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ARTICLE *in* BIOCHEMISTRY · FEBRUARY 2006

Impact Factor: 3.02 · DOI: 10.1021/bi051351z · Source: PubMed

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Vegetative Insecticidal Protein (Vip1Ac) of *Bacillus thuringiensis* HD201: Evidence for Oligomer and Channel Formation†

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ABSTRACT: The binding component (Vip1Ac) of the ADP-ribosylating vegetative insecticidal protein (Vip) of *Bacillus thuringiensis* HD201 was isolated from the supernatant of cell cultures. Vip1Ac protein solubilized at room temperature ran as oligomers on SDS−PAGE. These oligomers were not resistant to heating. Mass spectroscopic analysis of this high molecular mass band identified it as Vip1Ac. The protein formed in artificial lipid bilayer membranes channels with two conductance states of about 350 and 700 pS in 1 M KCl. The channel conductance showed a linear dependence on the bulk aqueous KCl concentration, which indicated that the channel properties were more general than specific. Zero-current membrane potential measurements showed that the Vip1Ac channel has a slightly higher permeability for chloride than for potassium ions. Asymmetric addition of Vip1Ac to lipid bilayer membranes resulted in an asymmetric voltage dependence, indicating its full orientation within the membrane. The functional role of Vip1Ac and its relationship to other ADP-ribosylating toxins are discussed.

The Gram-positive bacterium *Bacillus thuringiensis* is a common soil microorganism that has been used as a biological pesticide for several decades. Its insecticidal activity is mainly contributed to parasporal crystalline δ-endotoxin inclusions, formed during sporulation (*1*), and to some extent to the vegetative insecticidal proteins (Vip) (*2*, *3*). The detailed molecular mechanisms mediating the insecticidal activity of *Bacillus*-produced δ-endotoxins have been described as a multistep process, which initiates upon ingestion of the protein crystals. The insecticidal δ-endotoxins are solubilized in the insect midgut and subsequently undergo site-specific proteolysis from their N- and C-terminus. These activated polypeptides bind to receptors in the midgut epithelium and form ion channels, inducing osmotic lysis of the epithelium cells and subsequent death of the larvae (*4*).

Besides these crystalline proteins *B. thuringiensis* produces two known classes of vegetatively expressed insecticidal proteins. These include the binary toxins Vip1 and Vip2 with coleopteran specificity and Vip3 exhibiting lepidopteran specificity (*2*, *5*, *6*). Vip toxins, so-called because of their production during vegetative growth phase, extend their insecticidal properties especially against the Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and the European corn borer (*Ostrinia nubilalis*), both widespread corn pests, less affected by the δ-endotoxins (*2*). The binary Vip toxin produced by *B. thuringiensis* HD201 belongs to the group of A−B toxins with an A moiety (Vip2Ac) for the enzymatic reaction and the B moiety (Vip1Ac) responsible for the translocation of component A across the cell membrane (*7*). Both polypeptides function separately, with the membrane-binding 66 kDa Vip1Ac creating the pathway into the cytoplasm for the 45 kDa Vip2Ac, a NAD-dependent ADP-ribosyltransferase which likely targets arginine 177 of monomeric G-actin, but not polymerized F-actin, hereby disrupting the integrity of the cytoskeleton, resulting in a rounding up and death of the target cells (*3*, *8*). Both δ-endotoxins and vegetative insecticidal proteins are currently used in different forms of commercially available biopesticides for agriculture and mosquito control (*9*).

In this study, we demonstrate the purification of Vip1Ac from *B. thuringiensis* HD201. Vip1Ac was obtained in its native form from a *Bacillus* culture. Furthermore, we present here the first biophysical characterization of the oligomeric, channel-forming component of the Vip toxin, Vip1Ac, using artificial lipid bilayer membranes, which demonstrate that it is a channel-forming component.

MATERIALS AND METHODS

Protein Purification. Insecticidal protein was purified from supernatants of *B. thuringiensis* strain HD201. The cultures were grown for 12 h at 30 °C in Luria−Bertani broth and centrifuged at 6000*g* for 20 min. Proteins present in the supernatant were precipitated with ammonium sulfate (70% saturation) and collected by centrifugation at 10000*g* for 40 min. The pellet was then resuspended in 15 mL of 10 mM Tris-HCl, pH 9.0, and dialyzed overnight at 4 °C against 150 mM NaCl and 10 mM Tris-HCl, pH 9.0. The dialysate was concentrated to 1 mL using Centricon Plus-20 filter

† This work was financially and technically supported by the Deutsche Forschungsgemeinschaft (SFB 487, project A5; FZT-82, Rudolf-Virchow-Center for Experimental Biomedicine, and GRK 1141/1) and the Fonds der Chemischen Industrie.

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columns (Millipore, Schwalbach, Germany) and denatured using 8 M urea. After 30 min incubation at room temperature, the sample was fractionated on a HiTrap Q anion-exchange column (Amersham Bioscience, Freiburg, Germany) using a linear salt gradient from 0 to 1 M NaCl in 10 mM Tris-HCl, pH 9.0, at a flow rate of 1 mL min^{-1}. Fractions were collected with a FC-100 fraction collector (Amersham Bioscience, Freiburg, Germany) and inspected for the presence of Vip1Ac using 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Fractions possibly containing Vip1Ac were combined and concentrated to 100 μL using Centricon YM-30 filter devices (Amersham Bioscience, Freiburg, Germany). The combined fractions showed several bands on SDS−PAGE, which means that it was impossible to relate one single band to the channel-forming activity. Preparative SDS−PAGE was performed to identify the channel-forming protein. The gels were cut in different slices according to different molecular mass bands of the proteins. The slices were eluted with the Roti-Elution-Kit (Carl Roth, Karlsruhe, Germany) and tested for channel-forming activity.

SDS−PAGE. SDS−PAGE was performed according to the Laemmli gel system (*10*). The gels were stained with Coomassie brilliant blue or with silver stain (*11*).

Lipid Bilayer Experiments. Black lipid bilayer membranes were formed as described previously (*12*). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.5 mm^2. Membranes were formed by painting onto the hole a 1% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. The aqueous salt solutions (Merck, Darmstadt, Germany) were buffered with 10 mM MES to pH 5.5, 10 mM Tris, pH 9, and 10 mM CAPS, pH 11. Control experiments revealed that the pH was stable during the time course of the experiments. Vip1Ac was reconstituted into the lipid bilayer membranes by adding concentrated solutions to the aqueous phase to one side (the cis -side) of a membrane in the black state. Zero-current membrane potentials were measured with a Keithley 617 electrometer 5−10 min after a 5-fold salt gradient was established across the membranes (*13*). The temperature was kept at 20 °C throughout.

RESULTS

Isolation and Purification of Vip1Ac. Vip1Ac protein has a putative N-terminal signal peptide, which is believed to direct its secretion into the culture medium (*14*). To purify the soluble proteins of a *Bacillus* cell culture supernatant, we performed an ammonium sulfate precipitation of a HD201 overnight culture supernatant, dissolved the pellet, and dialyzed the protein against 150 mM NaCl and 10 mM Tris-HCl, pH 9.0. The protein was concentrated and dissolved in 10 mM Tris buffer, pH 9, including 8 M urea. Subsequently, it was applied to a HiTrap Q anion-exchange chromatography column. The different FPLC fractions of the gradient were collected and analyzed for protein content by SDS−PAGE and for the presence of pore-forming activity by reconstitution experiments with lipid bilayer membranes (see below). During gradient elution the major part of contaminating proteins was separated from Vip1Ac, which eluted in a relatively broad peak from 570 to 700 mM NaCl (data not shown). SDS−PAGE analysis of the combined fractions presenting pore-forming activity revealed the presence of a high molecular mass band (presumably oligomeric Vip1Ac), which was resistant to SDS but not to boiling in sample buffer (see Figure 1). Nano-LC mass spectroscopic analysis of the high molecular mass band identified the high molecular mass band as Vip1Ac oligomers. A single gel band was analyzed by nano-LC-MS/MS as described elsewhere (*15*). Data interpretation of the derived MS/MS data sets was done by the mascot algorithm[1] (*16*). Seven partial peptides could be identified with 100% identity from the mass spectra (data not shown) when compared to the known sequence of Vip1Ac (*17*). Further purification of Vip1Ac was achieved by excision of the oligomer band from preparative SDS−PAGE and subsequent extraction with 10 mM Tris-HCl and 150 mM NaCl, pH 9.0.

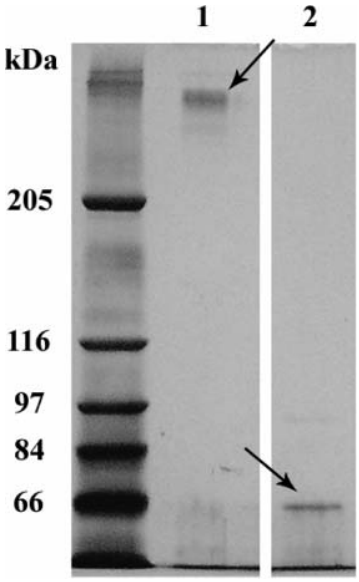

FIGURE 1: 6% SDS−PAGE analysis of the combined FPLC fractions presenting pore-forming activity and preparative gel elution. Lane 1: 10 μL of concentrated eluate, unboiled, revealed the presence of a high molecular mass band which was identified by mass spectroscopic analysis to be Vip1Ac. The arrow shows the position of the Vip1Ac oligomers. Lane 2: 10 μL of eluate, boiled. The arrow indicates the monomeric $Vip1Ac_{66}$. Lanes were brought together for better comparison. The gel was silver stained.

Purified Vip1Ac Forms Well-Defined Channels Exhibiting Two Conductance States at Higher Salt Concentration. Vip1Ac isolated from the supernatant of a *B. thuringiensis* overnight culture was purified to homogeneity as described above. The addition of the protein oligomers to the aqueous phase (concentration about 10 ng/mL) bathing a black lipid bilayer resulted in a very fast reconstitution of channels. Figure 2A shows a current recording in the presence of Vip1Ac. Vip1Ac reconstituted rapidly into planar lipid bilayers and formed well-defined channels (see Figure 2A). Analysis revealed two conductance states of about 350 and 700 pS in 1 M KCl and 10 mM MES, pH 5.5 (applied voltage 20 mV). Interestingly, these two conductance states were only visible at high electrolyte concentration. At salt

[1] http://www.matrix-science.com; http://www.cbs.dtu.dk/services/SignalP-2.0/; http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/.

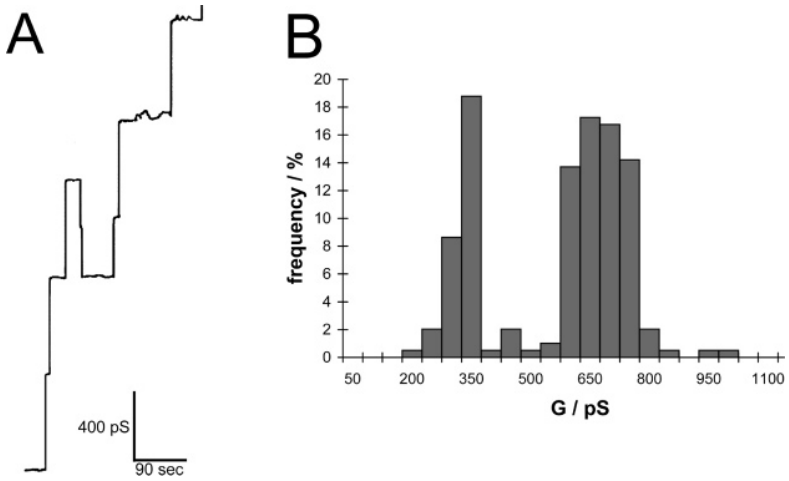

FIGURE 2: (A) Current recording of a diphytanoylphosphatidylcholine/*n*-decane membrane in the presence of Vip1Ac. The aqueous phase contained 1 M KCl, 10 mM MES (pH 5.5), and ~10 ng/mL Vip1Ac. The applied membrane potential was 20 mV; $T = 20$ °C. (B) Histogram of the probability of the occurrence of certain conductivity units (conductance steps) observed with membranes formed of diphytanoylphosphatidylcholine/*n*-decane in the presence of Vip1Ac. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; $T = 20$ °C. The average conductance of the left-hand and the right-hand maxima was 350 and 700 pS, respectively, for total 180 single events.

Table 1: Conductance of the Vip1Ac Channel in Different Salts and KCl Solutions of Different Concentrations[a]

	channel conductance *G* (pS)		
salt concn	Vip1Ac	C2-II*	Ib**
50 mM KCl	75	nd[b]	nd
150 mM KCl	150	nd	nd
300 mM KCl	200	55	30
500 mM KCl	160/300	80	nd
1 M KCl	350/700	150	85
3 M KCl	960/1900	380	180
1 M LiCl	160/300	60	35
1 M KF	220/400	nd	nd

[a] The membranes were formed of diphytanoylphosphatidylcholine dissolved in *n*-decane. The aqueous solutions were buffered (10 mM MES) and had a pH of 5.5. The applied voltage was 20 mV, and the temperature was 20 °C. The average channel conductance was calculated from at least 100 single events; concn indicates the concentration of the aqueous salt solution. The channel conductance *G** and *G*** of C2-II and Ib are given for comparison and were taken from refs *18* and *21*, respectively. [b] Not described.

concentrations below 500 mM KCl only one conductance state was observed (see Discussion). The pH of the aqueous salt solutions had a small influence if any on the conductance of the Vip1Ac channel. Table 1 shows a summary of the channel conductance of Vip1Ac in different KCl concentrations and in various salt solutions. The data indicate that the channel conductance was approximately a linear function of the bulk aqueous KCl concentration.

Selectivity of the Channel Formed by Vip1Ac. The selectivity of the Vip1Ac channel was determined in zero-current membrane potential measurements in the presence of salt gradients. After incorporation of a large number of channels in membranes bathed in 100 mM KCl, 5-fold salt gradients were established across the membranes by the addition of small amounts of concentrated KCl solution to one side of the membrane. In all cases, the more diluted side of the membrane became negative (on average −12.5 mV), which indicated preferential movement of anions through the Vip1Ac channel; i.e., it is anion selective. This result is consistent with the conductance data where anions had a somewhat stronger influence on the conductance of Vip1Ac channels as compared to cations (see Table 1). Analysis of the zero-current membrane potentials using the Goldman−Hodgkin−Katz equation showed that the permeability ratio P_{cation} over P_{anion} was about 0.36.

The Vip1Ac Channel Is Voltage-Dependent in an Asymmetric Manner. In conductance recordings Vip1Ac exhibited significant channel flickering at higher positive voltages applied to the cis side (side of protein addition), meaning it showed rapid transitions between open and closed configuration. This could be caused by voltage-dependent closing of the channel, and therefore we increased in single- and multichannel experiments the applied transmembrane voltage (see Figure 3).

Vip1Ac was added in a concentration of ~10 ng/mL to one side of a black diphytanoylphosphatidylcholine/*n*-decane membrane (the cis side), and the conductance increase was followed for about 25 min until stationary phase was reached. At this point, we applied different positive and negative potentials (with respect to the cis side) to the membrane starting from 20 mV. Then we repeated the experiment with increasing potentials. Figure 3B shows the results with 90 mV with a limited number of channels. We applied +90 mV to the cis side of the membrane and observed a stepwise decrease of the membrane current indicating the complete closing of the channel at high positive potentials. For negative potentials at the cis side, the current did not decrease even when the membrane potential was as high as 120 mV. This result indicated asymmetric insertion of Vip1Ac into the membranes. The addition of protein to both sides of the membrane resulted in a symmetric response to the applied voltage (data not shown). The data of the experiment of Figure 3B and similar experiments were analyzed in the following way: the membrane conductance (G) as a function of voltage, V_m, was measured when the opening and closing of channels reached an equilibrium, i.e., after the exponential decay of the membrane current following the voltage step V_m. G was divided by the initial value of the conductance (G_0, which was a linear function of the voltage) obtained immediately after the onset of the voltage. The data of Figure 3A correspond to the asymmetric voltage dependence of

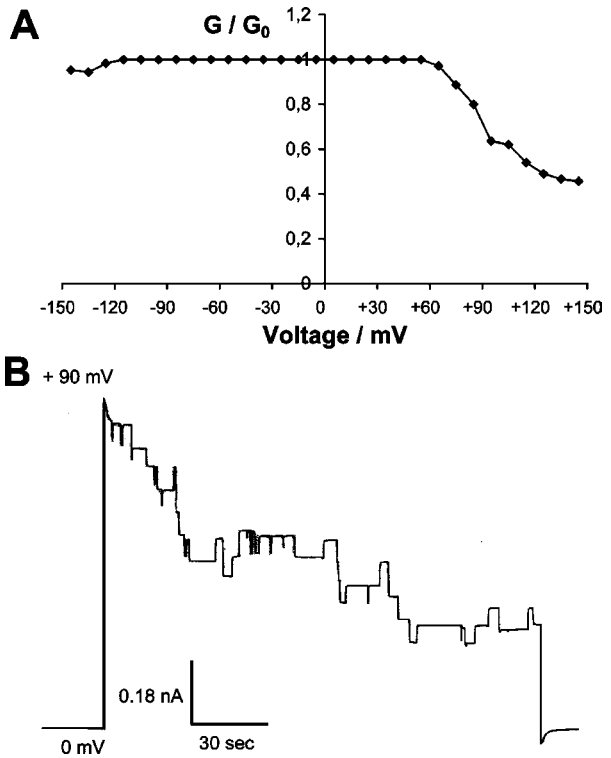

FIGURE 3: (A) Ratio of the conductance G at a given membrane potential (V_m) divided by the conductance G_0 at 10 mV as a function of the membrane potential V_m, applied to the cis side of the membrane, the side of addition of the protein. The closed squares show measurements in which 10 ng/mL Vip1Ac was added to the cis side of the membranes. The aqueous phase contained 1 M KCl, 10 mM MES, pH 5.5, and 10 ng/mL Vip1Ac. The membranes were formed from diphytanoylphosphatidylcholine/*n*-decane. $T = 20$ °C. Means of three experiments are shown. (B) Study of the voltage dependence of Vip1Ac when only a small number of channels were reconstituted. Protein was added to the cis side of the membrane, and reconstitution of channels was followed for about 20 min. When equilibrium was reached, +90 mV was applied to the cis side of the membrane, and the membrane current was measured as a function of time. The aqueous phase contained 1 M KCl, 10 mM MES, pH 5.5, and 10 ng/mL Vip1Ac. $T = 20$ °C.

Vip1Ac (mean of three membranes) when the protein was added to the cis side. The results indicated full orientation of the Vip1Ac channel when the protein was added only to one side of the membrane. This result is in clear contrast to the observed voltage dependency of other related channel-forming toxins such as iota toxin (Ib), C2 toxin (C2-II), and anthrax toxin (PA) (*18*−*20*) where channel closure is observed at high negative voltages applied to the cis side of the membrane.

DISCUSSION

The binary *B. thuringiensis* HD201 vegetative insecticidal protein (Vip) belongs to a novel class of binary actin−ADP-ribosylating toxins distinct from classical A−B toxins known so far. The protein nature of this toxin and the possibility of genetic engineering offer a great potential for pesticide improvement. In particular, it is expressed in different plants as systemic and specific biopesticides (e.g., VipCotton; Syngenta Corp.).

The toxin consists of two components, the enzymatic ADP-ribosyltransferase Vip2Ac and the binding component Vip1Ac. The latter shares significant sequence identity with the binding components C2-II of the C2 toxin from *Clostridium botulinum* (29% identity) and Ib of the iota toxin from *Clostridium perfringens* (31% identity), suggesting that these proteins have similar modes of action. The transport of several different bacterial toxins is accompanied with the formation of channels in artificial and biological membranes. Examples are channel formation by the C2 toxin (*21*), iota toxin (*18*), and anthrax toxin (*22*). In the present study we demonstrate for the first time that Vip1Ac forms channels consisting of oligomers in artificial lipid bilayer membranes.

Properties of the Vip1Ac Channel. Vip1Ac was isolated from the supernatants of cell cultures. Vip1Ac formed channels that had two conductance states of about 350 and 700 pS in 1 M KCl, indicating presumably a simultaneous reconstitution of two Vip1Ac channels. Two conductance states were only observed at high salt concentrations present in the aqueous phase bathing a black lipid membrane. Under these conditions hydrophobic molecules, such as activated $Vip1Ac_{66}$, strongly tend to aggregate, thus favoring the simultaneous reconstitution of two channels in artificial membranes. On the other hand, we cannot exclude the possibility that two different Vip1Ac oligomers exist at higher salt concentrations, thus forming channels with two different conductance states. Similar observations were made for the alpha toxin of *Staphylococcus aureus*, which can easily change its state of oligomerization (hexamer/heptamer), thus switching between a "high-" and "low-conducting" configuration (*23*, *24*). SDS−PAGE analysis in combination with mass spectroscopic studies of the Vip1Ac protein band clearly proved that Vip1Ac formed SDS-stable but heat-labile oligomers in solution. Aggregation of the toxin monomers to an SDS-insoluble oligomer in solution is a prerequisite for channel formation; this is also the case for the alpha toxin of *S. aureus* (*25*), C2 toxin from *C. botulinum* (*26*), and anthrax toxin of *Bacillus anthracis* (*27*).

The Vip1Ac channel was found to be moderately anion selective, which is in contrast to the cation-selective channels formed by C2-II or PA (*21*, *22*). The putative channel-forming domain of Vip1Ac contains two negatively (E340 and E345) and two positively charged amino acids (K351 and H363), which probably all contribute to the selectivity together with charged residues within the vestibule of the channel. Its conductance is a linear function of the bulk aqueous salt concentration, which means that there does not exist any indication for the presence of positive point charges, which would result in a dependence of the channel conductance on the square root of the aqueous KCl concentration similar to the situation in the C2-II channel (*21*).

The Vip1Ac Channel Shows Voltage-Dependent Gating. Starting with about +60 mV applied to the cis side of the membrane (the side of protein addition), the current through the channels decreased in an exponential fashion (Figure 3B). For opposite polarity at the cis side of the membrane, the current was not influenced. This result indicated asymmetric insertion of Vip1Ac into the membrane. Possibly, a large hydrophilic part of the binding component is exposed to the aqueous phase on the cis side of the membrane (i.e., the side of the addition of the protein), whereas the more hydrophobic channel-forming domain crosses the membrane and leads to a transmembrane channel, similar to that being suggested for PA (*28*, *29*). This asymmetric distribution of the protein results in an asymmetric response toward the sign of the

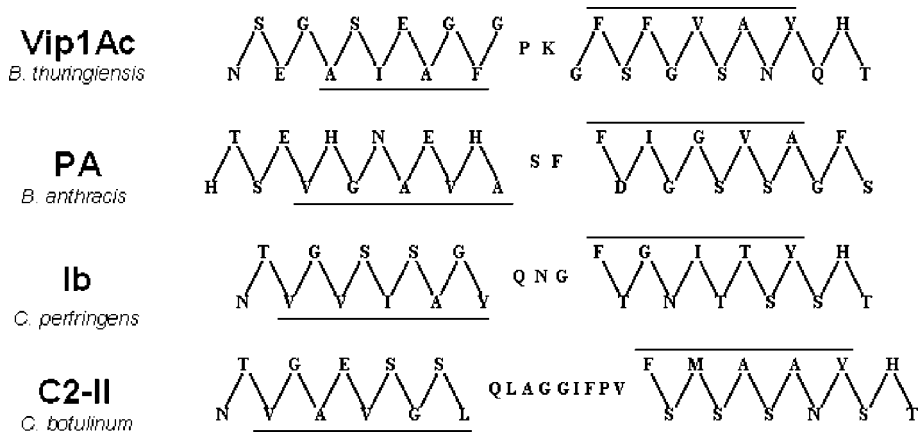

FIGURE 4: Comparison of the putative channel-forming domains of Vip1Ac, PA (H328−S354), Ib (N334−T359), and C2-II (N302−T333). The amino acids are given in the one-letter code. Residues oriented toward the lipid bilayer are underlined. The sequence of Vip1Ac is shown from N338 to T364. The putative amphipathic β-strands are similar to those proposed by Leppla, and the alignment was performed according to the known domain structure of PA (*36*).

membrane potential. Charged residues may be responsible for voltage-dependent gating as we could show for C2 toxin, where E307 is clearly responsible for channel gating (*30*). The interesting point is that the Vip1Ac channels started to close at high positive voltages applied to the cis side of the membrane, while the binding components of other channel forming toxins such as the C2-II (*20*, *31*), Ib (*18*), and PA (*19*) exhibited voltage-dependent gating only when negative potentials were applied to the cis side.

Biological Implications of the Channels Formed by Vip1Ac. The detailed molecular mechanisms mediating the insecticidal activity of *Bacillus*-produced vegetative insecticidal proteins still remained elusive. In principle, it can be described as a multistep process which initiates upon ingestion of the binary protoxin (Vip1/Vip2) by the susceptible larvae. The alkaline pH (9−11) of the insect midgut juice and the presence of trypsin-like proteases favor proteolytic cleavage of the protoxin binding component Vip1. Activated Vip1 binds to a specific cell surface receptor on the insect midgut brush border membrane. Oligomer formation following binding seems to be an essential prerequisite for pore formation. In support of this hypothesis we found in our studies SDS-resistant oligomers of Vip1Ac on SDS−PAGE (see Figure 1). Interestingly, the Vip1Ac channel showed only little dependence on the pH of the aqueous solutions in in vitro experiments. The oligomers result finally in the formation of pores providing a putative pathway for the 45 kDa Vip2Ac ADP-ribosyltransferase entering the cytosol and subsequently targeting the cytoskeleton by ADP-ribosylation of actin. It has to be noted that Finkelstein and co-workers recently presented some evidence that LF may be transported through the PA channel across the endosomal membrane (*32*).

The classical cellular uptake mechanisms of related binary actin−ADP-ribosylating toxins were shown for C2 toxin from *Clostridia* in 1989 by Simpson. C2-II binds to a receptor on the cell surface and enters cells via receptor-mediated endocytosis (*33*) mediating the uptake of C2-I. Endocytosed C2 toxin reaches the acidic early endosomal compartment, and C2-I is released into the cytoplasm (*34*). Besides this endosomal pathway there is evidence for a "direct" delivery of the enzyme component into the cytoplasm, when cells are preincubated with both toxin components (e.g.,C2-II and C2-I) and subsequently shifted to acidic pH (*34*).

The magnitude of the transmembrane potential of acidic vesicles is difficult to assess, but there is no question that the interior of the vesicle is positively charged with respect to its surrounding. Under these conditions Vip1Ac integrated in the endosomal membrane would be closed and consequently could not serve as a translocation pathway for the enzymatic component Vip2Ac. One interesting point is that the insect larval midgut juice is highly alkaline (pH ∼10), causing a strong outward proton gradient across the midgut brush order membrane of insect cells (e.g., pH 7/pH 10). The strong electrochemical potential created by the proton gradient across the midgut brush border membrane could mean that despite the existing cell membrane potential (from about −60 to −70 mV) the Vip1Ac channel present on the external surface of the cell would be open under in vivo conditions. This led us to the presumption that the enzyme component (Vip2Ac) might be "directly" delivered into the cytoplasm of the midgut cells via a channel formed by Vip1Ac. Further analysis of the effective proton conductance of the Vip1Ac channel and its dependency on different proton electrochemical gradients is in progress.

Inhibition of Channel Function. The primary sequence of Vip1Ac shares significant homology with the primary sequences of PA, C2-II, and Ib. It has been found that the binding components of anthrax and C2 toxins form oligomers (probably heptamers) in the target cell membrane and in artificial lipid bilayers (*26*, *35*). In previously performed studies we have shown that the function of both channels could be blocked by 4-aminoquinolones (*19*, *20*). Unlike PA and C2-II, 4-aminoquinolones did not block the channel formed by Vip1Ac, indicating that Vip1Ac lacks a specific binding site for these substances (data not shown).

Structural Implications of Vip1Ac. PA contains a flexible loop forming an amphipathic β-hairpin with alternating hydrophobic and hydrophilic residues (*35*). Benson et al. (*36*) showed that the loops from seven protomers combine to form a transmembrane, 14-stranded β-barrel where the hydrophobic residues face the lipid and the hydrophilic residues face the lumen of the channel (see Figure 4). Similar antiparallel, amphipathic β-strands of variable length are conserved in Vip1Ac, Ib, and C2-II (see Figure 4). One interesting point

is that the putative channel-forming domain of Vip1Ac contains two negatively charged residues in the channel-forming domain (E340 and E345), resulting in a somewhat balanced distribution of charges with the positively charged residues (K351 and H363) and a weak anion selectivity of Vip1Ac. The position of the positively charged lysine at the "exit" of the transmembrane β-barrel is presumably responsible for the asymmetric voltage response of the channel.

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BI051351Z