Insertion of Anthrax Protective Antigen into Liposomal Membranes

**EFFECTS OF A RECEPTOR**

Received for publication, October 20, 2006, and in revised form, November 14, 2006. Published, JBC Papers in Press, November 14, 2006, DOI 10.1074/jbc.M609869200

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Protective antigen (PA), the receptor-binding component of anthrax toxin, heptamers and inserts into the endosomal membrane at acidic pH, forming a pore that mediates translocation of the enzymic components of the toxin to the cytosol. When the heptameric pre-insertion form of PA (the prepore) is acidified in solution, it rapidly loses the ability to insert into membranes. To maximize insertion into model membranes, we examined two ways to bind the protein to large unilamellar vesicles (LUV). One involved attaching a His tag to the von Willebrand factor A domain of one of the PA receptors, ANTXR2, and using this protein as a bridge to bind PA to LUV containing a nickel-chelating lipid. The other involved using a His tag fused to the C terminus of PA to bind the protein directly to LUV containing the same lipid. Both ways enhanced pore formation at pH 5.0 strongly and about equally, as measured by the release of K⁺. Controls showed that pore formation in this system faithfully reproduced that in vivo. We also showed that binding unmodified ANTXR2 von Willebrand factor A to the prepore in solution enhanced its pore forming activity by slowing its inactivation at acidic pH. These findings indicate that an important role of PA receptors is to promote partitioning of PA into the bilayer by maintaining the prepore close to the target membrane and presumably in the optimal orientation as it undergoes the acidic pH-dependent conformational transition to the pore.

Anthrax toxin is a tripartite A-B toxin system, composed of two catalytic moieties, edema factor (EF)² and lethal factor (LF), and a single receptor-binding/pore-forming moiety, protective antigen (PA). PA (83 kDa) binds to cell-surface receptors and is cleaved by furin or a furin-like protease to an active, 63-kDa form (PA₆₃) (2). PA₆₃ oligomerizes into a heptameric, receptor-bound prepore, which contains high-affinity binding sites for EF and/or LF (3). The entire toxin-receptor complex is internalized by receptor-mediated endocytosis, and within the endosome the prepore undergoes an acidic pH-dependent conformational rearrangement to form a cation-selective, transmembrane pore (4). The PA pore mediates translocation of EF and LF across the endosomal membrane into the cytosol, where EF, an 89-kDa calmodulin-dependent adenylate cyclase, elevates levels of cAMP (5), and LF, a 90-kDa zinc protease, inactivates mitogen-activated proteins kinase kinases (6).

Two cellular receptors for PA have been identified: anthrax toxin receptor/tumor endothelial marker 8 (called ANTXR1) and capillary morphogenesis protein 2 (called ANTXR2) (7, 8). Both are type-I transmembrane proteins, containing a signal sequence, an extracellular von Willebrand factor A (VWA) domain, and a single-pass transmembrane region. The VWA domains of ANTXR1 and ANTXR2 contain a conserved metal ion-dependent adhesion site motif. The ANTXR2 VWA domain (abbreviated as R2 below) binds PA with high affinity (KD ≈ 170 pm when liganded by Mg²⁺) (9). X-ray structural studies of complexes consisting of R2 bound to PA or to the heptameric prepore have revealed that R2 binds not only to PA domain 4 (receptor binding domain) but also to domain 2 (10, 11). Domain 2 contains a mobile loop, the 2β2–2β3 loop (residues 303–322), and the seven 2β2–2β3 loops of PA heptamer are believed to move to the base of the structure during the low pH-induced conformational rearrangement to form a 14-strand transmembrane β-barrel (12). Binding of R2 to the PA prepore shifts the threshold of prepore-to-pore conversion to a more acidic pH range (10). Whole-cell voltage-clamp measurements of PA pore current have shown that ANTXR2 mediates PA

*This work was supported by National Institutes of Health Grants RO1 AI 22021, AI 48489, and PO1 AI 56013. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

²The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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pore formation on the plasma membrane of cultured cells when they are exposed to acidic conditions (13).

Liposomes have been widely used as model systems to study protein-membrane interactions. In an early study, the PA pre-pore was found to permeabilize unilamellar asolectin vesicles (lacking receptors) to K⁺ under acidic conditions (14). Later studies suggested that PA did not partition efficiently into liposomal membranes. In an attempt to make liposomes a more useful model system for studying this toxin, we have now devised ways to bind PA to the surface of large unilamellar vesicles (LUV). Synthetic Ni²⁺-chelating lipids provide a convenient way to attach His-tagged proteins to liposomal membranes (15–17), and we generated a His-tagged form of R2 (His-R2) to enable it to bind to LUV containing Ni²⁺-chelating lipids (DOGs-NTA-Ni) and serve as a bridge between PA and the membrane. We also prepared PA containing a His tag at its C terminus, enabling it to bind directly to the surface of LUV containing the Ni²⁺-chelating lipid. The effects of binding PA to LUV on toxin assembly and pore formation were examined and are reported below.

MATERIALS AND METHODS

Plasmid Construction—ANTXR2 cDNA encoding residues Ser³⁸-Cys²¹⁸ was inserted into pET15b at NdeI and BamHI sites, which resulted in a construct pET15b-ANTXR2(Ser³⁸-Cys²¹⁸) that expressed a N-terminally His₆-tagged ANTXR2 VWA domain. His-R2, ANTXR2 cDNA encoding residues Ser³⁸-Cys²¹⁸ was amplified by PCR (Forward primer: ggt ggt gaa ttc tgc aga cga gcc ttt g; Reverse primer: ggt ggt ctc gag tca atg gtt atg agt atg aca tga cgg aag tag tat tag atg att) to add a His₆ sequence after Cys²¹⁸. The amplified DNA was inserted into pGEX-4T at EcoRI and XhoI sites to generate a construct, pGEX-4T-ANTXR2(Ser³⁸-Cys²¹⁸)-His₆, that expresses a GST-selective enzyme (Orion Research) connected to a microcomputer pH/mV/Temp meter 6171. The signal was transmitted by a data transmitter (DATAQ) and displayed with DATAQ software. The channel-forming peptide, gramicidin A (5 µM), was added to entrapped K⁺ inside liposomes. Therefore, a control, buffer alone, was always included in the experiments, enabling the results obtained from different pools of liposomes differed in terms of the liposome concentration, the amounts of residual K⁺ outside liposomes, and entrapped K⁺ inside. Therefore, a control, buffer alone, was always included in the experiments, enabling the results obtained from the same pool of liposome preparation to be compared.

His-tagged Protein-Liposome Binding Assay—LUV (200 nm) prepared in present study can be easily collected by centrifugation at 100,000 × g for 30 min (17, 20). Purified His-tagged proteins were incubated with liposomes at indicated conditions. Membranes were pelleted at 100,000 × g for at least 30 min in an airfuge, and the pellet was analyzed by SDS-PAGE.

Time-lapse Intensity Measurements of NBD Emission—PA mutants G305C and N306C were labeled with NBD (N,N’-dimethyl-N(iodoacetoyl)-N’-(7-nitrobenz-2-oxa-1,3-diazol)ethylenediamine) as described (21). The liposomes (DOPC:DOGs-NTA-Ni = 100:8) were incubated with indicated proteins at pH 8.5 for 30 min at room temperature, and the incubated protein-liposome mixture (total volume, 1.8 ml) was subjected to three freeze-thaw cycles and extrusion through a 200-nm pore size polycarbonate filter (Nucleopore Inc.).
was transferred to a cuvette with a stirring bar in an ISS K2 fluorometer. The NBD was excited at 488 nm, and emission was recorded at 544 nm. Crossed polarizers on excitation, emission beams, and a 520-nm filter were used to reduce the background because of scatter. After addition of 0.2 ml of 1M pH 8.5 buffer or pH 5.0 buffer to the cuvette, a sharp decrease of the background scatter signal was observed because of dilution. This was followed by a specific signal resulting from shifting NBD from a polar (solution) to a non-polar environment (liposomal membrane).

RESULTS

Acidic Conditions Cause Rapid Inactivation of the PA Prepore—It is well documented that purified PA prepore and nPA form ion-conductive pores in planar phospholipid bilayers and unilamellar asolectin vesicles under acidic conditions (14, 22–24). The heptameric prepore rapidly loses the ability to form ion-conductive pores when exposed to acidic pH, however, limiting the partitioning into membranes. As shown in Fig. 1, in the absence of membranes the prepore loses a large fraction of its ability to induce K⁺ leakage from KCl-loaded DOPC LUV within 15 s after being exposed to pH 5.0 buffer, and this activity is almost completely lost within 1 min. We observed a similar inactivation when prepore was acidified before being added to planar bilayers*. Under acidic conditions, the prepore converts to an SDS-resistant state and forms aggregates, suggesting mechanisms for this inactivation.

His-R2 Mediates Binding of PA to Liposomes Containing Ni²⁺-chelating Lipids—In principle it should be possible to increase the partitioning of PA into liposomes by binding the protein to the membrane surface before it is exposed to low pH. To examine the use of a receptor domain to mediate binding of PA to membranes, we first prepared an N-terminally His-tagged form of the ANTXR2 VWA domain (His-R2) and measured its binding to DOPC LUV doped with various levels of the Ni²⁺-chelating lipid (DOGs-NTA-Ni). The binding showed a clear dependence on DOGs-NTA-Ni up through 16 mol % (Fig. 2A). The binding reached equilibrium by 15 min, and there was no nonspecific binding of His-R2 with extended incubation times. Imidazole effectively inhibited the binding, consistent with a Ni²⁺-mediated interaction (supplemental Fig. 1). The behavior of a C-terminally His-tagged ANTXR2 VWA domain (R2-His) was identical to that of the N-terminally tagged pro-

* B. Krantz, R. Melnyk, and R. Collier, unpublished data.

FIGURE 1. PA prepore is rapidly inactivated at pH 5.0. Samples of purified prepore (4 μM) were incubated at pH 5.0 for 15, 30, or 60 s and then added to KCl-charged LUV at pH 5.0 and assayed for ability to release K⁺. The final concentration of heptamer was 3 nM. Untreated heptamer (0 s) and buffer alone controls were included.

FIGURE 2. His-R2 binds to LUV containing DOGs-NTA-Ni and mediates the binding of PA and the assembly of toxic complexes. A, purified His-R2 (40 μM) was incubated at room temperature for 30 min in buffer (20 mM Tris-Cl, 150 mM NaCl, pH 7.5) with or without DOPC LUV (total lipid, 0.2 mg/ml) containing 0, 0.5, 1, 2, 4, 8, or 16 mol % DOGs-NTA-Ni. The final concentrations of DOGs-NTA-Ni were 0, 0.625, 1.25, 2.5, 5, 10, and 20 μM, respectively. The liposomes were pelleted by centrifugation (30 min at 100,000 × g), and liposome-bound His-R2 was examined by SDS-PAGE and quantified by densitometry. B, PA (5 μM), His-R2 (10 μM), and liposomes (8% DOGs-NTA-Ni/DOPC; total lipid, 1 mg/ml) were mixed as indicated and incubated with 1 mM MgCl₂ (pH 8.5). After 30 min at room temperature, the liposomes were pelleted by centrifugation, and membrane-associated proteins were analyzed by SDS-PAGE. C, PA (5 μM), nPA (5 μM), His-R2 (10 μM), LF₅-DTA (5 μM), and liposomes (8% DOGs-NTA-Ni/DOPC; total lipid, 1 mg/ml) were mixed as indicated and incubated at pH 8.5 in the presence of 1 mM MgCl₂. At 30 min, the membranes were pelleted by centrifugation, and the liposome-bound proteins were examined by SDS-PAGE.
We incubated PA with His-R2 and LUV containing 8% DOGs-NTA-Ni and then isolated the membranes by centrifugation and analyzed their associated proteins by SDS-PAGE. As shown in Fig. 2B, binding of PA to liposomes was at background levels in the absence of His-R2 and increased dramatically in its presence. Next, we tested the requirements for binding of LFN-N$_5$-DTA to liposomes (Fig. 2C). LFN-N$_5$-DTA, a fusion protein consisting of the enzymatic domain of DTA fused to the C terminus of the LFN$_5$ enters cells via the same pathway as LF and EF and blocks protein synthesis. Binding of LFN-N$_5$-DTA to nickel-containing liposomes was observed in the presence of His-R2 and trypsin-activated PA (nPA) (Fig. 2C). Native PA did not functionally replace the activated form, consistent with the fact that LF and EF bind to oligomers of PA$_63$ which form only after proteolytic activation of PA. The results suggest that nPA associated with liposomes via His-R2, and after dissociation of PA$_63$ oligomerized on the liposomal membrane, allowing LFN-N$_5$-DTA to bind.

His-R2-dependent, PA-mediated Pore Formation—As shown in Fig. 3A, binding to His-R2 greatly increased the activity of nPA in releasing entrapped K$^+$ from DOPC liposomes containing 8% DOGs-NTA-Ni (pH 5.0). Within 60 s K$^+$ release in the presence of His-R2 reached a level close to that seen following addition of gramicidin. In the absence of His-R2, the level of release by nPA was much lower. His-R2-dependent K$^+$ release was strongly dependent on acidic pH and showed a sharp threshold at pH $\sim$5.5 (Fig. 3B). In the absence of His-R2, the release was slower and more limited, and the threshold was about one pH unit higher. These results are consistent with the observation that binding of ANTXR2 VWA domain to PA prepore shifts the pH threshold of prepore-pore conversion to a more acidic pH range (10). Free and His-R2-complexed prepore gave results similar to those with free and His-R2-complexed nPA, respectively, in releasing K$^+$ as a function of pH.

We characterized two PA mutants as controls in the K$^+$ release assay. D512K, an oligomerization-deficient PA mutant (data not shown), consistent with the locations of the N and C termini of the ANTXR2 VWA domain; adjacent to each other and diametrically opposite the conserved metal ion-dependent adhesion site motif, the focal point of PA binding (10).

FIGURE 3. His-R2 facilitates PA-dependent K$^+$ release from LUV charged with a Ni$^{2+}$-chelating lipid. Pore formation was monitored by K$^+$ release from LUV containing 8% DOGs-NTA-Ni, as described under “Materials and Methods.” A, representative K$^+$ release traces generated by buffer alone, nPA (40 nm), and nPA (40 nm) + His-R2 (80 nm) at pH 5.0. B, PA pore formation as a function of pH in the presence of buffer alone, nPA (40 nm), or nPA (40 nm) + His-R2 (80 nm). Percentage K$^+$ release was calculated as K$^+$ release before gramicidin addition/total K$^+$ release $\times$ 100. The signal from the buffer alone reflected extravesicular K$^+$ in the liposome preparation and spontaneous K$^+$ leakage. The traces shown are representative ones from three independent experiments.

FIGURE 4. His-R2-dependent K$^+$ release requires PA oligomerization, conversion to the pore conformation, and membrane insertion. A, representative K$^+$ release curves from LUV containing 8% DOGs-NTA-Ni and generated at pH 5.0 by buffer alone, nPA (40 nm) + His-R2 (80 nm), or nD512K (40 nm) + His-R2 (80 nm). B, representative K$^+$ release curves from LUV containing 8% DOGs-NTA-Ni, generated at pH 5.0 by buffer alone, wild-type prepore (PA$_{wt}$)$_7$ (3 nm), (DNI)$_7$ (3 nm), and (WT:DNI)$_7$ (3 nm). All forms of PA heptamer were complexed with His-R2. C, fluorescence intensity of NBD-labeled nPA-G305C (0.5 mm) + His-R2 (1 mm) + G305C (3 mm) - His-R2 (1 mm) + G305C (3 mm) and (WT:DNI)$_7$ (3 nm). The proteins were incubated with liposomes (8% DOGs-NTA-Ni/DOPC, 0.5 mg/ml total lipid) at pH 8.5 for 30 min at room temperature, in the presence of 1 mM MgCl$_2$. Subsequently, the pH was either kept at pH 8.5 or rapidly lowered to pH 5.0 by adding 1/10 volume of 1 M pH buffers (1 M Tris-Cl, pH 8.5, or 1 M NaAc, pH 5.0). The solution was continuously stirred in the cuvette. NBD fluorescence (excitation at 488 nm, emission at 524 nm) was recorded. G305C, nPA-G305C; N306C, nPA-N306C; L, liposome (DOGs-NTA-Ni/DOPC 8%). The baseline-level curves, which are not labeled, for clarity, represent the following controls: G305C + His-R2 + L (pH 8.5); G305C + L (pH 5.0); G305C + L (pH 8.5); N306C + His-R2 + L (pH 5.0); and G305C (pH 5.0).
A Liposomal System to Study Anthrax Toxin

DISCUSSION

Many intracellularly acting toxins initially bind to a receptor on the surface of host cells and later translocate across the endosomal membrane under the influence of acidic pH. In some of these proteins (e.g. diphtheria toxin) the pH-dependent membrane insertion process has been studied extensively in model membranes devoid of receptors (27–32). In the case of anthrax toxin, the PA preprope has been shown to form ion-conductive pores (channels) in both planar phospholipid bilayers and liposomes in the absence of receptors, and much information about the properties of these pores has been obtained recently from studies in planar bilayers (24, 33). Although liposomes offer advantages over planar bilayers in addressing some aspects of pore formation and function, they have not been as useful in studying PA preprope insertion, in part because of the apparent inefficiency of insertion revealed in earlier studies (14).

In the current study we have addressed this problem and developed ways to overcome it by binding PA to vesicles in

branes by either mode, nPA gave greater activity than unbound nPA lacking His tag (Fig. 3A). nPA-His behaved similarly to PA lacking the His tag in terms of oligomerization and ANTXR2 binding (data not shown), and both had low activity in releasing K\(^{+}\) from DOPC liposomes lacking the Ni\(^{2+}\)-chelating lipid (Fig. 5C). Because PA that was bound to liposomes directly via its C terminus was as active in pore formation as PA bound via ANTXR2 VWA bridge, His-R2 apparently functioned primarily as a membrane anchor in this system.

R2 Lacking a His Tag Inhibits Aggregation of Heptameric PA in Solution and Facilitates Pore Formation—When we performed controls to test the possibility that R2 lacking a His tag might affect pore formation by PA preprope, we found that indeed it increased K\(^{+}\) release from liposomes (Fig. 6A). However, R2 did not bind to liposomes at pH 4–9 (data not shown), suggesting that, unlike His-R2, it did not mediate binding of PA to LUV. We therefore examined an alternative possibility; binding of R2 might slow the inactiva-
tion of heptameric PA\(_{63}\). We incubated the heptamer with excess R2 at pH 5.0 for various brief periods and then added the mixture to DOPC liposomes and monitored the kinetics of K\(^{+}\) release. As shown in Fig. 6B, in comparison with the data in Fig. 1, R2 significantly retarded the inactivation process. Also, we examined the effect of R2 on aggregation of the preprope by monitoring turbidity at 340 nm after lowering the pH from pH 8.5 to 5.0 (Fig. 6C). Although R2 did not significantly change the final level of aggregation, it caused a delay in the formation of aggregates, on the order of minutes. Consistent with the known high affinity of R2 for PA, the maximal shift in kinetics was observed with a relatively small molar excess of the receptor domain. Bovine serum albumin at molar ratio of the protein to PA (8:1) did not affect the aggregation kinetics (data not shown).

PA Bound to Liposomes via a His tag on Its C Terminus Mediates Efficient Pore Formation—To probe the role of the ANTXR2 VWA domain in this system, we compared attaching PA to liposomes via a ANTXR2 VWA bridge protein with attaching it directly via a His tag on the C terminus of PA. nPA-His and His-R2-complexed nPA showed approximately equal levels of binding to DOPC liposomes containing 8% DOGs-NTA-Ni (Fig. 5A) and similarly high levels of activity in promoting K\(^{+}\) release (Fig. 5B). Also, when attached to mem-

To demonstrate the insertion of PA\(_{63}\) into liposomal membranes directly, we attached an environment-sensitive dye (NBD) to either of two sites within the protein and monitored its fluorescence emission intensity during pore formation. The intensity of NBD fluorescence at 544 nm increased upon a shift from a polar to a non-polar environment, and earlier we monitored insertion of various residues of the 14-strand \(\beta\)-barrel into membranes of Chinese hamster ovary-K1 cells after replacing those residues with Cys and reacting the protein with a thiol-reactive form of NBD (21). In that study, NBD attached to an introduced Cys at position 305 (facing the lipid bilayer) showed a strong increase in fluorescence, whereas at position 306 (facing the aqueous lumen) the fluorophore showed little change. In the current study we observed a strong increase of fluorescence intensity at pH 5.0 with NBD-PA(G305C) during pore formation but not with NBD-PA(N306C) (Fig. 4C). The increase seen with the labeled PA (G305C) was dependent on low pH, His-R2, and liposomes. These data correlate results in the LUV system with those in cells and support the \(\beta\)-barrel model of PA insertion.

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**FIGURE 6.** R2 facilitates pore formation in liposomal membranes by slowing inactivation of heptameric PA at pH 5.0. A, representative K⁺ release curves from liposomes generated at pH 5.0 by buffer alone, prepore (3 nM), and prepore (3 nM) complexed with R2 (40 nM). B, prepore (4 μM) complexed with R2 (56 μM) was treated with 1/10 volume of 1 mM pH 5.0 buffer (final pH is 5.0) and after 15, 30, 60, and 120 s of treatment, the low pH-treated (PA₆₃)₇ complexes were assayed for ability to release K⁺ from LUV. Pre pore-R2 complexes that had not been treated at low pH treatment (0 s) were included as a control. C, prepore (64 nM) was incubated in buffer (1 mM MgCl₂, 20 mM Tris-HCl, 150 mM NaCl, pH 8.5) with or without R2 at R2/PA molar ratios of 0, 1, 2, 4, and 8. After 60 min at room temperature, 1 mM pH 8.5 or 5.0 buffers were added to the solutions with continuous stirring. The intensity (I) of scattered light at 340 nm was then monitored with time in a R2 fluorometer and was expressed as I-I₀ at 340 nm. Inset, plot of t₁⁄₂ values of PA aggregation versus R2/PA molar ratio.

advance of pore formation. Besides making liposomes a more useful system to study anthrax toxin, the results shed light on the role of receptors in toxin action.

Within seconds after the pH was lowered to 5.0, the prepore converted in solution to an inactive form, lacking the ability to permeabilize DOPC LUV membranes to K⁺. Concurrently the heptameric PA₆₃ complex became resistant to dissociation by SDS and fell out of solution, generating visible aggregates. Therefore, acidic pH triggered a conformational rearrange ment in the prepore, yielding a short-lived intermediate that was competent to insert into membranes and then converted to an inactive form. The structures of the active and inactive forms are unknown. Conceivably the active form corresponds to the pore, containing a fully formed 14-strand β-barrel, as a transiently soluble intermediate, and the inactive form corresponds to aggregates of this form. However, the pathway and the end product(s) may be more complex. Regardless, the pathway leading to inactivation in solution clearly competes with the pathway leading to pore formation, and this can cause partitioning into membranes to be inefficient.

In an attempt to rectify this problem, we sought ways to bind PA to the surface of LUV in advance of acidification. Availability of a full-length functional receptor was still a limiting factor, but the use of metal-chelating lipids in combination with a poly His-tagged receptor ecto-ligand-binding domain appeared to be an alternative. Incorporating a His-tagged poliovirus receptor into liposomes via nickel-chelating lipids was recently used to study non-enveloped virus cell entry (34, 35). We used a similar approach, based on the availability of the purified PA-binding domain (VWA) of ANTXR2 and the ability to produce a His-tagged form of this protein efficiently in *Escherichia coli*. LUV doped with a Ni²⁺-chelating lipid could bind His-R2, and this protein in turn could serve as a receptor for monomeric PA or prepore. The interaction between monomeric PA and R2 shows a Kᵦ ≈ 0.2 nM, and because the prepore can bind seven copies of R2, its linkage to R2 on liposomes would be expected to be effectively irreversible. Although the Kᵦ for the interaction between hexa-His tag and Ni²⁺-NTA was about 1 μM (36), in the present study the His-R2 binding to DOGs-NTA-Ni was optimized by using excess DOGs-NTA-Ni relative to His-R2, which allowed at least 90% His-R2 bind to liposomes (data not shown). In addition, when prepore was saturated with R2, the interaction of the entire complex with DOGs-NTA-Ni-containing membranes would be expected to be effectively irreversible because of the multi-point contact of the seven His-R2 molecules with the nickel-containing lipid.

We showed that the efficiency of pore formation in doped LUV was strongly enhanced when PA was bound to vesicles via the His-R2 bridge protein, as judged by the kinetics of K⁺ release. The insertion half-time was on the order of several seconds, as measured by K⁺ release or enhancement of fluorescence of NBD at position 305 and was much more rapid than that observed with unbound PA. Thus the insertion was largely complete before the complex could proceed down the pathway to inactivation (if such a pathway is in fact operative with membrane-bound PA.) Consistent with observations in other systems, the binding to His-R2 shifted the pH threshold of conversion to the pore to a lower range (9, 13, 37); mutations in PA known to affect heptamerization or prepore-to-pore conversion inhibited pore formation in the predicted way (3, 25); and membrane insertion by a fluorescently tagged form of PA gave results consistent with prior findings in cells and with the β-barrel model of pore formation (21). Also, binding of LF₃ΔN- DTA was shown to depend on proteolytic activation of PA (3). As judged by these various criteria, doped DOPC LUV containing His-R2 as receptor represent a valid model of the membrane insertion process in vivo.
An almost identical enhancement of pore formation was observed when PA was bound to doped LUV via a His tag appended to its C terminus. This implies that an important function of the PA receptor is to concentrate PA close to the surface of the membrane during prepore-to-pore conversion. Orientating the prepore with respect to the membrane is likely also to be important, but in our experiments binding either directly or via His-R2 occurred via the PA C-terminal domain 4, so the membrane-penetrating β-barrel would form on the surface of the pore facing the membrane. Thus, our results do not discriminate between this and alternative orientations. Binding via the His-R2 bridge protein would place the prepore farther away (~40 Å) from the membrane surface than binding via the C-terminal His tag on PA, but this did not diminish the efficiency of insertion. Furthermore, in the native ANTXR2 receptor there is an additional domain between the VWA domain and the transmembrane domain, so that ANTXR2-bound PA prepore is at an even greater distance from the membrane surface in vivo. The 14-strand β-barrel of the pore is predicted to be ~100 Å long (38), with the transmembrane segment adjacent to the insertion tip, allowing a significant “reach” of prepore not immediately adjacent to the membrane surface. There is evidence in vivo that the receptor dissociates from the PA heptamer after conversion to the pore (37), and binding to the ANTXR1 receptor affects the pH threshold of this conversion to a lesser extent than ANTXR2 (37). Our results do not speak to the question of whether partitioning into the membrane of membrane-bound nPA varies significantly with small changes in the pH at which prepore-to-pore conversion occurs. The ability to bind PA to liposomes doped with metal-chelating lipids, as shown here, makes such vesicles useful tools to study this and other questions pertaining to the action of anthrax toxin.

Acknowledgment—We thank Ruth-Anne Pimental for help with protein preparation.

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