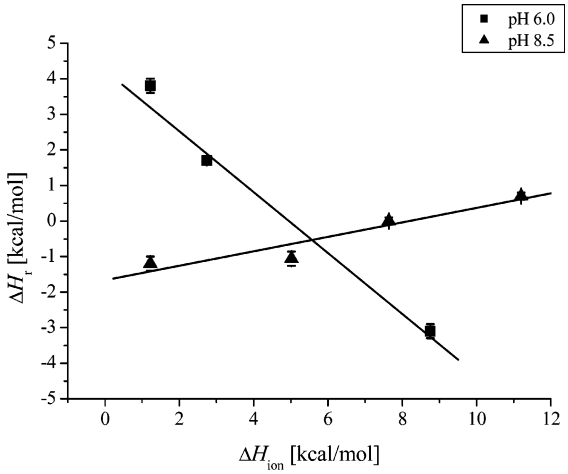

FIGURE 4: Plot of the enthalpy change of the binding reaction vs the ionization enthalpy change of different buffers at pH 6.0 (■) and pH 8.5 (▲). Experiments were performed at 30 °C in 20 mM buffer solutions. The ΔH_{ion} values used for pH 6.0 were potassium phosphate, 1.22 kcal/mol (*51*); PIPES, 2.74 kcal/mol (*51*); imidazole, 8.75 kcal/mol (*51*); and those for pH 8.5 were potassium phosphate, 1.22 kcal/mol (*51*); HEPES, 5.02 kcal/mol (*51*); Tricine, 7.64 kcal/mol (*51*); TRIS, 11.2 kcal/mol (*52*).

that ΔS_{conf} is large and positive at pH 6.0 (37 cal/K mol) and, thus, the main contributor to the driving force of the binding reaction. At higher pH values ΔS_{conf} decreases, and at pH 8.5, ΔS_{conf} has a small negative value (−8 cal/K mol).

Effect of Buffer Ionization and pH on Binding Enthalpy. Potential protonation/deprotonation effects coupled to allosamidin binding to ChiB were investigated by testing the contribution from buffer ionization to ΔH_r (*53*). ITC experiments were carried out at identical buffer concentrations at pH 6.0 with three reaction buffers and at pH 8.5 with four reaction buffers having different ionization enthalpies at 30 °C. The ΔH_r obtained was plotted as a function of the ionization enthalpy of the buffer (Figure 4) and fitted to eq 7 (*53*).

$$\Delta H_r = \Delta H_{ind} + n\mathrm{H}^+ \cdot \Delta H_{ion} \quad (7)$$

In this equation, ΔH_{ind} is the buffer independent enthalpy change and $n\mathrm{H}^+$ is the number of protons taken up or released by the enzyme upon ligand binding (*53*). The slope of the linear regression indicates that at 0.9 protons are transferred from the enzyme−ligand complex to the buffer ($n\mathrm{H}^+ = -0.90 \pm 0.03$) at pH 6.0 and that 0.2 protons are taken up by the enzyme−ligand complex from the buffer during formation of the ChiB−allosamidin complex ($n\mathrm{H}^+ = 0.20 \pm 0.05$) at pH 8.5. The intercepts give the buffer-independent enthalpy change to be 4.2 ± 0.2 kcal/mol at pH 6.0 and -1.7 ± 0.4 kcal/mol at pH 8.5.

DISCUSSION

Carbohydrate Binding to Glycoside Hydrolases and Lectins. Carbohydrate structures have an indisputable importance in biology. Their complexity ensures a high specificity in binding glycoside hydrolases or carbohydrate-binding proteins, lectins. Focus on the thermodynamics of carbohydrate binding to such enzymes has proved vital to better understanding the binding mechanisms. Parametrization of the entropy term has previously been described for the binding

of four mannose oligosaccharides to the lectin concanavalin A (conA) and a lectin from *Dioclea grandifora* (*54*). All binding reactions were enthalpically driven with entropic penalties mainly dominated by ΔS_{solv}. For example, methyl 3-*O*-(α-D-mannopyranosy1)-α-D-mannopyranoside binding to conA was found to have ΔH of −7.4 kcal/mol, ΔS_{solv} of 28.2 cal/K mol, and $\Delta S_{conf} = -25.0$ cal/K mol. In another study, the binding of a trimeric xylosaccharide (X_3) and hexameric xylosaccharide (X_6) to an inactive xylanase was also found to be enthalpically driven with entropic penalties as well at 298 K. (For X_3 $\Delta G = -7.0$ kcal/mol, $\Delta H = -10.5$ kcal/mol, $\Delta S = -11.6$ cal/K mol, and $\Delta C_p = -94$ cal/K mol; these data imply that $\Delta S_{solv} = 24$ cal/K mol and that $\Delta S_{conf} = -27.7$ cal/K mol, using the calculation method presented above; for X_6 binding $\Delta G = -8.8$ kcal/mol, $\Delta H = -15.0$ kcal/mol, $\Delta S = -20.4$ cal/K mol, and $\Delta C_p = -380$ cal/K mol; calculated, $\Delta S_{solv} = 97$ cal/K mol and $\Delta S_{conf} = -109.7$ cal/K mol for X_6 binding (*55*).) In this latter study, it is especially interesting to note that while ΔS_{solv} increases from 24 cal/K mol to 97 cal/K mol on going from a trimer to a hexamer, the ΔS_{conf} decreases from −27.7 cal/K mol to −109.7 cal/K mol. Both results are expected since longer saccharides will expulse more water than shorter ones and be more flexible, and, hence, lose more conformational freedom upon complexation. In another example, Garcia-Hernandez et al. showed that binding of chitotriose to lysozyme also is driven by enthalpy with an entropic penalty dominated by ΔS_{conf} ($\Delta G = -6.8$ kcal/mol, $\Delta H = -15.2$ kcal/mol, $\Delta S = -27$ cal/K mol, and $\Delta C_p = -120$ cal/K mol; calculated, $\Delta S_{solv} = 26{,}8$ cal/K mol and $\Delta S_{conf} = -45{,}8$ cal/K mol) (*56*).

Even though it appears that a vast majority of carbohydrate-binding events come with an entropic penalty, it has been suggested that isofagamine binding to almond β-glucosidase was entropically driven as determined by a van't Hoff analysis ($-\ln K_i$ vs $1/T$) (*57*). It was later confirmed through direct ITC measurements that even though the main contribution to the driving force was enthalpy change, indeed a positive entropy change was associated with binding ($\Delta G = -10.6$ kcal/mol, $\Delta H = -9.7$ kcal/mol, $\Delta S = 3$ cal/K mol, and $\Delta C_p = -50$ cal/K mol; calculated, $\Delta S_{solv} = 12{,}8$ cal/K mol and $\Delta S_{conf} = -1.8$ cal/K mol) (*58*). In this case, the ΔS_{conf} penalty is much less dominant than in the examples described above. The Davies group has followed up this work with a study of no less than 18 glycoside hydrolase inhibitors binding to the *Thermotoga maritima* family 1 β-glucosidase (*Tm*GH1) (*59*). Eleven of these inhibitors displayed a positive entropy change while all 18 had a negative enthalpy change (parametrization of the entropy change could not be done because ΔC_p values were not reported for this study). Thus, while binding in all cases was at least partly enthalpically driven, the contribution of the entropic term varied considerably. For the strongest binding inhibitor, the entropic contribution to ΔG was larger than the enthalpic contribution ($\Delta G = -10.9$ kcal/mol, $\Delta H = -4.6$ kcal/mol, and $\Delta S = 21$ cal/K mol). The present study provides an example of a case where the entalpic term is unfavorable, and entropic contributions are dominating in part of the pH spectrum that was tested.

Driving Force of Allosamidin Binding to ChiB. At pH 6.0, the binding of allosamidin to ChiB is strictly entropically driven (44 ± 1 cal/K mol) with an enthalpic penalty (3.8 ± 0.2 kcal/mol). This is in contrast to what is normally observed for interactions between carbohydrates and glycoside hydrolases and lectins (see above). The parametrization of the entropy change shows that ΔS_{conf} (37 cal/K mol) contributes more to the driving force of the binding process than ΔS_{solv} (15 cal/K mol). The positive ΔS_{conf} is rather surprising because ligand binding to glycoside hydrolases tends to lead to rigidification of flexible regions that interact with the ligand (*60*−*62*). ChiB contains several loops with relatively high *B*-factors near the substrate-binding groove, but these loops primarily interact with ligands binding in aglycon subsites (*27*, *43*). Allosamidin binds to the glycon subsites and interacts only with residues that have low *B*-factors in the structure of the ligand-free enzyme.

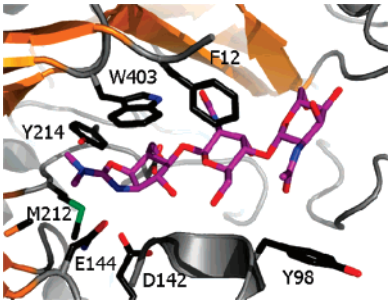

FIGURE 5: Crystal structure of the active site of ChiB with allosamidin bound (*27*) (PDB code 1e6r). E144 and D142 are both within hydrogen bond distance of the allosamizoline.

At higher pH values, the allosamidin−ChiB complex shows typical enthalpy−entropy compensation, that is, as the strength of the intermolecular forces increases, the degree of freedom for the complex decreases (*63*). As the weak interactions in the complex become stronger (i.e., lower ΔH_r), the entropic term becomes less favorable. The decrease in the entropic term, ΔS_r, with increasing pH is due to the loss of 45 cal/K mol in ΔS_{conf} in going from pH 6.0 to 8.5. The increase in ΔS_{solv} (29 cal/K mol) over the same pH range does not cover the ΔS_{conf} loss. All in all, the results show that entropic effects play a major role in driving allosamidin binding. ΔS_{solv} contributes at all pH values tested, whereas ΔS_{conf} contributes at pH values up to 7.5. At high pH, the reduction of entropic effects is compensated by a more favorable enthalpic term, and only at high pH do enthalpic effects contribute to the binding reaction at all.

Proton Transfer to and from the Allosamidin−ChiB Complex. The structure of allosamidin has been solved in complex with various family 18 chitinases (*21*, *23*, *25*, *27*) (Figure 5). These complexes show that binding is dominated by interactions between the allosamizoline group and highly conserved residues in the −1 subsite that are crucial for catalytic activity and substrate binding. The most prominent of these residues is Asp142 (ChiB numbering), which is the middle residue in the characteristic DXDXE sequence motif that includes the catalytic acid (Glu144 in ChiB). Asp142 interacts with the positive charge on allosamizoline while being hydrogen bonded to Glu144 (Figure 6). The explanation for the observed decrease in K_d with increasing pH is most likely that it is due to the titration of Asp142 and/or Glu144 and of allosamidin itself (*21*, *23*, *25*, *27*).

Calculations of electrostatic interactions and pK_a values in ChiB have shown that the Asp140−Asp142−Glu144 triad contains two protons at pH 6.1, regardless of ligand

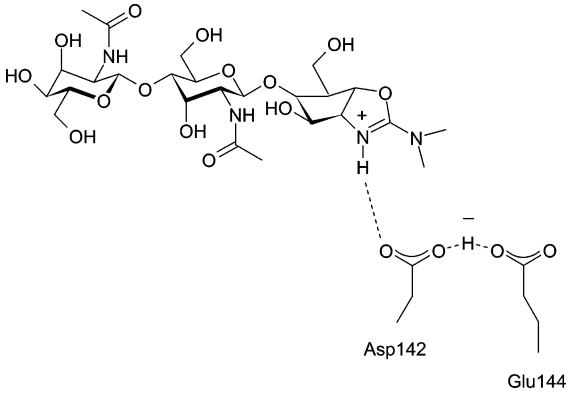

FIGURE 6: Schematic overview of the allosamidin interaction with Asp-142 and Glu-144 of ChiB (*27*, *43*). Upon complexation with allosamidin at lower pH, the Asp142−Glu144 diad will loose a proton, and the formed negative charge will participate in an electrostatic−electrostatic interaction with protonated allosamidin. (The negative charge is delocalized over carboxylic functional groups of the diad.) At higher pH, both the diad and allosamidin will become less protonated and the complex will take up a proton from the buffer to maintain the interaction. See text for details.

binding (*43*). Both calculations and structural analyses showed that these protons reside on the Asp142 and Glu144 diad in enzyme complexes and that there is a strong hydrogen bond between Asp 142 and Glu144. Optimal binding of a charged ligand such as allosamidin would require the deprotonation of the 142−144 diad (Figure 6) (*27*, *43*). Calculations for the ligand-free enzyme indicated that the lowest pK_a in the diad is that of Glu144 (pK_a = 7.1; (*43*)). The pK_a of allosamidin has not been measured yet, while 2-amino-oxazolines have been found to have pK_a values around 8.6 (*64*). Allosamidin displays similarities to these compounds. At pH 6.0, allosamidin will then most likely be protonated. The buffer dependency of the binding reaction at pH 6.0 shows that 0.9 protons are transferred from the allosamidin−ChiB complex to the buffer. This is most likely due to a deprotonation of the 142−144 diad, resulting in the formation a negative charge that can participate in an electrostatic−electrostatic interaction with the positively charged allosamidin (Figure 6). The fact that 0.2 protons are transferred to the enzyme−inhibitor complex from the buffer solution at pH 8.5 (where the 142−144 diad is likely to be largely deprotonated in the native enzyme) could be taken to imply that approximately 20% of the allosamidin molecules that bind to the enzyme need to pick up a proton from the solvent to maintain the electrostatic−electrostatic interaction. That is, 20% of the allosamidin molecules in solution are deprotonated, whereas the rest of the allosamidin units that complex with ChiB are protonated when in solution. This would suggest that the pK_a value of allosamidin is close to 9.0 rather than 8.5.

pH Dependency of Binding. Studies of pH dependency presented above show that at higher pH values, the entropic effects of complex formation become less favorable, whereas enthalpic effects become much more favorable, the net effect being a clear decrease in K_d. A similar dependency of binding on pH has been observed previously for K_i values estimated from simple IC_{50} measurements (0.16 μM, pH 6.0 and 0.04 μM, pH 8.0 vs 0.2 μM, pH 6.1 and 0.04 μM, pH 8.0) (*23*). Vaaje-Kolstad et al. also found that the pH dependency of K_i was strongly reduced upon mutating Asp142 in ChiB to Asn, showing that titration of the enzyme is an important component of the pH dependency (*23*). The proton-transfer studies show that one reason for ΔH_r becoming more favorable at increased pH is that the deprotonation penalty diminishes as a result of ionization of the 142−144 diad in the ligand-free enzyme. The pH dependency of ΔS_{conf} is not easy to explain in molecular terms but may, in general terms, be linked to the enthalpy−entropy compensation of allosamidin binding as discussed above. The increase in ΔS_{solv} with increasing pH may be explained on the basis of a developing negative charge due to the deprotonation of the Asp142−Glu144 diad. This negative charge will require increased solvation (*65*). Thus, at higher pH, the active site of ChiB will bind more water molecules than that at lower pH, and more water will be expulsed upon allosamidin binding, leading to an increase in the entropy gain. Dunitz has shown that the release of a highly ordered water molecule may generate a favorable entropy change up to 7 cal/K mol (*66*). Increased solvation of the empty active site at higher pH is also consistent with the observed decrease in ΔH_r with increased pH. Water expulsed from the active site upon allosamidin binding will contribute favorably to the enthalpy of binding by formation of stronger hydrogen bonds when returning to bulk solvent (*67*).

In this study, ITC has been used to measure the thermodynamics for the binding of allosamidin, the best known inhibitor of family 18 chitinases, to ChiB of *Serratia marcescens*. Analysis of the different energetic contributions showed that allosamidin binding to ChiB differs from what is normally observed for carbohydrate−protein binding reactions in that the reaction comes with an enthalpic penalty and a large and positive conformational entropy change at low pH. These results provide expanded insight into the repertoire of energetic contributions used in natural glycoside hydrolase−inhibitor interactions and add to the knowledge base needed for future inhibitor design.

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REFERENCES

1. Henrissat, B., and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases, *Curr. Opin. Struct. Biol. 7*, 637−644.
2. Vinetz, J. M., Valenzuela, J. G., Specht, C. A., Aravind, L., Langer, R. C., Ribeiro, J. M. C., and Kaslow, D. C. (2000) Chitinases of the Avian Malaria Parasite Plasmodium gallinaceum, a Class of Enzymes Necessary for Parasite Invasion of the Mosquito Midgut, *J. Biol. Chem. 275*, 10331−10341.
3. Herrera-Estrella, A., and Chet, I. (1999) Chitinases in biological control, *EXS 87*, 171−184.
4. Palli, S. R., and Retnakaran, A. (1999) Molecular and biochemical aspects of chitin synthesis inhibition, *EXS 87*, 85−98.
5. Shibata, Y., Foster, L. A., Bradfield, J. F., and Myrvik, Q. N. (2000) Oral Administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse, *J. Immunol. 164*, 1314−1321.
6. Renkema, G. H., Boot, R. G., Muijsers, A. O., Donker-Koopman, W. E., and Aerts, J. M. (1995) Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins, *J. Biol. Chem. 270*, 2198−2202.
7. Chou, Y. T., Yao, S., Czerwinski, R., Fleming, M., Krykbaev, R., Xuan, D., Zhou, H., Brooks, J., Fitz, L., Strand, J., Presman, E., Lin, L., Aulabaugh, A., and Huang, X. (2006) Kinetic Characterization of Recombinant Human Acidic Mammalian Chitinase, *Biochemistry 45*, 4444−4454.

8. Donnelly, L. E., and Barnes, P. J. (2004) Acidic mammalian chitinase - a potential target for asthma therapy, *Trends Pharmacol. Sci. 25*, 509−511.
9. Kawada, M., Hachiya, Y., Arihiro, A., and Mizoguchi, E. (2007) Role of mammalian chitinases in inflammatory conditions, *Keio J. Med. 56*, 21−27.
10. Zhu, Z., Zheng, T., Homer, R. J., Kim, Y. K., Chen, N. Y., Cohn, L., Hamid, Q., and Elias, J. A. (2004) Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation, *Science 304*, 1678−1682.
11. Saguez, J., Dubois, F., Vincent, C., Laberche, J. C., Sangwan-Norreel, B. S., and Giordanengo, P. (2006) Differential aphicidal effects of chitinase inhibitors on the polyphagous homopteran *Myzus persicae* (Sulzer), *Pest. Manag. Sci 62*, 1150−1154.
12. Banat, B. M. A., Kameyama, Y., Yoshioka, T., and Koga, D. (1999) Purification and characterization of a 54 kDa chitinase from *Bombyx mori*, *Insect. Biochem. Mol. Biol. 29*, 537−547.
13. Blattner, R., Gerard, P. J., and SpindlerBarth, M. (1997) Synthesis and biological activity of allosamidin and allosamidin analogues, *Pestic. Sci. 50*, 312−318.
14. Izumida, H., Nishijima, M., Takadera, T., Nomoto, A. M., and Sano, H. (1996) The effect of chitinase inhibitors, cyclo(Arg-Pro) against cell separation of *Saccharomyces cerevisiae* and the morphological change of *Candida albicans*, *J. Antibiot. (Tokyo*) *49*, 829−831.
15. Takaya, N., Yamazaki, D., Horiuchi, H., Ohta, A., and Takagi, M. (1998) Cloning and characterization of a chitinase-encoding gene (chiA) from *Aspergillus nidulans*, disruption of which decreases germination frequency and hyphal growth, *Biosci. Biotechnol. Biochem. 62*, 60−65.
16. Vinetz, J. M., Dave, S. K., Specht, C. A., Brameld, K. A., Xu, B., Hayward, R., and Fidock, D. A. (1999) The chitinase PfCHT1 from the human malaria parasite *Plasmodium falciparum* lacks proenzyme and chitin-binding domains and displays unique substrate preferences, *Proc. Natl. Acad. Sci. U.S.A. 96*, 14061−14066.
17. Filho, B. P. D., Lemos, F. J. A., Secundino, N. F. C., Pascoa, V., Pereira, S. T., and Pimenta, P. F. P. (2002) Presence of chitinase and beta-N-acetylglucosaminidase in the *Aedes aegypti*. A chitinolytic system involving peritrophic matrix formation and degradation, *Insect. Biochem. Mol. Biol. 32*, 1723−1729.
18. Sakuda, S., Isogai, A., Matsumoto, S., and Suzuki, A. (1987) Search for microbial insect growth regulators. II. Allosamidin, a novel insect chitinase inhibitor, *J. Antibiot. (Tokyo*) *40*, 296−300.
19. Sakuda, S., Isogai, A., Matsumoto, S., Suzuki, A., and Koseki, K. (1986) The structure of allosamidin, a novel insect chitinase inhibitor, produced by *Streptomyces* Sp, *Tetrahedron Lett. 27*, 2475−2478.
20. Horn, S. J., Sørlie, M., Vaaje-Kolstad, G., Norberg, A. L., Synstad, B., Vårum, K. M., and Eijsink, V. G. H. (2006) Comparative studies of chitinases A, B and C from *Serratia marcescens*, *Biocatal. Biotransform. 24*, 39−53.
21. Bortone, K., Monzingo, A. F., Ernst, S., and Robertus, J. D. (2002) The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism, *J. Mol. Biol. 320*, 293−302.
22. Koga, D., Isogai, A., Sakuda, S., Matsumoto, S., Suzuki, A., Kimura, S., and Ide, A. (1987) Specific-inhibition of Bombyx-Mori chitinase by allosamidin, *Agric. Biol. Chem. 51*, 471−476.
23. Vaaje-Kolstad, G., Houston, D. R., Rao, F. V., Peter, M. G., Synstad, B., van Aalten, D. M. F., and Eijsink, V. G. H. (2004) Structure of the D142N mutant of the family 18 chitinase ChiB from *Serratia marcescens* and its complex with allosamidin, *Biochim. Biophys. Acta 1696*, 103−111.
24. Rao, F. V., Houston, D. R., Boot, R. G., Aerts, J. M., Sakuda, S., and Van Aalten, D. M. (2003) Crystal structures of allosamidin derivatives in complex with human macrophage chitinase, *J. Biol. Chem. 278*, 20110−20116.
25. Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B., and Dijkstra, B. W. (1995) Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis, *Biochemistry 34*, 15619−15623.
26. Tews, I., vanScheltinga, A. C. T., Perrakis, A., Wilson, K. S., and Dijkstra, B. W. (1997) Substrate-assisted catalysis unifies two families of chitinolytic enzymes, *J. Am. Chem. Soc. 119*, 7954−7959.
27. van Aalten, D. M. F., Komander, D., Synstad, B., Gåseidnes, S., Peter, M. G., and Eijsink, V. G. H. (2001) Structural insights into the catalytic mechanism of a family 18 exo-chitinase, *Proc. Natl. Acad. Sci. U.S.A. 98*, 8979−8984.
28. Karasuda, S., Yamamoto, K., Kono, M., Sakuda, S., and Koga, D. (2004) Kinetic analysis of a chitinase from red sea bream, *Pagrus major*, *Biosci. Biotechnol. Biochem. 68*, 1338−1344.
29. Baldwin, R. L. (1986) Temperature dependence of the hydrophobic interaction in protein folding, *Proc. Natl. Acad. Sci. U.S.A. 83*, 8069−8072.
30. Livingstone, J. R., Spolar, R. S., and Record, M. T., Jr. (1991) Contribution to the thermodynamics of protein folding from the reduction in water-accessible nonpolar surface area, *Biochemistry 30*, 4237−4244.
31. Murphy, K. P., Privalov, P. L., and Gill, S. J. (1990) Common features of protein unfolding and dissolution of hydrophobic compounds, *Science 247*, 559−561.
32. Sturtevant, J. M. (1977) Heat capacity and entropy changes in processes involving proteins, *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236−2240.
33. Tomme, P., Creagh, A. L., Kilburn, D. G., and Haynes, C. A. (1996) Interaction of polysaccharides with the N-terminal cellulose-binding domain of *Cellulomonas fimi* CenC. 1. Binding specificity and calorimetric analysis, *Biochemistry 35*, 13885−13894.
34. Gomez, J., Hilser, V. J., Xie, D., and Freire, E. (1995) The heat capacity of proteins, *Proteins 22*, 404−412.
35. Gomez, J., and Freire, E. (1995) Thermodynamic mapping of the inhibitor site of the aspartic protease endothiapepsin, *J. Mol. Biol. 252*, 337−350.
36. Murphy, K. P. (1994) Hydration and convergence temperatures: on the use and interpretation of correlation plots, *Biophys. Chem. 51*, 311−326.
37. Baker, B. M., and Murphy, K. P. (1997) Dissecting the energetics of a protein-protein interaction: the binding of ovomucoid third domain to elastase, *J. Mol. Biol. 268*, 557−569.
38. Murphy, K. P., Xie, D., Thompson, K. S., Amzel, L. M., and Freire, E. (1994) Entropy in biological binding processes: estimation of translational entropy loss, *Proteins: Struct., Funct., Genet. 18*, 63−67.
39. Gurney, R. W. (193) *Ionic Processes in Solution*, McGraw-Hill, New York.
40. Parker, M. H., Lunney, E. A., Ortwine, D. F., Pavlovsky, A. G., Humblet, C., and Brouillette, C. G. (1999) Analysis of the binding of hydroxamic acid and carboxylic acid inhibitors to the stromelysin-1 (matrix metalloproteinase-3) catalytic domain by isothermal titration calorimetry, *Biochemistry 38*, 13592−13601.
41. Pey, A. L., Thorolfsson, M., Teigen, K., Ugarte, M., and Martinez, A. (2004) Thermodynamic characterization of the binding of tetrahydropterins to phenylalanine hydroxylase, *J. Am. Chem. Soc. 126*, 13670−13678.
42. Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B., and Watanabe, T. (1999) The third chitinase gene (chiC) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases, *Biochem. J. 343*, 587−596.
43. Synstad, B., Gåseidnes, S., van Aalten, D. M. F., Vriend, G., Nielsen, J. E., and Eijsink, V. G. H. (2004) Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase, *Eur. J. Biochem. 271*, 253−262.
44. van Aalten, D. M. F., Synstad, B., Brurberg, M. B., Hough, E., Riise, B. W., Eijsink, V. G. H., and Wierenga, R. K. (2000) Structure of a two-domain chitotriosidase from *Serratia marcescens* at 1.9-angstrom resolution, *Proc. Natl. Acad. Sci. U.S.A. 97*, 5842−5847.
45. Cederkvist, F. H., Zamfir, A. D., Bahrke, S., Eijsink, V. G., Sørlie, M., Peter-Katalinic, J., and Peter, M. G. (2006) Identification of a high-affinity-binding oligosaccharide by (+) nanoelectrospray quadrupole time-of-flight tandem mass spectrometry of a noncovalent enzyme-ligand complex, *Angew. Chem., Int. Ed. 45*, 2429−2434.
46. Horn, S. J., Sørbotten, A., Synstad, B., Sikorski, P., Sørlie, M., Varum, K. M., and Eijsink, V. G. H. (2006) Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*, *FEBS J. 273*, 491−503.
47. Horn, S. J., Sikorski, P., Cederkvist, J. B., Vaaje-Kolstad, G., Sørlie, M., Synstad, B., Vriend, G., Varum, K. M., and Eijsink, V. G. H. (2006) Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides, *Proc. Natl. Acad. Sci U.S.A. 103*, 18089−18094.

48. Krokeide, I.-M., Synstad, B., Gåseidnes, S., Horn, S. J., Eijsink, V. G. H., and Sørlie, M. (2007) Natural substrate assay for chitinases using high-performance liquid chromatography: A comparison with existing assays, *Anal. Biochem. 363*, 128−134.
49. Brurberg, M. B., Nes, I. F., and Eijsink, V. G. H. (1996) Comparative studies of chitinases A and B from *Serratia marcescens*, *Microbiology 142*, 1581−1589.
50. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter, *Anal. Biochem. 179*, 131−137.
51. Fukada, H., and Takahashi, K. (1998) Enthalpy and heat capacity changes for the proton dissociation of various buffer components in 0.1 M potassium chloride, *Proteins 33*, 159−166.
52. Todd, M. J., and Gomez, J. (2001) Enzyme kinetics determined using calorimetry: a general assay for enzyme activity? *Anal. Biochem 296*, 179−187.
53. Baker, B. M., and Murphy, K. P. (1996) Evaluation of linked protonation effects in protein binding reactions using isothermal titration calorimetry, *Biophys. J. 71*, 2049−2055.
54. Chervenak, M. C., and Toone, E. J. (1995) Calorimetric analysis of the binding of lectins with overlapping carbohydrate-binding ligand specificities, *Biochemistry 34*, 5685−5695.
55. Zolotnitsky, G., Cogan, U., Adir, N., Solomon, V., Shoham, G., and Shoham, Y. (2004) Mapping glycoside hydrolase substrate subsites by isothermal titration calorimetry, *Proc. Natl. Acad. Sci. U.S.A. 101*, 11275−11280.
56. Garcia-Hernandez, E., Zubillaga, R. A., Chavelas-Adame, E. A., Vazquez-Contreras, E., Rojo-Dominguez, A., and Costas, M. (2003) Structural energetics of protein-carbohydrate interactions: Insights derived from the study of lysozyme binding to its natural saccharide inhibitors, *Protein Sci. 12*, 135−142.
57. Bülow, A., Plesner, I. W., and Bols, M. (2000) A large difference in the thermodynamics of binding of isofagomine and 1-deoxynojirimycin to beta-glucosidase, *J. Am. Chem. Soc. 122*, 8567−8568.
58. Zechel, D. L., Boraston, A. B., Gloster, T., Boraston, C. M., Macdonald, J. M., Tilbrook, D. M. G., Stick, R. V., and Davies, G. J. (2003) Iminosugar glycosidase inhibitors: structural and thermodynamic dissection of the binding of isofagomine and 1-beta-glucosidases, *J. Am. Chem. Soc. 125*, 14313−14323.
59. Gloster, T. M., Meloncelli, P., Stick, R. V., Zechel, D., Vasella, A., and Davies, G. J. (2007) Glycosidase inhibition: an assessment of the binding of 18 putative transition-state mimics, *J. Am. Chem. Soc. 129*, 2345−2354.
60. Davies, G. J., Tolley, S. P., Henrissat, B., Hjort, C., and Schulein, M. (1995) Structures of oligosaccharide-bound forms of the endoglucanase V from *Humicola insolens* at 1.9 Å resolution, *Biochemistry 34*, 16210−16220.
61. Varrot, A., Schulein, M., and Davies, G. J. (2000) Insights into ligand-induced conformational change in Cel5A from *Bacillus agaradhaerens* revealed by a catalytically active crystal form, *J. Mol. Biol. 297*, 819−828.
62. Zou, J., Kleywegt, G. J., Stahlberg, J., Driguez, H., Nerinckx, W., Claeyssens, M., Koivula, A., Teeri, T. T., and Jones, T. A. (1999) Crystallographic evidence for substrate ring distortion and protein conformational changes during catalysis in cellobiohydrolase Ce16A from *Trichoderma reesei*, *Structure. 7*, 1035−1045.
63. Cooper, A., Johnson, C. M., Lakey, J. H., and Nollmann, M. (2001) Heat does not come in different colours: entropy-enthalpy compensation, free energy windows, quantum confinement, pressure perturbation calorimetry, solvation and the multiple causes of heat capacity effects in biomolecular interactions, *Biophys. Chem. 93*, 215−230.
64. Matoga, M., Laborde-Kummer, E., Langlois, M. H., Dallet, P., Bosc, J. J., Jarry, C., and Dubost, J. P. (2003) Determination of pKa values of 2-amino-2-oxazolines by capillary electrophoresis, *J. Chromatogr., A 984*, 253−260.
65. Yee, E. L., Cave, R. J., Guyer, K. L., Tyma, P. D., and Weaver, M. J. (1979) Survey of ligand effects upon the reaction entropies of some transition-metal redox couples, *J. Am. Chem. Soc. 101*, 1131−1137.
66. Dunitz, J. D. (1994) The entropic cost of bound water in crystals and biomolecules, *Science 264*, 670.
67. Clarke, C., Woods, R. J., Gluska, J., Cooper, A., Nutley, M. A., and Boons, G. J. (2001) Involvement of water in carbohydrate-protein binding, *J. Am. Chem. Soc. 123*, 12238−12247.

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