Solution NMR Structures of Pyrenophora tritici-repentis ToxB and Its Inactive Homolog Reveal Potential Determinants of Toxin Activity*

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Afua Nyarko1,2, Kiran K. Singarapu1,3, Melania Figueroa3, Viola A. Manning4, Iovanna Pandelova4, Thomas J. Wolpert5, Lynda M. Ciuftetti6, and Elisar Barbar15

From the 1Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, the 2Center for NMR and Structural Chemistry, Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad 500007, India, and the 3Department of Botany and Plant Pathology and Center for Genome Research and Biocomputing, Oregon State University, Corvallis, Oregon 97331

Background: ToxB is a proteinaceous toxin but its homolog toxb has no toxic activity.

Results: Both adopt a β-sandwich fold stabilized by two disulfide bonds but differ in the dynamics of one sandwich half.

Conclusion: Toxicity is correlated with decreased compactness, increased flexibility, and polymorphism in an active site loop.

Significance: ToxB activity depends on interplay between internal dynamics and interactions with putative targets.

Pyrenophora tritici-repentis Ptr ToxB (ToxB) is a proteinaceous host-selective toxin produced by Pyrenophora tritici-repentis (P. tritici-repentis), a plant pathogenic fungus that causes the disease tan spot of wheat. One feature that distinguishes ToxB from other host-selective toxins is that it has naturally occurring homologs in non-pathogenic P. tritici-repentis isolates that lack toxic activity. There are no high-resolution structures for any of the ToxB homologs, or for any protein with >30% sequence identity, and therefore what underlies activity remains an open question. Here, we present the NMR structures of ToxB and its inactive homolog Ptr toxb. Both proteins adopt a β-sandwich fold comprising three strands in each half that are bridged together by two disulfide bonds. The inactive toxb, however, shows higher flexibility localized to the sequence-divergent β-sandwich half. The absence of toxic activity is attributed to a more open structure in the vicinity of one disulfide bond, higher flexibility, and residue differences in an exposed loop that likely impacts interaction with putative targets.

We propose that activity is regulated by perturbations in a putative active site loop and changes in dynamics distant from the site of activity. Interestingly, the new structures identify AvrPiz-t, a secreted avirulence protein produced by the rice blast fungus, as a structural homolog to ToxB. This homology suggests that fungal proteins involved in either disease susceptibility such as ToxB or resistance such as AvrPiz-t may have a common evolutionary origin.

Pyrenophora tritici-repentis, the causal agent of tan spot of wheat (Triticum aestivum), is a plant pathogenic fungus that produces host-selective toxins that induce cell death and disease susceptibility only in hosts that have a toxin sensitivity gene/locus (1–5). To date, three host-selective toxins have been defined from P. tritici-repentis, the necrosis-causing Ptr ToxA (ToxA), and the chlorosis-causing Ptr ToxB (ToxB) and Ptr ToxC (ToxC). ToxA and ToxB are proteinaceous toxins, and the genes encoding them have been cloned and characterized (6–9). Functional studies of ToxA indicate that it localizes to the cytoplasmic compartments and to chloroplasts where it exerts a toxic effect (10). Structural and mutagenic analyses reveal a single-domain protein composed of a β-sandwich fold that contains an Arg-Gly-Asp motif on a solvent exposed loop, which is important for toxin uptake and biological activity (11–14).

Although progress has been made in understanding the role of ToxA in the development of tan spot, thus far no putative functional site for ToxB has been identified, and its impact on disease development is less well understood. This is in part because its amino acid sequence is less than 30% identical to any protein with known structure, and thus does not reveal any identifiable motif(s). ToxB is synthesized as an 87-amino acid preprotein that upon cleavage of its secretory signal peptide results in a 64-amino acid mature protein. It appears to have a complex evolutionary history as suggested by the presence of homologs in non-pathogenic P. tritici-repentis isolates and several pathogenic ascomycetes (15). For example, some homologs found in isolates of Pyrenophora bromii (Pb ToxB) are not toxic.
to the *P. bromi* host, bromegrass, but are toxic to ToxB-sensitive wheat cultivars, whereas other homologs such as a naturally occurring variant of ToxB, Ptr toxb (toxb), found in some non-pathogenic *P. tritici-repentis* isolates have no toxic activity (16, 17).

Assessment of the biological activity of ToxB/toxb chimeric and site-directed mutant proteins has demonstrated complexity in the ToxB structure-function relationship (16). Chimeric proteins that contain combinations of three broadly defined regions (N-terminal, central, and C-terminal) (see Fig. 1A) identify the N-terminal region of ToxB as crucial for toxin activity and also show the importance of both the central and C-terminal regions for full activity (16). Within the N-terminal regions of ToxB and toxb, there are only two amino acid differences: a Val-to-Thr at position 3, and an Ala-to-Val at position 12. ToxB mutants with either a V3T or A12V substitution also show reduced toxin activity (Fig. 1B).

In an attempt to understand the residue specific contributions to activity, we report here the three-dimensional NMR structures of ToxB and toxb proteins and analyze the structural features required for toxin activity. The global solution structures are virtually identical. However, toxb displays increased flexibility and an expanded structure, which together with an increased propensity of toxb to disulfide bond reduction, and residue differences in a putative active site, suggest that absence of the central and C-terminal regions (N-terminal, central, and C-terminal) (see Fig. 1) identifies the N-terminal region of ToxB as crucial for toxin activity and also show the importance of both the central and C-terminal regions for full activity (16). In active ToxB, these disulfide bonds are highly resistant to reduction even in high concentrations of a reducing agent and temperatures of 100 °C (16). To determine whether toxb is more prone to reduction than ToxB, we recorded relaxation delays of 15.4, 30.9, 61.8, 77.2, 92.6, 108.1, 123.5, and 138.9 ms. To determine whether toxb is more prone to reduction than ToxB, we recorded relaxation delays of 15.4, 30.9, 61.8, 77.2, 92.6, 108.1, 123.5, and 138.9 ms. T2 values were determined by fitting the measured peak heights versus time profiles to the relationship: $I = I_0 \exp(-t/T_2)$, where $t$ is the relaxation delay, $I$ is the intensity of the peak measured at time $t$, and $I_0$ is the initial intensity of the peak. Data were analyzed with the rate analysis interface of NMRview (22).

A series of 1H-15N NOEs were collected on lyophilized samples of 15N-labeled ToxB or toxb dissolved in D$_2$O. Amide protons were considered to be involved in hydrogen bonding if they were still visible after the first round of HSQC experiments. For each hydrogen bond, two distance restraints were applied for H$_3$N(i)-O(j) and N(ij)-O(j).

**Structure Calculation**—NMR structures of ToxB and toxb were calculated using NOEY data refined with identical protocols. Initial structures were calculated with CYANA (version 3.0) (23) using resonance assignments, NOEY peak lists, hydrogen bond restraints from hydrogen/deuterium exchange experiments, and dihedral angle restraints derived from TALOS+ (24) using 1H, 15N, 13C$_\alpha$, 13C$_\beta$, and 13C chemical shifts. The automated structures generated by CYANA were used along with the assigned peaks to further refine the structures. The root mean square deviations (r.m.s.d.)$^6$ were calculated with MOLMOL (25). The PSVS server (26) was used to check the quality of the structures.

The ensembles of structures were analyzed using in-house r.m.s.d.-based structure comparison algorithm.$^7$ For ease of comparison, Pro$^{49}$ insertion in toxb was not included in the analyses.

**RESULTS**

**Disulfide Bond Stability**—Sequence comparison of mature ToxB and toxb shows 78% identity (27), with conservation of 51 amino acids including four cysteines that form two disulfide bonds (Fig. 1A). In active ToxB, these disulfide bonds are highly resistant to reduction even in high concentrations of a reducing agent and temperatures of 100 °C (16). To determine whether toxb is more prone to reduction than ToxB, we compared their reducing and non-reducing SDS-PAGE patterns (Fig. 1C). In both conditions, ToxB migrates as a single

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$^6$ The abbreviation used is: r.m.s.d., root mean square deviation.

$^7$ P. A. Karplus, personal communication.
NMR Structures of ToxB and toxb

A sequence alignment of ToxB (accession no. AY007692) and toxb (accession no. AY083456). Amino acids that are different between the two proteins are shaded. Stars indicate identical residues; colon indicates conserved residues; period indicates semi-conserved. 

B, semi-quantitative biological activities of chimeras composed of the N-terminal, central, or C-terminal regions of ToxB (T) and toxb (t) show them all to have activities between the fully active native ToxB (TTT) and the inactive native toxb (ttt). Intermediate activity is also observed for ToxB mutants, V3T and A12V. All chimeras and mutants that include the N-terminal region of toxb show two bands on SDS-PAGE (as can be seen in Fig. 4A of Ref. 16). C, reducing (R) and non-reducing (N) SDS-PAGE of purified ToxB and toxb proteins. The size of the molecular mass marker is indicated on the left of the gel.

band in the oxidized form. In non-reducing conditions, the predominant band in toxb migrates similar to the oxidized form of ToxB; there are very faint bands at ~13 and 26 kDa, which may represent disulfide cross-linked dimers and tetramers. Multimeric forms of toxb were not detected in samples on which MALDI-TOF and gel filtration were performed (data not shown). Therefore, it appears that a very small population of toxb may have reduced cysteines that are prone to intermolecular disulfide bond formation leading to multimerization at high protein concentrations. Consistent with this interpretation, the disulfide bonds in toxb are more prone to reduction; under reducing conditions, only about half of toxb is present in the oxidized form and half in an ~14-kDa slower migrating band that we assign to reduced toxb. A disulfide cross-linked dimer would not exist under reducing conditions and the increased proportion of the ~14-kDa band in the reduced toxb sample relative to the similar migrating band present under non-reducing conditions supports the conclusion that the slower band is the reduced form of toxb.

Reducing SDS-PAGE for all chimeras show that only those that include the N-terminal region of toxb have a second slower migrating band (Fig. 1B), indicating that one or both of the two amino acid differences in the N-terminal is responsible for the increased ease in reduction. Interestingly, the slower migrating band is only present in reducing SDS-PAGE of the V3T substituted ToxB mutant protein and not in the A12V, even though both mutations lead to reduced activity. This suggests that an altered protein conformation in the vicinity of residue 3 may increase disulfide bond accessibility allowing for reduction of a population of molecules and thus the slower migrating band.

Solution Structures of Active and Inactive ToxB Homologs—Both ToxB and toxb show well resolved spectra consistent with an ordered structure (Fig. 2A). Differences in chemical shifts for the same amino acids in the sequence suggest small differences in their local environment. Interestingly, their global structures are virtually indistinguishable. ToxB adopts a β-sandwich fold consisting of six anti-parallel β-strands corresponding to residues 2–8 (β1), 14–19 (β2), 22–28 (β3), 34–40 (β4), 43–46 (β5), and 57–62 (β6) (Fig. 2C). Strands β1, β2, and β6 form one-half of the sandwich, and β3, β4, and β5 form the other. A disulfide bond between Cys2 in β1 and Cys13 in β5 bridges the halves; a second disulfide is formed between Cys18 in β2 and Cys64 in ToxB at the C terminus. The propensity to form disulfide bridges was initially inferred from the Cβ chemical shifts of the cysteines, which were higher than the 29–32 ppm range expected for Cβ chemical shifts of reduced cysteines (28). Specific disulfide bonding patterns were unambiguously assigned from weak NOE interactions between the side chain β protons of Cys2 and Cys43, and Cys18 and Cys64 (Cys65 in toxb). Inactive toxb has nearly identical structure (Fig. 2D), except for a slightly longer β5 (43–47) and shorter β6 (58–61). Subtle differences between the structures were identified by analysis of the ensemble structures as discussed below.

The ensembles of 20 energy-minimized conformers for both structures (Fig. 2B and E) show well defined regular secondary structure regions with small backbone displacements, but a less well defined loop region connecting β3 and β4. The average global r.m.s.d. values for the ToxB and toxb ensembles are 0.19 and 0.28 Å, respectively, for backbone residues in regular secondary structure and 0.78 and 0.63 Å for all heavy atoms.
(Table 1). A plot of residue-level r.m.s.d. values computed from iterative superposition of the structures and using only a designated subset of atoms is shown in Fig. 2F. Overall, the r.m.s.d. values are lower in ToxB than toxb, except for the long loop connecting β5 and β6 corresponding to residues 47–55 which has an average r.m.s.d. value of 0.9 Å in ToxB versus 0.4 Å in toxb. Backbone atoms within residues 41–44 and C-terminal residues 62–64/65 are poorly defined only in toxb (average r.m.s.d. values of 1.0 and 1.8 Å, respectively, with corresponding values in ToxB of 0.4 and 0.7 Å).

Differences between ToxB and toxb ensembles of structures (overlay in Fig. 2E) are highlighted in Fig. 3A. Differences between the ToxB and toxb ensembles (r.m.s.d. differences shown as gray area plot) are attributed to those regions that have higher values than the corresponding values for ToxB and toxb, shown in blue and orange, respectively. Residues 16–17, 27, 38–39, 44, 51–52, 56–57, and 60–61 have values greater (difference > 0.3) than the r.m.s.d. values of the individual proteins. Residues with values significantly greater (difference > 0.5) than the r.m.s.d. values of the individual proteins are mapped onto the ribbon representation of ToxB (Fig. 3A, right).

ToxB and toxb have 13 non-identical amino acid residues (shaded in Fig. 1A) and in Fig. 3B these are mapped onto the
NMR Structures of ToxB and tox b

### Table 1

| Statistics of NMR structures of ToxB and tox b |
|-----------------------------------------------|
| NOE-derived distance constraints              |
| ToxB (Å)                                      |
| tox b (Å)                                     |
| Intra-residue \( (i = j) \)                    |
| 205                                           |
| 303                                           |
| Sequential \( ([i − j] = 1) \)                 |
| 382                                           |
| 273                                           |
| Medium range \( 1 < |i − j| ≤ 5 \)            |
| 182                                           |
| 132                                           |
| Long range \( |i − j| > 5 \)                    |
| 577                                           |
| 368                                           |
| Total                                         |
| 1346                                          |
| 1076                                          |
| Dihedral angle constraints \( \psi \)         |
| 40                                            |
| 46                                            |
| H-bonding constraints                          |
| 21                                            |
| 19                                            |
| No. of contacts per residue                    |
| 22.5                                          |
| 18.2                                          |
| No. of long range contacts per residue         |
| 9.0                                           |
| 5.7                                           |
| Average r.m.s.d. to the mean CYANA coordinates \( Å \) |
| 0.19 ± 0.03                                   |
| 0.28 ± 0.06                                   |
| Regular secondary structures backbone         |
| 0.78 ± 0.10                                   |
| 0.63 ± 0.15                                   |
| Residues 2–63 backbone                         |
| 0.37 ± 0.17                                   |
| 0.40 ± 0.11                                   |
| Residues 2–63 backbone heavy atoms             |
| 0.92 ± 0.17                                   |
| 0.80 ± 0.15                                   |
| PROCHECK raw score \( \psi \)                 |
| -0.77/0.87                                   |
| -0.61/-0.71                                   |
| PROCHECK Z-scores /all dihedral angles/        |
| -2.71/5.14                                   |
| -2.08/-4.20                                   |
| MOLPROBITY raw score/Z-score                  |
| 35.07/4.49                                   |
| 34.28/4.36                                   |

### Ramachandran Plot Summary for Ordered Residues \( \% \)

| CYANA target function                  |
|----------------------------------------|
| Conformer \( \% > 0.5 \)               |
| 0.81 ± 0.10                            |
| 0.95 ± 0.12                            |
| Average number of dihedral-angle violations per CYANA conformer \( \% > 0.5 \) |
| 0.0                   |
| 0.0                   |
| Average number of Van der Waal violations per CYANA conformer \( \% > 0.5 \) |
| 0.0                   |
| 0.0                   |

### Restraint violations

| CYANA target function \( Å \) |
|--------------------------------|
| 0.81 ± 0.10                   |
| 0.95 ± 0.12                   |
| Conformer \( \% > 0.5 \)       |
| 0.0                           |
| 0.0                           |
| Average number of distance violations per CYANA \( Å \) |
| 0.0                           |
| 0.0                           |
| Average number of Van der Waal violations per CYANA conformer \( Å \) |
| 0.0                           |
| 0.0                           |

### Statistics of NMR structures of ToxB and tox b

- **ToxB** and **toxb**
- **Necessary for chlorosis-inducing activity of ToxB**
- **Greater distance between Thr3 and Gly63 abolishes interstrand**
- **The main chain carbonyl oxygen of Ala12**
- **Val in the loop region separating**
- **The most significant differences between ToxB and tox b**
- **ToxB Homologs—**

**Dynamics of ToxB and tox b—**

- **Steady-state 1H-15N heteronuclear NOEs**, sensitive to the mobility of individual N-H bond vectors on the picosecond–nanosecond time scale, were measured for each backbone N-H to assess the potential contributions of changes in dynamics to absence of activity. Both proteins are well ordered with ToxB showing homogeneous dynamics throughout the protein and tox b showing more flexibility in specific regions. Taken together, the dynamics data suggest increased flexibility in regions of tox b localized to the β3-loop-β4 and β5-loop-β6 segments and a more expanded overall structure.
the other β-strands (Fig. 4F). Conservation is highest at the beginning and end of β1 (Cys5, Asn6), the end of β2 (Cys18), the end of β4 (Ala39), β5 (Cys43, Gly44), and the C-terminal residues (Gly62 and Cys64). Two conserved residues within loops, Asn10 (within the loop connecting β1 and β2), and Gln50 (within the loop connecting β5 and β6), are not anticipated to contribute to the stability of the fold but could instead play a key role in targeting interactions with partner proteins. The most variable amino acids are located in the β3-loop-β4 segment.

Structural similarity searches of the Protein Data Bank performed with the DALI (30) and PROFUNC (31) servers identify the avirulence protein AvrPiz-t (Protein Data Bank code 2LW6) (32), produced by the causal agent of rice blast disease, Magnaporthe oryzae, as structurally similar to ToxB (sequence identity of 20%, Z-score 5.0, r.m.s.d. of 2.3 Å) (Fig. 5A). Of these residues, only 38 and 51 are different in toxb (Lys/Arg and Val/Ser). The other residues are primarily in spatial proximity to the Cys18-Cys64 disulfide bond; Gly17, which precedes Cys18, is close to Ala39 and Gly61.

Interestingly, inactive toxb is more flexible than active ToxB primarily in residues in β3, β4, and the loop connecting β5 and β6, all of which are located on one-half of the sandwich fold. These regions with higher flexibility correlate with segments of the protein with the most sequence variable residues (β3-turn-β4 segment and the long loop connecting β5 to β6) (Fig. 4E). Such correlation is also observed for a large set of proteins...
FIGURE 4. Dynamics of ToxB and toxb. Plots of steady-state heteronuclear NOE ($I_{\text{sat}}/I_{\text{unsat}}$) and $T_2$ values of individual amino acid residues in ToxB (A and C) and toxb (B and D). The arrows correspond to segments that make up the six $\beta$-strands. E, regions that are significantly more rigid in ToxB (shown in yellow) are mapped onto the ribbon representation of the ToxB structure. $\beta$3 and $\beta$4 are more rigid in ToxB than in toxb. F, the degree of amino acid conservation of ToxB homologs mapped onto the ribbon structure of ToxB (color-coded gradient from blue (variable) to magenta (conserved)). Highly conserved residues (except Gly$^{44}$) are labeled. Sequences from the ToxB protein family were analyzed with ConSurf, a bioinformatics tool for estimating the evolutionary conservation of amino acid positions in proteins. N, N-terminal; C, C-terminal.

TABLE 2

Fungal species with ToxB sequence homologs

ToxB homologs were obtained by a tblastn search of the NCBI GenBank™ nucleotide collection (nr/nt) and the Joint Genome Institute fungal genome database with the mature peptide sequence of ToxB. GenBank™ nos. and genome scaffold locations of homologues are listed in the reference column.

| Organism | Reference | Host | Lifestyle |
|----------|-----------|------|-----------|
| **Class Dothideomycetes** | | | |
| Pyrenophora tritici-repentis | AAM00019.1, AAO73335.1 | Wheat | Necrotroph |
| Pyrenophora bromi | ABR23218.1, ABR23223.1, ABR23217.1, ABR23224.1, ABR23221.1, ABR23220.1, ABR23219.1, ABR23222.1 | Bromegrass | Necrotroph |
| Pyrenophora teres f. teres | Pyrtt1.189019-1311-886.reverse | Barley | Necrotroph/Hemi-biotroph |
| Setosphaeria turcica | Settl1.scaffold_1-1483719-1483288.reverse | Maize | Hemi-biotroph |
| Cochliobolus sativus | Cocs1.scaffold_24-1312-881.reverse | Wheat and barley | Hemi-necrotroph |
| Botryosphaeria dothidea | Botdo1.NODE_6797_length_74690_cov_64.742096-26451-25985.reverse | Broad range of tree species; also endophyte |
| **Class Sordariomycetes** | | | |
| Colletotrichum fioriniae | Gloac1.scaffold_2-291201-290770.reverse | Broad plant host range; also endophyte | Flexible |
| Colletotrichum gloeosporioides | Gloac1.scaffold_14-472062-472507 | Broad host range phyto-pathogen |
| Colletotrichum higginsianum | ELA28482.1, Glocl1.scaffold_5-482195-481750.reverse | Brassica and Raphanus | Hemi-biotroph |
| Magnaporthe oryzae | Glocl1.scaffold_12-782516-782974 | Rice and wheat | Hemi-biotroph |
| Macrophomina phaseolina | CCFl0075.1 | Broad host range phyto-pathogen | Necrotroph |
representing various folds and functional classes (33). Sequence variation in the dynamic region enables the adaptation of the protein to multiple binding substrates while keeping the native fold and is proposed to provide the balance between mobility and chemical specificity (33).

There is growing evidence that allosteric activation/deactivation of proteins in the absence of changes in protein structure is modulated by changes in protein dynamics. The catabolite-activating protein is one such example (34). Other examples include γD-crystallin for which mutations that abolish activity do so by changing the protein dynamics, but not the global structure. Specific examples are the P23T mutant whose altered motional behavior initiates association (35) and the W24R mutant whose increased dynamics increase susceptibility to protease digestion (36). Another example is guanylate kinase, where a single mutation completely changes the protein
NMR Structures of ToxB and toxb

dynamics and function but not the global structure of the protein (37). ToxB appears to belong to this class of proteins whose activity could also be modulated by changes in dynamics rather than structure. An increase in flexibility as observed with toxb implies a larger conformational entropic penalty, which could result in weaker or abolished binding to putative targets and loss of activity (34).

Compactness in the Vicinity of the Cys^{18}^-Cys^{64} Disulfide Bond and Toxin Activity—A variety of evidence points to the possible involvement of changes in compactness in the vicinity of Cys^{18}^-Cys^{64} disulfide bond in regulating activity. First, inactive toxb is more susceptible to disulfide bond reduction as shown by the presence of two bands in SDS-PAGE for toxb only, one for the oxidized protein and one for the reduced. Active ToxB in comparison shows only a single band for the oxidized. Second, chimeric proteins that contain the N terminus of ToxB all resolve as a single band on SDS-PAGE, whereas those that contain the N terminus of toxb resolve as two bands (summarized in Fig. 1B) (16). In the N-terminal chimeric proteins, there are only two amino acid differences between ToxB and toxb: a Val-to-Thr at position 3, and an Ala-to-Val at position 12. When these residues are mutated one at a time, the Val^2 to Thr substitution shows increased propensity to disulfide bond reduction, whereas the Ala^12 to Val substitution shows no change (16). Our explanation for this observation is that a Val to Thr substitution results in a slightly expanded structure, as observed in toxb (Fig. 3C) where the disulfide bond is farther from Gly^{63}. The more open structure of inactive toxb is supported by lower T_2 values due to slower tumbling (Fig. 4) and faster elution of toxb compared with ToxB in gel filtration studies (data not shown). Therefore, activity could be correlated with amino acid substitutions that increase the conformational mobility in one-half of the β-sandwich and that expand the structure in the vicinity of the Cys^{18}^-Cys^{64} disulfide bridge.

Loop 1 Is a Proposed Site for Interactions with Putative Host Targets—Substitution of Ala^{12} with Val in toxb does not change the hydrogen bonding pattern of the protein and has no observable effect on the stability of the loop between β1 and β2 (loop 1). Interestingly, however, this substitution in ToxB renders the protein partially inactive (16). As the Ala-to-Val substitution is not predicted to impact the overall structure and dynamics of the protein, the most plausible explanation for the partial loss in activity is that loop 1 is an important site for host interactions, and the Ala/Val substitution disrupts the interaction surface. Thus far, no putative interacting site(s) or partners have been identified for ToxB or any of its homologs. Further support for loop 1 as an interacting surface comes from two variants of ToxB (P. bromi ToxBs) from P. bromi, the sister species to P. tritici-repentis. P. bromi ToxB1, an active toxin, and P. bromi ToxB2, an inactive toxin, have virtually identical sequences (94.5% identical), are 77–78% identical to ToxB (Fig. 5D) (17), and are expected to adopt a similar fold to the P. tritici-repentis ToxB structures. The most significant difference between the P. bromi ToxB variants is substitution of a neutral Gln with a positively charged Arg, which based on the structure of ToxB reported here, map to loop 1 (the other non-identical residues Leu^{36}/Ser^{36}, and Thr^{49}/Ser^{49} map to β4, and the loop region between β5 and β6, respectively, and are conservative changes expected to have minimal effect on the overall structure and dynamics). The expectation that this single substitution is responsible for inactivation of the P. bromi ToxB homologs supports our inference that loop 1 is an interacting surface where substitutions could impact interactions with a partner and modulate toxin activity. This position is occupied by negatively charged Glu in ToxB. We predict that a change of Gln or Gln to an Arg is enough to disrupt total activity, whereas a change of Ala to Val at a neighboring position in this loop abolishes some of the activity (Fig. 1) (16) but requires increased dynamics caused by additional amino acid differences in toxb to abolish full activity.

Implications of Structural Similarity between P. tritici-repentis ToxB and M. oryzae AvrPiz-t—ToxB has a similar fold to the avirulence effector protein, AvrPiz-t produced by the rice blast fungus, M. oryzae. AvrPiz-t and ToxB are both effectors contributing to the virulence of the fungus that produces them. ToxB is described as a host-selective toxin because its application to wheat tissue causes cell death only on wheat genotypes that contain a dominant gene for toxin sensitivity. Cell death induced by ToxB supports virulence of P. tritici-repentis because it is a necrotrophic pathogen. AvrPiz-t confers virulence to the biotrophic fungus, M. oryzae, by suppressing defense in rice (38). However, if rice contains the dominant Piz-t gene for resistance to M. oryzae, recognition of AvrPiz-t by the product of the Piz-t gene leads to cell death, disease resistance, and a lack of virulence by M. oryzae. Thus, host cell death is shared between susceptibility to P. tritici-repentis and resistance to M. oryzae. The structural homology between ToxB and AvrPiz-t suggests the tantalizing possibility that they also share a common mechanism for causing cell death, and thus, that the gene conferring sensitivity to ToxB, and consequently disease susceptibility in wheat, shares properties of a resistance gene. A shared function between ToxB and AvrPiz-t would provide significant support to the emerging hypothesis that genes that confer resistance to biotrophic pathogens can also confer susceptibility to necrotrophic pathogens (5, 39–41).

Concluding Remarks—In summary, analyses of the structural features of P. tritici-repentis ToxB and its non-toxic homolog P. tritici-repentis toxb identify three potential processes that could render the protein inactive: increased flexibility in one sandwich half, expanded structure in the vicinity of Cys^{18}^-Cys^{64} disulfide, and differences in a solvent-exposed loop region that likely impact interactions with a putative partner. All features are necessary for complete absence of chlorosis-inducing activity and would explain why activity is not fully abolished in chimeras that include either the N-terminal region (region with active site and high resistance to disulfide reduction in ToxB) or both the central and C-terminal regions (regions that display increased dynamics in toxb) (Fig. 1) (16). Sequence-based analyses of variants of P. bromi ToxB, closely related homologs of P. tritici-repentis ToxB, suggest that toxb activity is abolished by a change of Gln/Arg in loop 1 that significantly alters the electrostatic surface of this loop. Taken together, it appears that nature employs two mechanisms to modulate toxic activity: either by introducing a dramatic change at the interface loop as in an inactive form of P. bromi ToxB or by allosteric coupling of
increased protein dynamics and expansion with small perturbations of the active site as in toxb.

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