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Bussink, A.P.

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A SINGLE HISTIDINE RESIDUE MODULATES ENZYMATIC ACTIVITY IN ACIDIC MAMMALIAN CHITINASE

Anton P. Bussink*, Jocelyne Vreede#, Johannes M.F.G. Aerts* & Rolf G. Boot*

* Department of Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
# Van ’t Hoff Institute for Molecular Sciences, University of Amsterdam, Amsterdam, The Netherlands.

Mammals express two active chitinases, chitotriosidase and AMCase. Only AMCase displays an extremely acidic pH optimum, consistent with its observed presence in the gastro-intestinal tract. A structural model of AMCase reveals the presence of a conserved histidine residue in the active site. Mutational analyses and molecular dynamics simulations show that His187 is responsible for the acidic optimum and suggest pH dependent modulation of the reaction mechanism that is unique to AMCases. Concluding, His187 is a crucial structural component of the active site of AMCase and this unique feature may serve as a lead for the development of specific inhibitors.

**INTRODUCTION**

Chitinases are enzymes able to hydrolyse chitin, the linear polymer of β-1,4-linked N-acetyl glucosamine (GlcNAc), which is synthesized by a variety of lower organisms for structural purposes [1]. Despite the absence of endogenous chitin, two active chitinases are expressed in mammals. Investigations into the lysosomal storage disorder Gaucher disease serendipitously resulted in the identification of the first mammalian chitinase, chitotriosidase [1-3]. Soon after the second chitinase was discovered which due to its acidic pH optimum was named Acidic Mammalian Chitinase (AMCase) [4]. Both chitinases are 50 kDa proteins, consisting of a 39 kDa catalytic region separated from a chitin-binding domain by a hinge region [4,5]. They both show considerable homology to chitinases from lower organisms and are members of glycoside hydrolase family 18 according to the classification by Henrissat [6]. In man, chitotriosidase is the sole chitinase present in the circulation, consistent with its recently observed anti-fungal activity [7]. Despite this function in the innate immune system, in man a frequent mutation occurs, rendering approximately 6% of most ethnic groups completely deficient in the enzyme [8]. Investigations into the tissue-specific expression of chitinase mRNA and protein have revealed marked inter-species differences between mice and man [9]. In both species AMCase is expressed in the lung and gastro-intestinal tract. The occurrence of acidic chitinases in the stomach of *Xenopus* and *Gallus* species suggests the protein to have evolved as a result of a gene duplication early in tetrapod evolution [10].

A study of the kinetics of the enzyme revealed the protein to be able to catalyze the hydrolysis of chitin substrates as well as the reverse synthesizing reaction due to a phenomenon common to family 18 glycoside hydrolases known as transglycosylation [11,12]. Importantly, AMCase has recently attracted considerable attention due to a report linking the enzyme to pathogenesis of asthma [13]. The protein was reported to be elevated in a mouse model of bronchial asthma. Moreover, inhibition with the transition-state analogue
Allosamidin ameliorated the Th2 driven, IL13 dependent inflammation, suggesting chitinase activity to play a role in the disease, even in the absence of chitin. Several genetic variants were later proposed to be partly responsible for predisposition to the disease [14]. The precise role of AMCase in immune-mediated diseases is still far from clear since a later report suggested chitinase activity to exert a beneficial effect by negatively regulating chitin-induced tissue infiltration of innate immune cells associated with allergy [15].

Crystal structures of several family 18 chitinases have been solved, including that of chitotriosidase. The protein adopts a (β/α)₈ barrel fold, one of the most versatile folds known. Despite its fairly compact structure, the molecule shows an elongated cleft thought to be capable of binding long chitin-oligomers, compatible with the enzymes presumed endo-chitinolytic activity [16]. Co-crystallization experiments of chitotriosidase with the chitin analogue allosamidin, a micromolar inhibitor, revealed extensive electrostatic interactions and hydrogen bonding as well as hydrophobic stacking interactions between aromatic residues and the hydrophobic acetyl moiety of the chitin-analogue to allow for substrate binding [17].

The reaction mechanism of family 18 glycoside hydrolases was previously shown to proceed via substrate assisted catalysis, based on experiments performed on a bacterial chitinase, Chitinase B [18]. The role of Asp117, located two amino acids from the catalytic Glu119, in substrate binding is thought to be pivotal, in that it changes conformation upon binding of the substrate. pKa calculations combined with mutational analysis performed on a homologous chitinase later indicated Asp115 and Asp117 to share a proton at all physiologically relevant pH's in the native enzyme, with Asp117 capturing the proton upon binding of the substrate, leaving Asp115 deprotonated [19,20]. Subsequently, Glu119 is responsible for protonation of the glycosidic bond, after which hydrolysis takes place.

No structural information has been reported on AMCase to date. However, sequence homology within family 18 chitinases suggests a common reaction mechanism. This study investigates the molecular origin of the acidic optimum of Acidic Mammalian Chitinase by adopting a molecular modelling approach combined with molecular dynamics (MD) simulations, revealing adaptions necessary for functioning in an acidic environment.

**Material and Methods**

**Homology modelling & Molecular dynamics**

The models of human and murine AMCase were based on the crystal structure of native chitotriosidase 1LQ0 (resolution 2.20 Å). Initial modelling of the AMCase structures was accomplished online using the SWISSPROT server [21]. In order to construct a model of the H187N mutant, the histidine at position 187 was converted into an asparagine using the program Deepview [21]. Both the native and modified models were subjected to energy minimalization in GROMACS version 190.
3.3.1 with the GROMOS96 forcefield [22] using the steepest decent method. The quality of the models based on a variety of stereochemical parameters was determined by PROCHECK [23]. Surface electrostatic potentials of the modelled structures were calculated and visualized using Deepview [21]. MD was performed as described in the supplemental information.

**Mutagenesis, Recombinant Protein Expression and Enzymatic Assays**

The H187N point mutation was introduced directly into the wild-type murine AMCase cDNA in the expression plasmid, pcDNA3(1), using the high fidelity QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s protocol. Large-scale production and purification of the wild-type and mutant cDNA expression plasmids were performed using Promega Wizard Plus Midiprep kits. Recombinant expression in COS-7 cells was accomplished as described earlier [3]. For the enzyme activity assay with 4-MU-substrates, 25 μl medium and 100 μl substrate mixtures were incubated for one hour at 37 °C. The substrate mixtures contained 0.111 mM 4MU-deoxychitobiose and 1 mg/ml BSA in McIlvain buffer, at different pH. Reactions were stopped with 2.0 ml 0.3 M glycine NaOH buffer pH 10.6 and the formed 4MU was detected fluorometrically (excitation at 445 nm; emission at 366 nm). Only less than 10% difference in the duplicates was allowed. One unit (U) of activity is defined as 1 nmol of substrate hydrolyzed per hour.

**RESULTS AND DISCUSSION**

**Homology Comparisons and Overall AMCase Structure**

In order to gain insight into structure function-relationships we aligned both chitinases of various species. The alignment shown in figure 1 shows substantial overall conservation, in particular of the active site sequence, yet orthologue specific amino acids exist at various positions. In order to assess their importance in the catalytic mechanism we created models for AMCase based on the published chitotriosidase structure [16]. The overall structure shows high similarity with the experimentally determined crystal structure of chitotriosidase. This is reflected in a low root mean square deviation (RMSD) for Cα atoms between the mouse AMCase model and chitotriosidase structure of 0.54 Å. PROCHECK detected no major deviations from optimal geometry.

**Amino Acid Differences Between AMCase and Chitotriosidase**

Amino acid substitutions resulting in increased protein stability or increased enzymatic activity at low pH should be distinguished. The majority of substitutions in AMCases compared to chitotriosidase reside on the protein surface. This suggests they are adaptions conferring stability rather than adaptions directly modulating the reaction mechanism. Indeed, calculation of the surface potential of
both mouse and human AMCase shows that its substitutions result in a predominantly negative charge at the surface, as compared to chitotriosidase (figure 2). This most likely restricts intramolecular repulsion as a consequence of excessive protonation at low pH. Evolution of digestive lysozymes has followed a similar pattern [24].

Additionally, since AMCase contains two additional cysteines compared to chitotriosidase, a third disulfide bond most likely enhances the fold integrity in an acidic environment. Indirect evidence for this is provided by the observation that AMCase shows a different electrophoretic behaviour in the absence of a reducing agent, suggesting a difference in disulphide bonding between the two mammalian chitinases [4].

Figure 1. Alignment of the 39 kDa domain of vertebrate chitotriosidase and AMCase. h: homo sapiens; cf: canis familiaris; b: bos taurus; m: mus musculus; r: rattus rattus; gg: gallus gallus. Boxing is according to RISLER similarity scoring.
Figure 2. Surface potential of murine AMCase, human chitotriosidase and human AMCase. Red and blue represent a negative and positive surface charge, respectively (-/+ 2.5 kT/e).

Influence of Histidine 187

The mapping of sequence differences onto the modelled structures revealed that only a single paralogue specific substitution, His187, is located close to the active site. This histidine is conserved in all AMCases (figure 1). Replacing His187 with an asparagine in murine AMCase, the amino acid conserved at this position in chitotriosidase, revealed a clear difference in pH-dependent activity compared to wild type enzyme. Strikingly, activity at low pH is nearly abolished, whereas the near-neutral activity is affected far less (figure 3).

Figure 3. pH activity profile of wild-type and mutagenized His187Asn murine AMCase. Activity was measured with 4MU-deoxychitobioside as substrate as described in Materials and Methods.
In order to elucidate the role of this residue, MD simulations were performed on several systems: AMCase (both human and murine) with fully protonated His187, AMCase with deprotonated His187, the mutant murine AMCase His187Asn and chitotriosidase. All systems show considerable rigidity of secondary structure during the 10 ns runs, as shown by r.m.s. fluctuation (RMSF, a measure for flexibility) of 0.05-0.25 nm, consistent with the compact, highly stabilized structure of the \((\beta/\alpha)_8\) barrel. As expected, in view of hydrolase activity, the catalytic glutamic acid (Glu119), is accessible to solvent. Importantly, the simulations show a clear salt bridge interaction between deprotonated Asp117 and protonated His187 that remains constant during the full 10 ns. This distance is increased and fluctuates in both the mutant and chitotriosidase, suggesting that this strong interaction is absent in these enzymes (figure 4). The results were highly similar simulating both murine and human AMCase, suggesting the role of His187 to be ubiquitous in both enzymes.

**Figure 4.** Distances in time between Asp117 and His187/Asn187. Distances were calculated for (4) systems: (1) murine AMCase with protonated His187; (2) murine AMCase with deprotonated His187; (3) His187Asn mutant murine AMCase; (4) chitotriosidase. The distances plotted are between the C\textsubscript{\(\gamma\)} atoms of both amino acids. The values shown are representative for each of three independent MD runs.

**Acid pH Optimum**

A mechanistic explanation for the acid optimum and role of His187 can be given based on the reaction mechanism postulated earlier [20]. One of the pivotal steps in catalysis is the rotation of Asp117, preceding binding of the substrate by allowing formation of an H-bond with the N-acetyl moiety of the chitinous substrate. This can only be achieved by disruption of the stabilized system in
which Asp115 and Asp117 share a proton. Such a role can easily be envisioned for protonated His187 based on the fact that it is located close to the Asp115 carboxylate (figure 5).

For the observed pH optimum of mouse AMCase to be explained by protonation of His187, the effective pKa should be dramatically decreased, however this is not without precedent. pKa values of histidines are known to vary greatly depending on their electrostatic environment [25]. In the case of *Bacillus circulans* xylanase, for instance, the pKa value of a histidine residue (His149) is estimated to be <2.3, based on NMR titration experiments [26]. This is likely in part due to the hydrophobic environment surrounding the residue. More importantly perhaps, the histidine is inaccessible to solvent. Similarly, in AMCase, the presence of several "acidic" hydroxyl (Tyr6) and carboxylic acid groups (Asp115 and Asp117) in close vicinity may result in a change in effective pKa of the histidine (figure 5). Furthermore, visual inspection of the AMCase simulations shows that His187 is inaccessible to bulk water in both protonation states. The lack of stabilizing water molecules close to His187 is likely to favor a neutral state at low pH. Although our study renders proof for an important role of His187 in the extremely acidic pH optimum of mouse AMCase, other structural features most likely also have an impact. For example, the acidic activity of human AMCase is not as pronounced as that of mouse AMCase, despite the presence of His187 [4,12]. As the surface potential of mouse AMCase is significantly lower (figure 2), the loss of structural stability at very low pH may limit the extreme acidic activity of human AMCase. Concluding, His187 represents a major difference in the active site of AMCase
compared to that of chitotriosidase. The amino acid allows activity at extremely low pH, provided that the overall protein fold is stable at this extreme condition. The unique His187 feature of AMCases may serve as a lead for the development of specific inhibitors.

SUPPLEMENTAL INFORMATION

Preparation of the systems for MD included selection of the appropriate amino-acid protonation states, solvation of the protein structure in a periodic, cubic box, and addition of polar and aromatic hydrogen atom at acidic pH. For His187, we performed simulations of the fully protonated (HisH) and the partially protonated form (HisB), with the proton at nitrogen NE2. Subsequently, SPC water molecules were added [27], water molecules residing in hydrophobic cavities were removed and charge neutralization was accomplished by exchanging waters with chloride ions. Prior to actual MD the systems were subjected to another round of energy minimization, followed by 20 ps of MD with position restraints on heavy protein atoms and an unconstrained equilibration run of 1 ns. Both temperature and pressure in the systems was kept constant, at 300 K and 1 bar, respectively, using the Berendsen-thermostat and -barostat. Bonded interactions were described with the GROMOS96 forcefield [28], van der Waals interactions and short range electrostatic interactions were treated with a cutoff of 1.0 nm and electrostatic interactions were treated with the particle mesh Ewald method [29]. Using the LINCS algorithm to constrain bonds [30] allowed for a timestep of 2 fs. Prepared as such, the dynamics of each system were sampled during three independent MD runs of 10 ns, initiated from different starting velocities. From the resulting trajectories distances between atoms were calculated using tools included in the GROMACS software package.

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