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Title: Thermodynamic analysis of allosamidin binding to the Human chitotriosidase

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Keywords: Isothermal titration calorimetry; human chitinase, inhibition, enzyme mechanism

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Abstract: Human chitotriosidase (HCHT) is one of two active family 18 chitinases produced by humans, the other being acidic mammalian chitinase (AMCase). The enzyme is thought to be part of the innate human defense mechanism against fungal parasites. Recently it has been shown that levels of HCHT bioactivity and protein are significantly increased in the circulation and lungs of systemic sclerosis patients and for this reason is a suggested therapeutic target. For this reason, we have undertaken a detailed thermodynamic investigation using isothermal titration calorimetry of the binding interaction of HCHT with the well-known family 18 chitinase inhibitor allosamidin. The binding is shown to be strong (K_d = 0.20 ± 0.03 μM and $\Delta G_r°$ = -38.9 ± 0.4 kJ/mol) and driven by favorable changes in enthalpy ($\Delta H_r°$ = -50.2 ± 1.2 kJ/mol) and solvation entropy ($-T\Delta S_{solv}°$ = -41.8 ± 4.4 kJ/mol). It is accompanied with a large penalty in conformational entropy change ($-T\Delta S_{conf}°$ = 43.1 ± 4.2 kJ/mol).

Dear editor,

Please find enclosed our manuscript "Thermodynamic analysis of allosamidin binding to the human chitotriosidase", which we would like to be considered for publication in *Thermochimica Acta*. The manuscript details the inhibitor binding energetics to the human chitotriosidase, a part of the innate immune systeme. Such information is important for inhibitor design for such important enzymes, and to better understand its enzyme mechanism.

On behalf of all authors, I declare that neither this nor any manuscripts describing the contents of this paper in any related form have been submitted for publication in another journal or published as conference proceedings.

We hope that you will find our manuscript acceptable for publication and we are looking forward to your reply.

Yours sincerely,

Morten Sørlie

Dear Editor,

We are grateful for the thorough refereeing of our manuscript. We have revised the manuscript according to the very helpful suggestions made by Reviewer #2, as outlined below. We hope that the reviewers and the editor find these changes satisfactory and that our manuscript now is acceptable for publication in Thermochimica Acta.

Yours sincerely,

Morten Sørlie

Reviewers' comments:

Reviewer #1: Differences in thermodynamic signatures of allosamidin binding, a strong GH18 enzyme inhibitor, to GH18 enzymes are quite interesting.
The manuscript is generally well written and clearly presented.
I believe that this article is acceptable for publication in this Journal.

Reviewer #2: Reviewer #2 TCA-D-13-00122 Thermodynamic analysis of allosamidin binding to the human chitotriosidase. Eide, K.B., Lundmark, S.J., Sakuda, S., and Sørlie. M.

The manuscript highlights the thermodynamic parameters of the chitanase inhibitor, allosamidin, binding to human chitotriosidase. Thermodynamic analysis of allosamidin binding has been performed with a number of chitotriosidase enzymes, but this appears to be the first attempt at measuring these parameters with this particular enzyme. Results of this work are compared to parameters previously determined for other Family 18 chitinases and used as a basis for structure-based discussion. The work performed follows generally accepted protocols and the manuscript is well organized. There appears to be some calculation errors and data provided needs to be checked for accuracy. The apparent errors do not significantly change the data or the conclusions drawn from the data. For the most part, the manuscript is well written, although a few areas could use some clarity.
Highlights are fine. Additional comments are listed below.

* In Table 1 a few values appear to be miscalculated for HCHT-allosamidin. The deltaG value appears to be calculated incorrectly. If calculate deltaG using the relationship -RTlnKa or deltaH-TdeltaS, get a value of -37.6 kJ/mol using the data provided. There could also be an issue with -TdeltaSconf as the -TdeltaS value would be -14.2, not 12.6 kJ/mol using the data provided and equation. Check the values to ensure correct calculations and adjust as necessary. Fortunately these potential errors do not impact data greatly. Also order the equations sequentially; equation 3 is actually equation 4, if placed in order. What is listed as equation 6 should be equation 3.

Response: We greatly appreciate the detection of the calculation errors. ΔG is correctly calculated while it is $-T\Delta S_r°$ that is calculated incorrectly (and hence $-T\Delta S_{conf}°$). This has now been corrected. Moreover, the order of the Equations has been corrected.

* Materials and Methods, page 4, list temperatures used as 15, 17.5, 20, and 37 °C. No mention of using 30 °C and based on Figure 2, looks like used 20, 25, 30, and 37 °C. Confirm the actual temperatures used and correct in text as needed.

Response: It is right as the Reviewer states, temperatures of 20, 25, 30, and 37 °C have been used. This has now been corrected.

* Table 1 and Figure 3 present much of the same data. While Figure 3 is more visually appealing, no comments are made with respect to the crystal structures shown in Fig. 3. Pick either Table 1 or Figure 3; suggest Table 1 as it contains additional data.

Response: We have removed Figure 3 as suggested by the Reviewer, and changed the numbering of what was Figure 4 to Figure 3.

* Discussion section, page 9, line 3: "The favorable enthalpy change for…" sentence is wordy and difficult to follow. Need to clarify. In addition, discussion ends rather abruptly and would benefit from a concluding sentence or two.

Response: The sentence:

“The favorable enthalpy change for allosamidin binding HCHT is also interesting with respect to inhibition as a therapeutic target since evidence point to that inhibitors that are enthalpic optimized have been shown to be better drugs than their entropic optimized competitors [28].”

have been changed to:

“With respect to inhibition as a therapeutic target, evidence points to that it is beneficial to have binding driven by enthalpy changes compared to entropy changes [28], which is the case for allosamidin binding to HCHT.”

Moreover, we have added the following final sentence in the Discussion section:

“In summary, thermodynamic signatures for allosamidin binding to HCHT yield valuable information on mode of actions for this enzyme that are characteristic for family 18 chitinases such as endo-activity and directionality.”

Highlights:

- Large differences in thermodynamic signatures for family 18 chitinase inhibition
- Allosamidin binds tight to HCHT
- Binding driven by enthalpy change and desolvation

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Thermodynamic analysis of allosamidin binding to the Human chitotriosidase

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ABSTRACT

Human chitotriosidase (HCHT) is one of two active family 18 chitinases produced by humans, the other being acidic mammalian chitinase (AMCase). The enzyme is thought to be part of the innate human defense mechanism against fungal parasites. Recently it has been shown that levels of HCHT bioactivity and protein are significantly increased in the circulation and lungs of systemic sclerosis patients and for this reason is a suggested therapeutic target. For this reason, we have undertaken a detailed thermodynamic investigation using isothermal titration calorimetry of the binding interaction of HCHT with the well-known family 18 chitinase inhibitor allosamidin. The binding is shown to be strong (K_d = 0.20 ± 0.03 μM and $\Delta G_r°$ = −38.9 ± 0.4 kJ/mol) and driven by favorable changes in enthalpy ($\Delta H_r°$ = –50.2 ± 1.2 kJ/mol) and solvation entropy ($-T\Delta S_{solv}°$ = −41.8 ± 4.4 kJ/mol). It is accompanied with a large penalty in conformational entropy change ($-T\Delta S_{conf}°$ = 43.1 ± 4.2 kJ/mol).

Keywords: Isothermal titration calorimetry; human chitinase, inhibition, enzyme mechanism.

1. Introduction

Chitin is the second most abundant biopolymer in nature and common as a structural component in crustaceans, arthropods, fungi, and parasitic nematodes. It is an insoluble, linear polysaccharide consisting of repeated units of β-1,4-*N*-acetylgucosamine (GlcNAc). Chitin is degraded by enzymes called chitinases. They can be classified in two different glycoside hydrolase families, family 18 and 19, depending on structure and mechanism [1]. The human genome codes for eight family 18 chitinases [2], this despite mammals neither synthesizes chitin nor uses it as nurture. Two of the human chitinases, human chitotriosidase (HCHT, Fig. 1) and acidic mammalian chitinase (AMCase), have shown enzymatic activity and are believed to be part of the innate immune system [3; 4]. Both enzymes occur in two isoforms; 39 kDa and 50 kDa, where the 39 kDa isoform lack a C-terminal proposed chitin binding domain [5]. By post-translational modification or RNA-processing the 50 kDa isoform can be converted to the 39 kDa variant[5]. Both forms have shown chitinolytic activity.

Inhibition of AMCase has been observed to ameliorate asthma [6] while HCHT so far has not been a target for inhibition due to its fungistatic effect both *in vivo* and *in vitro* [4]. Even so, recent studies have shown that levels of HCHT bioactivity and protein are significantly increased in the circulation and lungs of systemic sclerosis patients. For this reason HCHT have been suggested as a therapeutic target [7]. It is therefore of interest to obtain information on the thermodynamics of HCHT inhibition, and to obtain a better understanding on the enzyme mechanism of HCHT. In this work, we have used isothermal titration calorimetry (ITC) to obtain the thermodynamic signature for the allosamidin binding, a well-known family 18 chitinase inhibitor, to HCHT (Fig. 1). These results are compared to

other chitinase-allosamidin binding equilibriums with respect to the different architectures of the catalytic centers and their roles in these enzymes.

2. Experimental

2.1. Proteins and Chemicals

HCHT (39 kDa) was overexpressed in *Pichia pastoris* and purified as described elsewhere [8]. Allosamidin was isolated from *Streptomyces sp*. and the purity was controlled by ^{1}H NMR as described elsewhere [9]. Previously, the structure of allosamidin has been verified by both NMR and crystallography [10].

2.2. Isothermal titration calorimetry experiments

ITC experiments were performed with a VP-ITC system from Microcal, Inc (Northampton, MA) [11]. Solutions were thoroughly degassed prior to experiments to avoid air bubbles in the calorimeter. Standard ITC conditions were 250 μM of allosamidin in the syringe and 15 μM of HCHT in the reaction cell in 20 mM potassium phosphate buffer of pH 6.0. Aliquots of 8 μL were injected into the reaction cell at 180 s intervals at temperatures of 20, 25, 30, and 37 °C with a stirring speed of 260 rpm. The titrations were normally complete after 22-27 injections. At least three independent titrations were performed for each binding reaction. The concentration of other buffers, PIPES and imidazole, was also 20 mM with a pH of 6.0. The heats of ionization of the buffers are as following: potassium phosphate; 1.22 kcal/mol, PIPES; 2.74 kcal/mol, and imidazole; 8.75 kcal/mol [12].

2.3 Analysis of calorimetric data

ITC data were collected automatically using the Microcal Origin v.7.0 software accompanying the VP-ITC system [11]. Prior to further analysis, data were corrected for heat of dilution by subtracting the heat remaining after saturation of binding sites on the enzyme. Data were fitted using a non-linear 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 reaction enthalpy change ($\Delta H_r°$) of the reaction. The value of n was found to be between 0.9 and 1.1 for all reactions. The equilibrium binding dissociation constant (K_d), reaction free energy change ($\Delta G_r°$) and the reaction entropy change ($\Delta S_r°$) were calculated from the relation described in Equation 1.

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

Errors are reported as standard deviations of at least three experiments at each temperature. A description of how the entropic term is parameterized has been described in detail previously [13; 14].

3. Results

3.1 Binding of allosamidin to HCHT.

The binding of allosamidin to HCHT (Fig. 1) at pH 6.0 (20 mM potassium phosphate buffer) at different temperatures (15-37 °C) was studied using ITC. Fig. 2 show a typical ITC thermogram and theoretical fit to the experimental data at t =30 °C. At this temperature,

HCHT binds allosamidin with a K_d of = 0.20 ± 0.03 μM (ΔG_r° = −38.9 ± 0.4 kJ/mol, Table 1). The reaction is accompanied by an enthalpic change (ΔH_r°) of –50.2 ± 1.2 kJ/mol and an entropic change (ΔS_r°) of –37 ± 4 J/K mol. The change in the heat of reaction, as determined by Equation 2, was determined to be −602 ± 63 J/K·mol.

$$\Delta C_{p,r}^{o} = \left(\frac{\partial \Delta H_r^{o}}{\partial T} \right) \quad (2)$$

3.2 Effect of buffer ionization.

By testing the contribution from buffer ionization to the observed ΔH_r°, potential protonation/deprotonation effects in binding between allosamidin and HCHT can be measured [15]. In addition to the binding reaction in the potassium phosphate buffer, ITC experiments were carried out in 20 mM of PIPES and imidazole, at pH 6 and 30 °C as well. The obtained ΔH_r° was plotted as a function of the ionization enthalpy of the buffer (Fig. 2) and fitted to the following Equation (3).

$$\Delta H_r^\circ = \Delta H_{ind}^\circ + nH^+ \bullet \Delta H_{ion} \quad (3)$$

In this equation, ΔH_{ind}° is the buffer independent enthalpy change and nH^+ is the number of protons taken up or released by the enzyme upon ligand binding [15]. The slope of the linear regression indicates that 0.79 protons are transferred from the enzyme-ligand complex to the buffer (nH^+ = 0.79 ± 0.07) at pH 6 (Fig. 2). The buffer-independent enthalpy change is by the intercept given to be −48.5 ± 1.4 kJ/mol.

3.3 Parameterization of the Entropic Term.

The entropic term, $\Delta S_r°$, can be viewed as the sum of translational, solvation, and conformational entropic changes [16] as seen in Equation 4.

$$\Delta S_r° = \Delta S°_{mix} + \Delta S°_{solv} + \Delta S°_{conf.} \quad (4)$$

By recognizing that the entropy of solvation is close to zero for proteins near $T = 385$ K, $\Delta C_{p,r}$ can be related to the solvation entropy change ($\Delta S°_{solv}$) of the binding reaction at $t = 30$ °C as described by Equation 5 [16; 17; 18].

$$\Delta S^{o}_{solv} = \Delta C^{o}_{p,r} \ln\left(\frac{303\,\mathrm{K}}{385\,\mathrm{K}}\right) \quad (5)$$

Using this relationship, a $\Delta S°_{solv}$ of 138 ± 15 J/K mol can be calculated representing −41.8 ± 4.4 kJ/mol ($-T\Delta S°_{solv}$) of the total free energy change of −38.9 kJ/mol for the binding reaction (Table 1). Since binding of allosamidin to the chitinases is associated with partial proton transfers (section above), it is important to assess the temperature dependence of $\Delta H_{ind}°$. At pH 6.0 (where there is proton transfer), this is the same within experimental errors to when potassium phosphate is used as buffer.

Furthermore, the translational entropy change ($\Delta S°_{mix}$) of the reaction can be calculated as a ‘cratic’ term, a statistical correction that reflects mixing of solute and solvent molecules and the changes in translational/rotational degrees of freedom (Equation 6) [16]:

$$\Delta S^{o}_{mix} = R \ln\left(\frac{1}{55.5}\right) \quad (6)$$

Using this approach, a $\Delta S°_{mix}$ of –33 J/K mol can be calculated corresponding to a $-T\Delta S°_{mix}$ of 10.0 kJ/mol. This then allows for the calculation of the conformational entropy change ($\Delta S°_{conf}$) to be 152 ± 14 J/K mol as described by Equation 3, corresponding to a $-T\Delta S°_{conf}$ of 43.1 ± 4.2 kJ/mol.

4. Discussion

The observed thermodynamic signature of allosamdin binding to HCHT can be compared to other allosamidin-chitinase systems that have been previously studied (Table 1) [13; 19]. ChiA and ChiB of *Serratia marcescens* are both processive enzymes (remaining attached to the substrate during hydrolysis) with initial binding at end of the polymer (exo-mode) at opposite directions; ChiA from the reducing end and ChiB from the non-reducing end [20; 21; 22]. Moreover, both enzymes have linear paths of surface exposed aromatic amino acids starting from the chitin-binding domains and extending into the catalytic domain. Towards the active site, ChiA has a Phe-Tyr-Trp-Trp-Trp-Phe motif in going from the –6 subsite to the +2 subsite in the active site [23] where Trp^{167} in subsite –3 is important for its processive action [22]. HCHT is similar to ChiA to this respect with a Trp-Tyr-Trp-Trp-Trp-Trp motif (Fig. 1) [24], and has also been shown to be processive [8]. ChiB is similar to HCHT in that is has the same last three aromatic amino acids (Trp-Trp-Trp) in going from the −1 subsite to the +2 subsite [25]. Here, subsites +1 (Trp^{97}) and +2 (Trp^{220}) are important for its processive mechanism [20].

All three chitinases bind allosamidin to subsites from −3 to −1 [24; 26; 27] at equal strengths ($\Delta G_r° \approx -39$ kJ/mol, Table 1), still there are large differences in their thermodynamic signatures. HCHT and ChiA are similar with enthalpy and solvation entropy changes making the most favorable contributions to the free energy change, albeit to a larger extent for HCHT than ChiA. The favorable enthalpy change for allosamidin to ChiA has previously been explained by its interaction with residues that are responsible to maintain the substrate bound during processive hydrolysis [19], i.e. Trp^{167}. In ChiB, this is the product release site, the +1 and +2 subsites remain bound to the substrate after processibe hydrolysis, and here the binding of allosamidin takes place with an unfavorable enthalpy change ($\Delta H_r° =$

18.5 kJ/mol). This suggests that HCHT may degrade chitin with the same directionality as ChiA, from the reducing end, and that subsites +1 (Trp^{99}) and +2 (Trp^{218}) takes part in product displacement as the similar Trp^{275} and Phe^{396} do for ChiA. With respect to inhibition as a therapeutic target, evidence points to that it is beneficial to have binding driven by enthalpy changes compared to entropy changes [28], which is the case for allosamidin binding to HCHT.

The most striking observation is the large difference in the conformational entropy change of 88.3 kJ/mol for allosamidin binding between the most favorable (ChiB with $-T\Delta S_{\mathrm{conf}}° = -45.2 \pm 2.0$ kJ/mol) to the least favorable (HCHT with $-T\Delta S_{\mathrm{conf}}° = 43.1 \pm 4.2$). Again, this suggests that the residues interacting with allosamidin, in HCHT and ChiB, respectively, have different roles in their substrate interactions.

Even though HCHT and ChiA both are processive, have similar aromatic motif in the active site, and likely degrade the substrate from the same direction, there is a notable difference in the solvation entropy changes where HCHT has twice the magnitude ($-T\Delta S_{\mathrm{solv}}°$ = −41.8 kJ/mol) as observed for ChiA ($-T\Delta S_{\mathrm{solv}}°$ = −20.4 kJ/mol). By inspection of the active sites of the crystal structures looking from negative subsites (Fig. 3), it is clear that both enzymes have substrate clefts where an α/β insertion domain (darker grey) makes up the "left wall" of the cleft. Still, there is a clear difference in that ChiA has two small α-helices (going from Gly^{196} to Glu^{222}, lighter gray) that forms a "right wall" of the cleft and two amino acids (Tyr^{418} and Thr^{419}, lighter gray) that forms a wall at the end of the active site that HCHT does not have. This makes the active site of HCHT more open compared to that of ChiA. In point of fact, the observed solvation entropy change of HCHT is equal to that of the nonprocessive endochitinase ChiC ($-T\Delta S_{\mathrm{solv}}°$ = −39.3 kJ/mol, [19]) of *Serratia marcescens* that do have a shallow and open active site [29]. This may hint at HCHT having more endo-character than ChiA. Both enzymes have previously been shown to have endo-activity towards the water

soluble chitosan [8; 30]. Endo-activity may promote increased substrate binding because the enzyme may not be solely dependent on finding chain ends.

In summary, thermodynamic signatures for allosamidin binding to HCHT yield valuable information on mode of actions for this enzyme that are characteristic for family 18 chitinases such as endo-activity and directionality.

Table 1

Thermodynamic parameters for binding of allosamidin binding to HCHT at t = 30 °C, as determined by isothermal titration calorimetry.

$\Delta G_r^{\circ a}$	$\Delta H_r^{\circ a}$	$-T\Delta S_r^{\circ a}$	$-T\Delta S_{solv}^{\circ a,b}$	$-T\Delta S_{conf}^{\circ a,c}$	$-T\Delta S_{mix}^{\circ a,d}$	$\Delta C_{p,r}^{\circ e,f}$
HCHT-allosamidin						
−38.9 ± 0.4	−50.2 ± 1.2	11.3 ± 1.2	−41.8 ± 4.4	43.1 ± 4.2	10.0	−602 ± 63
ChiA-allosamidin[g]						
−39.3 ± 0.9	−23.4 ± 0.9	−15.9 ± 1.7	−20.4 ± 3.1	−5.2 ± 3.5	10.0	−255 ± 52
ChiB-allosamidin[h]						
−38.0 ± 1.0	18.5 ± 0.9	−56.5 ± 1.7	−21.0 ± 1.1	−45.2 ± 2.0	10.0	−263 ± 16

[a] (kJ/mol); [b] $\Delta S_{solv}^{o} = \Delta C_p \ln(T_{293\,K}/T_{385\,K})$ [16; 17; 18]; [c] derived using $\Delta S_r^{\circ} = \Delta S_{solv}^{o} + \Delta S_{mix}^{o} + \Delta S_{conf}^{o}$; [d] $\Delta S_{mix}^{o} = R\ln(1/55.5) = -33$ J/K mol ("cratic" term) [24]; [e] (J/K mol); [f] these data are derived from the temperature dependence of ΔH_r°; [g] from Baban *et al*. [19]; [h] from Cederkvist *et al*. [13].

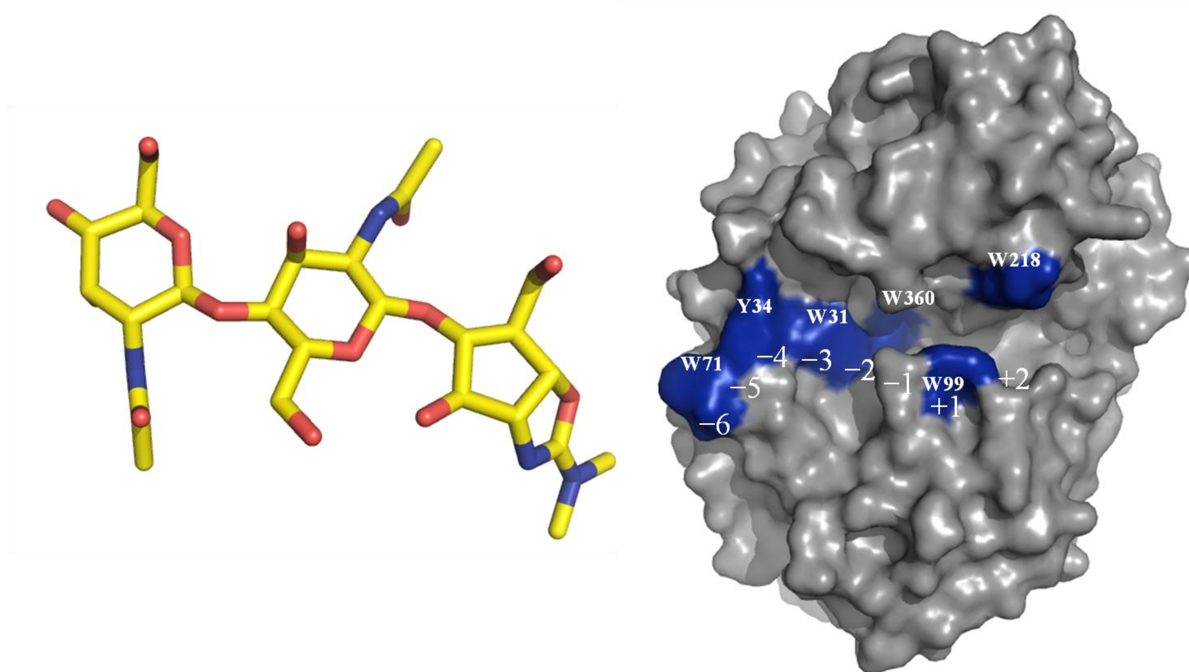

Fig. 1. Crystal structures of allosamidin (left) and HCHT (right) (pdb code 1hkk, [24]). In HCHT, the side chains of solvent-exposed aromatic amino acids corresponding to individual subsites are colored blue and labeled. Allosamidin binds in subsites –3 to –1.

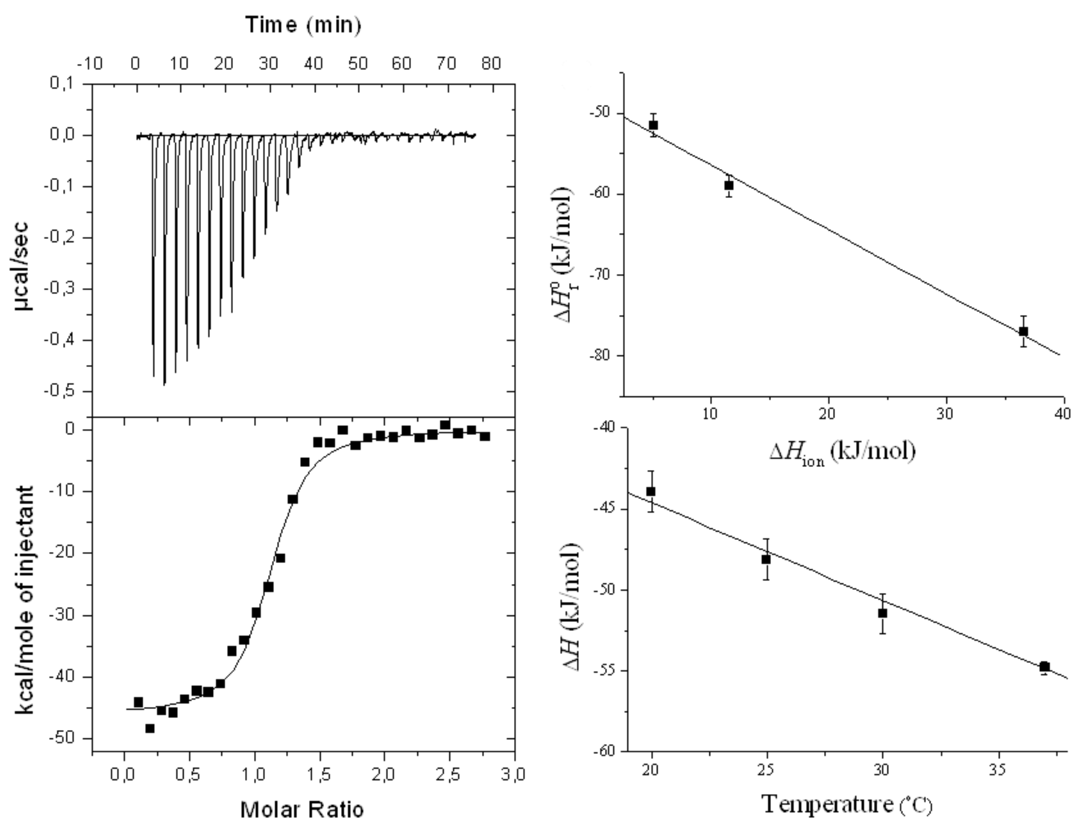

Fig. 2. Left panel, thermogram (top) and binding isotherm (bottom) for the titration of allosamidin (at t = 30 °C in 20 mM potassium phosphate buffer at pH 6.0). Right panel (top), the plot of the enthalpy changes of the binding reaction vs. the ionization enthalpy change of different buffers at pH 6.0. Experiments were performed at 30 °C in 20 mM buffered solutions of potassium phosphate, PIPES, and imidazole, yielding a transfer of 0.79 ± 0.07 protons upon formation of the HCHT-allosamidin complex. The buffer-independent enthalpy change $\Delta H_{ion}°$ of −48.5 ± 1.4 kJ/mol. Right panel (bottom), temperature dependence of allosamidin binding to HCHT at pH 6.0. The plot of $\Delta H_r°$ vs. temperature yields the change of heat capacity ($\Delta C_{p,r}$) as the slope. The value of $\Delta C_{p,r}$ is −602 ± 63 J/K mol.

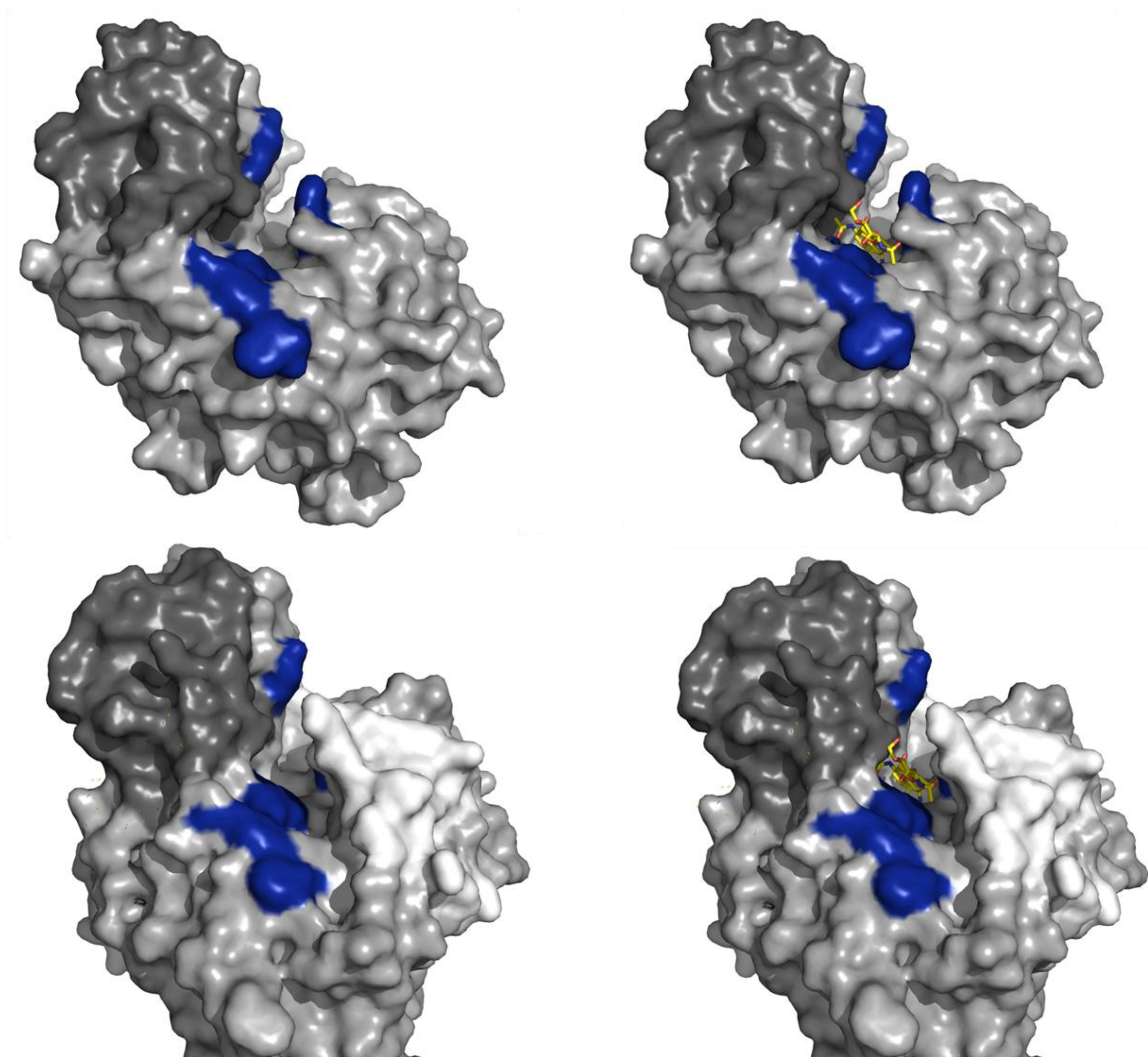

Fig. 3. Crystal structures of the active sites of HCHT (top) and ChiA (bottom) with and without allosamidin (the side chains of solvent-exposed aromatic amino acids going from subsite –6 to +2 are colored blue). From the structures, it is clear that the active site of HCHT is more open than that of ChiA. HCTH (pdb code 1hkk, [24]) and ChiA, (pdb code 1ctn, [26]).

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Table 1

Thermodynamic parameters for binding of allosamidin binding to HCHT at t = 30 °C, as determined by isothermal titration calorimetry.

$\Delta G_r^{\circ a}$	$\Delta H_r^{\circ a}$	$-T\Delta S_r^{\circ a}$	$-T\Delta S_{solv}^{\circ a,b}$	$-T\Delta S_{conf}^{\circ a,c}$	$-T\Delta S_{mix}^{\circ a,d}$	$\Delta C_{p,r}^{\circ e,f}$
			HCHT-allosamidin			
–38.9 ± 0.4	–50.2 ± 1.2	11.3 ± 1.2	–41.8 ± 4.4	43.1 ± 4.2	10.0	–602 ± 63
			ChiA-allosamidin[g]			
–39.3 ± 0.9	–23.4 ± 0.9	–15.9 ± 1.7	–20.4 ± 3.1	–5.2 ± 3.5	10.0	–255 ± 52
			ChiB-allosamidin[h]			
–38.0 ± 1.0	18.5 ± 0.9	–56.5 ± 1.7	–21.0 ± 1.1	–45.2 ± 2.0	10.0	–263 ± 16

[a] (kJ/mol); [b] $\Delta S_{solv}^{o} = \Delta C_p \ln(T_{293\ K}/T_{385\ K})$ [16; 17; 18]; [c] derived using $\Delta S_r^{\circ} = \Delta S_{solv}^{o} + \Delta S_{mix}^{o} + \Delta S_{conf}^{o}$; [d] $\Delta S_{mix}^{o} = R\ln(1/55.5) = -33$ J/K mol ("cratic" term) [24]; [e] (J/K mol); [f] these data are derived from the temperature dependence of ΔH_r°; [g] from Baban *et al*. [19]; [h] from Cederkvist *et al*. [13].

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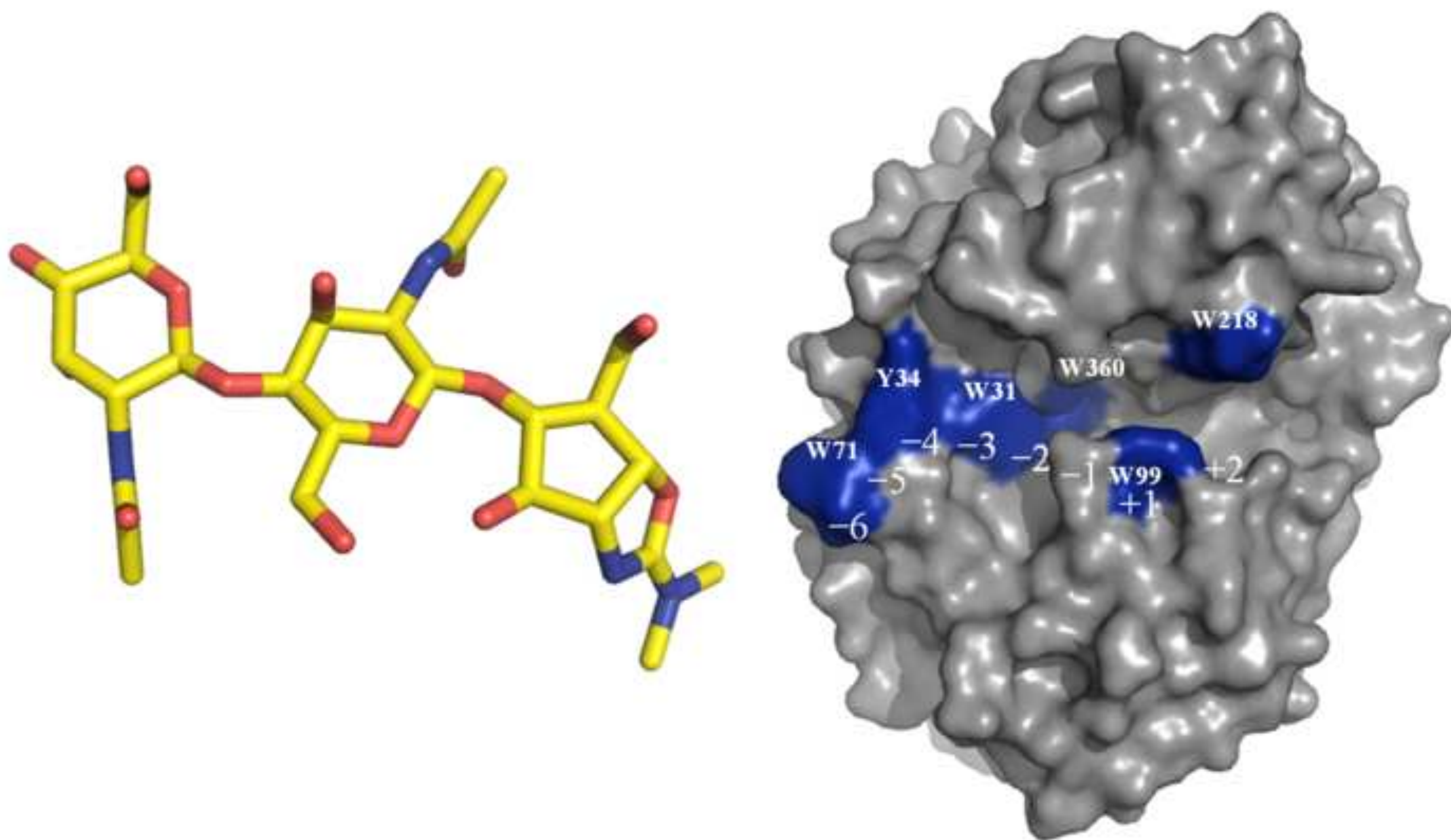

Fig 2 Thermogram.tif
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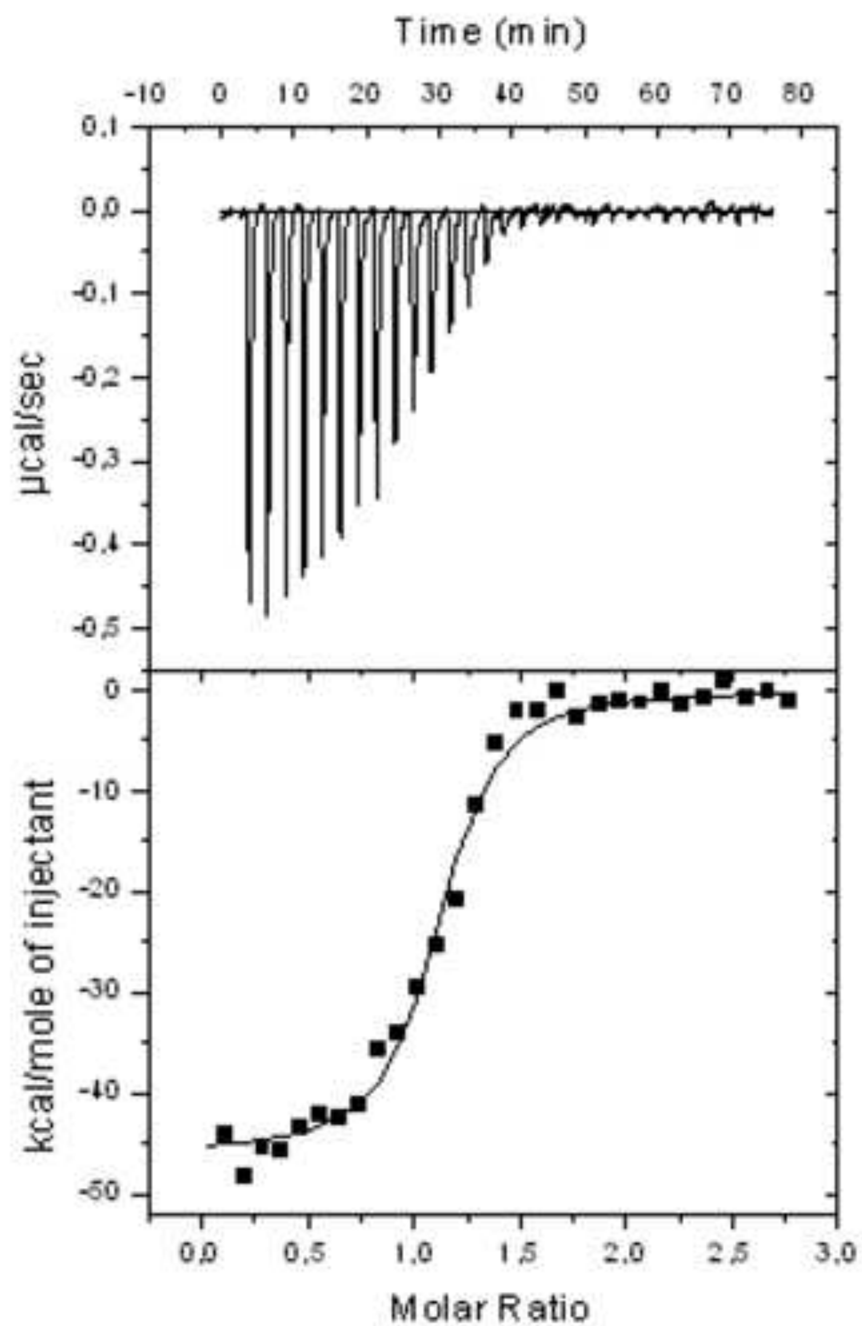

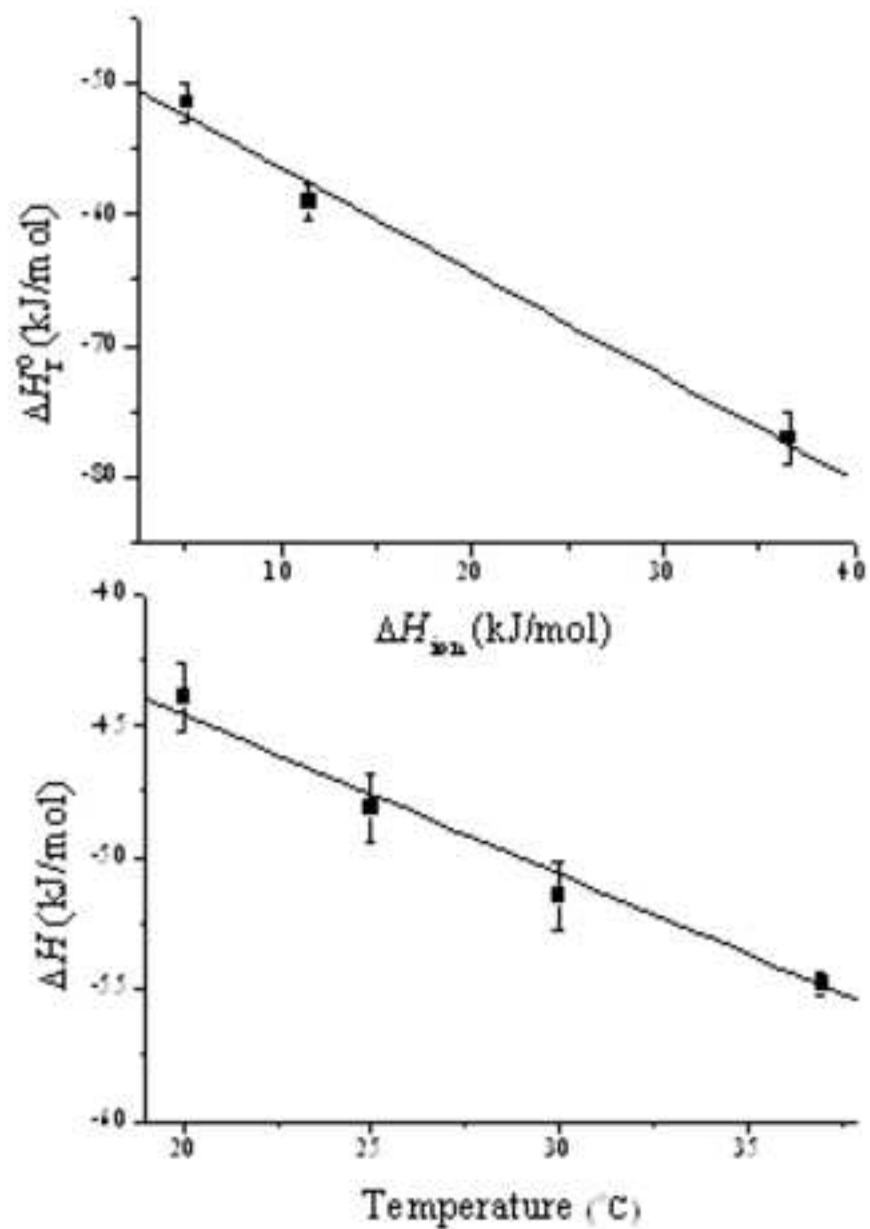

Fig 3 dSolv.tif

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