Structure of Human Chitotriosidase

IMPLICATIONS FOR SPECIFIC INHIBITOR DESIGN AND FUNCTION OF MAMMALIAN CHITINASE-LIKE LECTINS*

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Chitin hydrolases have been identified in a variety of organisms ranging from bacteria to eukaryotes. They have been proposed to be possible targets for the design of novel chemotherapeutics against human pathogens such as fungi and protozoan parasites as mammals were not thought to possess chitin-processing enzymes. Recently, a human chitotriosidase was described as a marker for Gaucher disease with plasma levels of the enzyme elevated up to 2 orders of magnitude. The chitotriosidase was shown to be active against colloidal chitin and is inhibited by the family 18 chitinase inhibitor allosamidin. Here, the crystal structure of the human chitotriosidase and complexes with a chito-ligosaccharide and allosamidin are described. The structures reveal an elongated active site cleft, compatible with the binding of long chitin polymers, and explain the inactivation of the enzyme through an inherited genetic deficiency. Comparison with YM1 and HCGp-39 shows how the chitinase has evolved into these mammalian lectins by the mutation of key residues in the active site, tuning the substrate binding specificity. The soaking experiments with allosamidin and chito-ligosaccharides give insight into ligand binding properties and allow the evaluation of differential binding and design of species-selective chitinase inhibitors.

Family 18 chitinases hydrolyze chitin, an abundant polymer of N-acetylglucosamine (NAG). These enzymes have been identified in bacteria (1), fungi (2), insects (3), plants (4), viruses (5), and protozoan parasites (6). Humans were thought not to possess or process chitin, and therefore chitinases have been proposed to be targets for the development of inhibitors with chemotherapeutical potential as insecticides, fungicides, and antimalarials (6–9). Recently, however, it was discovered that humans have a chitinase activity that was found to be elevated up to 2 orders of magnitude in the plasma of patients suffering from Gaucher disease, a rare genetic disorder that is caused by a mutation in the glucocerebrosidase gene (10, 11). Purification of this activity from a Gaucher spleen revealed that, although transcribed from a single gene, the enzyme occurs in two major forms of 39 and 50 kDa. The subsequent cloning of its cDNA from a macrophage library showed that the 50-kDa form can be converted to the 39-kDa form post-translationally or by RNA processing (12–15). The enzyme is able to cleave chitotriose (and was termed chitotriosidase) but also hydrolyzes colloidal chitin to yield chitobiase and is thus thought to be an exochitinase (12, 16). Although the 39-kDa form is sufficient for the chitinolytic activity, the additional C-terminal domain has been shown to play a role in processing colloidal chitin and has been proposed to be a chitin binding domain, similar to other domains observed in the structures of chitinase A/B from Serratia marcescens (14, 17, 18). The chitotriosidase gene appears not to be essential, as about 35% of humans are heterozygous (6% homozygous) for an inactivated form of the gene (11, 15).

Although the enzyme has been characterized in detail, its function has not been fully defined. The observations that it is able to degrade both colloidal chitin and chitin in the cell wall of the fungal pathogen Candida albicans have prompted the hypothesis that the chitotriosidase plays a role in defense against chitinous human pathogens (15, 16). Indirect support for this hypothesis has recently come from a study showing that humans deficient in chitotriosidase activity are more susceptible to nematodal infections (19). Parasitic nematodes synthesize chitin during several stages of their lifecycle, and a human chitotriosidase could interfere with these processes. Thus, although the chitotriosidase is not essential from a general metabolic point of view, it may play a key role as a pathogen-defense protein. This implies that although chitinases in the pathogenic organisms themselves may be targets for the design of small molecule inhibitors with chemotherapeutical potential, it would be beneficial to exclude inhibitors that show strong activity against the human chitotriosidase. The most potent inhibitor currently known, the pseudotrisaccharide allosamidin (3), inhibits all family 18 chitinases with Ki values in the nanomolar to micromolar range, the human enzyme being inhibited at a Ki of 0.4 μM (16).

Here, we describe the crystal structures of human chitotriosidase in two crystal forms (at 2.35- and 2.1-Å resolution), a complex with chitobiase, and a soaking experiment with allosamidin. The chitotriosidase structure provides insight into the molecular basis of the inherited genetic inactivation of the enzyme and the evolution of the chitinase to form a structural
scaffold for several mammalian lectins. By comparison of the active site with that of chitinases from pathogenic organisms, we show that it should be possible to design allosamidin derivatives or other small molecules that are selective inhibitors.

MATERIALS AND METHODS

Purification and Crystallization—Human recombinant chitotriosidase was purified from the medium of stably transfected baby hamster kidney cells using the full-length human chitotriosidase cDNA cloned in the pNUT vector (20). 1.5 mg of freeze-dried human chitinase was dissolved in 6 ml of 50 mM Tris/HCl, pH 8.7, and dialyzed against 1 l of the same overnight at 4 °C. It was then spin-concentrated to 7.7 mg/ml. Vapor diffusion crystallization experiments were set up by mixing 0.5 μl of protein with an equal volume of 0.1 M Tris/HCl, pH 8.5, 30% PEG 4000. After 5 weeks of equilibration at room temperature, rod-shaped crystals grew to a size of 0.1 * 0.5 * 0.2 mm. Crystals were cryoprotected by a 10-s immersion in mother liquor with 10% glycerol and then frozen in a 100 K nitrogen gas stream.

Native I

Native II

NAG2

Allosamidin

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<th>NAG2</th>
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R. M. S. deviations from ideal geometry:

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TABLE I

Details of data collection and structure refinement for native chitotriosidase and complexes

Values between brackets are for the highest resolution shell. All measured data were included in structure refinement.

RESULTS AND DISCUSSION

Overall Structure and Interaction with Chitin—The structure of the 39-kDa form of human chitotriosidase was solved by molecular replacement and refined against 2.35 Å diffraction data to an R-factor of 0.216 (Rfree = 0.262) in space group P2₁ with good overall stereochemistry (Table I, Fig. 1). The structure consists of two domains. The core domain has a (β/α)₈ barrel as observed in the other family 18 chitinase structures for hevamine (31), chitinases A (ChiA) (17) and B (ChiB) (18)
FIG. 1. Overview of chitotriosidase structure and comparison with other chitinases. A, stereo image of the final human chitotriosidase structure. The backbone is shown as a gray ribbon. The α/β domain is colored blue. Residues 344–372, which are deleted in the inherited mutated form of the enzyme, are colored green. Asp-136, Asp-138, and Glu-140 are shown as a sticks model with carbons colored yellow. Solvent-exposed aromatic side chains lining the active site cleft are shown as purple sticks. NAG₂, as seen in the complex (Table I), is shown in a sticks representation with orange carbon atoms. B, stereo image of the active site. The chitotriosidase backbone is shown as a gray ribbon. Solvent-exposed aromatics and residues interacting with NAG₂ are shown as sticks with carbons colored green for the apo structure and carbons colored purple for the chitotriosidase-NAG₂ complex. A simulated-annealing Fo-Fc, ϕβφc map for NAG₂ as observed in the chitotriosidase-NAG₂ complex is shown in blue, contoured at 3σ. NAG₂ is shown in a sticks representation with orange carbon atoms. C, overall comparison with other chitinases. Molecular surfaces (calculated with PyMOL) are shown for currently known complexes of chitinases with chitooligosaccharides: hevamine with NAG₁ (31), ChIA with NAG₂ (32), ChIB with NAG₂ (29), and the human chitotriosidase with a model of NAG₂. The TIM barrel core of the enzymes is orientated as in panel A. The catalytic glutamic acid is colored red, and exposed aromatic side chains lining the active site cleft are colored blue. The chitooligosaccharides are shown as sticks with green carbons.
from *S. marcescens* and CTS1 from *Coccidioides immitis* (28), although helix/H9251 is missing (Fig. 1A). An additional/H9252 domain, composed of six antiparallel/H9252-strands and one α-helix, is inserted in the loop between strand/H9252 and helix/H9251, which gives the active site a groove character (Fig. 1C). Like all other family 18 chitinases, the chitotriosidase has the D/X/D/X/E motif at the end of strand/H9252 with Glu-140 being the catalytic acid (Figs. 1A and 2). Two disulfide bridges were observed between Cys-26–Cys-51 and Cys-307–Cys-370, which are also present in the YM1 structure (23).

The native chitotriosidase structure was also refined using diffraction data from a second crystal form (space group P2₁, Table I). These crystals were obtained by adding 40% ethylene glycol to the precipitant solution. Ethylene glycol acts as an inhibitor of chitotriosidase, and the structure shows the presence of three molecules of ethylene glycol bound in the active site. One of them is hydrogen-bonded to Asp-138, which is essential for catalysis in chitinases (27, 29). This ethylene glycol molecule may inhibit the chitotriosidase by blocking the side chain of Asp-138 in the native position, preventing the binding of the substrate (attempts to bind NAG2 failed). The P4₃2₁₂ and P2₁ structures are essentially identical and superpose with a root mean squares deviation of 0.5 Å on Ca atoms.

In further discussions below, the structure in space group P4₃2₁₂ will be used when referring to the native human chitotriosidase structure.

Soaking experiments with chitooligosaccharides NAG₃, NAG₄, or NAG₅ were carried out at pH 10.6 to prevent acid-catalyzed hydrolysis of the substrate. Nevertheless, hydrolysis occurred, and the experiments consistently resulted in electron density maps where only an ordered N-acetylglucosamine dimer could be observed, lying in subsites –2 and –1 (Fig. 1B). The chitotriosidase structure shows little change upon NAG₂ binding. The most relevant is the side chain of Asp-138, which rotates toward the side chain of Glu-140, as seen in other studies with chitinases (29, 31–33) (Fig. 1B). Other side chains (Fig. 1B) undergo minor conformational changes, probably required to accommodate and stabilize the binding of the oligosaccharide. At 2.8-Å resolution (Table I), it is not possible to unambiguously distinguish a boat (as seen in the ChiA-NAG₈ (32) and ChiB-NAG₅ (29) complexes) from a chair (as seen in the hevamine-NAG₃ complex (31)) conformation in the H₁₁₀₀₁₁ subsite. However, in our complex, the H₁₁₀₀₁₁ subsite is not occupied, and we have therefore modeled the H₁₁₀₀₂₁ sugar in the chair conformation.

Through superposition of the ChiB-NAG₅ complex (29) and the ChaA-NAG₈ complexes (32) and ChiB-NAG₈ (29) complexes) from a chair (as seen in the hevamine-NAG₃ complex (31)) conformation in the –1 subsite. However, in our complex, the +1 subsite is not occupied, and we have therefore modeled the –1 sugar in the chair conformation.
Structure of Human Chitotriosidase

Although narrowing somewhat at the far reducing end of the cleft appears to be fully extended over one face of the enzyme, meric chitin (1). In contrast, the chitotriosidase active site which explains their synergism in the degradation of polymeric sites also appear to be blocked at opposite ends (at the reducing end of the substrate binding cleft, is replaced with an aspartic acid, whereas the nearby Trp-72 is replaced with a glutamic acid (Figs. 2 and 3)). On the other side of the cleft, Trp-218 is changed to a lysine, and Tyr-190 is replaced by the enzyme is produced with residues Val-344-Gln-372 missing. The chitotriosidase structure presented here allows us to understand why deletion of this region inactivates the enzyme. Figs. 1A and 2 show that residues 344–372 correspond to the C-terminal half of helix α7, the entire strand β8, and almost the entire β8-α8 loop. Deletion of these secondary structure elements could lead to misfolded, and therefore inactive, protein. More importantly perhaps, Trp-358, which lies at the end of strand β8, is completely conserved in all active family 18 chitinases (Fig. 2). Inspection of chitinase structures in complex with chitooligosaccharides (29, 32) shows that this tryptophan serves as an “anvil” onto which the –1 sugar is pressed, whereas specific hydrogen-bonds with other residues may force the sugar in the boat conformation required for the attack of the N-acetyl group on the anomic carbon (29) (Fig. 1A). Thus, deletion of Trp-358 could in itself lead to a completely inactive enzyme.

Comparison with the Mammalian Lectins YM1 and HCgp-39—Apart from the human chitotriosidase, two other family 18 chitinase-like mammalian proteins have been described recently. YM1 and HCgp-39 both show a high degree of sequence similarity to family 18 chitinases (23, 35) (Fig. 2). The structure of YM1 has been determined by x-ray crystallography, revealing density for a single glucosamine in the active site (23). As was predicted from multiple sequence alignments (Fig. 2), two key residues in the active site were mutated (Asp-136 to Asn and Glu-140 to Gln), which renders the protein completely inactive as a chitinase. Binding experiments and crystallographic analysis further showed that YM1 does not interact with polymeric carbohydrates but rather with monomers (23, 36), although the YM1 structure did not reveal why the chitinase-like protein had lost its ability to interact with carbohydrate polymers. The human chitotriosidase structures presented here allow us to explain this loss of affinity (Fig. 3A). Apart from the elimination of catalytic activity through the mutations in the active site, there are several non-conservative substitutions in the chitin binding cleft. Tyr-24, near the non-reducing end of the substrate binding cleft, is replaced with an aspartic acid, whereas the nearby Trp-72 is replaced with a glutamic acid (Figs. 2 and 3A). On the other side of the cleft, Trp-218 is changed to a lysine, and Tyr-190 is replaced by the smaller valine. The trend in these mutations is that almost half of the exposed aromatic residues, discussed earlier and shown in Fig. 1, have been non-conservatively replaced, indeed in most cases with charged amino acids. Thus, although all the residues required for interaction with monomeric carbohydrates are still present in the core of the active site, the cleflining residues required for the binding of polymeric substrate have been removed. This explains the observed carbohydrate binding specificity (monomers only) for YM1 (36).

Similar to YM1, the human cartilage protein HCgp-39 has all the signs of an inactivated chitinase. Although its sequence suggests a family 18 chitinase-like structure, two key active site residues, the equivalents of Asp-136 and Glu-140 in the chitotriosidase, have been mutated to Ala and Leu, respectively, and HCgp-39 does not have detectable chitinase activity (37). Binding studies, however, have shown that unlike YM1, HCgp-39 binds chitin with high affinity and has therefore been proposed to be a chitin-specific lectin (35). A structure for HCgp-39 is not available. However, sequence alignment shows that unlike YM1, all of the solvent-exposed aromatics are conserved in HCgp-39 with the exception of Tyr-190, which is not a conserved residue in other chitinases either (Fig. 2). Thus, like the human chitotriosidase, the active site cleft of HCgp-39 would be lined with exposed aromatic residues and could support the binding of longer chitin oligomers.
FIG. 3. Comparison of active site details and interaction with allosamidin. In A, the substrate binding pockets of the human chitotriosidase and YM1 are compared. Protein backbones are represented by a gray ribbon. A model of NAG$_n$, in equivalent position to that in Fig. 1, is
Implications for Selective Inhibitor Design—The most potent chitinase inhibitor currently known is allosamidin, a pseudotrisaccharide (consisting of two β(1,4)-linked N-acetyl allosamine sugars coupled to allosamizoline, Fig. 3B) isolated from Streptomyces (3, 38). All family 18 chitinas tested are inhibited by this molecule with $K_i$ values in the nanomolar to micromolar range (39, 40); it inhibits the chitotriosidase with a $K_i$ of 0.4 μM (16). Allosamidin has been shown to have a fungicidal effect by interfering with fungal cell separation (2, 9) and to act as a malaria transmission blocker as it strongly inhibits Plasmodium falciparum chitinase required for insect invasion (6, 8).

However, the recent implication that the human chitotriosidase, although not essential from a housekeeping point of view, may play a role in defense against pathogens (15, 19) suggests that a broad spectrum chitinase inhibitor might have negative side effects if administered to humans. Thus, if chitinase inhibitors, such as allosamidin, are further developed to enhance their fungicidal, insecticidal, and antimalarial potential, inhibition of the human chitotriosidase should be reduced to weak levels. We addressed this issue by soaking chitotriosidase crystals with allosamidin and positioning the inhibitor according to a 2.7Å difference map (Fig. 3B). Comparison of this complex with the one obtained for the chitinase CTS1 from the fungal pathogen C. immitis (28), which is the causative agent of the respiratory disease known as San Joaquin Valley fever in the southern Americas, suggests a strategy for improving the efficacy of antifungal molecules. It is apparent that the overall shape of the active site in the –1 subsite is nearly identical for the chitotriosidase and CTS1 complexes with allosamidin and also for complexes of the inhibitor with hevamine (27) and ChiB (29). The allosamizoline moiety binds deep in the active site, interacting closely with, hydrogen-bonds to the oxygen of the $N$-acetyl group on the –2 sugar, is a valine in the fungal enzyme, creating a small void (Fig. 3B). Thus, there appears to be scope for the development of allosamidin derivatives with differential specificity based on the changes around the –2 and –3 subsites. In this context, it is worth noticing that such derivatives have already been reported, although so far all changes in the allosamizoline sugars appear to reduce chitinase inhibition (39, 41, 42).

Conclusion—The structure of the human chitotriosidase and its complexes described here have provided further clues for understanding the possible functions of the protein and its closely related homologues, YM1 and HCgp-39. The structure reveals that a long cleft runs across one face of the protein and its differential specificity based on the changes around the –2 and –3 subsites. In this context, it is worth noticing that such derivatives have already been reported, although so far all changes in the allosamizoline sugars appear to reduce chitinase inhibition (39, 41, 42).

REFERENCES
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