Structural studies of a functional form of pneumolysin: a virulence factor from *Streptococcus pneumoniae*

Stephen Kelly and Mark J. Jedrzejas
Department of Microbiology, University of Alabama at Birmingham, AL 35294 USA

A key step in the understanding of human disease arising from gram-positive bacteria lies in the mechanisms of the cholesterol-dependent cytolysins (CDCs). Pneumolysin (PLY), a CDC from *Streptococcus pneumoniae*, is of special importance due to the severe impacts of pneumococcal infections on mortality worldwide [1–2]. We have overexpressed, purified, characterized, and crystallized pneumolysin for x-ray diffraction studies. Crystals have been obtained in the presence of cholesterol in an effort to produce a three-dimensional structure of PLY in its fully functional form with the enzyme bound to its activator [3]. The circular dichroism studies of PLY in solution with an excess of cholesterol show a change in the far UV spectrum consistent with an increase in the β-sheet structures for the enzyme. Such a change is expected of the fully functional enzyme that is able to form pores in the membranous environment, which is the functional target for this cytolysin. The sedimentation velocity analysis of the cholesterol-bound enzyme shows hydrodynamic properties indistinguishable from the cholesterol-free form. The determination of the three-dimensional structure of this pneumococcal cytolysin is in progress.

We have crystallized the functional form of PLY in the presence of a cholesterol activator as part of an ongoing effort to understand the structure/function relationship as well as the mechanism of action and virulence of the cholesterol-dependent cytolysins. The structure of PLY complexed with cholesterol will likely reveal the changes in secondary and tertiary structure that occur in the transition between the nonfunctional aqueous form and the membrane- and cholesterol-receptor-bound forms. This structure may lead to a more complete understanding of membrane pore formation and of the precise mechanism of these structural changes that occur in this cholesterol-dependent pneumococcal cytolysin that are currently under debate.

The CD studies revealed measurable differences in secondary structure between the native pneumolysin (which is cholesterol free) and its cholesterol activator complexed form (Table 1). The pneumolysin sample containing cholesterol at a 10 mM concentration is sufficient to induce a decrease in the composition of α-helices in PLY from 16% to 12% accompanied by an increase in random coil from 24% to 27%. The changes in the β-sheet conformation were not detected (Table 1). However, these changes in secondary structure were either not measurable or did not occur when PLY was incubated with only 1 mM of cholesterol activator. This likely shows that the pneumolysin binding to the cholesterol activator at concentrations lower than 10 mM is not sufficient to induce the conformational changes that would produce the observable shift in its secondary structure during the transition from the cholesterol-free PLY to the cholesterol-bound (and likely membrane-bound) conformation. These changes are expected to occur in domains 3 and 4 of the PLY enzyme [4]. Any significant changes in the secondary structure, such as a shift in the percent compositions of α-helices and β-sheets, would likely be reflected in the CD spectra. Such changes were observed in perfringolysin O, a similar enzyme in another gram-positive bacteria *Clostridium perfringens*, when bound to cholesterol and/or liposomal membranes [5]. Furthermore, it is also likely that in addition to binding of the cholesterol activator, membrane insertion followed by the oligomerization of PLY also influences the structural changes in PLY.

Data reveal that pnemolysin complexed with cholesterol shows slight differences in secondary structure from native pneumolysin. Cholesterol at 10 mM is sufficient to induce a change in the percent α-helical composition of PLY, whereas 1 mM cholesterol is not.

Table 1. Circular Dichroism Studies of Pneumolysin

Secondary structure element	Pneumolysin	Pneumolysin – cholesterol (1 mM)	Pneumolysin – cholesterol (10 mM)
α-helices	16%	16%	12%
β-sheets	59%	60%	61%
β-turns	0%	0%	0%
random coil	25%	24%	27%

In order to further investigate the properties of this cholesterol-free and cholesterol-bound enzyme, band and boundary sedimentation studies were performed. These hydrodynamic experiments revealed no significant difference between the activator-free pneumolysin and the PLY-cholesterol complex. However, for both conditions, two distinct species were observed in solution: one corresponding to a monomer and the other (less prevalent) corresponding to the dimer population. These data show that monomers and dimers of PLY exist for both conformations of the enzyme. It is, therefore, likely that the shapes of both PLY forms (cholesterol-free and cholesterol-bound) are similar enough not to be distinguishable with the band and boundary sedimentation velocity analysis.

Structural information obtained from the x-ray crystallographic studies of this PLY conformation will undoubtedly lead to a better understanding of this toxin and its interactions with the membrane receptor. This may explain the structural changes that occur in PLY upon binding to the cell receptor, during target membrane insertion, and with pore formation as well as the relationships between its structure and function in particular. Knowledge of the enzyme's structure could likely allow for the design of inhibitory compounds as specific to PLY, which might have therapeutic utility not only against pneumococci but perhaps also against other pathogenic bacteria having cholesterol-dependent cytolysins as well.

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