Structure of Ustilago maydis Killer Toxin KP6 α-Subunit

A MULTIMERIC ASSEMBLY WITH A CENTRAL PORE*

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Ustilago maydis is a fungal pathogen of maize, some strains of which secrete killer toxins. The toxins are encoded by double-stranded RNA viruses in the cell cytoplasm. The U. maydis killer toxin KP6 contains two polypeptide chains, α and β, having 79 and 81 amino acids, respectively, both of which are necessary for its killer activity. The crystal structure of the α-subunit of KP6 (KP6α) has been determined at 1.80-Å resolution. KP6α forms a single domain structure that has an overall shape of an ellipsoid with dimensions 40 Å × 26 Å × 21 Å and belongs to the αβ-sandwich family. The tertiary structure consists of a four-stranded antiparallel β-sheet, a pair of antiparallel α-helices, a short strand along one edge of the sheet, and a short N-terminal helix. Although the fold is reminiscent of toxins of similar size, the topology of KP6α is distinctly different in that the αβ-sandwich motif has two right-handed βαβ split crossovers. Monomers of KP6α assemble through crystallographic symmetries, forming a hexamer with a central pore lined by hydrophobic N-terminal helices. The central pore could play an important role in the mechanism of the killing action of the toxin.

Toxins are protein molecules that disrupt cell functions in a number of ways, some by making ion channels in cell membranes and others by interacting with membrane channels and/or receptors. Three-dimensional structures of several of these toxins have been determined, including cardiotxin (1, 2), δ-endotoxin (3), hemolysin (4), anthrax toxin (5), colicins (6), and diphtheria toxin (7). The tertiary structures of toxins vary widely from being almost entirely β-sheet, as in cardiotoxin, to nearly all α-helical, such as colicins. Ustilago maydis is a fungal pathogen of maize and is one of a number of fungi that secrete cellular killer toxins (8). These killer toxins are encoded by double-stranded RNA viruses in the cell cytoplasm (9). In the absence of an immunity or resistance gene, these toxins are lethal to the organism of origin and sometimes to closely related organisms but not to plant or animal cells. The U. maydis strains P1, P4, and P6 secrete toxins KP1, KP4, and KP6, respectively, all of which are low molecular weight (∼100 amino acids) polypeptides (10–12). The crystal structure of the U. maydis KP4 toxin (a single polypeptide chain of 105 amino acids) has been determined and shown to possess a α/β-sandwich fold (13) having a central five-stranded anti-parallel β-sheet and two α-helices (14). It appears to kill sensitive cells by blocking Ca2+ channels (14). The SMK1 secreted by the halo tolerant yeast Pichia farinosa also has a similar αβ-sandwich structure (15). Williopsis mrakii killer toxin, having a four-stranded antiparallel β-sheet structure similar to that of βγ crystalline (16, 17), inhibits β-glucagon synthesis.

The U. maydis KP6 killer toxin gene has been cloned, sequenced, and expressed, and the protein was characterized (10). The toxin is unique in that two polypeptide chains, KP6α and KP6β, are necessary for its killer activity (18). The 1234-base pair double-stranded RNA P6M2 codes for a pretoxin of 219 amino acids, which is post-translationally cleaved by two endopeptidases, yielding α- and β-subunits having 79 and 81 amino acids, respectively, as determined by cDNA sequence, N-terminal protein sequence analysis, and by mass spectrometry.2 It has been proposed that the KP6 toxin may act by either interfering with the K+ channel or binding to its own membrane receptor (10, 19), thus depleting cellular K+ levels and eventually killing the cells. Here we present the crystal structure of the α-subunit of KP6 toxin determined by the isomorphous heavy atom replacement method and refined at a 1.80-Å resolution. We show that the symmetry-related trimeric or hexameric assembly of the subunit creates a central pore that could have functional implications.

EXPERIMENTAL PROCEDURES

KP6α was purified to homogeneity as described previously (18, 20). The selenomethionine derivative was prepared by growing cells in a medium supplemented with 20 mg/ml selenomethionine. Native protein crystals were obtained by the hanging drop vapor diffusion technique (21). Hanging drops containing 5 µl of 10 mg/ml protein and 1 µl of 85% ammonium sulfate in 10 mM MES buffer, pH 6.0, were equilibrated against 1 ml of reservoir solution containing 18–22% ammonium sulfate in 10 mM MES buffer. Single crystals shaped as hexagonal rods having typical dimensions of 0.15 × 0.30 × 0.30 mm3 were obtained in about 20 days at room temperature. The crystal of the selenomethionine-substituted protein was obtained under the same conditions as the native crystal, but it took longer to grow to a size of 0.1 × 0.2 × 0.2 mm3. The heavy atom derivative was prepared by soaking a single native crystal in 200 µl of 0.5 mM PIP (di-µ-iodosibis(ethylenediamine) diplatinum(II) nitrate) solution in MES buffer, pH 6.0, for 36 days. Three room temperature data sets for native, platinum derivative, and selenomethionine-substituted protein crystals were collected with the in-house RAXIS IIC image plate area detector, receiving x-rays from a Rigaku RU-200 rotating anode generator operated at 50 kV and 90 mA. Graphite-monochromated CuKα radiation was used. Crystals used for

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‡‡ The abbreviations used are: SMK, salt-mediated killer toxin; MES, 2-(N-morpholino)ethanesulfonic acid; PIP, di-µ-iodosibis(ethylenediamine) diplatinum(II) nitrate.

§§ J. Bruenn, unpublished results.
The solution for this crystal was 0.5 mm for 36 days.

Phasing power \( = \Sigma (I - \langle I \rangle)^2/\langle \Sigma (I)^2 \rangle \)

No. of reflections phased 1742 1184 1852

Phasing power (3.0 Å, overall/last shell)
Before phase combining 1.59/1.62 1.86/1.60 1.55/1.16
After phase combining 1.70/1.61 2.44/1.97 1.52/1.01

Mean figure of merit (cent/acent)
Before phase combining 0.27/0.16 0.32 0.53/0.33
After phase combining 0.48/0.26 0.40 0.58/0.37

Overall 0.678b

After solvent flattening
No. of reflections 1792

R factor 0.310

Mean FOMb 0.842

Correlation coefficient 0.936

a SIR, single isomorphous replacement.
b SAS, single anomalous scattering.
c Phasing power = root mean square \( |F_{\text{H}}|/|E| \) where \( |F_{\text{H}}| \) is the heavy-atom structure factor amplitude and \( E \) is the residual lack of closure error.
d Figure of merit \( = -2\langle P(\alpha)\rangle^\alpha/\langle 2\langle P(\alpha) \rangle \rangle \), where \( P(\alpha) \) is the phase probability distribution.
e For 1793 reflections.
f FOM, figure of merit.

Data collection was carried out using four datasets from native, PIP derivative, and selenomethionine protein crystals, respectively. After solvent flattening, MIRAS maps for both hands of the heavy atom positions were calculated, and the correct hand was chosen based on the quality of the maps and evidence of right-handed \( \alpha \)-helices. Chain-tracing and protein model building were performed manually on a SGI Elan workstation using CHAIN (27). The location of the selenium position played a key role in the tracing of the chain by identifying the only methionine in the \( \beta \)-strand near the C terminus. Identification of the N-terminal \( \alpha \)-helix also helped to complete the model building process. The complete polypeptide chain of 79 residues was built into this MIRAS electron density map prior to refinement. A 2\( F_o - F_c \) density map of the single methi-
one-containing β-strand is shown in Fig. 1 along with the independent determination of the selenium position from the cross-difference Fourier with the phases from the PIP derivative alone.

The model was refined with XPLOR (28) using the simulated annealing procedure. The resolution was gradually extended to 1.8 Å. Positions and isotropic parameters of a total of 690 non-hydrogen atoms including 77 solvent water oxygens and 1 sulfate ion were refined in the final model, using the 6838 reflections (F ≥ 2σF) in the range 1.8–8.0 Å. Some of these densities modeled as water oxygens could possibly be other ions present in the crystallization medium. The agreement between side chain densities and the amino acid sequence is unambiguous, except in a few cases. Residues Asp77 and His86 are located at a turn which is exposed to the outer surface; consequently, their average B values are relatively high. The C-terminal Lys79 also has a high B average value probably because of dynamical disorder.

RESULTS AND DISCUSSION

Quality of the Model—The numbers from the final cycle of refinement are provided in Table III. The random positional error estimated from the Luzzati plot (29) is less than 0.20 Å. The average B values remain low throughout most of the residues of the structure. The agreement between side chain densities and the amino acid sequence is unambiguous, except in a few cases. Residues Asp77 and His86 are located at a turn which is exposed to the outer surface; consequently, their average B values are relatively high. The C-terminal Lys79 also has a high B average value probably because of dynamical disorder. Although the refinement results shown are based on the unit weight scheme, nonunit weight schemes were also attempted.

The atomic coordinates and the electron density maps resulting from these two modes of refinement were virtually identical.

Secondary and Tertiary Structures of a KP6α Polypeptide—A monomer of the KP6 killer toxin α-subunit shown in Fig. 2a forms a single domain structure having an overall shape of an ellipsoid of dimensions 40Å × 26Å × 21Å. The structure has a single split ββ motif that belongs to the α/β sandwich family (30). It consists of a four- (β1, β2, β3, and β4) stranded antiparallel β-sheet, a pair of antiparallel α-helices (α2 and α3) that lies approximately 20° to the β-strands above one side of the sheet, a strand β2 along one edge of the sheet, and a short N-terminal helix α1 on the other side of the β-sheet. The twist of the β-sheet is left-handed, as it usually occurs (13). Among the three linkages of the four-stranded antiparallel β-sheet, one belongs to a hairpin connection (β1 to β2), and the other two are right-handed βαβ split crossover connections (β1 to β3 and β2 to β4) via α2 and α3, respectively (13, 30). All the secondary structural elements are connected through six loops. However, only two of them are β turns; one is a β type II turn (13) (Leu29, Ser30, Lys41, and Ser42) connecting the helix α2 with β3, and the other one is a β type I turn (13) (Ser68, Ser77, Leu84, and Asp85) connecting α3 with β4. There are eight cysteines in the structure as indicated in Fig. 2, a and b, all of which form intrachain disulfide bridges linking these secondary structural elements into a compact domain. Helices α2 and α3 are linked to the β-sheet through disulfide bridges: α2 to β4 through Cys34 and Cys51 in a right-handed conformation and α3 to β2 through Cys23 and Cys47 in a left-handed conformation. The remaining two disulfide bridges are both right-handed, one formed between the N-terminal helix α1 and the loop connecting it to β1 (Cys35 and Cys55) and the other between the two longest antiparallel strands β1 and β2 (Cys16 and Cys74). The Cα-Cα distances between a pair of cysteines range from 4.05 to 5.86 Å, depending on the conformation of each individual disulfide bridge. Besides disulfide bonds, the hydrogen bonding network holds the structure together. The hydrogen bonds involving main chain atoms among the five antiparallel β-strands are schematically illustrated in Fig. 2c. A total of 44 hydrogen bonds are formed among main chain atoms, ranging from 2.67 to 3.14 Å, with an average bond length of 2.93 Å. There are 16 hydrogen bonds between main chain and side chain atoms and 7 hydrogen bonds among side chain atoms ranging from 2.60 to 3.42 Å, with an average value of 2.99 Å. All water molecules are hydrogen-bonded to protein atoms either directly or indirectly through another solvent atom. No exposed cluster of hydrophobic side chains was found in the monomer. Three regions in this molecule interact through hydrophobic side chains that appear to stabilize the tertiary structure. As shown in Fig. 2b, among the three hydrophobic clusters, two located between the two antiparallel α-helices and the β-sheet are formed by residues Ala20, Leu27, Ala30, and Leu68 at the top of the molecule (opposite of the N terminus) and by Leu39, Phe53, Leu57, Phe61, and Met72 at the lower middle section of the molecule. The third region, located near the N terminus, is comprised of residues Ala3, Phe4, and Phe8 forming a hydrophobic pocket with neighboring symmetry-related molecules. There are four

| TABLE III |
| --- |
| **Refinement results of KP6α** |
| Resolution range (Å) | 8.0–1.8 |
| No. of reflections used (F > 2σF) | 6838 |
| Weighing scheme | unit weight |
| Percentage of observed data used | 96. |
| No. of protein-atoms + SO4 2- | 613 |
| No. of solvent water | 77 |
| Crystallographic R (F ≥ 2σF) | 0.164 |
| Rmerge (F ≥ 2σF) | 0.211 |
| RMS deviation from ideal geometry |
| Bond (Å) | 0.008 |
| Angle (°) | 1.309 |
| Average B values (Å²) |
| Whole protein | 17.6 |
| Backbone | 15.1 |
| Side chains | 24.6 |
| Solvent | 39.2 |
| Ramachandran plot summary: |
| Residues in most favored regions | 91.4% |
| In additional allowed regions | 8.6% |
| In disallowed regions | 0.0% |

![Fig. 1. The final 2Fo − Fc electron density map and the refined model of the methionine-containing β-strand (β2) of the KP6α molecule. Contoured at 1.5σ. The position of the independently determined selenium atom is indicated by a large sphere.](image)
histidines in the molecule; although all four are located on the surface, three of them reside on the helical side of the molecule (His21, His38, and His64), and His50 resides on the \( \beta \)-sheet side, as shown in Fig. 2. Because the pH of the crystallization buffer was 6.0, one of four of these histidines was deprotonated, which made it possible to coordinate to a heavy metal ion. The surface of the protein is hydrophilic except for the N-terminal helix-\( \alpha_1 \).

The topology of KP6\( \alpha \) along with the other recently determined killer toxins SMK (15) and KP4 (14) is illustrated schematically in Fig. 2d. Although all of them have striking similarity in folding motifs, i.e. \( \alpha/\beta \) sandwich motifs, there are distinct differences among them; KP6\( \alpha \) has two right-handed \( \beta/\alpha \) split crossovers, whereas both SMK and KP4 have two left-handed crossovers. A survey (30) has shown that there are more known right-handed \( \alpha/\beta \) sandwich proteins than the left-handed ones. Whether the handedness of connections has any significance in the biological function is not clear at this moment, but it is evident that all resulting structures are unique in their specific functions. Although the KP4 and KP6 killer toxins are both produced by \emph{U. maydis}, the difference in their folding connections may indicate that they are not functionally similar.

**Assembly of KP6\( \alpha \) Molecules**—As described above, the hydrophobic region with exposed phenyl rings of Phe\(^4\) and Phe\(^8\) of the monomer is located in the N-terminal helix-\( \alpha_1 \). The topology of KP6\( \alpha \) along with the other two recently determined killer toxins SMK (15) and KP4 (14) is illustrated schematically in Fig. 2d. Although all of them have striking similarity in folding motifs, i.e. \( \alpha/\beta \) sandwich motifs, there are distinct differences among them; KP6\( \alpha \) has two right-handed \( \beta/\alpha \) split crossovers, whereas both SMK and KP4 have two left-handed crossovers. A survey (30) has shown that there are more known right-handed \( \alpha/\beta \) sandwich proteins than the left-handed ones. Whether the handedness of connections has any significance in the biological function is not clear at this moment, but it is evident that all resulting structures are unique in their specific functions. Although the KP4 and KP6 killer toxins are both produced by \emph{U. maydis}, the difference in their folding connections may indicate that they are not functionally similar.

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The network of intermolecular salt bridges and hydrogen bonds links the monomers to form a solvent-filled funnel-like structure in which the \( \beta \)-sheet of each molecule spreads on the inner surface and the N-terminal \( \alpha_1 \)-helix sits close to the tip of the funnel (Fig. 4, \( a \) and \( b \)). The funnel is 9–10 Å across at the top, has a 6-Å diameter opening in the middle and narrows to a 4.2-Å diameter barrel bordered by three symmetry related phenyl rings arising from the Gly\(^7\)-Phe\(^8\)-Gly\(^9\) string of residues at the N-terminal \( \alpha_1 \)-helix. More than 50 well ordered water molecules have been located in the funnel. Fig. 4, \( a \) and \( b \) shows the KP6\( \alpha \) trimer with phenyl rings at the tip of the funnel and water oxygens inside of the funnel. The assembly of the hexamer brings the two narrow funnel tips face to face, thus forming an hourglass shaped structure as shown in Fig. 5. The distance between two planes of intratramer salt bridges is about 34 Å.

The analysis of total accessible surfaces for the monomer, the trimer, and the hexamer bears out the above description of molecular association. The details are listed in Table IV. The total accessible surface area for KP6\( \alpha \) monomer is 4783 Å\(^2\). Because of the formation of the trimer and the hexamer, total accessible surface areas for each monomer decreased by 21.2 and 24.2%, respectively. The loss of accessible surface area/monomer because of oligomerization is 1014 Å\(^2\) for the trimer and 1157 Å\(^2\) for the hexamer. These numbers are in good agreement with the values of \(-800\) Å\(^2\) for a homodimer and \(-1000\) Å\(^2\) for a heterocomplex of a 10-kDa protein calculated by Jones and Thornton (31) for 59 different protein-protein complexes. Among lost accessible surfaces, the loss of hydrophobic surface contributed the most, followed by the charged surface and the polar surface. As an overall effect of oligomeric association of KP6\( \alpha \), the percentage of the hydrophilic surface area, consisting of charged and polar residues, increased from 73.0% for the monomer to 75.6 and 78.6% for the trimer and the hexamer, respectively, making them more soluble in an aqueous medium. By forming hexamer from trimer, the gain or loss of both accessible hydrophilic and hydrophobic surfaces are in small magnitudes, \(-3\)%, indicating a weak tendency to form the hexamer from the trimer.

Implications of the Quaternary Structure—It is conceivable that the crystallographic monomer, the trimer, or the hexamer of KP6\( \alpha \), in association with one or more polypeptides of KP6\( \beta \) forms a molecular assembly that interacts with either the K\(^+\) channel or another receptor on the cell membrane or even by itself penetrates the membrane and causes disruption of the cellular ion balance. No conclusive biochemical evidence is available yet on any of these possibilities. The KP6\( \alpha \) hexamer has an inner pore opening of 4.2 Å, large enough for monovalent cations such as K\(^+\) to pass through and a length of 34 Å, similar to the thickness of the membrane bilayer. Although neither KP4 nor SMK structures exhibit similar oligomeric association, the distantly related all \( \beta \)-strand \( \gamma \)-cardiotoxin (32) is trimeric. However, the central opening of the trimer narrows to less than 0.5 Å where interfacial atoms are virtually at van der Waals contact distances (32). It was proposed that \( \gamma \)-cardiotoxin in its oligomeric state could either interact with a membrane receptor or could insert itself into the membrane forming an ion channel (32). To our knowledge, no other member of this class of toxins is known to have a central pore like the one in the KP6\( \alpha \) hexamer.

Cardiotoxins are known to act by depolarizing the Ca\(^+\) channel. KP4 was shown to kill cells by blocking divalent cation channels but not Na\(^+\) or K\(^+\) channels (14). KP4 crystallizes as a monomer and a single polypeptide chain is known to possess the killer action (14). Structural data suggest that its participation in channel formation is unlikely. The mechanism of pH-dependent killer action of SMK is still unknown (15). The monomer containing the \( \alpha \)- and the \( \beta \)-peptides forms a dimer in the crystal (15). Despite the overall similarity of folds of monomers of these toxins, there appears to be diversity in the mechanism of killing action, possibly stemming from diverse oligomeric states of their functional entities. KP6 is distinctive in the sense that it kills by depleting the K\(^+\), unlike any other toxin in the family. The architecture of the KP6\( \alpha \) hexamer...
provides a pore structure that has right dimensions to function as a $\text{K}^+$ channel, either outside or within the cell membrane bilayer. However, the outer surface of the hexamer as well as the monomer has polar and charged residues, accounting for the solubility of the toxin in aqueous medium. In order for the KP6 assembly to enter the membrane bilayer, the outer polar surface must be concealed. It is conceivable that the $\beta$-subunit plays a critical structural role in this aspect because it is known that both subunits are necessary for the killer action of the toxin. Any large rearrangement in the tertiary structure of KP6 on complexation with the $\beta$-subunit is unlikely, because

![Image](50x167 to 297x602)

![Image](308x155 to 555x729)

**FIG. 4.** Views of a funnel-shaped KP6 trimer with phenyl rings at the tip of the funnel and water oxygens inside of the funnel. *a,* along the crystallographic 3-fold axis. *b,* along the direction perpendicular to the 3-fold axis.

**FIG. 5.** The KP6 hexamer. *a,* viewed along the crystallographic 3-fold rotation axis. *b,* viewed along the direction perpendicular to the 3-fold axis.

**TABLE IV**

| Accessible surface area ($\text{Å}^2$) | Total | Charged | Polar | Hydrophobic |
|--------------------------------------|-------|---------|-------|-------------|
| Monomer                              | 4783  | 1455    | 2038  | 1289        |
| Trimer                               | 11,306| 3060    | 5484  | 2763        |
| ↓ vs. monomer (↓ 21.2%)               |       |         |       |             |
| Hexamer                              | 21,748| 6137    | 10,957| 4655        |
| ↓ vs. monomer (↓ 24.2%)               |       |         |       |             |
| ↓ vs. trimer (↓ 3.8%)                 |       |         |       |             |
of its compact nature that is rigidly held by four disulfide bridges. Formation of higher order oligomeric states either by the KP6α hexamer or by an α-β complex to hide hydrophilic surfaces from the lipid environment is also a plausible scenario. Further insight into the mechanism of action of KP6 may come from the crystal structure of an active complex of α- and β-subunits of the toxin.

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