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The structure of chitinases and prospects for structure-based drug design

Jon D. Robertus, P. John Hart, Arthur F. Monzingo, Edward Marcotte, and Thomas Hollis

Abstract: Many fungi, including pathogenic strains, require proper chitin metabolism to assure normal cell wall replication. Chitinase hydrolyzes chitin; inhibition of endogenous chitinases or application of extracellular chitinases can disrupt fungal division. It is possible that chitinase inhibitors could be used as antifungal agents. We have solved the X-ray structure of a class II chitinase from barley and proposed a mechanism of action. The enzyme has a structural core similar to lysozyme and probably acts in a similar catalytic manner. The enzyme structure can, in principle, be used to identify small molecules that will bind avidly to the active site and act as inhibitors. Those inhibitors that embody transition state geometry are likely to be particularly effective.

Key words: chitinase, mechanism of action, drug design.

Résumé : Plusieurs champignons, incluant des souches pathogènes, ont besoin d'un métabolisme de la chitine approprié pour assurer une reproduction normale de la paroi cellulaire. La chitinase hydrolyse la chitine; l'inhibition des chitinases endogènes ou l'application de chitinases extracellulaires peut bloquer la division fongique. Il est possible que des inhibiteurs de la chitinase puissent être utilisés comme substances antifongiques. Les auteurs ont déchiffré la structure, aux rayons X, d'une chitinase de classe II chez l'orge, et proposent un mécanisme d'action. L'enzyme possède une structure centrale semblable à celle du lysozyme et agit probablement d'une façon catalytique comparable. La structure de l'enzyme peut, en principe, être utilisée pour identifier de petites molécules qui s'attachent fortement au site actif pour agir comme inhibiteurs. Ces inhibiteurs, qui montrent un état géométrique de transition, ont des chances d'être particulièrement efficaces.

Mots clés : chitinase, mécanisme d'action, conception de médicaments.

[Traduit par la rédaction]

Fungal infestations are clearly a major problem in agricultural production and storage; they also constitute a major problem in human pathogenesis. These run from relatively benign but stubborn skin infections like ring worm, to life-threatening lung infections like coccidioidomycosis (San Joaquin Valley fever), caused by *Coccidioides immitis* (Cole and Kirkland 1991). There is a general recognition that fungal infections are difficult to treat and there is a long-standing desire to develop agents that can kill fungi or retard their growth. Also, there is presently great interest in trying to improve the process of drug or antibiotic design using detailed knowledge of the molecular structure of biological macromolecules.

The vast majority of drugs and antibiotics are small molecules that bind as ligands to enzymes or other proteins and act as inhibitors of their natural functions. A well-known example is penicillin. It binds to and inactivates the transpeptidase enzyme used by various bacteria to cross-link polysaccharide strands that form the bacterial cell wall. Penicillin does not kill the bacteria like a poison but prevents the

daughter cells of a replicative division from forming intact walls. Since the bacterial cytoplasm is normally under substantial osmotic pressure, the incomplete walls cannot prevent the daughter cells from literally exploding. One factor that makes penicillin an exceptional antibiotic drug is that humans lack cell walls and consequently lack any analog of the transpeptidase enzyme; inhibitors of the transpeptidase have no direct effect on human metabolism. This is not so for all drugs. For example, many antibiotics are inhibitors of steps in protein synthesis and may be as toxic to humans as to an invading pathogen.

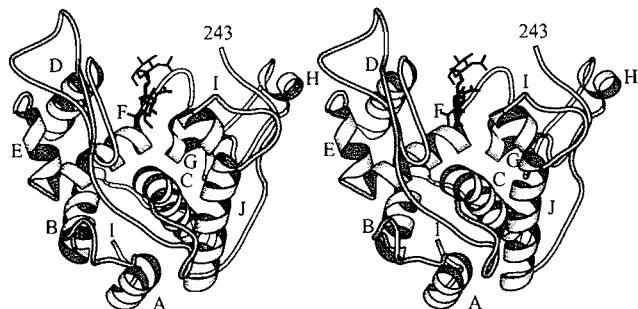
As mentioned above, there are a large number of fungal pathogens that cause diseases in humans and they tend to be difficult to treat (Beneke 1991). Fungi are eucaryotes and many of their enzymes are similar enough to human analogs that drugs aimed at them will also adversely affect human tissue. However, like bacteria, fungi have cell walls and it is reasonable to suppose that small molecule inhibitors of fungal cell wall metabolism might prove to be efficacious antibiotics.

Many fungal cell walls contain chitin, an insoluble β -1,4-linked polymer of *N*-acetylglucosamine (NAG). Not surprisingly, fungi contain chitin synthases, but they also contain chitinases that hydrolyze chitin and are required for proper cell division. Fungal division requires that chitin-containing cell walls need to be rearranged. Even yeast,

Received August 19, 1994.

J.D. Robertus, P.J. Hart, A.F. Monzingo, E. Marcotte, and T. Hollis. Department of Chemistry and Biochemistry, University of Texas, Austin, TX 78712, U.S.A.

Fig. 1. A ribbon drawing of the barley endochitinase backbone. The amino terminus is labeled 1 and the carboxy terminus is 243; ten α -helical segments are labeled A–J. A triNAG oligosaccharide is shown as a dark bonded skeletal model. The position is based on that observed in hen egg white lysozyme, oriented over chitinase in a least-squares operation matching common structural elements.



which has chitin only in its bud scar, requires chitinase for cell separation (Kuranda and Robbins 1991). The importance of proper cell wall metabolism to fungal growth is emphasized by the fact that extracellular endochitinases are often effective in preventing the growth of fungal mycelia (Roberts and Selitrennikoff 1986, 1988; Leah et al. 1991). Chitinases are most effective attacking the accessible nascent chitin fibers produced at the apex of growing hyphal tips of filamentous fungi (Mauch et al. 1988; Roberts and Selitrennikoff 1988). Clearly, either fungal chitin synthases or chitinases would be good targets for antibiotic design. Toward this end, our laboratory has begun a broad investigation of chitinase enzymes.

Exochitinases remove NAG units from the nonreducing end of the unbranched chitin polymer but are fairly rare. Far more common are endochitinases, which cleave chitin internally. These are found in higher plants, fungi, and bacteria. Some endochitinases also exhibit lysozyme activity; that is, they are able to cleave the β -1 \rightarrow 4 bond between the *N*-acetyl muramic acid and NAG found in peptidoglycan.

Endochitinases are generally monomeric proteins between 25 and 40 kDa. Four classes have been proposed based on amino acid sequences (Shinshi et al. 1990; Collinge et al. 1993). Classes I, II, and IV are homologs in that they contain a major catalytic domain of about 26 kDa. Class II enzymes, like that from barley, contain only this catalytic domain. Class I and IV endochitinases also have an N-terminal cysteine-rich domain of about 50 residues, which anchors the enzyme to the insoluble chitin matrix. The N-terminal domain is linked to the chitinase domain by a flexible glycine/proline-rich hinge segment. Class III chitinases show no apparent sequence similarity to enzymes in class I, II, or IV and may be structurally unrelated to them. Class III proteins are common in fungi and occur in some higher plants as well. These proteins often have three domains as seen in the archetypal enzyme from yeast (Kuranda and Robbins 1991). It shows (i) an amino terminal catalytic domain of about 300 residues, (ii) a Ser-Thr rich region of about 170 residues, which may be very heavily glycosylated, and (iii) an anchoring domain of about 80 residues. In either major family of chitinase, it is the catalytic domain that is of structural interest and that would be expected to bind inhibitors that could interfere with

normal fungal cell wall metabolism.

Several laboratories have attempted to crystallize chitinases for X-ray analysis, but this has generally proven to be unrewarding. It may be that the flexible hinge region, which links the catalytic domain to the anchoring domain of most chitinases, allows too much conformational freedom to permit good crystallization. We purified the chitinase from barley (Leah et al. 1991), because it is the only class II enzyme known and in essence consists only of the catalytic domain. The protein formed excellent crystals and allowed the structure to be solved by X-ray diffraction to 0.28 nm resolution (Hart et al. 1993).

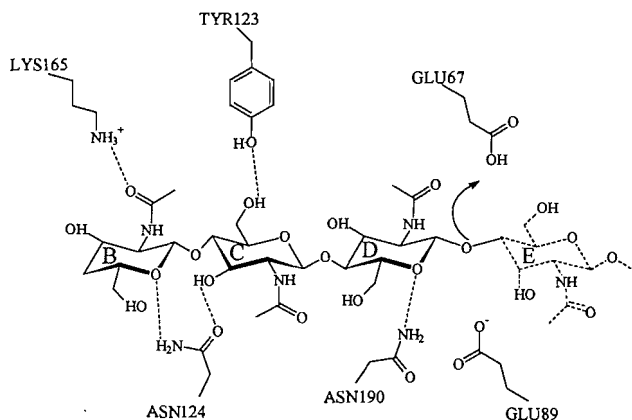
Figure 1 shows a ribbon drawing of the barley chitinase. The protein is a compact globular structure roughly $4 \times 4.5 \times 4.5$ nm. It has three disulfide bonds; the cysteine pairs are 23 to 85, 97 to 105, and 204 to 236. Cys223 is a free thiol that reacted to form the sole mercury derivative site. The enzyme has 10 helical segments, which compose 47% of the linear sequence. An elongated cleft running the length of the molecule is clearly evident and presumed to be the region responsible for substrate binding and catalysis.

A major goal of the structural analysis of enzymes is to understand the mechanism of substrate binding and catalysis. A clue to the importance of key residues is often provided by examining those residues that are invariant in a family of related proteins. Unfortunately, the class I, II, and IV chitinases are very closely related, and so many amino acids are conserved among them that it is difficult to ascertain which are crucial based on their evolutionary persistence. However, our analysis of the structure did provide an unexpected finding. We have recently shown that barley chitinase has elements of secondary structure that are conserved in all the known lysozyme structures, hen egg white (HEWL), T4 phage (T4L), and goose egg white (GEWL). This relationship was also detected by a computer search of the Brookhaven Crystallographic Data Base, including the recently deposited coordinates for barley chitinase (Holm and Sander 1994). The helices labeled C and F and the antiparallel loop immediately preceding helix D in Fig. 1 are found in identical positions in chitinase, HEWL, T4L, and GEWL. These structural elements define a common oligosaccharide-binding site. The binding of oligosaccharides has been observed crystallographically for HEWL (Kelly et al. 1979) and the corresponding position for a trisaccharide bound to chitinase is shown in Fig. 1.

The superposition of chitinase with lysozymes suggests that the chitinase mechanism is similar and probably identical to that of lysozyme. Based on its position in the common active site structure, Glu67 is the crucial acid, protonating the leaving group sugar. It is the homolog of Glu35 in HEWL. A schematic of the putative interaction between chitinase and a triNAG molecule is shown in Fig. 2. Notice that it is possible to define a number of interactions, typically hydrogen bonds, between the substrate and the enzyme. Interactions of this type determine the affinity between protein and ligand.

Initial rate kinetics for an enzyme can often measure k_{cat} , the turnover, and K_m , the substrate concentration producing half maximal velocity. K_m is often taken as a rough measure of the dissociation constant between the Michaelis complex and the free enzyme and substrate forms. It is very difficult to measure K_m for chitinases, because the natural substrate

Fig. 2. Schematic of hypothetical triNAG binding to barley chitinase. Hen egg white lysozyme has a substrate binding site homologous to chitinase and has been observed crystallographically to bind a trisaccharide. The lysozyme site has been postulated to have six sugar binding sites, labeled A–F, with bond cleavage between sites D and E. The trisaccharide binds into B–D, as labeled. The triNAG seen in lysozyme has been rotated into the chitinase active site and seen to make the interactions indicated. A hypothetical NAG in the leaving group E site has been shown in dashed bonds. Glu67, the putative catalytic acid, is well positioned to protonate the leaving group O4.



is insoluble and the concentration cannot be varied easily. The class of enzymes is consequently not well characterized.

Koga and co-workers used di through hexa NAG substrates to carry out a kinetic analysis of the yam chitinase (Koga et al. 1989). They showed k_{cat} was no more than 50/min for tetraNAG but only 0.1/min for triNAG. This is consistent with values for HEWL, which show a k_{cat} of 15/min for hexaNAG and of 30/min for (NAG-NAM)₃ (Imoto et al. 1972). K_m for yam chitinase was about 25 μ M for tetrasaccharide and nearly 1 mM for trisaccharide. Disaccharides are not reactive. We have attempted to soak diNAG at 500 mM into barley chitinase crystals and found that the disaccharide will not bind even at this high concentration. This emphasizes that short substrates will not bind to the chitinase active site. Binding is optimized for long polymers, and cleavage to shorter oligosaccharides aids product release. This low affinity of chitinases for short oligosaccharides makes excellent sense for the enzyme's catalytic purposes but will effect the strategy of inhibitor design for this enzyme class.

Inhibitor binding

Enzyme inhibitors, particularly competitive inhibitors, often bear a strong physical resemblance to the substrate that the protein has evolved to bind. All enzymes increase catalytic rates, which is equivalent to saying that they preferentially bind the transition state of the reaction they catalyze (Kraut 1988). An inhibitor molecule that incorporates the geometry or charge distribution of the transition state is called a transition state analog. These often bind to enzymes 10⁴ times stronger than substrates (Wolfenden 1976).

Lysozyme has a well-studied mechanism of action and

Fig. 3. Schematic of the proposed mechanism of action of chitinase. The mechanism is essentially identical to that of lysozyme. The C4 oxygen of the leaving group sugar is protonated by a glutamic acid. This is Glu67 for chitinase and Glu35 for hen egg white lysozyme. Cleavage of the glycosidic bond creates a positive charge on the potential aldehyde sugar that is spread between C1 and O5 and is called an oxycarbonium. This transition state structure is locally flat; the charge may be stabilized by a carboxylate that is not shown. It would be Asp52 in hen egg white lysozyme and may be Glu89 in chitinase. Water attacks the oxycarbonium while re-protonating the mechanistic glutamic acid to form products.

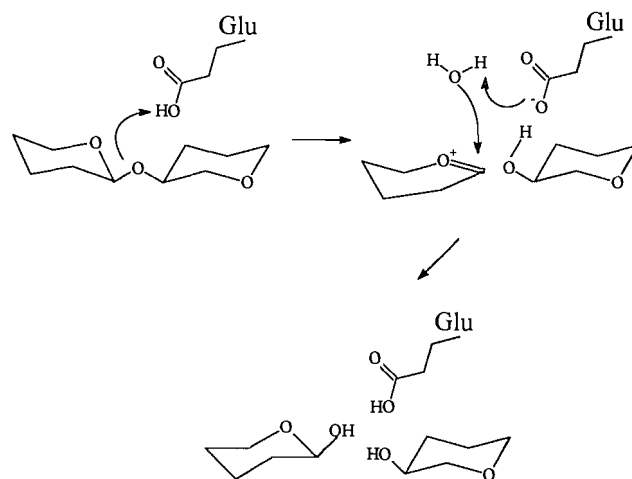
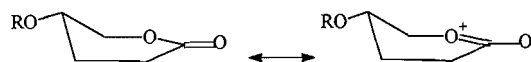
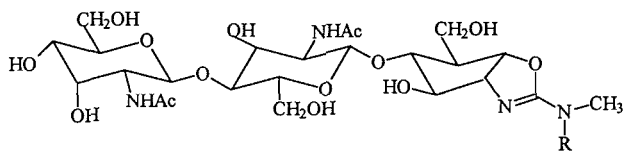


Fig. 4. The geometry of glucolactones. Glucolactones are known inhibitors of lysozyme. They have the locally flat geometry of the mechanistic transition state; resonance forms also exhibit the positive charge on O5 seen in the transition state. The lactones bind more tightly than boat form sugars at the catalytic site, because the enzyme has evolved to make favorable interactions with the transition state geometry.



one that undoubtedly resembles chitinases. Using the example of HEWL one sees the substrate binding at six sites labeled A through F; hydrolysis occurs between sugars D and E. Glu35, the analog of barley chitinase Glu67, donates a proton to the glycosidic bond, allowing the E sugar to leave with an alcohol at the C4 position (Fig. 3). The cleaved bond develops positive charge on the D sugar, which delocalizes to form an oxycarbonium ion. This causes sugar D, normally in a chair configuration, to flatten out. The transition state of the reaction is flat. This geometry is mimicked by lactones, which act as inhibitors of lysozyme (Ford et al. 1974). Fig. 4 shows the geometry of the lactone, emphasizing that, like the transition state of the lysozyme/chitinase reaction, the carbon at position 1 has planar sp^2 geometry, compared with the tetrahedral configuration of the sugar. A resonance form of the lactone also has the positive charge distribution spread between C1 and O5. This causes the lactone analog of tetraNAG to bind HEWL 30 times more tightly than does the unmodified saccharide (Ford et al. 1974).

Fig. 5. The structure of allosamidin. The trisaccharide analogs are inhibitors of many chitinases. They have NAG units to bind in the substrate cleft and the oxazoline ring probably mimics transition state geometry. R = Me for allosamidin; R = H for demethylallosamidin.



Allosamidin and derivatives are chitinase inhibitors isolated from microbial sources such as *Streptomyces* (Sakuda et al. 1986). The structures of these modified trisaccharides are shown in Fig. 5. There is no experimental evidence concerning the nature of allosamidin inhibition, but it is not unlikely that the planar oxazoline ring may act as a transition state mimic, while the two NAG residues bind to the enzyme in the manner of an extended substrate.

Structure-based drug design

The most common way to produce drugs is to screen thousands of chemical compounds that may inhibit a given enzyme. Often the compounds are based on natural products, which have some desired property, and generally they resemble a known substrate for an enzyme. Organic synthesis is used to modify the initial drug "lead" in a random fashion and subsequent testing of the compounds guides the direction of drug design. Recently there has been interest in using the known structure of a target protein to predict or design inhibitors in what will hopefully be a short cut compared with more traditional methods.

Drug design must consider two principal problems. One is the design of an inhibitor that fits tightly into the target protein and inactivates its normal function. The second problem is that while a drug must be a strong inhibitor compound, to be used therapeutically, it must also have good bio-availability (preferably oral), have low toxicity, and reasonable residence time. These constraints may limit the chemistry of inhibitor design. The progress of this field of structure-based design was the subject of a recently reviewed meeting (Robertus 1994).

Structure-based inhibitor design has two main forms. One is the use of computer programs to find compounds in a data base that are likely to be complementary in shape and charge to the active sites of enzymes. Programs have been written to search the vast data banks of existing chemical compounds and computationally fit them into the active site of the protein of interest, provided that protein structure has been described by high resolution crystallography. The most commonly used program of this type is DOCK (DesJarlais et al. 1988). It has been used to find a moderately strong inhibitor of the HIV protease (DesJarlais et al. 1990) and inhibitors with micromolar dissociation constants from thymidylate synthase (Shoichet et al. 1993).

A second form of structure-based inhibitor design is the construction of hypothetical molecules that will be complementary to the active site based on the shape and charge distribution. The compound designed in this way may not exist and indeed may be very difficult or impossible to synthesize.

A balance must be struck between the theoretical structure of an ideal drug and a realistic assessment of the chances of procuring it. The program GRID, developed at Oxford by P.J. Goodford, can be used in this way (Goodford 1985). GRID was used to design inhibitors of thymidylate synthase in a project that is the archetype of the algorithm widely used in the field (Appelt et al. 1991). That is, once a lead compound is discovered by search or by design, it can be diffused into the crystalline enzyme, and the mode of binding analyzed by X-ray crystallography. This may suggest alterations that will improve inhibitor binding. The new compound can then be tested kinetically and crystallographically in the same way as the initial lead compound. This process is referred to as design by iterative crystallographic analysis.

These powerful new methods of inhibitor design can be applied to chitinases. In particular it would be useful to learn the structure of chitinases from pathogenic fungi so that a systematic program of antibiotic design could be undertaken. The rationale can be developed on the barley enzyme, however. Some inhibitor design principles can be seen by examining the interactions shown in Fig. 2. For example, Lys165 donates a hydrogen bond to a substrate amide. If an inhibitor had a carboxylate at the amide position, it might make a very favorable ion pair that would allow the inhibitor molecule to bind much more tightly than a natural sugar. The importance of transition state geometry has been stressed above. An inhibitor might be designed with lactone or similar geometry at the E site and should have a positive charge to interact with Glu89.

The active site of the chitinases does not seem to bind short oligosaccharides very avidly, although molecules designed by the principles outlined above might be useful. Instead it may be prudent to carry out a DOCK search of chemical data bases to find compounds unrelated to the sugar substrates. Such molecules may adventitiously bind very strongly to the enzyme surface. This was the case when the dye phenolphthalein was predicted by DOCK to bind to thymidylate synthase and served as the lead compound for the development of strong inhibitors that bear no structural resemblance to natural substrates (Shoichet et al. 1993). Although these inhibitors may not resemble sugars, they would still place appropriate chemical groups in a position to interact favorably with the enzyme residues.

Acknowledgments

This work was supported by grants GM30048 and GM35989 from the National Institutes of Health and by grants from the Foundation for Research and the Welch Foundation.

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