



# Lysenin

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## Abstract

Lysenin is a pore-forming toxin present in the coelomic fluid of the earthworm *Eisenia fetida*. Pore-forming toxins (PFTs) are proteinaceous virulence factors produced by many pathogenic bacteria. Following the general mechanism of action of PFTs, lysenin is secreted as a soluble monomer that binds specifically to the membrane receptor sphingomyelin. After attachment, lysenin forms a 9-copy oligomer (nonamer) prepore on the lipid bilayer before membrane insertion. The biological role of lysenin is still unclear, however the most plausible theory is that it is part of an immune-avoiding mechanism. There are many proposed technological applications proposed for lysenin, and understanding its molecular role in bacterial infection could help in developing different antibiotic strategies to solve the problem of multiple drug resistance in bacteria.

## Monomer

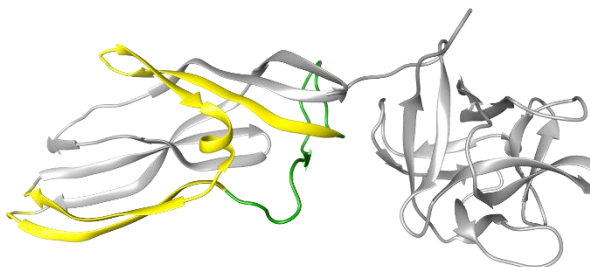
Lysenin is a protein produced in the *coelomocyte-leucocytes* of the earthworm *Eisenia fetida*.<sup>[1]</sup> This protein was first isolated from the coelomic fluid in 1996 and named lysenin (from lysis and *Eisenia*).<sup>[2]</sup> Lysenin is a relatively small water-soluble molecule with a molecular weight of 33 kDa (Figure 1). Using *X-ray crystallography*, lysenin was classified as a member of the *Aerolysin* protein family by structure and function.<sup>[3]</sup> Structurally, each lysenin monomer consists of a receptor binding domain (grey globular part on right of Figure 1) and a Pore Forming Module (PFM; rest of the molecule, Figure 1); domains shared throughout the aerolysin family.<sup>[3]</sup> The lysenin receptor binding domain shows three *sphingomyelin* binding motifs. The Pore Forming Module contains the regions (green and yellow in Figure 1) that undergo large conformational changes to become the  $\beta$ -barrel in the pore.<sup>[4]</sup>

## Membrane receptors

The natural membrane target of lysenin is an animal plasma membrane lipid called *sphingomyelin* located mainly in its outer leaflet, involving at least three of its *phosphatidylcholines* (PC) groups.<sup>[5]</sup> Sphingomyelin is

usually found associated with *cholesterol* in *lipid rafts*.<sup>[6]</sup> Cholesterol, which enhances *oligomerization*, provides a stable platform with high lateral mobility where monomer-monomer encounters are more probable.<sup>[5]</sup> PFTs have shown to be able to remodel the membrane structure,<sup>[7]</sup> sometimes even mixing lipid phases.<sup>[8]</sup>

The region of the lysenin pore  $\beta$ -barrel expected to be immersed in the hydrophobic region of the membrane is the 'detergent belt', the 3.2 nm high region occupied by detergent in *Cryogenic Electron Microscopy* (Cryo-EM) studies of the pore.<sup>[4]</sup> On the other hand, sphingomyelin/Cholesterol bilayers are about 4.5 nm height (Figure 2).<sup>[9]</sup> This difference in height between the detergent belt and the sphingomyelin/cholesterol bilayer implies a bend of the membrane in the region surrounding the pore, called *negative mismatch*.<sup>[10]</sup> This bending results in a net attraction between pores that induce pores aggregation.<sup>[11]</sup>



**Figure 1** | Lysenin water-soluble monomeric X-ray structure (PDB: 3ZXD). Receptor binding domain on right in grey. Pore Forming Module (PFM) on left with region *previously* thought to be responsible for  $\beta$ -barrel formation in green. Additional region *now* known to be important in  $\beta$ -barrel formation in yellow (from X-ray data).

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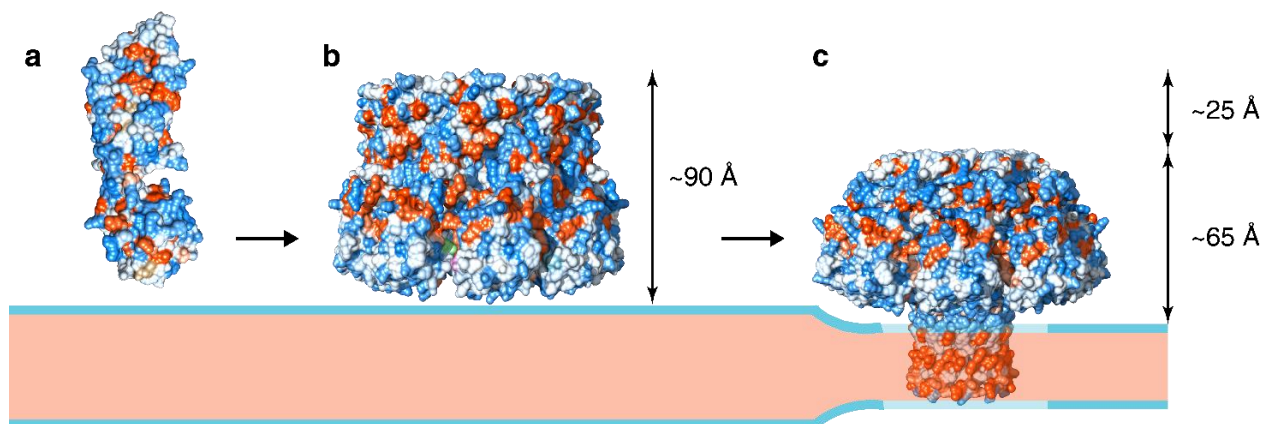
## Binding, oligomerization and insertion

Membrane binding is a requisite to initiate PFT oligomerization. Lysenin monomers bind specifically to sphingomyelin via the receptor binding domain.<sup>[3]</sup> The final lysenin oligomer is constituted by nine monomers without quantified deviations.<sup>[12]</sup> When lysenin monomers bind to sphingomyelin-enriched membrane regions, they provide a stable platform with a high lateral mobility, hence favouring the oligomerization.<sup>[5]</sup> As with most PFTs, lysenin oligomerization occurs in a two-step process, as was recently imaged.<sup>[11]</sup>

The process begins with monomers being adsorbed into the membrane by specific interactions, resulting in an increased concentration of monomers. This increase is promoted by the small area where the membrane receptor accumulates owing to the fact that the majority of PFT membrane receptors are associated with lipid rafts.<sup>[13]</sup> Another side effect, aside from the increase of monomer concentration, is the monomer-monomer interaction. This interaction increases lysenin oligomerization. After a critical threshold concentration is reached, several oligomers are formed simultaneously, although sometimes these are incomplete.<sup>[11][14]</sup> In contrast to PFTs of the **cholesterol-dependent cytolysin** family,<sup>[15]</sup> the transition from incomplete lysenin oligomers to complete oligomers has not been observed.

A complete oligomerization results in the so-called prepore state, a structure on the membrane. Determining the prepore's structure by X-ray or Cryo-EM is a challenging process that so far has not produced any results. The only available information about the prepore structure was provided by **Atomic Force Microscopy** (AFM). The measured prepore height was 90 Å; and the width 118 Å, with an inner pore of 50 Å (Figure 2).<sup>[14]</sup> A model of the prepore was built aligning the monomer structure (PDB: 3ZXD) with the pore structure (PDB: 5GAQ) by their receptor-binding domains (residues 160 to 297). A recent study in aerolysin suggests that the currently accepted model for the lysenin prepore should be revisited, according to the new available data on the aerolysin insertion.<sup>[11]</sup>

A **conformational change** transforms the PFM into the transmembrane **β-barrel**, leading to the pore state.<sup>[4]</sup> The trigger mechanism for the prepore-to-pore transition in lysenin depends on three glutamic acid residues (E92, E94 and E97), and is activated by a decrease in pH,<sup>[16]</sup> from physiological conditions to the acidic conditions reached after endocytosis. These three glutamic acids are located in an α-helix that forms part of the PFM, and glutamic acids are found in aerolysin family members in its PFMs. Such a conformational change produces a decrease in the oligomer height of 2.5 nm according to AFM measurements.<sup>[14]</sup> The main dimensions, using lysenin pore X-ray structure, are height 97 Å, width 115 Å and the inner pore of 30 Å (Figure 2).<sup>[4]</sup>



**Figure 2 |** Lysenin mechanism of action Scheme. **a**) Lysenin monomers are segregated as soluble proteins that bind specifically to sphingomyelin by its receptor binding domain. After binding, and reach a certain density, the oligomerization starts. **b**) After a complete oligomerization, the prepore is formed. The prepore model shown here was assembled from the monomer structure and aligned with the pore structure (PDB: 5GAQ) by their receptor-binding domains (residues 160 to 297). The height of the prepore was set to agree with the Atomic Force Microscopy measurements. **c**) Membrane inserted Lysenin assembly (PDB: 5GAQ). The height of the pore was measured from the detergent belt to the last residue, assuming that the detergent belt corresponds with the part of the pore surrounded by the membrane. The membrane was placed in the β-barrel of the pore to match with the detergent belt, that englobe all the hydrophobic residues of the β-barrel. The hydrophobic surface colour scale is according to the hydrophobicity scale of Kyte and Doolittle.



However, complete oligomerization into the nonamer is not a requisite for the insertion, since incomplete oligomers in the pore state can be found.<sup>[14]</sup> The prepore to pore transition can be blocked in crowded conditions, a mechanism that could be general to all  $\beta$ -PFTs. The first hint of crowding effect on prepore to pore transition was given by congestion effects in electrophysiology experiments.<sup>[17]</sup> High-Speed AFM studies incubating lysenin on sphingomyelin/cholesterol membranes has shown that under crowded conditions the prepore to pore transition gets blocked by steric interactions.<sup>[18][11]</sup>

## Insertion consequences

The ultimate consequences of lysenin pore formation are not well documented; however, it is through to induce **apoptosis** via three possible hypotheses:

- Breaking the sphingomyelin asymmetry between the two leaflets of the lipid bilayer by punching holes in the membrane<sup>[19]</sup> and inducing **lipid flip-flop** (reorientation of a lipid from one leaflet of a membrane bilayer to the other).<sup>[7]</sup>
- Increasing the calcium concentration in the cytoplasm.<sup>[20]</sup>
- Decreasing the potassium concentration in the cytoplasm.<sup>[21]</sup>

## Biological role

The biological role of lysenin remains unknown. It has been suggested that lysenin may play a role as a **defence mechanism** against attackers such as **bacteria**, **fungi** or small **invertebrates**.<sup>[22]</sup> However, lysenin's activity is dependent upon binding to sphingomyelin, which is not present in the membranes of bacteria, fungi or most invertebrates. Rather, sphingomyelin is mainly present in the plasma membrane of **chordates**.<sup>[23]</sup> Another hypothesis is that the earthworm, which is able to expel coelomic fluid under stress,<sup>[24][25]</sup> generates an avoidance behaviour to its **vertebrate** predators (such as birds, **hedgehogs** or **moles**).<sup>[26]</sup> If that is the case, the expelled lysenin might be more effective if the coelomic fluid reaches the eye, where the concentration of sphingomyelin is ten times higher than in other body organs.<sup>[27]</sup> A complementary hypothesis is that the pungent smell of the coelomic fluid - giving the earthworm its specific epithet *foetida* - is an **anti-predator adaptation**. However, it remains unknown whether lysenin contributes to avoidance of *Eisenia* by predators.<sup>[28]</sup>

## Applications

Lysenin's conductive properties have been studied for years.<sup>[29]</sup> Like most pore-forming toxins, lysenin forms a non-specific channel that is permeable to ions, small molecules, and small peptides.<sup>[30]</sup> There have also been over three decades of studies into finding suitable pores for converting into **nanopore sequencing systems** that can have their conductive properties tuned by point mutation.<sup>[31]</sup> Owing to its binding affinity for sphingomyelin, lysenin (or just the receptor binding domain) has been used as a fluorescence marker to detect the sphingomyelin domain in membranes.<sup>[32]</sup>

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Conflict of Interest: none declared.

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