Phenylalanine-427 of anthrax protective antigen functions in both pore formation and protein translocation

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The protective antigen (PA) moiety of anthrax toxin forms a heptameric pore in endosomal membranes of mammalian cells and translocates the enzymatic moieties of the toxin to the cytosol of these cells. Phenylalanine-427 (F427), a solvent-exposed residue in the lumen of the pore, was identified earlier as being crucial for the transport function of PA. The seven F427 residues were shown in electrophysiological studies to form a clamp that catalyzes protein translocation through the pore. Here, we demonstrate by a variety of tests that certain F427 mutations also profoundly inhibit the conformational transition of the heptameric PA prepore to the pore and thereby block pore formation in membranes. Lysine, arginine, aspartic acid, or glycine at position 427 strongly inhibited this acidic pH-induced conformational transition, whereas histidine, serine, and threonine had virtually no effect on this step, but inhibited translocation instead. Thus, it is possible to inhibit pore formation or translocation selectively, depending on the choice of the side chain at position 427; and the net inhibition of the PA transport function by any given F427 mutation is the product of its effects on both steps. Mutations inhibiting either or both steps elicited a strong dominant-negative phenotype. These findings demonstrate the dual functions of F427 and underline its central role in transporting the enzymatic moieties of anthrax toxin across membranes.

acidic pH | dominant-negative | endosome

Toxins that modify targets within the cytosolic compartment of mammalian cells play crucial roles in many bacterial diseases. Most such toxins have separable catalytic and receptor-binding moieties; the receptor-binding moiety binds to cell surface receptors and facilitates delivery of one or more catalytic moieties to the cytosol (1). Besides binding to receptors, the receptor-binding moieties of some of these toxins, including anthrax toxin, also can insert into membranes and form ion-conductive pores (channels) that function in A-chain transport. The mechanisms of pore formation and membrane translocation by these toxins are of great interest in understanding how proteins translocate across membranes and for developing a firmer foundation for medical applications of toxins.

Anthrax toxin is a tripartite intracellularly acting toxin, composed of two catalytic moieties, edema factor (EF) and lethal factor (LF), and a single receptor-binding/pore-forming moiety, protective antigen (PA) (2). 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) (3). PA₆₃ oligomerizes into a heptameric, receptor-bound prepore, which contains high-affinity binding sites for EF and LF. The PA prepore can bind up to three molecules of EF and/or LF competitively (4–8), and the resulting toxin-receptor complexes are internalized by receptor-mediated endocytosis and trafficked to endosomes. There, the prepore undergoes an acidification-triggered conformational rearrangement to a cation-selective, transmembrane pore (9). Although the structure of PA pore has not been determined, there is strong evidence that during prepore–pore conversion the 2β2–2β3

loops (residues 302–325) in domain 2 of the prepore move to the base of the structure to form a 14-strand transmembrane β-barrel, similar to that formed by *Staphylococcus aureus* α-hemolysin (10–12) (Fig. 1). This transition is believed to yield a mushroom-shaped pore, which mediates translocation of EF and LF across the endosomal membrane. Within the cytosol, EF, an 89-kDa calmodulin-dependent adenylate cyclase, elevates the intracellular cAMP level (13), and LF, a 90-kDa zinc protease, inactivates mitogen-activated protein kinase kinases (14).

Membrane translocation is the least well understood aspect of the action of intracellularly acting toxins, and anthrax toxin has recently emerged as a tractable system for studying this process. Phenylalanine-427 (F427), a residue in the 2β10–2β11 loop on the luminal aspect of PA, is essential for the protein transport activity of the protein and has become a focal point in understanding the process of translocation. Attention was first drawn to F427 when the crystallographic structure of the prepore was solved, revealing this residue to be luminal and solvent-exposed (15), and thus a candidate for interacting with translocating polypeptides (Fig. 1). Mutation of F427 to Ala was found almost to abolish the ability of PA to mediate the entry of a model enzymatic A-chain into Chinese hamster ovary-K1 (CHO-K1) cells (16). Furthermore, F427A PA showed a dominant-negative phenotype, which depended on its ability to co-oligomerize with the wild-type (WT) protein and form heptamers with attenuated transport activity (17). Subsequently, electrophysiological studies showed that pores formed by the F427A mutant in planar phospholipid bilayers were defective in catalyzing polypeptide translocation across these membranes (18). Evidence was presented that the seven F427 residues of the PA heptamer come into close proximity within the lumen of the pore and form a clamp, termed the Phe clamp, which catalyzes polypeptide translocation. The translocation defects caused by several F427 mutants in planar bilayers appeared to correlate approximately with the degree of impairment in mediating toxicity in CHO-K1 cells, suggesting that the biochemical defect might fully account for the loss of biological activity.

Here, we report that whereas certain F427 mutations primarily affect the translocation function of PA, others strongly inhibit the conformational transition of the prepore to the pore, and still others affect both steps. Thus, F427 participates in two sequential steps in the overall transport function of the toxin, and the net effect of any given mutation is the product of its effects on both steps. These findings have ramifications for understanding

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site on toxicity therefore involves effects on both the pore formation and the translocation steps in transporting substrate proteins across endosomal membranes. We found that replacement of F427 with Gly or a charged residue, such as Arg, strongly impaired prepore-to-pore conversion, and several other mutations moderately inhibited this step. Prepore-to-pore conversion and translocation showed similarities in side-chain dependence at position 427, in that hydrophobic residues, except Ile, were active in promoting both of these steps, and Gly or residues with a charged side chain blocked both steps. However, F427S, F427T, and F427H did not impair prepore-to-pore conversion but strongly inhibited translocation. Thus, at least for some mutations at this site, different mechanistic explanations must be invoked to explain the differential effects of F427 mutations on pore formation and translocation.

How might a F427 mutation inhibit prepore-to-pore conversion? A plausible mechanism is suggested by electron paramagnetic resonance measurements indicating that the F427 side chains are close to one another (≤ 10 Å) in the pore. Depending on the closeness of approach of side chains at this position within the pore, or in intermediate states in the process of prepore-to-pore conversion, hydrophobic and/or stacking interactions between aromatic (Trp, Tyr, and His) or aliphatic side chains (Leu), or hydrogen bonds between certain others (e.g., Ser, Thr, or His), could foster prepore-to-pore conversion, whereas electrostatic repulsion between charged side chains, e.g., Arg, could inhibit this conversion. The slightly weaker inhibition observed with F427D, relative to F427R and F427K, could reflect the high density of acidic residues in the pore lumen, implying a mildly acidic local pH, and thus partial protonation of an acidic residue at position 427. Consistent with this notion and the fact that Glu has a higher pK_a than Asp, the F427E mutation caused less inhibition of K^+ release than the F427D mutation (data not shown). The basis of the strong inhibitory effect of the F427G mutation is unclear, but the consistently lower activity of Ile (β -branched) relative to Leu (γ -branched) illustrates the exquisite sensitivity of the prepore-to-pore transition to side-chain geometry at position 427.

The selective inhibition of the translocation step by certain mutations, notably F427S, F427T, and F427H, could have various explanations. Whereas WT PA was found in single-channel (single-pore) conductance measurements to interact with LF_N in such a manner as to form a seal against ion passage, the F427A pores gave an incomplete seal and a flickering conductance pattern (18). An incomplete seal could disrupt the transmembrane gradient and hence the driving force for translocation. A similar mechanism may underlie the effects of the Ser and Thr mutations on translocation, because of their relatively small side-chain volumes. However the inhibitory effect of the F427H mutant may depend on its ability to be protonated, inasmuch as a positive charge at this position would be predicted to inhibit interactions with the positively charged N-terminal translocation leader sequences of LF and EF. It is also possible that the capacity of His, Ser, and Thr to form hydrogen bonds, between each other or with translocating polypeptides, may be related to their blockage of translocation.

The F427D, F427R, F427H, and F427G mutants exhibited a strong dominant-negative phenotype, with inhibitory potency similar to that of DNI. F427D, F427R, and F427G inhibited pore formation, whereas F427S and F427H specifically inhibited membrane translocation. This finding indicates that major blockage of either prepore-to-pore conversion or translocation can yield such a phenotype. Both types of the mutants therefore cooligomerize with WT to yield inactive complexes. Dominant-negative forms of PA have been of interest both as potential inhibitors of anthrax toxin and as components of conjugate vaccines containing capsular material from *Bacillus anthracis*

covalently linked to PA (25). The results presented here cast a new perspective on these applications.

Materials and Methods

Cell Culture and Media. CHO-K1 cells were from the American Type Culture Collection and were grown in Ham's F-12 medium supplemented with 10% calf serum, 2 mM glutamine, 500 units/ml penicillin, and 500 units/ml streptomycin sulfate, under a humidified atmosphere with 5% CO_2 .

Site-Directed Mutagenesis and Protein Purification. Mutations at F427 were generated by site-directed mutagenesis (QuikChange; Stratagene) and were confirmed by DNA sequencing. Recombinant PA, R2, His-R2, and LF_N -DTA were expressed and purified as described in ref. 19. R2 LF_N -DTA and prepore were prepared as described in ref. 26. Protein concentrations were determined by spectrophotometry (A_{280}) and densitometry on SDS/PAGE with BSA as standard.

Liposome Preparation. Liposomes were prepared as described in ref. 19. Briefly, DOPC, either alone or mixed with a Ni^{2+} -chelating lipids, DOGS-NTA- Ni (1,2-dioleoyl-*sn*-glycero-3-[[N(5-amino-1-carboxypentyl)iminodiacetic acid]-succinyl nickel salt]) (molar ratio, 100:8), in chloroform (Avanti Polar Lipids) was dried under N_2 gas to form a lipid film, followed by vacuum for 3 h to remove residual solvent. The dried lipid film was rehydrated with buffer to form multilamellar vesicles and subjected to three freeze-thaw cycles and extrusion through a 200-nm pore size polycarbonate filter (Nucleopore) in a mini extruder (Avanti Polar Lipids). The protocol yielded large unilamellar vesicles with an average diameter of 150–200 nm.

K^+ Release Assay. Liposomes containing 150 mM KCl, 10 mM Hepes (pH 7.4) were transferred into 150 mM NaCl, 20 mM Tris-HCl (pH 8.5) by buffer exchange, as described in ref. 19. The liposomes were added to K^+ release buffer (50 mM sodium acetate, 150 mM NaCl, pH 5.0) and after 1 min, PA prepore (3 nM) complexed with R2 (40 nM) was added. The solution was stirred continuously with a magnetic stirrer, and K^+ release was monitored with a K^+ -selective electrode (Orion Research). Rates of K^+ release were calculated in SigmaPlot by fitting the K^+ release curves to the double exponential equation $f = a*[1 - \exp(-b*x)] + c*[1 - \exp(-d*x)]$, in which b is the rate constant of K^+ release by PA and d is the rate constant of spontaneous K^+ leakage from liposomes.

Time-Lapse Intensity Measurements of NBD Emission. PA proteins carrying the mutation G305C were labeled with NBD as described (19, 26). The labeling efficiency was 90–100%. NBD emission of PA pores in the liposomal membranes was measured as described in ref. 19. Briefly, the liposomes were incubated with trypsin-activated PA (0.5 μ M) and His-R2 (1 μ M) at pH 8.5 for 30 min, and the protein-liposome mixtures were transferred to a cuvette with a stirring bar in an ISS K2 fluorometer. 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 scatter. After addition of 1/10 volume of 1 M sodium acetate (pH 5.0) to the cuvette, the shift of NBD label from a polar (solution) to a nonpolar environment (liposomal membrane) was monitored. Rates of NBD emission were calculated in SigmaPlot by fitting the curves to the single exponential equation $f = y_0 + a*[1 - \exp(-b*x)]$, in which b is the rate of NBD emission at 544 nm.

Rb^+ Release. As described in ref. 27, CHO-K1 cells were incubated overnight in complete F-12 medium containing 1 μ Ci/ml $^{86}Rb^+$. After removal of the excess $^{86}Rb^+$, the cells were incubated with PA prepore (1 μ g/ml) in F-12 medium at 4°C for 1 h. Unbound protein was removed by washing with cold PBS, and the cells were incubated with pH 5.0 or pH 8.0 buffer (140 mM NaCl, 20 mM Mes, 5 mM gluconic acid, pH 5.0 or pH 8.0) at 37°C for 5 min and subsequently incubated in 4°C for 30 min. Supernatants containing the released $^{86}Rb^+$ were collected and counted in a gamma-counter.

SDS-Resistant PA₆₃ Oligomer. To assay prepore samples in solution, prepore (3 mg/ml) was incubated with R2 (2 mg/ml) in 20 mM Tris-HCl, 150 mM NaCl (pH 8.5) at room temperature for 30 min. The solution was acidified by the addition of 1/10 volume of 1 M sodium acetate (pH 5.0) and incubated for 10 min. Samples were exposed to 1.25% SDS for 20 min and electrophoresed on SDS/PAGE, and the bands were visualized after Coomassie staining. To assay prepore samples bound to the plasma membrane, CHO-K1 cells were incubated with PA prepores (10 μ g/ml) for 1 h at 4°C. The cells were washed with cold PBS and incubated in 150 mM NaCl, 20 mM Mes, 5 mM gluconic acid, pH