The Roles of Three *Serratia marcescens* Chitinases in Chitin Conversion Are Reflected in Different Thermodynamic Signatures of Allosamidin Binding

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Binding of allosamidin to the three family 18 chitinases of *Serratia marcescens* has been studied using isothermal titration calorimetry (ITC). Interestingly, the thermodynamic signatures of allosamidin binding were different for all three chitinases. At pH 6.0, chitinase A (ChiA) binds allosamidin with a K_d value of 0.17 ± 0.06 μM where the main part of the driving force is due to enthalpic change ($\Delta H_r^\circ = -6.2 \pm 0.2$ kcal/mol) and less to entropic change ($-T\Delta S_r^\circ = -3.2$ kcal/mol). A large part of ΔH is due to allosamidin stacking with Trp^{167} in the -3 subsite. Binding of allosamidin to both chitinase B (ChiB) ($K_d = 0.16 \pm 0.04$ μM) and chitinase C (ChiC) ($K_d = 2.0 \pm 0.2$ μM) is driven by entropy ($\Delta H_r^\circ = 3.8 \pm 0.2$ kcal/mol and $-T\Delta S_r^\circ = -13.2$ kcal/mol for ChiB and $\Delta H_r^\circ = -0.6 \pm 0.1$ and $-T\Delta S_r^\circ = -7.3$ kcal/mol for ChiC). For ChiC, the entropic term is dominated by changes in solvation entropy ($\Delta S_{conf} = 1$ cal/K·mol and $\Delta S_{solv} = 31$ cal/K·mol), while, for ChiB, changes in conformational entropy dominate ($\Delta S_{conf} = 37$ cal/K·mol and $\Delta S_{solv} = 15$ cal/K·mol). Corresponding values for ChiA are $\Delta S_{conf} = 4$ cal/K·mol and $\Delta S_{solv} = 15$ cal/K·mol. These remarkable differences in binding parameters reflect the different architectures of the catalytic centers in these enzymes that are adapted to different types of actions: ChiA and ChiB are processive enzymes that move in opposite directions, meaning that allosamidin binds in to "product" subsites in ChiB, while it binds to polymer-binding subsites in ChiA. The values for ChiC are compatible with this enzyme being a nonprocessive endochitinase with a much more open and solvated substrate-binding-site cleft.

1. Introduction

Chitin, a β-1,4-linked polymer of *N*-acetylglucosamine (GlcNAc), is an abundant biopolymer in nature. It is the most important nonplant structural biopolymer, occurring in, e.g., the exoskeletons of invertebrates, fungal cell walls, and the digestive tracts of insects. Chitin is easily derived from waste products such as shrimp shells. Chitin does not accumulate in nature because the polymer is effectively degraded by different chitinases belonging to the glycoside hydrolase enzyme families 18 and 19.[1] As an example, genome-wide analysis of chitinase genes in the *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) genome database revealed the presence of 18 open reading frames (ORFs) encoding putative family 18.[2] Interestingly, chitinases have also been identified in mammalian species.[3] Chitinases play a pivotal role in the development of organisms that need to degrade and reshape chitinous structures during their life cycles. 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 plant protecting compounds[4,5] and medicines for allergic and inflammatory disorders.[6,7]

Several types of inhibitors for family 18 chitinases have been reported.[8–10] The best known of these is allosamidin, a pseudotrisaccharide produced by *Streptomyces* sp. that binds to the -3 to -1 glycon subsites, using nomenclature for sugar-binding subsites in glycosyl hydrolases, of family 18 chitinases (Figure 1).[11,12] The allosamizoline group of allosamidin resembles the oxazolinium ion intermediate structure that is formed in the -1 subsite during (substrate-assisted) catalysis in family 18 chitinases.[13–16] Although allosamidin binding depends on interactions with the by far most conserved part of family 18 chitinases, namely, the catalytically crucial -1 subsite, these enzymes show considerable differences in their binding constants for the inhibitor.[17] Such differences are likely to be due to differences in the architecture of the other binding subsites (-2 and -3) which may be adapted to the varying roles that different family 18 chitinases play in chitin degradation.

The Gram-negative soil bacterium *Serratia marcescens* has an efficient chitinolytic machinery consisting of three family 18 chitinases, A (ChiA), B (ChiB), and C (ChiC) (Figure 1).[18] ChiA and ChiB are processive enzymes that digest the chitin polymer in opposite directions, mainly producing $(GlcNAc)_2$, while ChiC is a nonprocessive endochitinase that hydrolyzes the polymer randomly, yielding longer chitooligosaccharides.[19,20] The crystal structures of ChiA[21,22] and ChiB[16,23] show that these processive enzymes have deep substrate-binding clefts. Both structural data and analyses of enzymatic reactions[20,24–26] suggest that ChiA degrades chitin chains from their reducing ends, meaning that the $+1$ and $+2$ subsites bind the disaccharide product, whereas the polymer is attached to the glycon subsites. Similar experimental data show a reciprocal situation for ChiB, meaning that the dimeric product binds in the -2 and -1 subsites.[16,20,23,24] The crystal structure of ChiC is not known, but sequence alignments show that the catalytic domain of this enzyme resembles that of hevamine (about 22% sequence identity).[13,27–29] Most importantly, these two enzymes lack the so-called α/β-domain that makes up one of the walls of the

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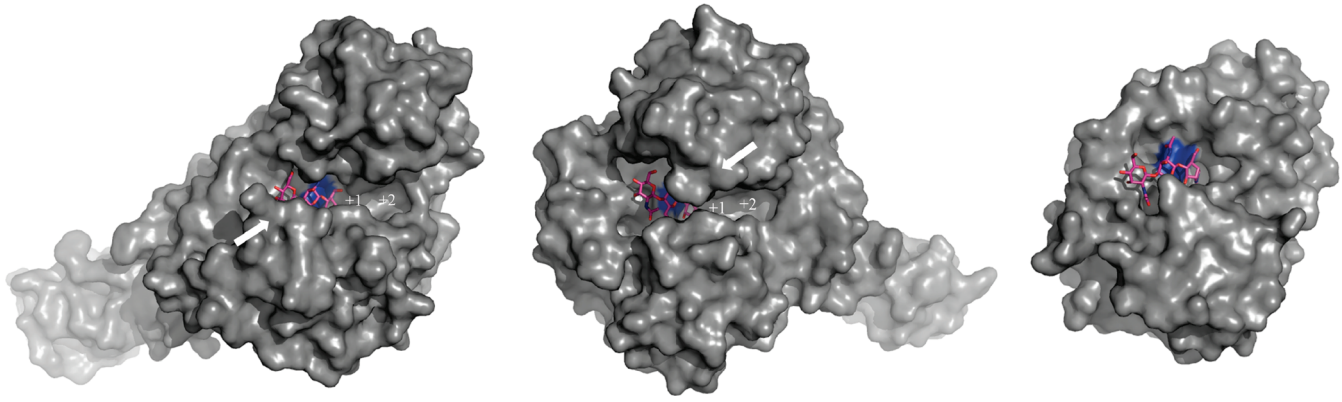

Figure 1. Crystal structures of allosamidin bound to the active sites of ChiA (top)[21] (pdb code 1ffq), ChiB (middle)[16] (pdb code 1e6r), and hevamine, which resembles ChiC (bottom)[13] (pdb code 1llo). A fully conserved tryptophan residue stacking with the allosamizoline moiety in the −1 subsite is highlighted in blue (ChiA, Trp^{539}; ChiB, Trp^{403}; hevamine, Trp^{253}). Before making the pictures, the three chitinases were structurally aligned and the three panels show identical views. Allosamidin binds in the glycon subsites (−3, −1) for all chitinases. "+1" and "+2" indicate the positions of aglycon subsites as derived from the structures of enzyme−substrate complexes (hevamine does not have similar aglycon subsites as ChiA and ChiB).[16,26] The white arrows indicate where extended loops in ChiA and ChiB make up "walls" in the substrate-binding clefts. These loops are predominantly in glycon (−) subsites for ChiA and aglycon (+) subsites for ChiB (see the Discussion for further details). Furthermore, it is clearly seen that ChiA and ChiB have more closed active sites than hevamine/ChiC.

deep substrate-binding clefts for ChiA and ChiB. Consequently, in contrast to ChiA and ChiB, the endoacting nonprocessive ChiC has a shallow substrate-binding groove (Figure 1).

Previously, we have investigated the thermodynamics of allosamidin binding to ChiB using isothermal titration calorimetry (ITC).[30] Now, we have studied the thermodynamics of allosamdin binding to the other two *S. marcescens* chitinases, ChiA and ChiC. The results reveal large differences between the three enzymes. This shows that enzymes with the same catalytic mechanism and substrate are designed to act very differently on this substrate and have subtle differences in their active site architectures that include differences in solvation characteristics and conformational dynamics.

2. Materials and Methods

Isolation of ChiB and Allosamidin. Wild-type ChiA and ChiB from *Serratia marcescens* were overexpressed in *Escherichia coli* and purified as described elsewhere.[31] ChiC was overexpressed and purified as described in Synstad et al.[28] Allosamidin was isolated from *Streptomyces sp*., and the purity was controlled by ^{1}H NMR as described elsewhere.[32] Previously, the structure of allosamidin has been verified by both NMR and crystallography.[10]

Isothermal Titration Calorimetry Experiments. ITC experiments were performed with a VP-ITC system from Microcal, Inc. (Northampton, MA).[33] Solutions were thoroughly degassed prior to experiments to avoid air bubbles in the calorimeter. Standard ITC conditions were 250 μM allosamidin in the syringe and 20 μM ChiA or ChiC in the reaction cell in 20 mM potassium phosphate buffer of pH 6.0, 7.5, or 8.5. The concentration of other buffers, PIPES and imidazole at pH 6.0, was also 20 mM. The heats of ionization of these buffers are as follows: potassium phosphate, 1.22 kcal/mol;[34] PIPES, 2.74 kcal/mol;[34] imidazole, 8.75 kcal/mol.[34] For a typical titration of ChiA or ChiC with allosamidin, the chitinase solution was placed in the reaction cell with a volume of 1.4214 mL, and aliquots of 8 μL of allosamidin solution 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.[33] All data were corrected for heat of dilution by subtracting the heat remaining after 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 reaction 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 reaction free energy change (ΔG_r°), the reaction entropy change (ΔS_r°), and the equilibrium binding dissociation constant (K_d) were calculated from the relation described in eq 1.

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

Errors are reported as standard deviations of at least three experiments at each temperature and buffer. Parameterization of the entropic term has been described in detail previously.[30,35]

3. Results

Binding of Allosamidin to ChiA, ChiB, and ChiC. The binding of allosamidin to ChiA, ChiB, and ChiC in 20 mM phosphate buffer at different temperatures (20−37 °C) and pH (6.0, 7.5, and 8.5) was studied by ITC. Figure 2 shows typical ITC thermograms and theoretical fits to the experimental data for allosamidin binding to the respective chitinases at pH 6.0.

At t = 30 °C and pH 6.0, allosamidin binds equally strong to ChiA and ChiB with a K_d value of 0.17 ± 0.06 and 0.16 ± 0.04 μM, respectively (Table 1). However, the two enzymes show striking differences when partitioning the binding energies into enthalpic and entropic terms. The binding of allosamidin to ChiA is mainly enthalpically driven ($\Delta H_r^\circ = -6.2 \pm 0.2$ kcal/mol and $-T\Delta S_r^\circ = -3.2$ kcal/mol), while for ChiB binding is completely entropically driven with an enthalpic penalty ($\Delta H_r^\circ = 3.8$ kcal/mol and $-T\Delta S_r^\circ = -13.2$ kcal/mol). Allosamidin binds about 10-fold less strongly to ChiC with a K_d value of 2.0 ± 0.2 μM. The entropy change is the main contributor to the binding strength ($\Delta H_r^\circ = -0.6$ kcal/mol and $-T\Delta S_r^\circ = -7.3$ kcal/mol). It is important to point out that, even though the quantity of the enthalpy change for binding of allosamidin

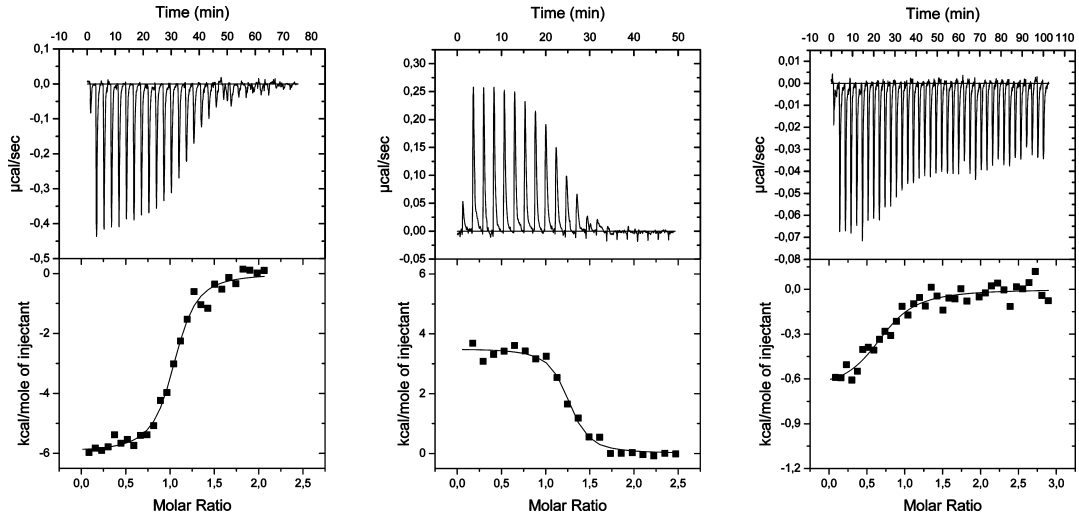

Figure 2. Thermograms (upper panel) and binding isotherms with theoretical fits (lower panel) obtained for the binding of allosamidin to ChiA (left), ChiB (middle, from Cederkvist et al.[30]), and ChiC (right) at pH 6.0 and $t = 30$ °C in 20 mM potassium phosphate.

TABLE 1: Thermodynamic Parameters Obtained for the Binding of Allosamidin to Family 18 Chitinases at $t = 30$ °C, in 20 mM Potassium Phosphate

pH	K_d[a]	ΔG_r°[b]	ΔH_r°[b]	ΔS_r°[c]	$-T\Delta S_r^{\circ}$[b]	$\Delta C_{p,r}^{\circ}$[c,d]
			ChiA			
6.0	0.17 ± 0.06	-9.4 ± 0.2	-6.2 ± 0.2	11 ± 1	-3.2	-61 ± 13
7.5	0.10 ± 0.01	-9.7 ± 0.1	-7.0 ± 0.1	9 ± 1	-2.7	-92 ± 13
8.5	0.12 ± 0.01	-9.6 ± 0.1	-7.8 ± 0.2	6 ± 1	-1.8	-125 ± 13
			ChiB[e]			
6.0	0.16 ± 0.04	-9.4 ± 0.1	3.8 ± 0.2	44 ± 1	-13.2	-63 ± 4
7.5	0.04	-10.3	0.3 ± 0.1	35	-10.6	-131 ± 8
8.5	0.033 ± 0.004	-10.4 ± 0.1	-1.2 ± 0.2	30 ± 1	-9.2	-190 ± 13
			ChiC			
6.0[f]	2.0 ± 0.2	-7.9 ± 0.1	-0.6 ± 0.1	24 ± 1	-7.3	-120 ± 15

[a] μM. [b] kcal/mol. [c] cal/K·mol. [d] These data are derived from the temperature dependence of ΔH_r° and not only at $t = 30$ °C. [e] From Cederkvist et al.[30] [f] Low signal-to-noise ratios in the thermograms precluded measurements for ChiC at pH 7.5 and 8.5.

to ChiC at this temperature and in this buffer is relatively low, the determination of both temperature and buffer dependency on this parameter (see below) supports this value. Both enthalpically and entropically, the binding energies for ChiC lie in between the "extremes" represented by ChiA and ChiB.

The K_d value for allosamidin binding to ChiA did not show pH dependency (Table 1). On the other hand, there is a clear enthalpy−entropy compensation when increasing the pH, with a decreasing entropic contribution and a more favorable enthalpic term. A similar enthalpy−entropy compensation was observed for ChiB (Table 1[30]). In the case of ChiB, K_d decreases 5-fold upon raising the pH from 6.0 to 8.5. The pH dependency of the enthalpy change is stronger for ChiB (−2.0 kcal/mol per pH unit) than for ChiA (−0.6 kcal/mol per pH unit). Poor signal-to-noise ratios in the thermograms at pH 7.5 and 8.5 precluded the determination of the pH dependency for allosamidin binding to ChiC.

It has recently been shown that Trp^{167} in the −3 subsite is important for the interaction between ChiA and the substrate (Figure 3).[25] There is no corresponding aromatic residue in either ChiB or ChiC. Allosamidin was titrated against the ChiA−W167A mutant in an ITC experiment to assess the effect Trp^{167} has on binding, and a K_d value of 2.4 ± 0.2 μM was observed, 20-fold higher than the binding constant for the wild-type enzyme at pH 8.5. The reduced binding affinity is caused by a reduction of ΔH_r° from -7.8 ± 0.2 to -3.4 ± 0.2 kcal/mol that is not fully compensated by a more favorable entropic term ($-T\Delta S_r^{\circ} = -4.4$ kcal/mol versus −1.8 kcal/mol for the wild type). The crystal structure of ChiA−WT−allosamidin (1ffq)[21] was compared to that of ChiA−W167A−allosamidin (1x6n)[36] to check that the change of a bulky tryptophan to an alanine does not cause a movement in a number of other residues in the active site upon allosamidin binding. The only noticeable structural consequence of the W167A mutation is the removal of the interaction between Trp^{167} and allosamidin. Otherwise, the structures of the ChiA−allosamidin complexes look the same. These results show that Trp^{167} makes a major contribution to allosamidin binding.

Effect of Buffer Ionization on Binding Enthalpy. 2-Aminooxazolines have pK_a values around 8.6.[37] The charged allosamizoline group of allosamidin interacts with the Asp−Glu diad in the −1 subsite of the family 18 chitinases (Figure 3). For ChiB, the lowest pK_a in this diad was calculated to be 7.1,[38] meaning that the diad is less than 10% charged at pH 6.0. Thus, at pH 6.0, the enzyme−allosamidin complex would need to lose almost one proton to permit formation of the strong, electrostatic−electrostatic interaction between the allosamizoline proton and a negative charge in the 142−144 diad. Deprotonation upon allosamidin binding to the three chitinases was determined through the buffer ionization enthalpy change dependency.[39] ITC experiments were carried out at identical buffer concentrations at pH 6.0 with three reaction buffers

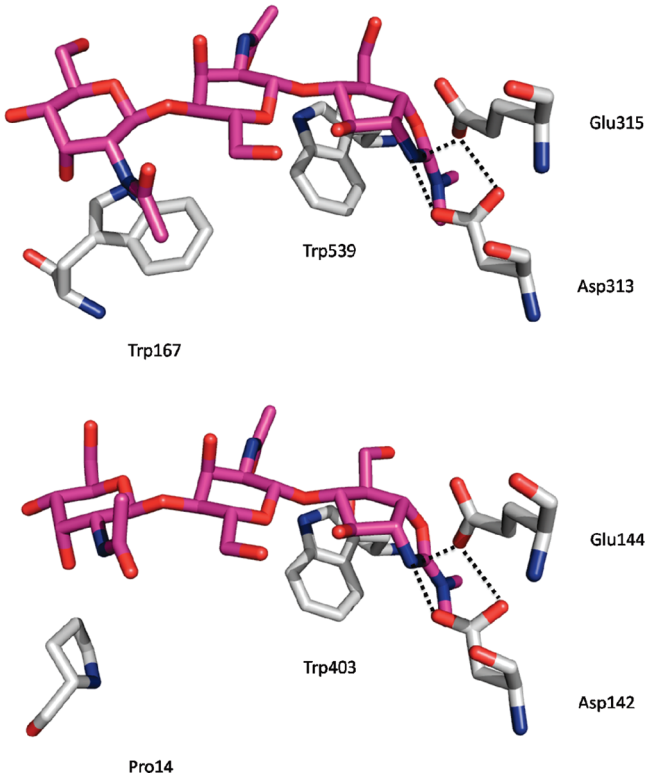

Figure 3. Crystal structures of allosamidin bound to the active sites of ChiA (top)[21] (pdb code 1ffq) and ChiB (bottom)[16] (pdb code 1e6r). Shown are the catalytic acids (ChiA−Glu^{315} and ChiB−Glu^{144}) and the aspartic acids (ChiA−Asp^{313} and ChiB−Asp^{142}) that interact with both the catalytic acid as well as the allosamizoline group. These residues are a part of the conserved DxxDxDxE motif of family 18 chitinases. Moreover, Trp$^{539/403}$ makes the −1 subsite. A significant difference between ChiA and ChiB is the presence of a tryptophan residue in the −3 subsite of ChiA (Trp^{167}) compared to a proline residue for ChiB (Pro^{14}).

having different ionization enthalpies at 30 °C. The ΔH_r value obtained was plotted as a function of the ionization enthalpy of the buffer (Figure 4) and fitted to eq 2.[39]

$$\Delta H_r^\circ = \Delta H_{ind} + n\mathrm{H}^+ \cdot \Delta H_{ion} \quad (2)$$

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−ligand complex upon binding.[39] The slopes indicate that, during binding at pH 6.0, 0.52 protons are transferred from the ChiA−allosamidin complex ($n\mathrm{H}^+ = -0.68 \pm 0.13$ with $\Delta H_{ind} = -5.8$ kcal/mol), 0.90 protons from the ChiB−allosamidin complex ($n\mathrm{H}^+ = -0.90 \pm 0.03$ with $\Delta H_{ind} = 4.2 \pm 0.2$ kcal/mol),[30] and 0.31 protons from the ChiC−allosamidin complex ($n\mathrm{H}^+ = -0.31 \pm 0.04$ with $\Delta H_{ind} = 0.2 \pm 0.1$ kcal/mol).

Parameterization of the Entropic Term. Determination of the temperature dependence of ΔH_r° yields the change in the reaction heat capacity ($\Delta C_{p,r}^\circ$). The changes in reaction heat capacities for both ChiA and ChiB are pH dependent ($\Delta C_{p,r}^\circ = -61 \pm 13$ kcal/mol at pH 6.0 and -125 ± 13 kcal/mol at pH 8.5 for ChiA; $\Delta C_{p,r}^\circ = -63 \pm 4$ kcal/mol at pH 6.0 and -190 ± 13 kcal/mol at pH 8.5 for ChiB). The binding of allosamidin to ChiC at pH 6.0 involves a change in reaction heat capacity of -125 ± 13 cal/K·mol, which is the largest $\Delta C_{p,r}^\circ$ among the three chitinases at pH 6.0.

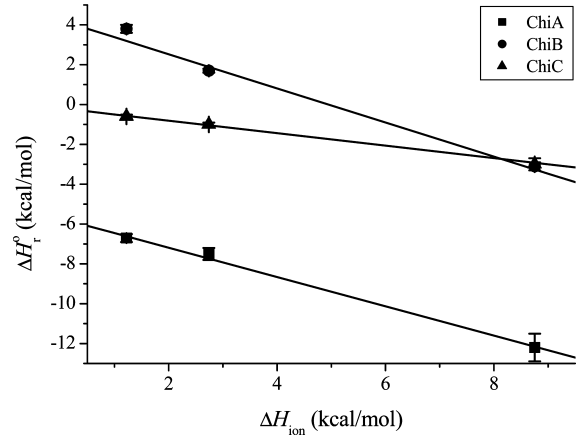

Figure 4. Plots of the enthalpy changes of the binding reaction vs the ionization enthalpy change of different buffers at pH 6.0 for ChiA (■), ChiB (●), and ChiC (▲). Experiments were performed at 30 °C in 20 mM buffer solutions. The ΔH_{ion} values used were as follows: potassium phosphate, 1.22 kcal/mol;[34] PIPES, 2.74 kcal/mol;[34] imidazole, 8.75 kcal/mol. For ChiA, $n\mathrm{H}^+ = -0.68 \pm 0.13$ and $\Delta H_{ind} = -5.8$ kcal/mol, for ChiB, $n\mathrm{H}^+ = -0.90 \pm 0.03$ and $\Delta H_{ind} = 4.2 \pm 0.2$ kcal/mol, and for ChiC, $n\mathrm{H}^+ = -0.31 \pm 0.04$ and $\Delta H_{ind} = 0.2 \pm 0.1$ kcal/mol.

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

pH	$\Delta S_r^{\circ a}$	$\Delta S_{mix}^{a,b}$	$\Delta S_{solv}^{a,c}$	$\Delta S_{conf}^{a,d}$
		ChiA		
6.0	11	−8	15	4
7.5	9	−8	24	−7
8.5	6	−8	32	−18
		ChiB[e]		
6.0	44	−8	15	37
7.5	35	−8	34	9
8.5	30	−8	46	−8
		ChiC		
6.0	24	−8	31	1

[a] cal/K·mol. [b] $\Delta S_{mix} = R \ln(1/55.5) = -8$ cal/K·mol ("cratic" term). [c] $\Delta S_{solv,calc} = \Delta C_p \ln(T_{298\ K}/T_{385\ K})$. [d] Derived using $\Delta S_r^\circ = \Delta S_{solv} + \Delta S_{mix} + \Delta S_{conf}$. [e] Data from Cederkvist et al.[30]

Furthermore, $\Delta C_{p,r}^\circ$ is proportional to ΔS_{solv}[40–42] when ΔS_r° is parametrized into three terms (eq 3).[43]

$$\Delta S_r^\circ = \Delta S_{solv} + \Delta S_{mix} + \Delta S_{conf} \quad (3)$$

Since binding of allosamidin to the chitinases is associated with partial proton transfers (section above), it is important to assess the temperature dependence of ΔH_{ind}. At pH 6.0 (where there is proton transfer), this is the same within experimental errors to when potassium phosphate is used as buffer. The results, summarized in Table 2, show that for ChiA ΔS_{solv} increases from 15 cal/K·mol at pH 6.0 to 32 cal/K·mol at pH 8.5. A similar trend was observed for ChiB albeit with a more positive entropic change at each tested pH (ΔS_{solv} of 15 and 46 cal/K·mol at pH 6.0 and 8.5, respectively). The binding of allosamidin to ChiC at pH 6.0 involves a change in solvation entropy of 31 cal/K·mol.

Since 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}), which may be calculated from ΔS_r° and ΔS_{solv} using eq 2. For allosamidin binding to ChiA, ΔS_{conf} equals 4 cal/K·mol at pH 6.0 and

decreases to a value of −18 cal/K·mol at pH 8.5 (Table 2). The conformational entropy change for ChiB at pH 6.0 is more positive than that for ChiA with a value of 37 cal/K·mol with a greater pH dependency than for ChiA (−8 cal/K·mol at pH 8.5). When allosamidin binds to ChiC at pH 6.0, ΔS_{conf} is 1 cal/K·mol (Table 2), which is the lowest value among the three chitinases.

4. Discussion

Despite the fact that the reaction intermediate analogue allosamidin interacts with several catalytically crucial residues in the −1 subsite that are fully conserved among family 18 chitinases, the thermodynamic signatures of allosamidin binding to the three family 18 chitinases compared in this study differ considerably. It is interesting to evaluate these differences in the context of the roles that these three enzymes have in chitin conversion. In particular, the opposite directionalities of ChiA and ChiB are intriguing.

At pH 6.0, the reaction free energy changes of allosamidin binding to ChiA and ChiB are of equal magnitude ($\Delta G_r = -9.4$ kcal/mol). In both enzymes, allosamidin binds in the −3 to −1 subsites. While allosamidin binding to ChiB provides a rare example of entropically driven ligand binding (see Cederkvist et al., 2007, for an extensive discussion), binding to ChiA is driven by favorable enthalpic changes. Thus, binding of allosamidin to "substrate-binding sites" in ChiA (i.e., where the chitin chain would be binding during hydrolysis) has a fundamentally different thermodymanic signature than binding of allosamidin to what are "product-binding sites" in ChiB (i.e., where chitobiose is released from the enzyme during hydrolysis). ChiA contains a prominent tryptophan residue in its −3 subsite (Trp^{167}), whereas a similar residue is absent in ChiB. Recent studies have shown that this residue is crucial for binding of the chitin chain during processive degradation.[25] We show here that this residue also makes a major contribution to the beneficial enthalpic effect of allosamidin binding to ChiA (representing an enthalpic gain of 4.4 kcal/mol), explaining the difference between ChiA and ChiB.

Another potential enthalpic contribution to allosamidin binding concerns the electrostatic interaction between the allosamizoline group and the Asp-Glu diad in the −1 subsite (Figure 3). Our data show that at pH 6.0 this diad is about 68 and 90% protonated before ligand binding in ChiA and ChiB, respectively. Thus, binding of allosamidin (which is protonated at pH 6.0) will yield an enthalpic deprotonation penalty. This explains why the binding enthalpy becomes more favorable as the pH increases, in both ChiA and ChiB. For ChiA, this electrostatic−electrostatic interaction comes in addition to the strong stacking interaction with Trp^{167}, which is independent of pH, since there are no titratable groups involved. Assuming that these two interactions are dominating determinants of the enthalpy of binding, one might expect that ΔH_r° for ChiB not only is less favorable than for ChiA but also is more pH-dependent, which is indeed observed (−0.6 kcal/mol per pH unit for ChiA versus −2.0 kcal/mol per pH unit for ChiB).

For both enzymes, enthalpy−entropy compensation is observed (Table 1): as the strength of the intermolecular forces increases (at higher pH), the degree of freedom for the complexes decreases.[44] Table 2 shows that the less favorable entropy of binding at higher pH is solely due to a less favorable ΔS_{conf}; ΔS_{solv} actually becomes more favorable at higher pH (discussed below). ChiB combines a stronger pH dependency of ΔH_r° with a stronger pH dependency of ΔS_{conf}. While it is not possible to rationalize why binding of allosamidin at pH 6 would lead to a positive ΔS_{conf} (see Cederkvist et al.[30] for a more elaborate discussion), it is interesting to speculate about why ΔS_{conf} is less positive for ChiA than for ChiB. In both enzymes, extended loops make up "walls" of the substrate-binding cleft to the extent that they almost create a tunnel (Figure 1). ChiB has loops affecting the aglycon (+) subsite that are not present in ChiA. Likewise, ChiA has a protruding loop that affects the glycon (−) subsites and that is absent in ChiB (marked with white arrows), resulting in more "open" aglycon subsites in ChiA than in ChiB. Since allosamidin binds in the glycon subsites, it is conceivable that its binding to ChiA reduces protein flexibility to a larger extent than in ChiB, which may explain why ΔS_{conf} is less positive for ChiA. Both in ChiA and ChiB, ΔS_{solv} contributes to the binding. The increase in ΔS_{solv} at increasing pH may be explained by increased deprotonation of the Asp−Glu diad. The resulting negative charge will require increased solvation, implying increased desolvation upon ligand binding.[45]

The thermodynamics of allosamidin binding to ChiC at pH 6.0 are completely dominated by the entropic term. In contrast to ChiA and ChiB, the entropic term for the endochitinase ChiC is dominated by a large and positive solvation entropy change of 31 cal/K·mol (versus 15 cal/K·mol, for ChiA and ChiB, respectively). The substrate-binding cleft of ChiC is shallow and open, and thus highly exposed to the solvent (Figure 1). It is thus reasonable to assume that the substrate-binding cleft of ChiC is more solvated than the more closed substrate-binding clefts of ChiA and ChiB, meaning that more water molecules are expulsed upon ligand binding. It is interesting to note that the ΔS_{solv} value for ChiC at pH 6.0 is similar to the ΔS_{solv} values for ChiA and ChiB at higher pH (Table 2). While this may be coincidental, it may also have to do with the fact that ChiC has a lower and more narrow pH optimum for activity (at a pH of about 3.9)[28] than ChiA and ChiB, which have remarkably broad pH optima around a pH of approximately 6.0.[18,31] Thus, one may expect that the Asp−Glu diad (residues 142 and 144 in ChiB) has another protonation and solvation state in ChiC than it has in ChiA and ChiB. In line with this, we found that allosamidin binding to ChiC at pH 6.0 involved the loss of only 0.31 protons (Figure 4), which is lower than the 0.68 protons lost by ChiA and the 0.90 protons lost by ChiB. It should be noted though that the difference in proton transfer between ChiA and ChiB is larger than expected on the basis of their similar pH-activity profiles. This adds uncertainty to the interpretation of the proton transfer results in the light of the pH optima for activity.

5. Conclusion

The present study reveals clear differences in the binding properties of the −3 to −1 subsites in three family 18 chitinases with different roles in the conversion of chitin. It is in itself remarkable that the same structural scaffold of the family 18 chitinases is used to create rather different enzymes that are processive (ChiA, ChiB) or not (ChiC) processive and that slide along the substrate in opposite directions (ChiA versus ChiB). The differences in the thermodynamic signatures for allosamidin binding show that there are subtle differences between the substrate-binding sites of these enzymes that are compatible with their functional differences.

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