Structural Basis for Inhibition of Aspergillus niger Xylanase by Triticum aestivum Xylanase Inhibitor-I*

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Plants developed a diverse battery of defense mechanisms in response to continual challenges by a broad spectrum of pathogenic microorganisms. Their defense arsenal includes inhibitors of cell wall-degrading enzymes, which hinder a possible invasion and colonization by antagonists. The structure of Triticum aestivum xylanase inhibitor-I (TAXI-I), a first member of potent TAXI-type inhibitors of fungal and bacterial family 11 xylanases, has been determined to 1.7-Å resolution. Surprisingly, TAXI-I displays structural homology with the pepsin-like family of aspartic proteases but is proteolytically nonfunctional, because one or more residues of the essential catalytical triad are absent. The structure of the TAXI-I-Aspergillus niger xylanase I complex, at a resolution of 1.8 Å, illustrates the ability of tight binding and inhibition with subnanomolar affinity and indicates the importance of the C-terminal end for the differences in xylanase specificity among different TAXI-type inhibitors.

The plant cell wall forms a structurally heterogeneous physiological barrier that in many cases must be perturbated before colonization by a pathogen can take place. To do so, fungal and bacterial pathogens are armed with a deconstructing mixture of enzymes that depolymerize polysaccharides in the plant cell wall (1). At the same time, plants can secrete proteins that inhibit these degradative glycosidases, including polygalacturonase-inhibitor proteins (2), pectin lyase inhibitor proteins (3), and two different classes of endoxylanase inhibitors (4, 5).

Endo-1,4-β-D-xylanases (EC 3.2.1.8, further referred to as xylanases) are key enzymes in the degradation of xylan, next to cellulose, the most abundant natural polysaccharide, and arabinoxylan, an important quality-determining nonstarch polysaccharide in cereals. Plant xylanases, a topic of recent work (6), are believed to have a regulatory function during processes of fertilization, seed germination, and programmed cell death. Microbial xylanases are increasingly better characterized because of their growing use in biotechnological processes to the benefit of processing and/or product quality parameters (e.g. bread making (7), pasta processing (8), wheat gluten-starch separation (9), and paper and pulp production (10, 11)). Based on amino acid sequence similarities, the data base of “carbohydrate-active enzymes” (12) groups xylanases mainly into glycosyl hydrolase (GH) families 10 and 11. GH family 10 xylanases possess a TIM-barrel (βα)8 topology, with the two catalytic residues located at the C termini of strands β-4 and β-7 (13). GH family 11 xylanases have a β-jelly roll structure in which the substrate-binding groove is formed by the concave face of the inner β-sheet (14). The only endogenous wheat xylanase characterized to date belongs to GH family 10.

The two-step reaction mechanism for the hydrolysis of the β-1,4-linkages between D-xylosyl residues in arabinoxylan by these retaining GHs is well understood. However, there is a paucity of information on the mechanism underlying their inhibition by naturally occurring inhibitors. To date, two distinct types of xylanase inhibitors have been isolated from wheat, i.e. Triticum aestivum xylanase inhibitor (TAXI) (4) and xylanase-inhibiting protein (XIP)-type endoxylanase inhibitors (5). At least two TAXI-type inhibitors, TAXI-I and TAXI-II, have been identified both with molecular masses of ~40 kDa but differing from one another in pI (8.9 and 9.3, respectively) (15). Both TAXI-I and TAXI-II type inhibitors occur in two molecular forms, A and B. After reduction with 2-mercaptoethanol, form B dissociates into a 10- and a 30-kDa fragment, whereas the molecular mass of form A (40 kDa) is not affected by this treatment. Because the N-terminal sequences of the 30- and 40-kDa polypeptides are identical, the 10- and 30-kDa polypeptides of form B, held together by one disulfide bond, are believed to be derived from form A by processing, possibly mediated by proteolytic enzymes (16). Xylanase inhibitor protein-I (XIP-I) is a glycosylated monomeric protein with a molecular mass of 29 kDa and a pI value of 8.7–8.9. XIP-I possesses a (βα)8 barrel fold and displays structural features typical of GH family 18 but lacks chitinase activity (17). There is no sequence homology between TAXI- and XIP-type inhibitors. Moreover they have different xylanase specificities. Both TAXI-type inhibitors specifically inhibit bacterial and fungal GH family 11 xylanases, with Ki values ranging from 12.3 to 20.1 nM. Although TAXI-I inhibition activity seems to be independent of the pH optima of the xylanases, TAXI-II inhibition of GH family 11 enzymes with low pH optima is weak or absent (15). XIP-I inhibits fungal xylanases from both GH families 10 and 11, with K_i values between 3.4 and 610 nM, with exception of

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The atomic coordinates and structure factors (codes 1T6E and 1T6G) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: GH, glycoside hydrolase; TAXI, Triticum aestivum xylanase inhibitor; ANXI, Aspergillus niger xylanase I; XIP, xylanase-inhibiting protein.
the GH family 10 Aspergillus aculeatus xylanase but does not affect bacterial GH family 10 and 11 xylanases (18). We describe here the x-ray structures of TAXI-I alone and TAXI-I in complex with Aspergillus niger xylanase I (ANXI). Despite very low sequence homology levels, TAXI-I is structurally homologous with the pepsin-like family of aspartic proteases. Reasons are put forward for a lack of proteolytic activity in TAXI-I and the ANXI-TAXI-I complex reveals the molecular basis for the tight binding inhibition. The extensive interactions provide a deeper understanding for the differences in TAXI-I and TAXI-II-type inhibition specificities. TAXI protein homologues are discussed and indicate a physiological function of TAXIs as plant protective microbial GH inhibitors.

**Materials and Methods**

**Crystalization**, Data Collection, and Phasing of TAXI-I—TAXI-I was purified from wheat (var. Soissons) flour as described previously (19). Crystals were obtained in 0.1 M sodium acetate buffer (pH 4.6) containing 23% (w/v) polyethylene glycol 4000 and 0.15 M ammonium sulfate, using the hanging drop vapor-diffusion method at room temperature. TAXI-I crystals belong to space group P2₁2₁2₁ (29) as templates. The orientation of the molecule and two xylanase molecules were independently positioned in the asymmetric unit. According to Matthews coefficient calculations (27), the asymmetric unit consists of three (Ne, Nf, and Ng) and four (Ng, Nf, Nb, and Nc) antiparallel b-sheets, whereas C3 is composed of mixed a-strands, whereas C3 is composed of mixed a-strands. The nomenclature of the secondary structure elements, as the secondary structure elements, were assigned by the Protein Data Bank (PDB) code 1ku1r (29) as templates. The orientation of the molecules was found with data between 15 and 4.0 Å. The positioning of the oriented molecules resulted in the first model of the ANXI-TAXI-I complex when space group P63 was used. Initial rigid body refinement was followed by cycles of maximum likelihood refinement with REFMAC (24) and manual rebuilding. Table I lists the refinement statistics. The final model exhibits good stereochemical parameters with all residues in the allowed regions of the Ramachandran plot. As for the native TAXI-I structure, three surface loop regions of TAXI-I cannot be defined by electron density.

**Results**

**Overall Structure of TAXI-I**—The structure of TAXI-I (residues 1–381) is numbered starting from the first residue of the mature protein as determined by gene sequencing (17). TAXI-I folds as a two b-barrel domain protein with a few helical segments, and the separate domains are divided by an extended cleft (Fig. 1A). A homology search with the program Dali (30) reveals a close structural relationship with the pepsin-like family of aspartic proteases and identified the architecture of the Rhizomucor miehei protease as the most similar structure (Z-score = 27.6).

The nomenclature of the secondary structure elements is illustrated in Fig. 1B. Loop regions will hereafter be referred to as LXyYz, with X and Y as the secondary structure elements, respectively, at the N and the C terminus of the connecting loop.

The six-stranded antiparallel b-sheet B (Na, Nj, Ni, Cz, and Ck) between the N and C domain forms the backbone of the molecule with each domain contributing three strands. In the N domain three b-sheets (N1–N3) are found to be packed orthogonally in a three-layered sandwich. Although N2 and N3 are composed of three (Ne, Nf, and Ng) and four (Ng, Nf, Nb, and Nc) antiparallel b-strands, respectively, sheet N1 consists of seven (Na, Nb, Ne, Nh, Nd, Ng, and Nf) mixed b-strands. A fairly similar image is seen in the C domain, three (C1–C3) b-sheets of which, C1 (Cl, Co, Cx, Cg, and Cw) and C3 (Ct, Cg, Cm, and Cn), are orthogonally packed in a two-layered sandwich. C1 and C2 (Cq, Cr, Cc, and Cu) are formed out of anti-parallel b-strands, whereas C3 is composed of mixed b-strands. Three long loops LNaH, LHeNe, and LNeNe₃ together forming

**Table I**

| Protein    | TAXI-I | TAXI-I-ANXI |
|------------|--------|-------------|
| Space group| P2₁2₁2₁| P63         |
| Wavelength used (Å) | 0.811 | 0.934 |
| Resolution limit (Å) | 1.7 (1.73–1.70) | 1.8 (1.90–1.80) |
| Cell parameters (Å) | | |
| a | 50.26 | 88.43 |
| b | 66.72 | 88.43 |
| c | 106.06 | 128.99 |
| X-ray source | X11 DESY | ID4-EH1 ESRF |
| Total observations | 892907 | 623496 |
| Unique reflections | 38462 (1925) | 104587 (15228) |
| Completeness of all data (%) | 95.7 (97.5) | 100 (99.9) |
| Mean I/σ(I) | 27.8 (3.9) | 9.3 (4.2) |
| Rsym value (%) | 3.4 (27.7) | 5.0 (17.5) |
| Resolution range (Å) | 30.0–1.70 | 76.7–1.8 |
| Number of reflections used | 36023 | 94039 |
| Number of reflections used in Rmerge set | 3939 | 10511 |
| Rmerge/Rmerge,c | 0.182/0.214 | 0.156/0.192 |
| Number of protein atoms | 2583 | 8102 |
| Number of solvent atoms | 330 | 1072 |
| Root mean square deviation | | |
| Bond angles (°) | 1.46 | 1.49 |
| Bond lengths (Å) | 0.013 | 0.013 |

a Values in parentheses indicate data in the highest resolution shell.

b Refinement was followed by cycles of maximum likelihood refinement with REFMAC (24) and manual rebuilding. Table I lists the refinement statistics. The final model exhibits good stereochemical parameters with all residues in the allowed regions of the Ramachandran plot. As for the native TAXI-I structure, three surface loop regions of TAXI-I cannot be defined by electron density.

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the outer rim of the N domain, are stabilized by disulfide bridges between Cys50 and Cys71, Cys55 and Cys80, and Cys66 and Cys92. The antiparallel topology of these three strands, together with the positions of the half-cysteines constituting the three disulfide bridges, makes the portion Cys50-Cys92 of TAXI-I look like a knottin domain. The small structural knottin motif is characterized by a triple-stranded /H9252-sheet to which three disulfide bonds between Cys1-Cys4, Cys2-Cys5, and Cys3-Cys6 (Cys1 being the first cysteine at the N-terminal end of the domain) in a “disulfide through disulfide knot” provide a rigid framework (31). In TAXI-I, however, none of the disulfide bridges “thread” through the macrocycle formed by the two other disulfide bonds and their interconnecting backbone. Therefore, this small cysteine-rich TAXI-I domain can be classified as knottin-like. Loop LNdHa is further connected to the end of /H9252-sheet Ng by a fourth disulfide bridge between Cys39 and Cys124. The TAXI-I structure also presents a disulfide bond between the \( \beta \)-strands Cr and Cv (Cys282-Cys327) of the C domain and one interdomain disulfide connection between Cys282 and Cys778. This interdomain bond is not only of particular interest with regard to the defined molecular forms A and B of TAXI-type inhibitors, in addition to hydrophobic interactions between side-chain atoms, it also procures the major maintaining force for the position of the C-terminal loop LCzCterm.

SDS-PAGE analysis under reducing circumstances on dissolved TAXI-I crystals indicates the presence in the crystal of both molecular forms A and B (data not shown). Hence, the absence of clearly defined electron density for residues His263, Ala264, Asn265, Glu266, Ala267, Pro268, and Val269 can be explained, because form B presumably results from a proteolytic cleavage of form A after Asn265 (16). These residues are part of loop LNHeCq connecting the longest \( \alpha \)-helix He present in the structure, with \( \beta \)-sheet C2, the outer rim of the C domain (Fig. 1). The loop LNHeCq is believed to protrude deeply into the solvent and hence to be very flexible. This freedom of movement can be inspected in the crystal packing, for which the N domain makes many more surface contacts with symmetry-related molecules than the C domain. It is also reflected in the slightly higher individual \( B \)-factors of the residues adjacent to this region. In our opinion, the partial cleavage has no profound effect on the overall architecture of TAXI-I, and hence both molecular forms can coexist in TAXI-I crystals.

Comparison with Pepsin Fold and Lack of Proteolytic Activity of TAXI-I—The structure of TAXI-I is highly similar to the core structure of R. miehei aspartic protease (PDB code 2rmp) (32) with a root mean square deviation of 1.78 Å for 241 pairs of C\(^{\alpha} \) atoms while sharing only 15.3% of sequence identity over the equivalenced positions. Differences between the structures are confined primarily to the loop regions. Similar statistics for secondary structure matching were found for other proteins of the pepsin-like family with an aspartic protease fold. According to the program Dali (30), fungal endothiapepsin, yeast and fungal acid protease, human and mouse renin, and plant phytepsin folds all display an equal strength in structural similarity and an equally low degree of sequence identity (max-
A structure-based sequence alignment of aspartic proteases deposited in the PDB reveals 23 strictly conserved residues. Each of them occupies a similar spatial position in all proteins, believed to serve as conformational determinants specifying the uniqueness of the fold. Therefore, it is striking that in TAXI-I only seven of them are conserved. Tyr16 and Trp36, in β-strand Nb and Nd, are involved in mediating two separate loops through hydrogen bonds. Tyr16 O'' is hydrogen-bonded (2.6 Å) to O'' to Glu354 on loop LNeNH in central sheet B. Trp36 fills a large void in the N domain, with N stabilizing the main-chain Tyr85 oxygen in loop LNeNH. Gly142, Gly180, and Gly349 are invariably located at a turn and ascertain the peculiarities of this fold. Lastly, Asp29 and Thr236 are part of the two catalytic triads known to constitute the active site of aspartic proteases.

With only a few exceptions, aspartic-type peptidases attack the scissile peptide bond by a nucleophilic water molecule activated by two Asp residues, located in two Asp-Thr-Gly motifs at the active site. With respect to the standard numbering system for mature pepsin, residues Asp32, Thr33, Gly34, Asp215, Thr216, and Gly217 are shown to be Asp29, Val30, Ala31, Ser235, Thr236, and Arg237 for the equivalent positions in TAXI-I (Fig. 2A). The replacement of Asp by Ser in position 235, together with a subtle change in the placement of the nucleophilic solvent molecule, normally situated medially between the two active-site Asp residues, might be expected to thwart the nucleophilic capability of the water molecule and hence render TAXI-I proteolytically inactive. A similar observation is seen in a model of the structure of bovine pregnancy-associated glycoproteins, a class of proteins structurally related to pepsin and for the greater part proteolytically inactive (33). In addition, the position of the Arg237 side chain, strongly hydrogen bonded to Asp29 through N* to O'' (2.86 Å) and N' to O'' (2.77 Å), is such that the P1-P1' binding position of a possible substrate is occupied. This completely covers the “active-site area” and hence prevents access by a possible substrate (Fig. 2A). Finally, another significant difference between pepsin-like aspartic proteases and TAXI-I is the position of the so-called “flap...
region” (34), a β-hairpin loop crucial for the substrate-specificity. A determining factor for the positioning of this region in pepsin is the orientation of Tyr14, which is juxtaposed with Trp36 and defined by an O5-O4 distance of 3.1 Å. In TAXI-I, this whole region is tilted inwards, driven by the aforementioned hydrogen bond between Trp36 N and the main-chain Tyr36 oxygen, resulting in a closure of the “substrate-binding area” (Fig. 2B).

**Interaction Interface**—The structure of the ANXI-TAXI-I complex reveals extensive interactions, resulting in the burial of 992 Å² of accessible surface area. Five TAXI-I loop regions (LHhCy, LHhCy, LHCy, and LCCterm) completely cover the deep substrate-binding and active site cleft of the xylanase through ionic, hydrogen-bonding, hydrophobic, and water-bridged contacts (Fig. 3A). Upon binding, no major conformational changes take place in the TAXI-I structure. In the xylanase, the “thumb” region is displaced (in accordance with the description by Törnönen et al. (35) based on the resemblance of the overall structure to a right-hand shape) with respect to the uncomplexed ANXI structure (PDB code 1ukr) (29). This region, a β-hairpin loop encompassing residues 110–130, points back toward the bottom of the cleft (or “hand palm”) with the shortest spanning distance of 4.3 Å (Pro110 O6 to Tyr14 O6). Upon intercalation of the loop LCCterm of TAXI-I, this distance is lengthened to 7.35 Å. Changes in this region have previously been observed when comparing native xylanase and substrate-liganded xylanase structures. They are driven by changes in torsion angles mainly concentrated in the area at the base of the thumb. In the ANXI-TAXI-I complex, this movement originates from side-chain rotations at the “tip of the thumb.” The side-chain χ-2-torsion angle of Arg115 is flipped by 180°, compared with the uncomplexed structure, forming three hydrogen bonds with TAXI-I main-chain oxygen atoms in loop LCCterm. In Glu118, a rearrangement of 94° in the χ-2-torsion angle results in two hydrogen bridges with O6 of Asp155 on the LHhCy loop of TAXI-I.

**Structural Basis for the Inhibition of Xylanase**—The active site of GH family 11 xylanases contains two conserved Glu residues located on either side of the extended open cleft. Hydrolysis of xylosidic substrates proceeds via a double displacement mechanism in which the nucleophilic catalyst Glu79 forms a covalent glycosyl enzyme intermediate, which is subsequently hydrolyzed by the acid/base catalyst Glu170 (numbers as described previously (29)). To this end, both Glu residues are part of an extended hydrogen bond network, which is capable of fine tuning their respective catalytical properties and of which the constituting residues function as substrate-binding and positioning sites. These sites, that bind the glycon or aglycone regions of the substrate, are pre-fixed by − and +, respectively, and the number that identifies each sub-site is related to the proximity to the site of bond cleavage, which takes place between substrates −1 and +1 (29).

In the ANXI-TAXI-I structure, the His374 imidazole ring of TAXI-I is located in between the two catalytic Glu residues of ANXI (Fig. 3B). The Nδ atom virtually takes the place of the xylose C-1 atom in subsite −1 during hydrolysis and is highly stabilized through ionic interaction with Glu79 O4 (distance 2.63 Å). Furthermore, the Nε2 atom is hydrogen bonded to Glu170 O4 and Asp37 O6 of the xylanase with respective distances of 3.16 and 2.69 Å. Additional contacts between Nδ4 and Tyr14 O6 (3.65 Å), main-chain oxygen and Arg115 Nε2 (2.95 Å), and main-chain N and Asp37 O4 (2.89 Å) make this His374 the key residue for the inhibition of this xylanase by TAXI-I. More contacts in the glycon sub-site −1 are made by two main-chain oxygen atoms of Phe77 and Thr79 in the LCCterm loop that form hydrogen bonds with Gln129 Nδ and Arg115 Nε2, of the xylanase. In the −2 glycon sub-site Leu292 on loop LHhCy, perfectly mimics the position of xylose −2. In a superimposition of the structure of a catalytically inactive Bacillus circulans xylanase mutant complexed with xyloobiose (PDB code 1bcx) (36) (in substrates −1 and −2) with the structure of the ANXI-TAXI-I complex (root mean square deviation of 0.6 Å for 181 xylanase Ca atoms), five Leu292 atoms (N, Cα, Cβ, Cγ, and Cδ) coincide with C-5, O-5, C-1, C-2 and O-2 of the xylose in subsite −2 (Fig. 3B). On the aglycone site, where substrates are believed to be determined by Trp172 (subsites +1 and +2) and Tyr89 (subsite +3), TAXI-I interferes with loop LHhCy to form four more hydrogen bonds. That between Gln187 oxygen and Trp172 Nε1 directly interferes with subsites +1 and +2, whereas three others (Gln190 Nε2-Ser84 O7, Gln190 Nδ2-Asp88 O5, and Ser81 O7-Ser73 O7) presumably prevent access to the aglycone end through steric hindrance.

**DISCUSSION**

TAXI-I displays significant sequence similarity with a carrot extracellular dextrin glycoprotein (37), a tomato xyloglucan-specific endoglucanase inhibitor protein (38) and with TAXI-like proteins (16) identified in barley (HXVI), rye (SCXI-I to SCXI-IV), and durum wheat (TDXI-I and TDXI-II). In addition, screening of the available data bases revealed that multiple TAXI-I homologous genes also occur in soybean, rice, and Arabidopsis thaliana (16). Several amino acids in the TAXI-I sequence, notably 10 of the 12 cysteines, are generally conserved among all TAXI-I-related sequences suggesting a fundamental structural similarity. Corresponding EST data from cereals show that TAXI-like genes are expressed in different tissues at different stages of plant development and under plant stress conditions. Based on a rapid increase of the carrot extracellular dextrin glycoprotein mRNA level in response to wounding, it was suggested to be a plant defense protein (37). Recent characterization of tomato xyloglucan-specific endoglucanase inhibitor protein revealed a possible plant defense function as an inhibitor of a microbial glucanase of GH family 12 (38). Moreover, GH family 12 glucanases share a common β-sandwich fold with GH family 11 xylanases. We therefore can conclude that to TAXI-like proteins a function as plant protective microbial GH inhibitor can be ascribed and that the TAXI-I protein structure presented here is the first structure of this newly identified class of plant proteins.

The ANXI-TAXI-I structure reveals a direct interaction of the inhibitor with the active site region of the enzyme and further substrate-mimicking contacts with binding subsites filling the whole substrate-docking region. It can be postulated that other members of this newly identified class of plant proteins use a similar interaction interface and hence that their specificities are determined by the residues constituting this area. In this regard, the observed difference in xylanase specificity between TAXI-I and TAXI-II can be attributed to the C-terminal loop. A TAXI-II candidate sequence (GenBank Accession Code BT009260) shows this loop to be lengthened by six extra C-terminal residues that seem the importance of loop LCCterm in the ANXI-TAXI-I complex structure may well interfere upon binding with xylanases. Furthermore, because His374 on this LCCterm loop is strongly hydrogen bonded to Asp37, known to be a determinant factor for the “acidic” GH family 11 hydrolases and replaced by Asn in xylanases that function optimally under more alkaline conditions, it can be postulated that a subtle change in the positioning of the loop LCCterm might affect the specificity of TAXI-II with respect to the xylanase pH optimum. Because it is known that the ambient pH is a determining factor for the positioning of this loop LCCterm, per-
Structural Basis for Inhibition of Xylanases

xylanases that thrive over a broad pH range. In this context, the presence in cereals of different classes of xylanase inhibitors, with different specificities and expression patterns, could be explained (16). XIP-I, representative for the other class of xylanase inhibitors isolated from wheat, is active against both GH family 10 and 11 fungal hydrolases (27). Both classes interfere with the activated enzyme by interacting at the active site by substrate mimicry (Payan et al. (47)).

The secondary structure-based superimposition of TAXI-I with *R. miehei* aspartic protease highlights a structural relationship between TAXI-type xylanase inhibitors and proteins of the pepsin-like family of aspartic proteases, which was not obvious from overall sequence homology. There are several reasons to speculate that TAXI-I divergently evolved in an early stage from a pepsin-like aspartic protease ancestor. The bilobular nature of the pepsin-like family of aspartic proteases with inherent pseudo 2-fold symmetry results from a tandem duplication and the fusion of a gene encoding an ancestral retroviral protease acting as a dimeric homologue of pepsins. Because duplicated genes are likely to be quickly lost as a result of Darwinian selection, unless their products acquire a function, it is tempting to believe that the proteolytic function was first preserved. Then, once duplicated, genes became separated by a burst of amino acid replacements that allowed a specific function to be established. A similar adaptability of the pepsin fold can be found in the multigene family of pregnancy-associated glycoproteins, where most members lost proteolytic activity. Nevertheless, all are capable of binding specific peptides or proteins, reflecting their possible function as carrier of targeting molecules (40). A second argument in favor of an early divergent evolution is the fact that TAXI-I shares an equally low degree of sequence identity for an equally high structural homology throughout the different members of the pepsin-like family of aspartic proteases independent of their different specific origin. Thirdly, the existence of two molecular forms for TAXIs, with apparently the same inhibitory activity (16), may be an evolutionary remainder of the presence of a plant-specific insert found in most pepsin-like plant aspartic proteases. To date, two different plant acid proteases have been structurally characterized, *i.e.* cardosin A from *Cynara cardunculus* L. (41) and phytepsin from *Hordeum vulgare* L. (42). In a superimposition of TAXI-I with barley phytepsin (PDB code 1qdm) (43), with a root mean square deviation of 2.48 Å for 264 pairs of Cα atoms, the TAXI-I loop Leu292-containing residue Asn265 virtually coincides with that surface loop of phytepsin where the plant-specific domain is attached. In plants, the saposin-like plant-specific insert is precisely removed during processing. For cardosin A, this well studied phenomenon proceeds through an asparaginyl-specific cleavage of an Asn-Gly bond (43). TAXI-I molecular form B most probably results from proteolytic cleavage of form A between Asn265 and Gly266 (16). Finally, an inherent pseudo 2-fold symmetry for TAXI-I is less obvious than in most members of the pepsin-like family of aspartic proteases and is disturbed by the presence of the knottin-like motif in the N domain. In a sequence or structural alignment with pepsin-like proteins this cysteine-rich TAXI-I portion is most divergent. Knottins are small individual proteins demanding a two-step folding process (44). In knottins, an extreme stability is achieved in the knotted disulfide topology, for which a wide variability in constituting residues is tolerated located in between the two catalytic residues (Glu79 and Glu779) of ANXI and is strongly hydrogen bonded to Asp37. In a superimposition of the structure of a catalytically inactive *B. circulans* xylanase mutant complexed with xylooligosaccharide (PDB code 1bxc) (36) (in subsite −1 and −2) with the structure of the ANXI-TAXI-I complex, Leu292 on loop Leu292 perfectly mimics the position of a xylose molecule in subsite −2.
Structural Basis for Inhibition of Xylanases

The fact that this domain is embedded in TAXI-I probably hampers the knot to be “tied.”

In conclusion, we demonstrated the ability of TAXI-I to strongly inhibit A. niger xylanase with His374 as a key residue and the shape and length of the C-terminal loop L_{C-term} as a possible determinant for xylanase specificity. These insights will help us to understand the influence, efficiency, and functionality of microbial xylanases in the aforementioned biotechnological applications. In addition, these results also hold promise for the further characterization of other TAXI-like proteins, which might provide more evidence for a role in plant defense.

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