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Lysenin-His, a sphingomyelin-recognizing toxin, requires tryptophan 20 for cation-selective channel assembly but not for membrane binding

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Abstract

Lysenin is a 297 amino acid long toxin derived from the earthworm Eisenia fetida which specifically recognizes sphingomyelin and induces cell lysis. We synthesized lysenin gene supplemented with a polyhistidine tag, subcloned it into the pT7RS plasmid and the recombinant protein was produced in Escherichia coli. In order to obtain lysenin devoid of its lytic activity, the protein was mutated by substitution of tryptophan 20 by alanine. The recombinant mutant lysenin-His did not evoke cell lysis, although it retained the ability to specifically interact with sphingomyelin, as demonstrated by immunofluorescence microscopy and by dot blot lipid overlay and liposome binding assays. We found that the lytic activity of wild-type lysenin-His was correlated with the protein oligomerization during interaction with sphingomyelin-containing membranes and the amount of oligomers was increased with an elevation of sphingomyelin/lysenin ratio. Blue native gel electrophoresis indicated that trimers can be functional units of the protein, however, lysenin hexamers and nanomers were stabilized by chemical cross-linking of the protein and by sodium dodecyl sulfate. When incorporated into planar lipid bilayers, wild type lysenin-His formed cation-selective channels in a sphingomyelin-dependent manner. We characterized the channel activity by establishing its various open/closed states. In contrast, the mutant lysenin-His did not form channels and its correct oligomerization was strongly impaired. Based on these results we suggest that lysenin oligomerizes upon interaction with sphingomyelin in the plasma membrane, forming cation-selective channels. Their activity disturbs the ion balance of the cell, leading eventually to cell lysis.

Keywords: Channel assembly, hemolysis, lysenin, oligomerization, plasma membrane, toxin

Abbreviations: BSA, bovine serum albumin; DSS, disuccinimidyl suberate; IPTG, isopropyl-1-thio-D-galactopyranoside; lysenin-His, lysenin fused with a polyhistidine tag at the N terminus; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TBST, Tris-buffered saline containing Tween 20; wt, wild type.

Introduction

Several toxins of prokaryotic and eukaryotic origin exert their cytolytic activity by binding to the cell surface via specific interaction with sphingomyelin. As more than half of the total amount of sphingomyelin in the cell resides in the plasma membrane, mostly in its outer leaflet [1,2], the use of sphingomyelin as a target ensures efficient binding of the toxins to the plasma membrane. After binding, toxin molecules self-assemble into transmembrane pores and subsequently kill the cell via osmotic shock, as has been described for equinatoxin II of the sea anemone Actinia equina [3,4]. The binding of toxins to sphingomyelin and their cytolytic activity often either require or are augmented by cholesterol. Thus, pleurotolysin and ostreolysin, toxins of the mushroom Pleurotus ostreatus, and VacA of Helicobacter pylori, bind to cholesterol- and sphingomyelin-containing membranes that probably acquire liquid-ordered phase [5–7]. Similarly, the Vibrio cholerae cytolsin displays dual specificity for cholesterol and sphingolipids [8]. In the plasma membrane, sphingolipids and cholesterol are believed to form distinct microdomains of liquid-ordered state, so-called rafts, which serve as signaling platforms for...
a subset of plasma membrane receptors [9–11]. Owing to the sphingomyelin- and cholesterol-dependent activity of a variety of pore-forming toxins, the lipid composition of rafts is likely to render them susceptible to the action of the proteins. Accordingly, it has been shown that perfringolysin O from Clostridium perfringens targets in vivo cholesterol accumulated in plasma membrane rafts of intact cells [12].

Recently, several proteins isolated from the coelomic fluid of the earthworm Eisenia fetida have been added to the list of sphingomyelin-dependent cytolytic toxins [13]. Among them lysenin, a unique 297 amino acid long protein recognizes sphingomyelin exclusively. The conserved tryptophan residues of the protein are crucial for this interaction [14,15]. The binding of lysenin to sphingomyelin is accompanied by oligomerization of the protein and both events are facilitated upon incorporation of cholesterol. The cholesterol effect can be attributed to the changes of sphingomyelin topology in the membrane yielding local concentration of sphingomyelin into discrete microdomains [14,16]. Accordingly, lysenin was applied for identification of sphingomyelin-rich domains in the plasma membrane [17].

The aforementioned oligomerization of lysenin is believed to be a prerequisite for the formation of pores in the membrane by the toxin [15,18]. As a result of pore formation, lysenin causes leakage of liposomes and lysis of erythrocytes in a sphingomyelin-dependent manner [14]. The cytolytic activity of lysenin has also been claimed to cause death of cultured mammalian cells and vertebrate spermatozoa [19,20]. It is of interest, however, that the first bioactivity of lysenin to be discovered was contraction of strips of rat aorta. Lysenin at 10^-9 M evoked contraction of aorta muscles intensity of which reached 60% of the maximum contraction induced by 60 mM KCl [21]. This phenomenon can be ascribed to lysenin-induced changes of sarcolemma permeability leading to ion influx and muscle contraction. These data prompted us to study whether lysenin can form transmembrane pores having ion channel activity. For these studies we prepared recombinant wild type lysenin with a polyhistidine tag (wt lysenin-His). Wt lysenin-His recognized sphingomyelin exclusively, evoked hemolysis and formed oligomers, thus resembling the activity of the native protein. When incorporated into planar lipid bilayers containing sphingomyelin, lysenin-His formed large-conductance cation-selective channels. Channel activity was characterized by various open/closed states. The protein mutated at tryptophan 20, although still able to bind sphingomyelin, was defective in oligomer and channel formation. We suggest that lysenin, upon binding to sphingomyelin-containing membranes, self-assembles into cation-permeable channels and the resulting intracellular ion imbalance can cause the death of cells.

Materials and methods

Preparation of synthetic lysenin gene

Synthetic lysenin gene was obtained from DNA oligos by PCR, as shown on-line in supplementary Figure 1, using the nucleotide sequence of lysenin cDNA cloned by Sekizawa et al. [22]. The oligos were divided into four groups consisting of: group I – oligos from U1 to U6 and from J1_2 to J5_J6; group II – from U6 to U11 and from J6_7 to J10_11; group III – from U10 to U15 and from J10_11 to J14_15; and group IV – from U15 to U20 and from J15_16 to J19_20. Mixtures of Ux oligos (250 pM of each oligo) of each group were phosphorylated with T4 polynucleotide kinase and mixed with equimolar joining oligonucleotides Jx in a solution containing ligation buffer [23]. The mixtures were heated to 60°C for 5 min and left for 20 min at room temperature. After that, T4 DNA ligase was added to each mixture and ligation was carried out at 16°C for at least 12 h. After ligation, 1 µl of the mixtures was taken for PCR amplification with pairs of 50 pM primers (U1, J5_6), (U6, J10_11), (U10, J14_15) and (U15, P2) for groups I, II, III and IV, respectively. Commonly, 18 cycles of amplification were required to obtain a sufficient amount of the necessary DNA fragment. The amplified DNA fragments obtained from group I and II after electrophoresis and isolation from polyacrylamide gel [24] were mixed together, the oligos U1 and J10_11 were added and PCR was carried out to join the two DNA fragments and to amplify their sum. Similarly, fragments obtained from groups III and IV of oligos were treated in the same manner. After that, both (I+II) and (III+IV) DNA fragments were mixed in one tube and amplified by PCR using the U1 and P2 oligos as primers. The final PCR product containing cDNA of lysenin was isolated from polyacrylamide gel, digested with EcoRI and HindIII restriction nucleases and cloned into pBlue-script plasmid (Stratagene, La Jolla, CA).

The nucleotide sequence of lysenin cDNA was modified by introducing six histidine codons at the 5′ end of the coding sequence. For this we used the sense primer 5′GGGGAAATTCATAGCTCATC ACCATCATCAGCTGCAAAAGCTGAGAAG G3′ designed to contain EcoRI and NdeI restriction sites, codons for the 6 × His tag, and the 5′ end of the open reading frame, and the antisense primer 5′GCAGCAGGTTAAGCTTACCGACCAC
5

Mutagenesis kit (Stratagene) with the sense primer lysenin-His was used to create the W20A point number for the sequence of pT7RS expression plasmid pT7RS (the GenBank accession NdeI/HindIII sites. Subsequently, the 900bp NdeI/HindIII fragment was cloned into the expression plasmid pT7RS (the GenBank accession number for the sequence of pT7RS expression vector is AY923866). This construct of full length lysenin-His was used to create the W20A point mutant of recombinant lysenin. W20A lysenin-His was amplified by PCR and the Quick Change Mutagenesis kit (Stratagene) with the sense primer 5’ GATGTTGGTGCAAGAAGGGCT ATGTATACG3’, and the antisense primer 5’ CGT ATACATAAGCTTCTTCTCGCTACTGACACCA CATC3’ designed to contain the W20A mutation (underlined).

Purification of recombinant proteins

For bacterial expression of the His-tagged wt and W20A lysenin, the coding sequences were subcloned into the bacterial expression vector pT7RS using EcoRI/HindIII restriction sites. The plasmids were used to transform the BL-21-DE3 strain of Escherichia coli. Transfectants were grown in LB medium containing 100 μg/ml ampicillin at 37°C to A600 0.6 when 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added. After 20 h at 25°C, bacteria were harvested, sonicated and lysed with 50 μg/ml lysozyme (20 min, 30°C) followed by 1% Triton X-100 and 10 μg/ml DNase (20 min, 30°C). The recombinant proteins were purified on a HIS-Select Nickel HC Affinity Gel column (Sigma-Aldrich, St Louis, MO). They were eluted from the column with 100 mM imidazole, 300 mM NaCl, 5 mM β-mercaptoethanol, 5 mM phosphate buffer, pH 8.0, and dialyzed against phosphate-buffered saline (PBS) containing 5 mM β-mercaptoethanol. The concentration of wt and W20A lysenin-His was established after 10% SDS-PAGE by quantifying the Coomasie-Blue stained bands using bovine serum albumin (BSA) as a standard.

Protein-lipid overlay assay

For analysis following lipids were used: dioleoyl phosphatidylcholine, sphingosine, Cα— and C16— ceramides, cholesterol, bovine brain or semisynthetic bovine brain sphingomyelin, and galactocerebrosides (all from Sigma-Aldrich). The molecular weight of the latter lipid was tentatively estimated as 1200 Da. Lipid samples of 1 μl containing various amounts of lipids (5-100 pmol) were spotted onto a nitrocellulose membrane (0.45 μm, Santa Cruz Biotechnology, Santa Cruz, CA) and allowed to dry for 30 min [25]. To enhance binding of the lipids to the membrane, the method of Taki and Ishikawa [26] was used. The membrane was blocked for 1 h at 20°C with 1% gelatin and 1% polyvinylpyrrolidone in Tris-buffered saline containing 0.03% Tween-20 (TBST), exposed for 2 h at 20°C to 3 μg/ml wt or W20A lysenin-His in 1% gelatin/TBST, and incubated with goat anti-His IgG-peroxidase (Sigma-Aldrich) in 1% gelatin/TBST (1 h, 20°C). Immunoreactive spots were visualized with the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Hemolytic assay

Serial dilutions of recombinant wt and W20A lysenin-His up to 200 ng/ml (6 nM) were incubated with 7 × 107 sheep erythrocytes in 1 ml of TBS for 1 h either at 4°C or at 20°C. After pelleting of nonlysed cells (200 g, 4°C, 5 min), the amount of released hemoglobin was estimated at 405 nm. The hemolysis caused by lysenin-His was expressed as the percentage of maximal hemolysis (100%) found after osmotic lysis of erythrocytes in distilled water.

Immunofluorescence

Sheep red blood cells (3 × 107/sample) were fixed with 1% glutaraldehyde/TBS (20 min, 4°C) and after quenching with 50 mM NH4Cl/TBS they were pelleted onto coverlips coated with poly-L-lysine (200 g, 4°C, 5 min). The presence of imidazole during incubation of lysenin-His with erythrocytes abrogated nonspecific interactions of the His tag with the negatively charged cell surface. Subsequently, cells were exposed to rabbit anti-His IgG (Santa Cruz Biotechnology; 30 min, 20°C) followed by goat anti-rabbit IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA). The samples were examined under a Nikon microscope [27]. Fluorescent cell images were collected with a digital Nikon DS-5M camera.

In controls, cell incubation with lysenin-His was omitted or lysenin-His denatured by 10 min heating at 60°C was applied. In both cases, no staining of the erythrocyte membrane was detected. In a series of experiments cells were treated with 70 μU/ml of bacterial sphingomyelinase (1 h, 37°C; Sigma-Aldrich) prior to fixation and incubation with the recombinant proteins.
Preparation of liposomes, erythrocyte ghosts and lysenin-His binding

To prepare small unilamellar vesicles, semisynthetic sphingomyelin and dioleoyl phosphatidylcholine with or without cholesterol were mixed in different molecular ratios and dried from a chloroform/methanol (1:1, by vol.) under nitrogen. The lipids were resuspended in 100 µl of PBS, vortexed and sonicated on ice for 30 min. Vesicles were pelleted were resuspended in 100 µl of PBS. For experiments, 4 µl of the liposome suspension was mixed with 8 µl of a solution containing 1 µg (2.5 µM) of lysenin-His, 50 mM NaCl, 20 mM imidazole and 50 mM phosphate buffers, pH 8.0. In the mixture, the total concentration of sphingomyelin and phosphatidylcholine was 1 mM. After 45 min incubation at 20°C, the liposomes were diluted with 300 µl of PBS and pelleted as above. In a series of experiments, prior to the centrifugation, the mixture was supplemented with 2 mM disuccinimidyl suberate (DSS, Pierce) and 2 h later, 20 mM Tris, pH 7.4, was added to stop cross-linking of the protein.

For preparation of sheep red blood cell ghosts, erythrocytes (7 × 10⁹/sample) were lysed in distilled water and washed five times with the water by centrifugation (15,000 g, 6 min, 4°C). The ghosts were suspended in 50 µl of solution containing 0.4 µg lysenin-His (0.24 µM), 40 mM imidazole and 50 mM phosphate buffer, pH 8.0, and 0.1–0.8 M NaCl. After incubation (30 min, 20°C), ghosts were washed with PBS, suspended in 20 µl SDS sample buffer and analysed by SDS-PAGE.

SDS-PAGE, blue native gel electrophoresis and immunoblotting

For SDS-PAGE, pellets of liposomes obtained after incubation with lysenin-His were dissolved in 45 µl of 2% SDS/1% β-mercaptoethanol sample buffer, boiled for 5 min and loaded onto 7% gel (14 µl). After electrophoresis and transfer to nitrocellulose, the proteins were visualized by immunoblotting with anti-His IgG-peroxidase and chemiluminescence. Monomers and oligomers of lysenin-His were quantified densitometrically in Fluor-S MultiImager equipped with Quantity One software (Bio-Rad). For normalization, the densitometric data were expressed in relation to the monomer level detected in liposomes at sphingomyelin/lysenin ratio 8:1 without cholesterol, and arbitrary equalized to 1.

For blue native gel electrophoresis, liposomes pelleted after incubation with lysenin-His were suspended in 12 µl of solubilization buffer containing 1% dodecyl-β-maltoside, 20% glycerol, 25 mM BisTris-HCl, 50 mM 6-amino-caproic acid, pH 7.0, and 0.5% Coomassie Blue Serva G. The samples were loaded onto a 7% polyacrylamide gel and developed for 5 h (160 V, 4°C) according to Cline’s protocols [http://www.hos.ufl.edu/cclineweb/Protocols/BNgel.htm]. Ferritin (880 kDa and 440 kDa) and BSA (132 kDa and 66 kDa) were applied as molecular weight standards. Subsequently, lanes of interest were dissected from the gel, incubated in SDS electrophoresis buffer for 10 min, mounted on the top of 9% SDS-PAGE gels and run for the second dimension analysis at 120 V. After transfer to nitrocellulose, the samples were immunoblotted with anti-His as above.

Electron microscopy

For ultrastructural studies, multilamellar liposomes were prepared by mixing semisynthetic sphingomyelin, dioleoyl phosphatidylcholine and cholesterol (1:2:2 molar ratio) or dioleoyl phosphatidylcholine and cholesterol (3:2 molar ratio). The lipids were dried under nitrogen, resuspended in 100 µl PBS, vortexed, sonicated for 2 min on ice and collected by centrifugation (15,000 g, 15 min, 4°C). The liposomes (6 mM total phospholipid content) were incubated with 7 µM wt or W20A lysenin-His for 45 min at 20°C. After incubation, the vesicles were treated with 1% glutaraldehyde in PBS (20 min, 20°C) and applied onto poly-L-lysine-coated formvar/carbon-treated grids. After 20 min, the samples were washed twice with PBS and once with water, and counterstained with 2% uranyl acetate. They were examined under a JEM-1200EX (JEOL) microscope.

Planar lipid bilayer technique and lysenin reconstitution

The planar lipid bilayer technique was applied as described previously [28]. In brief, planar phospholipid bilayers were formed in a 250 µm diameter hole which separated two chambers (cis 2 ml and trans 3 ml internal volume). The chambers contained 450/150 mM KCl, 5 mM Hepes, pH 7.0 (adjusted with KOH). The outline of the aperture was coated with a lipid suspension and dried with N₂ prior to bilayer formation to improve membrane stability. Planar phospholipid bilayers were painted using azolectin (L-α-phosphatidylyceroline, Sigma-Aldrich) or azolectin enriched with 3% sphingomyelin and 1% cholesterol – all in n-decane at a final concentration of 25 mg of lipid/ml. Formation and thinning of the bilayer were monitored by capacitance measurements. Final capacitance values ranged from 110–200 pF. Electrical connections were made using Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. Voltage was applied to the cis compartment of the chamber and
the trans compartment was grounded. Solutions of wt lysenin-His or W20A lysenin-His in 50 mM KCl, 20 mM Hepes, pH 7.2 (adjusted with KOH) were added to the trans compartment. In a series of experiments native lysenin isolated from Eisenia foetida (Peptides International, Louisville, KY) was used. All measurements were carried out at room temperature.

Data recording and analysis

The current was measured using a Bilayer Membrane Admittance Meter (model ID 562, IDB, Gwynedd, UK). Signal was filtered at 0.2 kHz (Low Pass Bessel Filter 4 Pole, Warner Instrument Corp.), digitized (A/D converter 1401, Cambridge Electronic Design, UK) and transferred to a PC for off-line analysis by the CED Electrophysiology Package V6.41 and pClamp6 software (Axon Instruments, Union City, CA) and plotted in Microcal Origin. All measurements were conducted in asymmetric ionic conditions (450/150 mM KCl, pH 7.0 cis/trans). Recordings were low-pass filtered at 200 Hz. Channel conductances were expressed as the mean ± SD.

Results

Expression of wt lysenin-His

The synthetic lysenin gene was constructed from partially overlapping DNA fragments obtained by PCR as shown on-line in supplementary Figure 1. The prepared cDNA of recombinant lysenin was cloned into pT7RS expression plasmid and confirmed by sequencing. The predicted amino acid sequence of the recombinant lysenin was identical to the sequence of native lysenin cloned from Eisenia foetida by Sekizawa et al. [22]. A tag consisting of six histidine residues was added without any linker at the N terminus of the protein to facilitate its purification and detection in further analysis. The lysenin-His cDNA was expressed in Escherichia coli that were induced with IPTG and cultured at 25°C. Under these conditions, most of the protein remained soluble and was purified under non-denaturing conditions in one step affinity chromatography on Ni$_{2+}$-agarose. The purified protein migrated as a 41 kDa band on SDS-PAGE and was recognized by anti-His antibody (see Figure 3B).

Wild type and W20A lysenin-His bind sphingomyelin selectively

The tryptophan residues of lysenin were found to be important for the protein activity [29]. Therefore, in an attempt to interfere with the protein properties, we introduced a point mutation in lysenin-His cDNA replacing the N-terminal-most tryptophan residue 20 with alanine (on-line supplementary Figure 1, shadowed). To analyse the ability of W20A lysenin-His to recognize and bind sphingomyelin, the protein-lipid overlay assay was applied. Both wt and W20A lysenin-His at 3 μg/ml detected as little as 5 pmol of sphingomyelin (Figure 1). The mutant lysenin-His retained selectivity for sphingomyelin binding found for wt lysenin-His. Aside from sphingomyelin, neither of the recombinant proteins recognized any other sphingolipid nor phosphatidyl-choline or cholesterol (Figure 1).

To assess whether the recombinant proteins were able to bind sphingomyelin of the plasma membrane, the proteins were incubated with sheep erythrocytes, whose membrane contains 51% sphingomyelin of total phospholipids [30]. Distinct staining of the erythrocyte surface was found when the cells were incubated with wt lysenin-His or with W20A lysenin-His (Figure 2). Both recombinant proteins labeled the cells with similar intensity. Pretreatment of erythrocytes with bacterial sphingomyelinase markedly reduced the staining, as shown in Figure 2 (inset) for wt lysenin-His.

Wild type, but not W20A lysenin-His, forms oligomers

Recombinant wt lysenin-His evoked hemolysis of sheep erythrocytes in a dose dependent manner,
resembling the activity of the native protein [15]. To cause 50% hemolysis at 4°C, 28 ng/ml of wt lysenin-His was required (Figure 3A, open squares). In contrast, W20A lysenin-His did not cause hemolysis even at 200 ng/ml both at 4°C and at 20°C (Figure 3A, closed circles, and results not shown).

This difference prompted us to compare the ability of wt and W20A lysenin-His to oligomerize. For this purpose, proteins were mixed with sphingomyelin/phosphatidylcholine liposomes, and after pelleting the samples were subjected to SDS-PAGE analysis. In the liposomes, the sphingomyelin content varied from 0.02 mM to 0.7 mM yielding sphingomyelin/lysenin ratio in the range from 8:1 to 280:1. When the ratio exceeded 40:1, a SDS-resistant oligomer of wt lysenin-His of about 280 kDa was assembled at the expense of the 41 kDa monomer (Figure 3B). Oligomerization of wt lysenin-His was facilitated by cholesterol and was observed already at sphingomyelin/protein ratio > 20:1 when liposomes were supplemented with cholesterol in an equimolar ratio to sphingomyelin. At the cholesterol presence, the oligomer of wt lysenin-His prevailed with minute amounts of the monomer left when the sphingomyelin/lysenin ratio reached 280:1 (Figure 3B). However, an increasing sphingomyelin/lysenin ratio facilitated the protein oligomerization regardless of the cholesterol presence in liposomes (Figure 3B). Studies performed with W20A lysenin-His confirmed that the protein bound to sphingomyelin-containing liposomes in amounts comparable to wt lysenin-His (Figure 3B). In contrast to wt lysenin-His, the vast majority of W20A lysenin-His bound to the liposomes existed as the monomer. The mutant lysenin formed only minute amounts of the SDS-resistant 280 kDa oligomer at sphingomyelin/protein ratio > 40:1 and the protein oligomerization was slightly promoted by cholesterol (Figure 3B). Similarly, the monomer of W20A lysenin-His prevailed after incubation of the protein with sheep erythrocyte ghosts instead of sphingomyelin-containing liposomes (Figure 3C). Oligomerization of W20A lysenin-His was moderately improved by increasing salt concentration up to 0.8 M during erythrocyte ghost binding. However, high salt concentrations, 0.1–0.8 M NaCl, impaired binding and oligomerization of wt lysenin-His during incubation with erythrocyte ghosts (Figure 3C).

The assembly of lysenin oligomers was further analysed using blue native gel electrophoresis which allowed us to isolate native protein complexes. Dissected lanes of the native gel were subsequently subjected to SDS-PAGE at denaturing conditions for analysis in the second dimension (Figure 4A). This approach confirmed that wt lysenin-His oligomerized efficiently upon binding to sphingomyelin-containing liposomes (sphingomyelin/protein ratio of 120:1), leaving small amounts of the monomer. However, the apparent molecular weight of the wt lysenin-His oligomer found in the native gel electrophoresis was the half of the value of the SDS-resistant oligomer and reached about 120 kDa (Figure 4A, compare with Figure 3B). In contrast, W20A lysenin-His bound to the liposomes mainly as the monomer (Figure 4A). Aside from the monomer, the protein formed very large oligomers of about 800 kDa and 620 kDa with traces of 120 kDa complex (Figure 4A). It is of note, that in the second run during SDS-PAGE, lysenin complexes and the monomer migrated at the same velocity. This indicates that after isolation at native conditions, including treatment of liposomes with a non-ionic detergent dodecyl-β-maltoside, the oligomers were decomposed by SDS.
Figure 3. W20A lysenin-His is devoid of hemolytic activity and inefficiently forms oligomers which are characteristic for wt lysenin-His. (A) Hemolytic activity of wt lysenin-His (open squares) and its lack in W20A lysenin-His (closed circles). Sheep erythrocytes (7 × 10⁷/ml) were incubated with the recombinant proteins at indicated concentrations for 1 h at 4°C. In controls, cells were suspended in H₂O to estimate 100% hemolysis. The results are mean ± SE from five experiments. (B, C) Wt lysenin-His forms a distinct SDS-resistant oligomer upon binding to sphingomyelin-containing liposomes (B) and sheep red blood cell ghosts (C). (B) Small unilamellar liposomes composed of SM/DOPC with or without cholesterol (1 mM total phospholipids) were incubated with 2.5 μM wt or W20A lysenin-His at the SM/lysenin ratio from 8:1 to 280:1. Pelleted liposomes were subjected to 7% SDS-PAGE under denaturing condition and analyzed for the presence of lysenin monomers and oligomers by immunoblotting with anti-His (upper panel). In lane “no liposomes” 0.2 μg of lysenin-His was applied. On the left, molecular weight standards are shown. Lower panel: Quantification of lysenin monomers (closed symbols) and oligomers (open symbols) based on a densitometric analysis of blots shown in the upper panel. Triangles, liposomes with cholesterol; circles, liposomes without cholesterol. Data are mean ± SE from three experiments. (C) Erythrocyte ghosts were incubated with 0.24 μM recombinant proteins in the presence of 0.1 – 0.8 M NaCl and analysed by SDS-PAGE and immunoblotting with anti-His. Data shown are representative of three experiments.
The tendency of W20A lysenin-His for multi-oligomerization was confirmed by chemical cross-linking of the liposome-bound protein with DSS of 11.4 Å spacer arm length. Under these conditions, large W20A lysenin-His complexes, which did not penetrate 4% SDS-stacking gel, were revealed (Figure 4B). In the case of wt lysenin-His, such large complexes were also assembled. Despite that, two oligomers of about 360 kDa and 250 kDa and some amounts of 41 kDa monomer of wt-lysenin-His were detected (Figure 4B).

When viewed under electron microscope, sphingomyelin-containing liposomes treated with wt lysenin-His were smaller than phosphatidylcholine vesicles after such treatment (Figure 5A, 5B). On some of the sphingomyelin-containing liposomes, a regular lattice composed of hexagonal units with an external diameter of 10 nm was seen (Figure 5B–5D). This pattern closely resembled the structures described for native lysenin [15] and was not found for W20A lysenin-His (not shown). Phosphatidylcholine liposomes were devoid of wt lysenin-His assemblies (Figure 5A). Accordingly, no binding of wt and W20A lysenin-His to these liposomes was found after SDS-PAGE analysis (not shown).

Taken together the data indicate that wt lysenin-His, but not W20A lysenin-His, exerts lytic activity which is correlated with the ability of the protein to form homogenous oligomers. Therefore, it was tempting to examine whether oligomerization of wt lysenin-His could involve ion channel formation.

Wild type, but not W20A lysenin-His, forms ionic channels in planar lipid bilayer

The addition of recombinant wt lysenin-His (1 µg/ml) to a bilayer composed of azolectin, sphingomyelin and cholesterol (96:4:1, by weight) caused fluctuations of the measured current with discrete conductance changes. Lysenin was added to the bilayers at various holding potentials (0, 50 and 90 mV) and the current changes induced by lysenin were observed at all applied potentials (Figure 6A). With an increase of the voltage, the duration of channel opening and closure increased. This may suggest that at higher voltages, lysenin molecules more easily formed permeable channels and eventually cooperativity of the channels occurred. Accordingly, the current amplitude recorded from a sphingomyelin-containing bilayer held at a constant voltage of 50 mV increased slowly in time after the bilayer treatment with wt lysenin-His. As seen in Figure 6B, the current amplitude of 5–8 pA appearing after 15–30 min after addition of 1 µg/ml wt lysenin-His to the experimental chamber increased 3-fold 1.5–2 h later. If in the same time-frame the bilayer was exposed to new doses of the protein, an increasing current was also recorded.
Channel formation induced by wt lysenin-His appeared to be a function of the protein concentration in the range of \(1-5 \mu g/ml\). At concentrations exceeding \(6 \mu g/ml\) the current increased rapidly until the membrane broke.

Wt lysenin-His formed channels only in membranes containing sphingomyelin, since no channel-like activity was recorded from bilayers composed of azolectin alone in the presence of \(2-6 \mu g/ml\) of the protein (Figure 6D).

Substitution of tryptophan 20 by alanine in lysenin-His abolished the ability of the protein to form channels in sphingomyelin-containing membranes (Figure 6E). Addition of \(2-6 \mu g/ml\) of W20A lysenin-His to an azolectin/sphingomyelin/cholesterol bilayer had no effect on current measurements (Figure 6E).

**Lysenin forms cation-permeable channels**

To characterize the current-voltage relationship for single wt lysenin-dependent channel openings, current-time traces were recorded at holding potentials ranging from 70 mV to \(-150 \text{ mV} (450/150 \text{ mM KCl, cis/trans})\) (Figure 7A). At positive voltage, frequent short-lasting channel closures were observed, while under negative voltage, up to \(-110 \text{ mV}\), the channels displayed longer openings of lower amplitude. However, the potential of \(-150 \text{ mV}\) induced prolonged closure of the channels. All the traces shown were recorded when the lysenin channels exhibited activities devoid of initial flickering observed immediately after incorporation of lysenin into the membrane.

The current-voltage plot derived from the recordings presented in Figure 7A was a straight line with a reversal potential \(U_{rev} = -39 \pm 2 \text{ mV}\) (Figure 7B), indicating that the channel is selective for cations. The most frequent single channel conductance was \(100 \pm 9 \text{ pS}\), as determined from 30 measurements for \(1 \mu g/ml\) wt lysenin-His. In experiments with higher concentrations of the protein (2-5 \( \mu g/ml\)) we observed cation-selective channels with conductances ranging from 350 pS to 890 pS and complex kinetics. Occasionally, we also found a similar behavior of the channels when \(1 \mu g/ml\) of wt lysenin-His was applied (not shown).

Since wt lysenin-His is a recombinant protein, the channel-forming properties of native lysenin isolated from *Eisenia fetida* were also examined. The native protein formed cation-selective channels of similar properties (not shown).
Discussion

The data presented in this report indicated that wt lysenin-His forms oligomers the size of which varied between 120 kDa and 280 kDa, depending on conditions of their isolation. On the other hand, lysenin-His mutated on tryptophan 20 failed to form correct oligomers and existed mainly as the monomer when bound to sphingomyelin-containing membranes. The ability of wt lysenin-His for oligomerization was correlated with the ability of the protein to assemble ion-permeable, cation-selective channels in a sphingomyelin-dependent manner. The formation of channels by wt lysenin-His required an interaction of the protein with sphingomyelin, as no channel activity was detected from azolectin membranes even at relatively high wt lysenin-His concentrations. However, sphingomyelin binding was not sufficient for the protein activity. Mutant W20A lysenin-His specifically recognized and bound sphingomyelin as determined by lipidooverlay assay, erythrocyte staining and liposome binding. Despite the sphingomyelin binding, the mutant lysenin was not able to assemble channels and lacked hemolytic activity. These data point to tryptophan 20 residue located in the N-terminus of lysenin-His as important to the lytic activity of the recombinant protein, but not required for specific sphingomyelin recognition by the protein. The crucial role of the conserved tryptophan residues of lysenin for both sphingomyelin binding and hemolytic activity of the protein was already shown by Kobayashi's group [29]. Accordingly, intrinsic tryptophan fluorescence of lysenin exhibited blue shift in the presence of sphingomyelin in membranes, suggesting that at these conditions the aromatic residues are exposed to a less polar environment [15,16]. However, contrary to those data, in our hands W20A lysenin-His retained the ability to bind sphingomyelin with a comparable sensitivity as wt lysenin-His, although it lost the hemolytic activity. When trying to find a reason for this discrepancy, one can consider that the recombinant W20A lysenin used by Kobayashi and co-workers that had lost the sphingomyelin-binding ability was fused at the N-terminus to a maltose-binding protein tag [29]. In comparison to only six histidine residues fused to the N-terminus of lysenin in our construct, maltose-binding protein adds more than 300 amino acids to the recombinant protein.
acids to the 297 amino acid long lysenin. Such an extended tag could have markedly affected the structure and properties of the N-terminal fragment of W20A lysenin. Hence, the significantly diminished binding of sphingomyelin by W20A lysenin fused to maltose-binding protein could ensue from the plausible constraints exerted on the mutant lysenin by the huge tag. In agreement with this assumption it was recently reported that deletion mutants of lysenin lacking up to 160 N-terminal amino acids, including tryptophan 20, bound sphingomyelin without causing hemolysis [31]. Altogether the data of Kobayashi’s group and the results presented here confirm that the N-terminus of lysenin including tryptophan 20 is of importance for the cytotoxic activity of the protein, being dispensable for sphingomyelin binding. On the basis of our results we suggest that the N-terminus of the protein is important for efficient lysenin oligomerization and/or membrane penetration.

Oligomerization is a typical stage during pore and channel formation by cytotoxins [32–34]. When studied by SDS-PAGE, an oligomer of wt lysenin-His of about 280 kDa was found (Figure 3B, 3C) in line with earlier reports [15,31]. The shift from 41 kDa monomer to 280 kDa oligomer of wt lysenin-His started at 40:1 sphingomyelin/lysenin threshold and was enhanced by increasing sphingomyelin/protein ratio and by cholesterol, although cholesterol was not required for the binding of the protein to sphingomyelin, as shown by SDS-PAGE and an overlay assay. A similar oligomer of wt lysenin-His of about 250 kDa, accompanied by 360 kDa one, was found after chemical cross-linking of the protein bound to liposomes. However, during studies employing blue native gel electrophoresis, a predominant 120 kDa oligomer of wt lysenin-His was detected. These data suggest that trimer of 120 kDa can be a functional unit of wt lysenin-His responsible for lytic activity of the protein. Accordingly, lytic-inactive W20A lysenin-His failed to assemble the 120 kDa trimer forming instead multi-oligomers of about 620 kDa and 800 kDa. Further studies are required to determine whether haxamer (250–280 kDa) or nanomer (360 kDa) of wt lysenin-His detected after chemical cross-linking and SDS-PAGE analysis represent higher form of organization of the basic trimeric unit of the protein or result from an action of the cross-linker as well as SDS. The latter suggestion is in agreement with reports that SDS led to non-specific oligomerization of the pore-forming Pseudomonas aeruginosa cytotoxin [35].

To explain the lytic/channel activity of lysenin three mechanisms can be considered. The presence of positively charged amino acids spread along the polypeptide chain of lysenin suggests that the protein can cover the surface of negatively charged membranes in a carpet-like manner, leading to membrane penetration.

Figure 7. Wild type lysenin-His forms cation-selective channels. (A) Single channel activity induced by wt lysenin-His (1 μg/ml) in an azolectin/sphingomyelin/cholesterol bilayer (96:4:1, by weight) and recorded at indicated holding potentials. Lipid bilayers were exposed to asymmetric ionic conditions (450/150 mM KCl, pH 7.0 cis/trans). All recordings were low-pass filtered at 200 Hz. The closed channel state is indicated by c. (B) Current/voltage relationship of the wt lysenin-His-induced single channel currents from the experiment shown in (A). The current/voltage relationship was linear for holding potentials in the range from −150 mV to 70 mV.
Accordingly, wt lysenin-His at 6 μg/ml no longer formed channels but caused membrane rupture. Fragmentation of liposomes by wt lysenin-His was also detected in ultrastructural studies when high doses of wt lysenin-His were used. These data indicate that the detergent-like action of lysenin can prevail at high concentrations of the protein while channels are functioning at lower protein content. This suggestion is in line with the suggestion that formation of ion channels, transmembrane pores and membrane damage can represent various stages of lytic toxin action [37,38]. The channel assembly requires formation of well-organized transmembrane pores. To this end, an analysis of the secondary structure of lysenin has indicated that the protein has no helices capable of forming transmembrane domain [39]. Nevertheless, lysenin shares several properties with equinatoxin II, a sphingomyelin-binding toxin known to form cation-selective channels of a toroidal pore structure. It was found that for equinatoxin II to bind to sphingomyelin-containing membranes, clusters of aromatic residues of the protein, including two tryptophan residues, are required [40]. After binding to the membrane, the protein aggregates. Upon oligomerization, a short N-terminal fragment of equinatoxin II inserts into the membrane which is concomitant with the formation of a toroidal lipid pore in the bilayer [41,42]. On the basis of ultrastructural studies, formation of toroidal pores by equinatoxin II was connected with reorganization of lipids into hexagonal phase HII [41]. We did not detect corresponding lipid structures in liposomes treated with wt lysenin-His (Figure 5 and data not shown). This, however, does not preclude insertion of wt lysenin-His into the membrane to form toroidal pores since melittin, another toxin forming such pores, also did not favor HII lipid phase formation and cleaved the liposomes into smaller vesicles [41], resembling action of wt lysenin-His. The lack of pore-forming capacity of W20A lysenin-His can ensue from its incorrect oligomerization and possible failure in membrane lipid distortion. Recent studies on α-hemolysin demonstrated that insertion of the toxin into the membrane and membrane lysis can be two uncoupled phenomena [43]. In an analogy, one can consider that W20A lysenin-His can also penetrate the membrane but fails to change the lipid architecture to induce membrane permeation. As demonstrated in this paper, lysenin forms cation-selective ion channels that can be detected by electrophysiological means. Their single channel conductance is 100 ± 9 pS. The cation selectivity of the lysenin channels is probably a result of the presence of clusters of negatively charged amino acids promoting passage of cations rather than anions. Further studies are needed to establish which parts of the protein may contribute to this interesting feature. The formation of cation-selective channels by lysenin can account for the observed long-lasting contraction of strips of aorta exposed to the toxin [21]. Presumably, this effect was related to the influx of calcium ions through the lysenin-formed channels. Formation of channels by lysenin in the plasma membrane of erythrocytes and other cells can lead to ion imbalance followed by osmotic lysis of the cells. It is worthy of note that several other cytolytic toxins also form ion channels in membranes, indicating that the channel activity of the toxins is relevant to their ability to kill cells [3,44–46]. It is a matter of further investigation how lysenin oligomerization influences channel kinetic properties, and its conductance and selectivity.

While the manuscript was completed, another report analysing hemolytic and microbicidal activity of recombinant lysenin was announced [47]. It was shown that lysenin oligomerization occurred on erythrocytes but not on bacterial membranes. Aiming to examine the interaction of lysenin with sphingomyelin-containing membranes, the authors found that in lipid planar bilayers lysenin formed ion channels, in agreement with our data on wt lysenin-His.

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References


Oligomers of lysenin form channels


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Supplementary Figure 1. Nucleotide sequence of synthetic lysenin gene. The coding strand synthetic oligonucleotides are in capital and lower-case letters, alternately, and are marked by symbols ‘’Ux’’ on the right margin of the same row; oligonucleotides joining the upper ones during ligation are written in bold capital letters and marked by symbols ‘’Jx_x/C27’’ on the right margin. The amino acid sequence of lysenin is given above the coding upper strand. Start and stop codons are in bold italics. The nucleotide sequence of oligonucleotide P2 which allowed amplification of the final PCR product is double underlined. Tryptophan 20 that was substituted with alanine in W20A lysenin-His is indicated by black background.

Oligomers of lysenin form channels