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The effect of carbohydrate binding modules and linkers on inhibitor binding to family 18 glycoside hydrolases

Kristine Bistrup Eide, Morten Sørlie *

Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway

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ABSTRACT

Enzyme catalyzed hydrolysis of glycosidic bonds is undertaken by glycoside hydrolases (GHs) in nature. In addition to a catalytic domain (CD), GHs often have carbohydrate-binding modules (CBMs) attached to the CD through a linker. Allosamidin binding to full-length GH18 *Serratia marcescens* ChiB and the catalytic domain only yield equal changes in reaction free energy (ΔG_r^o = −38 kJ/mol), enthalpy (ΔH_r^o = 18 kJ/mol), and entropy ($-T\Delta S_r^o$ = −57 kJ/mol). Interestingly, the change in heat capacity ($\Delta C_{p,r}$) was 3-fold smaller for full-length vs. the CD alone (−263 vs. −695 J/K mol). Allosamidin binding to the full-length isoform and the CD alone of the GH18 human chitotriosidase yielded different ΔG_r^o (−46.9 vs. −38.9 kJ/mol) due to differences in ΔH_r^o (−58.2 vs. −50.2 kJ/mol), while $-T\Delta S_r^o$ and (11.3 vs. 11.3 kJ/mol) and $\Delta C_{p,r}$ (−531 vs. −602 kJ/mol) are similar. The results combined show that the nature of the linker region and CBM affect the thermodynamic signatures of active site ligand binding.

1. Introduction

Enzymatic degradation of recalcitrant polysaccharides, such as cellulose and chitin, is of great biological and economical importance. In nature, enzymatic depolymerization of polysaccharides is accomplished by glycoside hydrolases (GHs). Chitin is degraded by enzymes called chitinases. These enzymes can be classified in two different GH families, family 18 and 19, depending on structure and mechanism [16]. Family 19 chitinases are mainly found in plants and actinomycetes, while family 18 chitinases occur in many different organisms, including bacteria and human. The chitinolytic machinery of the Gram negative soil bacterium *Serratia marcescens* is one of the best known enzyme systems for the conversion of insoluble polysaccharides, consisting of four chitin-active enzymes; chitinase A (*Sm*ChiA), chitinase B (*Sm*ChiB), chitinase C (*Sm*ChiC), and chitin binding protein (CBP21), a surface-active lytic polysaccharide monooxygenase (LPMO) [37]. The human genome codes for two active GH18 chitinases, acidic mammalian chitinase (AMCase) and human chitotriosidase (HCHT). HCHT, mainly found in circulation, is expressed and secreted in human macrophages, while AMCase is expressed in lung and stomach tissue [4,43,26]. Both enzymes are believed to be a part of the innate immune system [32,10].

Common for all GHs are a catalytic domain (CD) hydrolyzing the glycosidic bonds between different carbohydrate moieties. In addition, GHs often have a supplementary carbohydrate-binding module (CBM) with carbohydrate-binding activity attached to the CD through a linker region [5,13]. Removal of CBMs often result in severely impaired binding to polymeric substrate [38,36]. HCHT occurs in two isoforms; one with a catalytic domain only (abbreviated HCHT39) and one variant with a family 14 CBM, consisting of 49 amino acids, attached C-terminally through a 29-residue linker (abbreviated HCHT50) [26,21,5]. *Sm*ChiB occurs in a single isoform with a family 5 CBM attached through a C-terminal linker, about the same size (49 and 26 amino acids, respectively) as HCHT50 extending the positive subsite binding surface [34]. Previous results suggest that the CBM of HCHT50 also extends positive subsite surfaces as observed in *Sm*ChiB [29]. Still, *Sm*ChiB has a CBM with a flat surface more common for recalcitrant polysaccharide degradation, while the CBM of HCHT50 is associated with oligosaccharide binding [5]. Moreover, based on structural evidences available to date, bacterial GH18 chitinases appear to have their CBMs appended to the catalytic domain via a linker that is virtually fused against the surface of the catalytic domain, resulting in a compact, multi modular enzyme with an extended binding surface [25,34]. In other enzyme families, such as families GH6 and GH7 active on cellulose, this linker region can be found as a solvent-exposed, intrinsically disordered peptide sequence [24,40], preventing the acquiring of crystal structures. Interestingly, efforts to obtain a crystal structure of HCHT50 were fruitless until recently. The

* Corresponding author.
E-mail address: morten.sorlie@nmbu.no (M. Sørlie).

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structure of the catalytic domain were solved in 2002 [12] and only through a combined cross-seeding and micro-seeding cycle approach were Fadel et al. able to obtain the crystal structure of the full-length enzyme [11]. Even so, the linker-region could not be modeled due to a lack of interpretable electron density due to the flexibility of this region. This suggests that the linker region of HCHT50 is less fused with the catalytic domain as seen for example *Sm*ChiB.

Ligand binding to an enzymes active site is accompanied by conformational changes in the protein-ligand complex. Since CBMs and their linker regions vary for selected GH18s, it is interesting to see if their presence affect the thermodynamics of active site binding. Previously, we have used isothermal titration calorimetry (ITC) to obtain the thermodynamic signature for the allosamidin binding, a well-known family 18 chitinase inhibitor, to HCHT39 and *Sm*ChiB {Cederkvist, 2007 #79; Eide, 2013 #4}. In this work, we have investigated the binding of the same inhibitor to HCHT50 and *Sm*ChiB catalytic-domain only (*Sm*ChiB-CD) to assess the effect of the linkers and CBMs on GH18 active site ligand binding.

2. Experimental

2.1. Proteins and chemicals

HCHT50 was overexpressed in HEK293-6E cells and purified as described elsewhere [29]. For *Sm*ChiB-CD, mutagenesis of His_{10}-*Sm*ChiB-E144Q-G446 was performed using the QuickChange™ site directed mutagenesis from Stratagene (La Jolla, CA, USA), as described by the manufacturer. The primers used for the mutagenesis was designed as described by Westereng [39]. The template in the mutagenesis was His_{10}-*Sm*ChiB-E144Q [23]. Then His_{10}-*Sm*ChiB-G446 (*Sm*ChiB-CD) was mutated by use of the following primers in the PCR reaction: 5′GGACATCGACTGGGAGTACCCGCAAGC′3 (forward) and 5′GCTTG CGGGTACTCCCAGTCGATGTCC′3 (reverse). To confirm the desired mutation, the mutated gene was sequenced using GATC Biotechs (Conctance, Germany) LIGHTrun Sequencing service. When desired mutation was confirmed, the gene was transformed into *Escherichia coli* BL21Star (DE3) cells (Life Technologies, Carlsbad, CA, USA). *Sm*ChiB-CD was further expressed and purified by the same method as described by Hamre et al. [14]. Enzyme purity was verified by SDS-PAGE and estimated to be above >95% in all cases. Protein concentration was determined by using the Bradford-method from Bio-Rad. Allosamidin was a kind gift from Professor Shohei Sakuda, University of Tokyo. Allosamdin is isolated from *Streptomyces* sp. and the purity is controlled by ^{1}H NMR as described by Sakuda et al. [27]. The structure of allosamidin has previously been verified by both NMR and crystallography [28]. Buffers were made of potassium phosphate from Sigma-Aldrich (St. Louis, MO USA). The pH of final the solutions were controlled to be the desired one (pH 6.0) using glass electrode pH meter that was calibrated prior to use.

2.2. Isothermal titration calorimetry experiments

ITC experiments were performed with a VP-ITC system from Microcal, Inc (Northampton, MA) [41]. Solutions were made by using Milli-Q watere and 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 HCHT50 or *Sm*ChiB-CD 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.

2.3. Analysis of calorimetric data

ITC data were collected automatically using the Microcal Origin v.7.0 software accompanying the VP-ITC system [41]. 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 further 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 relationship 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. A description of how the entropic term is parameterized has been described in detail previously [6,42].

3. Results

3.1. Binding of allosamidin to HCHT50 and SmChiB-CD

The binding of allosamidin to HCHT50 and *Sm*ChiB-CD (Fig. 1) at pH 6.0 (20 mM potassium phosphate buffer) at different temperatures (20–37 °C) was studied using ITC. Fig. 2 shows typical ITC thermograms and theoretical fits to the experimental data at t = 30 °C and pH 6.0. At this temperature, HCHT50 bind allosamidin with a K_d of = 0.008 ± 0.003 μM ($\Delta G_r°$ = −46.9 ± 0.5 kJ/mol, Table 1). The stoichiometry (n) was found to be 1.10 ± 0.03. The reaction was accompanied by an enthalpic change ($\Delta H_r°$) of −58.2 ± 0.8 kJ/mol and an entropic change of ($\Delta S_r°$) of −37 ± 3 J/K mol ($-T\Delta S_r°$ 11.3 ± 0.9 kJ/mol). The change in the heat of the reaction, as determined by Eq. (2), was found to be −531 ± 13 J/K mol.

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

The binding of allosamidin to *Sm*ChiB-CD at t = 30 °C gave a K_d of 0.18 ± 0.03 μM (−39.1 ± 1.0 kJ/mol, Table 1). The stoichiometry (n) was found to be 0.95 ± 0.09. The reaction was accompanied by an enthalpic change ($\Delta H_r°$) of 18.4 ± 0.5 kJ/mol and an entropic change of ($\Delta S_r°$) of 189 ± 4 J/K mol ($-T\Delta S_r°$ = −57.5 ± 1.1 kJ/mol). The change in the heat of the reaction was determined to be −695 ± 40 J/K mol.

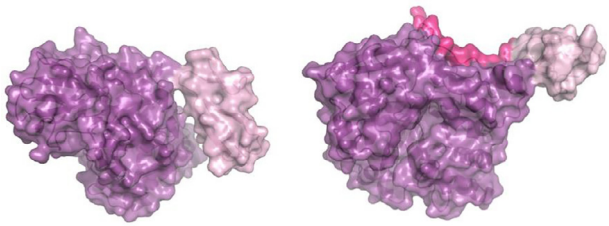

Fig. 1. Structure of HCHT50 (pdb code 5hbf) (left), and *Sm*ChiB (pdb code 1e6r [33]) (right). The catalytic domains are colored in magenta while the carbohydrate-binding modules are colored in pink. For *Sm*ChiB, the linker region is colored in hotpink. This region of HCHT50 could not be modeled due to a lack of interpretable electron density due to the flexibility of this region.