

Electrophysiological characterization of derivatized alpha-hemolysin (αHL) for single-molecule fluorescence studies in planar lipid membranes

M. Madison Taylor, Derek J. Bailey, Ashley R. Paulson, Lisa M. Keranen-Burden, Daniel L. Burden

Wheaton College, Chemistry Department
Wheaton, IL 60187

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Abstract: Previous biochemical and crystallographic investigations have established that *Staphylococcus aureus* alpha-hemolysin (αHL) forms conductive pores in lipid membranes by self-assembling in a series of steps from seven identical protein monomers. However, the assembly process, the translational dynamics of the pre-pore and pore, and the quaternary structure of αHL in lipid bilayers have not been directly visualized. Furthermore, visualization at the single-molecule level has not been simultaneously correlated with transmembrane current. Here, we report findings from electrical studies on a battery of eight site-directed mutants that are covalently linked to one of two fluorescent dyes (BODIPY or TMRIA). Ensemble and single-channel current measurements are performed using planar diphytanoyl phosphatidylcholine lipid bilayers. Channel conductance, voltage rectification ratio, and gating stability are used to compare mutants, fluorescently labeled mutants, and wild-type αHL. All mutated and labeled channels maintain pore-forming capability, although differences in conductance state, rectification ratio, and gating stability are observed. We present details of the electrical characterization and discuss initial work on simultaneous optical and electrical measurements in planar lipid membranes.

Introduction

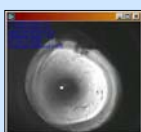
Staphylococcal alpha-Hemolysin (αHL) monomers aggregate on the surface of cell membranes to form toxic heptameric channels. In addition to cytolytic activity, αHL has been linked to increased antibiotic resistance in Methicillin Resistant *Staphylococcus aureus* (MRSA).¹ Recent studies have also demonstrated that antibodies against αHL can prohibit Staphylococcal pneumonia.^{2,3}

The mechanism of monomer aggregation and pore assembly, along with the translational dynamics of αHL in lipid membranes, have not been directly observed. In effort to visualize these processes, we made eight αHL mutants and covalently attached a fluorescent dye to strategically placed cysteine residues. Here, we explore the impact of these site-directed mutations and the associated fluorescent labels on the electrophysiological characteristics of the pore.

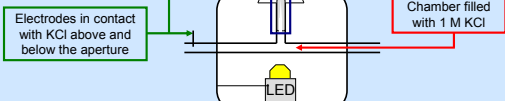
Experimental Setup

- Apertures are made by pushing the tip of a pin through PTFE tubing creating a 50-μm tapered hole
- The aperture is put into the sample chamber

¹ Caiazza, N. and O'Toole, G. (2003) *J. Bacteriol.* 185 (10), 3214-7
² DeLeo F. and Otto M. (2008) *J. Exp. Med.* 205 (2), 287-94
³ Buback, Wardenburg, J. and Schneewind, O. J. (2008) *J. Exp. Med.* 205 (2), 287-94

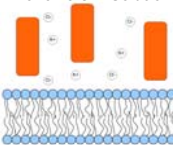


A schematic of the sample chamber used (left) and a picture of a 50 μm aperture in PTFE tubing (right)

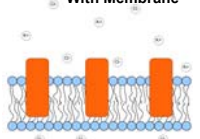


Proposed αHL Assembly Mechanism and Dynamics

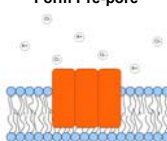
Monomers in Solution



Monomers Associate With Membrane



Monomers Aggregate Form Pre-pore

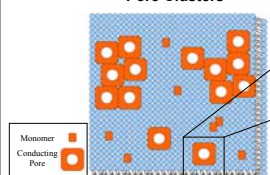


- Pore contains an extended hydrophobic β-barrel domain
- Pores are dynamic and open and close in random fashions. Generally, quiescent at high salt concentration.

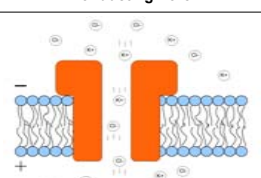
- Potential cooperativity of pore formation

Extracellular (Cis)
Lipid Membrane
Intracellular (Trans)
Crystal Structure: Song, L., et al. (1996) *Science*, 274, 1859

Pore Clusters

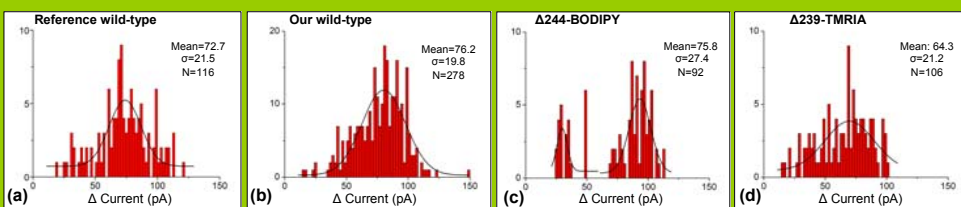


Conducting Pore

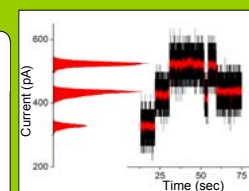
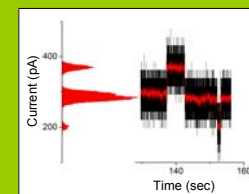


Adapted from: Walker, B., et al. (1992) *J. Biol. Chem.* 267, 21782

Channel Conductance

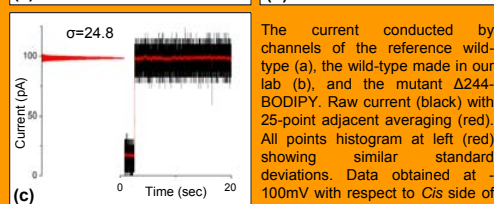
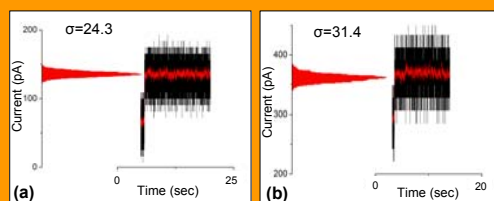


Histograms showing step current associated with single open channels for the reference wild-type (a), the wild-type made in our lab (b) and the mutants Δ244-BODIPY (c) and Δ239-TMRIA (d). No significant difference between the step size of the reference wild-type and our wild-type observed.

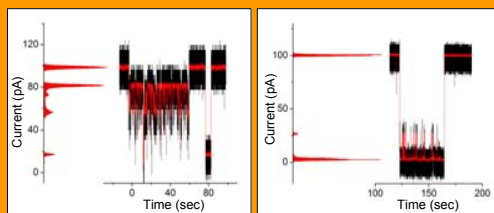


Transmembrane channel insertion currents of wild-type (top) and Δ244-BODIPY (bottom). Raw data (black) and 25-point adjacent averaging of current data (red). All points histogram at left (red) shows the magnitude of insertion currents for individual channels.

Gating Stability

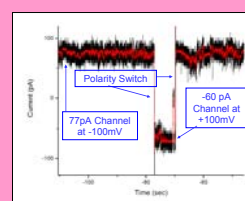


The current conducted by channels of the reference wild-type (a), the wild-type made in our lab (b), and the mutant Δ244-BODIPY. Raw current (black) with 25-point adjacent averaging (red). All points histogram at left (red) showing similar standard deviations. Data obtained at -100mV with respect to Cis side of the membrane.



Mutant Δ244-BODIPY. Raw current (black) with 25-point adjacent averaging (red). Similar instability can be seen in other labeled mutants as well. The all points histogram on the left of each image suggests the presence of small conformational alterations.

Voltage Rectification Ratio



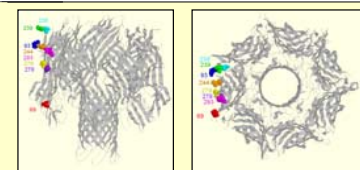
Ref. wild-type	1.35
Our wild-type	1.33
Δ238-TMRIA	1.3
Δ239 Unlabeled	1.27
Δ239-BODIPY	1.28
Δ239-TMRIA	1.23
Δ244-BODIPY	1.36
Δ279 Unlabeled	1.13

Rectification Ratio: $[77] \setminus |-60| = 1.28$

Example of raw data used to determine rectification ratio (left) and the voltage rectification ratio for several mutants (right).

Summary

Δ 69 No Label	Yes	Δ 244 No Label	Yes
Δ 69 TMRIA		Δ 244 TMRIA	Yes
Δ 69 BODIPY	Yes	Δ 244 BODIPY	Yes
Δ 93 No Label	Yes	Δ 278 No Label	
Δ 93 TMRIA		Δ 278 TMRIA	Yes
Δ 93 BODIPY	Yes	Δ 278 BODIPY	Yes
Δ 238 No Label	Yes	Δ 279 No Label	Yes
Δ 238 TMRIA	Yes	Δ 279 TMRIA	Yes
Δ 238 BODIPY		Δ 279 BODIPY	
Δ 239 No Label	Yes	Δ 293 No Label	Yes
Δ 239 TMRIA	Yes	Δ 293 TMRIA	Yes
Δ 239 BODIPY	Yes	Δ 293 BODIPY	Yes



Mutated residues on a single monomer of αHL:
S69C, N93C, A238C, S239C, N244C, S278C, S279C and N293C

Conclusions

- All materials tested (20 of 25) demonstrate channel forming activity
- Reference αHL and wild-type show no significant step-size difference
- Selected single-channel rectification ratios show no significant difference between mutants, labeled mutants and wild-type
- Some labeled mutants demonstrate a sub-population of altered conductance states (fast and slow time scales)
- More brief periods of instability observed in labeled mutants
- Single-molecule optical studies to directly visualize the channel dynamics are ongoing

Results of tests to determine the pore-forming capability of each of the mutants with each fluorescent label. "Yes" indicates pore-forming capability.