Interaction of Earthworm Hemolysin with Lipid Membranes Requires Sphingolipids*

(Received for publication, December 2, 1996, and in revised form, June 10, 1997)

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Lytic activity in the coelomic fluid of earthworm (Eisenia fetida fetida) has been ascribed to eiseniapore, a hemolytic protein of 38 kDa. Since receptors for eiseniapore on target cell membranes are not known, we used lipid vesicles of various composition to determine whether specific lipids may serve as receptors. Lytic activity of eiseniapore was probed by the relief of fluorescence dequenching from the fluorophore 8-amino-naphthalene-1,3,6-trisulfonic acid originally incorporated into the vesicle lumen as a complex with p-xylene-bis-pyridinium bromide. Hemolysin binds to and disturbs the lipid bilayer only when distinct sphingolipids consisting of a hydrophilic head group as phosphor-ylcholine or galactosyl as well as the ceramide backbone, e.g. sphingomyelin, are present. Cholesterol enhances eiseniapore lytic activity toward sphingomyelin-containing vesicles probably due to interaction with sphingomyelin. Leakage of vesicles was most efficient when the lipid composition resembled that of the outer leaflet of human erythrocytes. Presumably, an oligomeric protein pore formed by six monomers is responsible for leakage of sphingomyelin-containing vesicles. The secondary structure of eiseniapore did not change upon binding to lipid membranes. The lytic activity of eiseniapore was completely abolished after its denaturation or after preincubation with polyclonal antibodies. Our results suggest that the presence of specific sphingolipids is sufficient to mediate lytic activity of eiseniapore. This action contributes to our understanding of earthworm immune responses.

Coelomic fluid of the earthworm Eisenia fetida ssp. (Oligochaeta, Lumbricidae) containing more than 40 proteins exhibits several biological effects as follows: cytolytic, proteolytic, hemolytic, hemagglutinating, tumourstic, mitogenic, and bacteriostatic activities (1–10). Recently, efforts have been directed toward identifying the molecular nature and regulation of lytic activity. Based upon partial purification of coelomic fluid, cytolytic and hemolytic activities have been associated with a protein of an apparent molecular mass of about 42 kDa (11). We have isolated recently a hemolysin from the coelomic fluid of Eisenia fetida fetida, referred to as eiseniapore, for the first time (12). The molecular mass of this thiol-activated hemolysin was 38 kDa, and it exerted a strong lytic activity against erythrocytes.

At least two different mechanisms preventing homologous lysis by eiseniapore have to be considered. First, an eiseniapore-regulating factor (ERF) purified recently by us inhibits the eiseniapore hemolytic properties (12). Eiseniapore-regulating factor shares immunological properties with vitronectin, an inhibitor of complement and perforin lytic activities (13, 14). Indeed, vitronectin and eiseniapore interacted reversibly to suppress hemolytic activity, and eiseniapore-regulating factor acted as a potent inhibitor of complement-induced hemolysis. Identification of an eiseniapore-regulating factor and its properties confirms the hypothesis of Canicatti (15), which suggested that vitronectin and related structures act as possible protectors of autolysis in invertebrates. Second, another strategy that may prevent self-killing considers the absence of membrane-associated receptor(s), which are required for binding of eiseniapore to self-membranes. However, the receptor for eiseniapore and a mechanism of any subsequent interaction with the lipid bilayer are not identified. Glycoproteins of the cell membrane are suggested as receptors since various acetylated and methylated carbohydrates were effective inhibitors of hemolytic activity of coelom fluid (1). Moreover, preincubation of semi-purified hemolysin with sphingomyelin, a typical component of mammalian plasma membranes, strongly suppressed hemolysis of mammalian erythrocytes (4). These results prompted the hypothesis of a specific involvement of sphingomyelin in the hemolysin-mediated lysis of target cells (15). As a corollary, it is not known whether this lipid will act as a receptor for eiseniapore.

Here we present a comprehensive analysis concerning the interaction of eiseniapore from E. fetida fetida with unilamellar liposomes of various lipid composition. We discovered for the first time that distinct sphingolipids are an absolute requirement for both binding of eiseniapore to lipid membranes and their subsequent leakage. In the presence of these lipids we observed the formation of oligomeric pore-like structures of eiseniapore on liposomal membranes and rapid leakage of vesicles. While cholesterol alone did not mediate binding of nor leakage by eiseniapore, complexes of sphingomyelin and cholesterol enhanced its lytic activity. Our data suggest that eiseniapore hemolytic activity is optimized toward a lipid composition typically for the outer leaflet of the plasma membrane of mammalian erythrocytes. Most importantly, our results imply that membrane-(glyco)proteins, as receptors for eisenia-
porc, are not required as long as specific sphingolipids are present.

MATERIALS AND METHODS

Earthworms and Harvesting of Coelomic Fluid—Adult *E. fetida fetida* (58), synonym *E. fetida typica* (59) (Annelida, Oligochaeta, Lumbricidae), were collected from 15 localities throughout Northern Germany. Earthworms were washed, cultured, and covered with wet filter paper for 48 h at 6 °C. Coelomic fluid was obtained by puncturing the coelomic cavity with a glass microcapillary. The suspension pooled from 40 earthworms was centrifuged (13,000 × g, 17 min) and the supernatant was used immediately for purification of eiseniapore.

Isolation and Purification—Preparative PAGE ensures high yield purification of biologically active molecules. Since we found that SDS destroyed the lytic activity of eiseniapore, it was omitted. We found that eiseniapore was not impaired up to a pH of 9.5. Thus, we used the Ornstein-Davis system (40) (16, 17) as follows: upper and lower electrode buffer (25 mM Tris base, 192 mM glycine), electrophoresis buffer (25 mM Tris base, 192 mM glycine, 2 mM reduced glutathione, 1.5 mM thioglycolic acid), and sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 0.025% bromphenol blue, 2 mA reduced glutathione, 2 mM thioglycolic acid, 4 mM phenylmethylsulfonyl fluoride). Addition of reduced glutathione and thioglycolic acid was required to protect the biological activity of the protein. The monomer concentration for maximum resolution was determined empirically with native analytical gels. The system used for preparative electrophoresis was composed of a running gel (38-mL monomer volume of 7% acrylamide, 0.18% bisacrylamide) and a stacking gel (5-mL monomer volume of 4% acrylamide, 0.16% bisacrylamide). Preparative electrophoresis (model 491 Prep Cell, Bio-Rad) was conducted at 15 watts for 13.5 h with a flow rate of 1 ml/min. Fractions of the elution buffer were collected at 4 °C and tested for hemolytic activity of eiseniapore using a suspension of 2% sheep erythrocytes in a microtiter plate assay. Hemolytic fractions were monitored by using 5–10% SDS-PAGE (see below), and purified hemolysin was used for analysis and lyophilization.

Analytical Gel Electrophoresis—SDS-PAGE (nonreducing condition) was performed with 5–10% acrylamide gels in a vertical gel apparatus (Protein 2 x i Vertical Electrophoresis Cells; Bio-Rad). Samples were mixed by preparing equal volumes of sample and buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol) and subsequent boiling for 5 min. Gels were run at 250 V.

Polyclonal Antisera of Eiseniapore—Rabbits were immunized subdermally into the backs with 20 μg of isolated eiseniapore in incomplete Freund's adjuvant. An additional immunization was made 2 weeks later. Sera were purified by using a protein A-Sepharose column (Bio-Rad) with a final concentration of antibodies of 0.5 mg/ml. To probe neutralization, samples (80 μl) of eiseniapore (5 μg/ml) were diluted with 40 μl of antibody solution (100 μg/ml) and incubated for 20 min at 25 °C.

Circular Dichroism—CD spectra were recorded on a JASCO J-720 spectropolarimeter interfaced with an external water bath to maintain temperature control and connected to a PC for data recording and processing. Fused silica cells of 1 mm path length were used. Spectra were recorded from 185 to 250 nm using a scan speed of 10 nm/min. The spectra correspond to the average of three scans. For data processing, the base line was subtracted, and spectra were smoothed using the software package supplied with the spectropolarimeter. The protein concentration was 3 μM in 10 mM sodium phosphate, pH 7.2.

Tryptophan Fluorescence Spectroscopy—Fluorescence was measured at 25 °C with an Aminco Bowman Series 2 Luminescence Spectrometer (Rochester, NY) using 10-mm quartz cuvettes. Protein fluorescence intensity was measured at wavelengths of λex = 280 nm and λem = 330 nm for excitation and emission, respectively. Background intensity measured in the absence of protein was subtracted.

Electron Microscopy—Vesicle membranes in the absence (control) and presence of eiseniapore were fixed with 1% glutaraldehyde at room temperature. Fixation was stopped after 10 min by addition of 1% Triton X-100, pH 7.4. Specimens were visualized with 1% uranyl acetate and phosphotungstate, pH 7.0, applying a double-carbon film technique. As support, 400-mesh copper grids covered with a holey carbon film were used. Micrographs were taken with an EM 400T electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV with a magnification of 60,000 and 80,000 ×.

Preparation of Liposomes and Ghosts—Lipids (Sigma, Deisenhofen, Germany) of the desired composition (total lipid concentration 750 μmol/liter) were dissolved in 1 ml of chloroform/methanol (1:1) and subsequently dried under nitrogen at room temperature. Lipids were hydrated by the addition of phosphate-buffered saline (5.8 mM NaH2PO4/Na2HPO4, 150 mM NaCl, pH 7.4). If not stated otherwise small unilamellar vesicles (SUV) have been used. SUV were prepared by sonification of the lipid suspension on ice with a Branson sonifier (Danbury, CT) until the suspension became opalescent, usually for 8 min (18). Large unilamellar vesicles (LUV) of different composition were prepared after six freeze-thaw cycles by extrusion (extruder from Lipex Biomembranes Inc., Vancouver, Canada) through two stacked polycarbonate membranes (Nucleopore, CA) with 0.1-m pore size. ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, di-sodium salt)–DPX (p-xylene-bis-pyridinium bromide) (Molecular Probes, OR)-filled vesicles and ghosts, respectively, were used to measure eiseniapore-induced leakage of luminal aqueous contents. The incorporation of the water-soluble complex ANTS/DPX into vesicles and the leakage assay were performed following the method described in Ellens et al. (19). Vesicles contained 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 10 mM Tris-HCl, pH 7.4. Vesicles were separated from non-encapsulated ANTS/DPX complexes by chromatography on Sephadex G-75 with Tes buffer (100 mM NaCl, 2 mM Tes, 2 mM 1-histidine, and 1 mM EDTA, pH 7.4). The same buffer but with 0.1 mM EDTA only was used for the leakage assay. For preparing resealed ghosts we followed the approach of Schwob and Passow (20) with the modifications of Pomorski et al. (21). Briefly, lysis of 2 ml of ice-cold suspension of washed erythrocytes (90% packed) in 1.2 mM acetic acid, 4 mM MgSO4, 1.2 mM ATP, 1 mM CaCl2 (pH 7.0) was performed at 4 °C. After 5 min erythrocytes were spun down, and the pellet was resuspended in buffer (12.5 mM ANTS, 45 mM DPX, 50 mM Tris-HCl) and pH 7.0 was adjusted by addition of appropriate amounts of 0.1 M NaOH. Resealing of ghosts was performed by incubation at 37 °C for 45 min. Ghosts were separated from non-encapsulated fluorescence marker by gel filtration (Sephadex G-50 column) using 50 mM Tris-HCl, 85 mM NaCl, and 10 mM CaCl2, pH 7.0, as elution buffer. To study the influence of lysolipids on the interaction of eiseniapore with ghost membranes, ghosts (total lipid concentration 750 μmol/liter) were preincubated with l-α-lysopalmitoylphosphatidylcholine (C16:0, Sigma, Deisenhofen, Germany) for 7 min at 25 °C before gel filtration.

Leakage Measurements—Leakage measurements were performed using a Shimadzu RF5001PC spectrofluorometer (Duisburg, Germany) at various temperatures with the wavelengths set at λex = 355 nm and λem = 530 nm, respectively. Dilution of the ANTS/DPX complexes caused by their release from the vesicle or ghost lumen leads to complex dissociation and, by that, to the relief of fluorescence quenching of ANTS. Thus, leakage is accompanied by an increase of ANTS fluorescence intensity. To elucidate the percentage of leakage, at the end of each experiment Triton X-100 (Sigma, Deisenhofen, Germany) was added (final concentration 0.1 vol %) to allow infinite dilution and dissociation of the ANTS/DPX complexes.

EPR Measurements—To characterize membrane fluidity of liposomes, we recorded the EPR membrane spectrum of the spin-labeled fatty acid I(12, 3) (5-doxyl-stearic acid; Sigma, Deisenhofen, Germany). The paramagnetic NO moiety is localized in the hydrophobic membrane phase close to the head group region of lipids. The concentration of I(12, 3) was 1 mol % of endogenous lipids. EPR spectra were measured by a Bruker ECS 106 (Bruker, Karlsruhe, Germany) or by a Radiospectrometer RS 100 (Magnettech GmbH, Berlin, Germany) both equipped with a temperature controller. From the spectra the order parameter was measured which has been shown to characterize membrane fluidity (22); an increase in fluidity causes a decrease of this parameter. The order parameter S was calculated according to Equation 1.

$$ S = \frac{(A_2 - A_0)}{(A_2 - A_1)(A_1 + 2A_2)} $$
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RESULTS

Eiseniapore-induced Leakage of Liposomes—We measured leakage of small unilamellar vesicles (SUV) in the presence of eiseniapore using the ANTS·DPX assay. We observed no eiseniapore-mediated destabilization and, thus, leakage of liposomes consisted only of egg phosphatidylcholine (egg-PC) (Fig. 1, curve a, pH 7.4, 25 °C, molar lipid to protein ratio (L/P) = 50:1 (mol/mol)). Even at an L/P as low as 10:1, eiseniapore caused no leakage of egg-PC liposomes. Likewise, variation in temperature, 10–37 °C, and pH, 4.8–8.1, did not affect the stability of egg-PC membranes in the presence of eiseniapore. Furthermore, no eiseniapore-mediated release of ANTS·DPX was observed upon incorporation of phosphatidylserine (PS) and/or phosphatidylethanolamine (PE) up to 50 mol % into egg-PC liposomes (data not shown). When replacing egg-PC for dipalmitoylphosphatidylcholine also no lysis of liposomes was observed neither below nor above the phase transition of this phospholipid (data not shown) indicating that the phase state and/or membrane fluidity of the lipid bilayer did not determine the lytic activity of eiseniapore.

A profound leakage of liposomes was observed, however, in the presence of sphingomyelin (SM) (Fig. 1). At an egg-PC/SM ratio of 3:1 the extent of leakage after 5 min was approximately 10% (Fig. 1, curve b, 25 °C, pH 7.4, L/P = 50:1). An increase in ANTS·DPX leakage occurred when the lipid ratio was changed from 3:1 to 1:3 (Fig. 1, curves c and d). Leakage of sphingomyelin-containing vesicles was also found at much higher L/P ratios (Fig. 2, curve b, L/P 250:1). Destabilization of egg-PC/SM liposomes by eiseniapore was efficient at all L/P ratios investigated in the presence of cholesterol (e.g. L/P = 250:1, pH 7.4, 25 °C, Fig. 2). Both the extent (see also Fig. 4) and the kinetics of leakage were enhanced in liposomes

Fig. 1. Eiseniapore-induced leakage of egg-PC/SM liposomes (SUV) at pH 7.4, 25 °C, in Tes buffer (L/P = 50:1). Leakage was measured by the change of fluorescence upon release of the ANTS·DPX complex initially encapsulated into the liposomes (λex = 355 nm; λem = 530 nm). Lytic activity of eiseniapore was measured for various egg-PC/SM ratios: a, 1:0; b, 3:1; c, 1:1; d, 1:3. At time t = 0 the protein (final concentration 200 nM) was added to the liposome suspension (final concentration 10 μM). To elucidate the percentage of leakage, at the end of each experiment Triton X-100 was added (final concentration 0.1%) to allow infinite dilution. For details, see “Materials and Methods.”

Fig. 2. Eiseniapore-induced (final concentration 40 nM) leakage of liposomes (SUV, final concentration 10 μM) of different lipid composition at pH 7.4, 25 °C, in Tes buffer (L/P = 250:1). Egg-PC/Chol/SM ratio a, 1:1:3; b, 1:0:3; c, 1:1:2. For details, see legend to Fig. 1 and “Materials and Methods.”

composed of egg-PC/Chol/SM, but there was no leakage in the case of egg-PC/Chol (Fig. 2) nor PS/egg-PC/Chol liposomes. Since it is known that cholesterol exerts a profound influence on membrane fluidity, the latter observations support the preliminary conclusion that the membrane fluidity does not resemble a determinant of eiseniapore interaction with membranes.

The extent of lysis of sphingomyelin-containing liposomes by eiseniapore depends upon the molar lipid to protein ratio. The leakage process became accelerated, and the final extent of lysis was enhanced upon increasing the protein concentration relative to lipids as shown for PS/egg-PC/Chol/SM (1:1:2:4) vesicles in Fig. 3A (see also Fig. 4). For kinetics, we measured the half-time of vesicle lysis corresponding to 50% of the extent of final leakage. Dependence of half-time on the inverse of protein concentration can be fitted to a first approximation by a linear function (Fig. 3B, curve a). The extent of leakage is a function of molar lipid to protein ratio demonstrated for egg-PC/SM liposomes of various compositions (Fig. 4).

Recently, we discovered that eiseniapore caused hemolysis of human erythrocytes (12) which prompted the preparation of liposomes resembling in their lipid composition the erythrocyte outer leaflet. The main lipid components in the exoplasmic human erythrocyte membrane leaflet are cholesterol, phosphatidylcholine, and sphingomyelin (24, 25). Moreover, phosphatidylethanolamine and gangliosides (GA) are present, whereas phosphatidylserine is almost exclusively oriented to the cytoplasmic leaflet (25). To mimic the exoplasmic leaflet of the erythrocyte membrane liposomes were made of egg-PC/Chol/SM/PE/GA (12:17:10:3:1). Indeed, those vesicles allowed high eiseniapore activity (Fig. 4). For example, L/P as low as 260:1 facilitated a significant leakage of about 40% after 7 min incubation at 25 °C (pH 7.4). Indeed, those vesicles allowed high eiseniapore activity (Fig. 4). For example, L/P as low as 260:1 facilitated a significant leakage of about 40% after 7 min incubation at 25 °C (pH 7.4). At 15 °C, the extent was about 35% (data not shown). Remarkably, a spongolipid as sphingomyelin was required for eiseniapore to exhibit lytic activity. Neither gangliosides nor the phospholipids phosphatidylethanolamine and phosphatidylserine could replace sphingomyelin. The presence of phosphatidylserine in sphingomyelin-containing liposomes, however, stimulated eiseniapore-induced leakage (Fig. 4).
To elucidate whether high curvature of SUV is essential for eiseniapore-mediated leakage, we also probed large unilamellar vesicles (LUV). Using LUV composed of PS/egg-PC/Chol/SM (1:1:2:4), we observed a slight reduction in the extent of leakage when compared with SUV (25 °C, pH 7.4; Fig. 4, inset). This indicates that curvature of lipid membranes is not an essential determinant for destabilization of lipid membranes by eiseniapore.

To prove that leakage of lipid vesicles is specifically related to eiseniapore, we preincubated the protein with polyclonal antibodies (see “Materials and Methods”). After adding of ANTS•DPX-containing liposomes (egg-PC/Chol/SM, 1:1:2) no leakage was observed. Denaturation of eiseniapore by preincubation at 56 °C in each instance caused a complete loss of its lytic activity suggesting that the native conformation of eiseniapore is essential for its activity.

Eiseniapore-mediated release of ANTS•DPX from sphingomyelin-containing liposomes depended upon pH and temperature. As shown for PS/egg-PC/Chol/SM (1:1:2:4), liposomal leakage (Fig. 5A, 25 °C) at neutral pH reached a maximum. At acidic or alkaline pH, pH 4.8 and pH 8.1, respectively, extent of lysis as well as kinetics declined. While the eiseniapore-induced release of ANTS/DPX from vesicles was slow with a low final extent at 10 °C, leakage became faster and enhanced after increasing the temperature (Fig. 5B, measured for 25 and 37 °C, respectively).

By thin layer chromatography (data not shown) we verified that no destruction of lipids occurred during the time course of the experiments. Thus, we have no indication for a hydrolase activity of eiseniapore.

Eiseniapore-induced Leakage of Resealed Erythrocyte Ghosts—Since (i) eiseniapore caused hemolysis of human erythrocytes (12) and (ii) liposomes comparable in their lipid composition to the outer leaflet of erythrocytes are sensitive to eiseniapore (see above), we measured leakage of resealed human erythrocyte ghosts using the ANTS•DPX assay. As can be deduced from the kinetics and the extent of leakage (Fig. 6A, 25 °C, pH 7.4), eiseniapore-triggered release of fluorophore from ghosts was faster and more efficient in comparison to liposomes even for a lipid composition corresponding to the outer leaflet of human erythrocytes (see also Fig. 3B). Even at the low L/P of 16,000:1, we found a significant leakage (about 20% after 7 min). At a ratio of 100:1, leakage was high; after 1.5 min of the addition of eiseniapore, leakage attained approximately 80% close to its plateau (90%). To a first approximation the half-time of the kinetics (see above) depends also linearly upon the inverse of the eiseniapore concentration (Fig. 3B).

To determine whether the composition of the lipid phase was also crucial for the interaction with eiseniapore in the case of ghosts, we preincubated ANTS•DPX filled ghosts with 0.5 mol % lysophosphatidylcholine (C16:0). By employing radioactively labeled lysophosphatidylcholine, we showed the rapid insertion...
of those lipids into ghosts membrane (data not shown). The presence of 0.5 mol % lysophosphatidylcholine in ghost membranes caused a significant reduction of eiseniapore-mediated leakage (Fig. 6B). Similar to liposomes, we observed no leakage of ghosts upon denaturation of eiseniapore by heat pretreatment (data not shown).

**Binding of Eiseniapore to Liposomes**—To ascertain whether binding of eiseniapore to sphingomyelin-containing liposomes was reversible, we measured the ANTS·DPX leakage of liposomes after preincubation of eiseniapore with unlabeled vesicles. When eiseniapore was preincubated with sphingomyelin-free vesicles (egg-PC, egg-PC/Chol (1:1), or egg-PC/PS (1:1)) for 15 min at 25 °C, the subsequent addition of labeled egg-PC/Chol/SM (1:1:2) caused a release of ANTS·DPX that was similar in extent and kinetics to that of controls (preincubation in the absence of liposomes) (Fig. 7). However, after preincubation of eiseniapore with egg-PC/Chol/SM (1:1:2) liposomes subsequently added ANTS·DPX-labeled vesicles of the same lipid composition caused no lysis (Fig. 7). This suggests an irreversible binding of eiseniapore to sphingomyelin-containing liposomes in agreement with results obtained by electron microscopy (see below).

As shown by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of eiseniapore, irreversible binding to liposomes may be explained by the formation of high molecular weight complexes of eiseniapore. When the protein was incubated in the presence of sphingomyelin-containing vesicles, we observed bands of a molecular mass of about 228 kDa which is about six times...
higher than that of a monomer (38 kDa) (Fig. 8). This suggests the formation of oligomers consisting of six monomers. Bands of higher molecular weight complexes were not detected when eiseniapore was incubated with lipid vesicles lacking sphingomyelin.

Electron Microscopy—The interaction of eiseniapore with vesicles was further investigated by electron microscopy of glutaraldehyde-fixed negatively stained samples. In the presence of eiseniapore we observed circular structures in the lipid bilayer of liposomes provided sphingomyelin was incorporated into the membrane (Fig. 9). We suggest that these structures represent oligomeric complexes of eiseniapore. They were not observed in the absence of sphingomyelin. The outer and inner diameter of the pore-like structures are approximately 10 and 2–3 nm. In side views, eiseniapore complexes appear as square-like structures with a height of about 10 nm, which project from the edge of the vesicle membrane. Thus, eiseniapore complexes seem to form a cylindrical structure with a central hole oriented perpendicular to the surface of the vesicle membrane (Fig. 9, 5–d).

Molecular Components of Sphingomyelin Essential for Leakage—We elucidated which structural features of sphingomyelin are important for interaction with lipid membranes and investigated whether complexes of cholesterol and sphingomyelin are responsible for enhanced extent of leakage when cholesterol is incorporated into sphingomyelin liposomes. We measured the extent of ANTS-DPX leakage in the presence of different sphingolipids at various temperatures and molar lipid to protein ratios. Almost the same extent was measured when sphingomyelin was replaced by galactosylceramide in egg-PC liposomes, but the single chain galactosylsphingosine (psychosine) was ineffective in mediating leakage (Table I, 37 °C; L/P, 250:1). Therefore, the ceramide structure with two chains seems to be essential. However, the presence of ceramide alone was not sufficient because no lytic activity of eiseniapore was detected when sphingomyelin was replaced by the double-chain N-palmitoyl-D-sphingosine. Thus, the ceramide structure as well as a hydrophilic moiety seemed to be essential membrane components for eiseniapore to mediate leakage of liposomes.

No further increase of fluorophore release was observed when cholesterol was added to phosphatidylcholine liposomes containing galactosylceramide in egg-PC liposomes, e.g. by formation of complexes (see “Discussion”), may enhance eiseniapore lytic activity. Results similar to that shown in Table I were obtained at lower temperature (25 °C) (data not shown).

For the various liposomes, we measured the order parameter of EPR membrane spectra of the spin-labeled fatty acid I(12, 3) at 37 °C (Table I; spectra not shown). This parameter provides a measure of membrane fluidity. The order parameter did not
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**Table I**

**Eiseniapore-mediated leakage and membrane fluidity of liposomes containing different sphingolipids (10 mol %) at 37 °C, pH 7.4**

Leakage was monitored by the release of the ANTS·DPX complex at a L/P of 250:1. The extent of leakage was measured 5 min after addition of eiseniapore to liposomes. The membrane fluidity is characterized by the order parameter S of the EPR membrane spectrum of the spin-labeled stearic acid 1(12,3). The concentration of the fatty acid analogue was 1 mol % of lipid concentration. Spectra were recorded in the absence of eiseniapore.

| Lipid composition                                      | Extent of leakage | Order parameter |
|--------------------------------------------------------|-------------------|-----------------|
| Phosphatidylcholine:cholesterol:sphingomyelin (9:9:2)   | 47                | 0.709           |
| Phosphatidylcholine:sphingomyelin (9:1)                 | 25                | 0.630           |
| Phosphatidylcholine:cholesterol:galactosylceramide*    | 22                | 0.714           |
| Phosphatidylcholine:galactosylceramide* (9:9:2)        | 20                | 0.632           |
| Phosphatidylcholine:cholesterol:ceramide*              | 0                 | 0.715           |
| Phosphatidylcholine:ceramide* (9:9:2)                  | 0                 | 0.629           |
| Phosphatidylcholine:cholesterol:galactosylphosphingosine* (9:9:2) | 0            | 0.694           |
| Phosphatidylcholine:galactosylphosphingosine* (9:1)   | 0                 | 0.627           |

*Type 1, 98% α-hydroxy fatty acid.

**Characterization of Eiseniapore in the Presence of Liposomes**—A fluorescence spectrum typical for tryptophan residues was measured when eiseniapore was excited at 280 nm (Fig. 10A). Presumably, tryptophan residues are buried within the protein in a rather hydrophobic region. This is supported by the fluorescence maximum at 333 nm which is characteristic for an apolar environment. Addition of the aqueous quencher potassium iodide (20 mM final concentration) caused a reduction of approximately 8% in fluorescence intensity. No influence on fluorescence intensity nor on wavelength of its maximum was observed in the presence of liposomes which did not contain sphingomyelin. However, a significant decrease in eiseniapore-associated fluorescence was observed when egg-PC/Chol/SM liposomes were added (Fig. 10B). The wavelength of fluorescence maximum was only slightly shifted to higher values ($\lambda_{\text{max}} = 335 \text{ nm}$). Upon binding of eiseniapore to those liposomes, we found an enhanced accessibility of potassium iodide to tryptophan residues. The fluorescence intensity was decreased by approximately 30% in the presence of 20 mM KI.

The CD spectrum of eiseniapore was measured in the presence or absence of liposomes consisting either of egg-PC/Chol/SM or only of PC. No alteration of the CD spectrum of eiseniapore was observed after adding lipid vesicles even when sphingomyelin and cholesterol were incorporated (data not shown). The secondary structure of eiseniapore consists of about 37% β-sheet, 28% α-helix, 17% β-turn, and 18% random coil as estimated by the computer program CONTIN (26).

**Discussion**

We have shown that a purified hemolysin (eiseniapore) from the earthworm *E. fetida fetida* (58) is able to perturb the bilayer structure of liposomal as well as human erythrocyte membranes causing release of internal aqueous contents from each of them. Its lytic activity does not require any other component from the coelom fluid, and it is suppressed by corresponding polyclonal antibodies. However, this activity is unambiguously associated with its native structure; it was completely lost after denaturation of eiseniapore by heat pretreatment. An essential prerequisite for eiseniapore-mediated leakage of lipid membranes was the presence of distinct sphingolipids as sphingomyelin or galactosylceramide. Sphingolipids in sphingomyelin are essential and, presumably, sufficient to cause leakage of lipid membranes by eiseniapore. In the absence of sphingomyelin, no release of egg-phosphatidylcholine liposomal content was detected even in the presence of phosphatidylserine, phosphatidylethanolamine, and cholesterol, respectively. A rapid leakage of liposomes was observed only after incorporation of sphingomyelin which was maximum at neutral pH. Although more detailed investigations are warranted, we elucidated the following molecular features of sphingolipids as imperative for eiseniapore-mediated leakage: (i) a hydrophilic head group as phosphorylcholine or galactosyl and (ii) the double chain backbone of sphingolipids, the ceramide.

A significant enhancement of eiseniapore-mediated leakage of egg-PC/SM liposomes was observed when cholesterol was present. Cholesterol alone did not support lytic activity because (i) in the absence of sphingomyelin no liposomal release was observed, and (ii) leakage was not enhanced when sphingomyelin was replaced by galactocerebroside. These results suggest that the interaction, perhaps the formation of complexes, between cholesterol and sphingomyelin is responsible for increased leakage after incorporating cholesterol into egg-PC/SM liposomes. Recently sphingomyelin has been shown to form complexes with cholesterol (27, 28), whereas galactocerebroside does not (29, 30, see also Ref. 31). Remarkably, the cholesterol...
interaction of sphingomyelins and galactoceramides may depend significantly on the structure of the acyl chains (32).

We observed the highest extent of leakage when the lipid composition of liposomes simulated that of the outer leaflet of human erythrocyte ghosts. The lytic activity was even higher for resealed human erythrocyte ghosts. At present we do not know which components of the erythrocyte membrane are responsible for this additional stimulating effect on eiseniapore-mediated lysis. We precluded that the lytic activity of eiseniapore was related to a specific phase state of the bilayer. Furthermore, electrostatic interactions do not seem to play an essential role since phosphatidylserine alone could not render egg-PC liposomes susceptible for eiseniapore and was not required for leakage of SM liposomes. We cannot preclude the possibility that specific protein receptors may exist which enable eiseniapore to develop its lytic activity. However, as long as distinct sphingolipids are present those proteins are not required. Although no conclusive data are available, so far no indication for the existence of protein receptors for eiseniapore has been given. The present study points rather to sphingolipids as the relevant target for eiseniapore. Indeed, we found that eiseniapore-triggered hemolysis of erythrocytes of different mammalian species was closely related to the sphingomyelin content of the erythrocyte membrane.4

Our results do not only emphasize the essential role of sphingolipids for leakage but also strongly support that these lipids are necessary for binding. Preincubation of eiseniapore in the presence of liposomes of various lipid composition but without sphingomyelin did not affect the subsequent eiseniapore-mediated leakage of egg-PC/Chol/SM liposomes. In contrast, no leakage of labeled egg-PC/Chol/SM liposomes was found when eiseniapore was preincubated with unlabeled liposomes of the same composition. Thus, we surmise that binding of eiseniapore to liposomes containing sphingolipids as sphingomyelin is essentially irreversible. We have no evidence for substantial binding of eiseniapore to lipid membranes lacking specific sphingolipids.

Presumably, membrane-bound eiseniapore forms an oligomeric pore-like structure provided sphingolipids like sphingomyelin are present. These complexes seem to consist of six monomers. As already pointed out (33, 34) the formation of a dimer. For example, poly-C9 of the complement system (35), staphylococcal α-toxin (36), aerolysin, a cytolytic bacterial exotoxin produced by Aeromonas hydrophila (37), and the β-toxin of Clostridium perfringens (perfringolysin O) (38) exhibit a high content of β-sheet structure. We have no indication for a rearrangement in secondary structure of eiseniapore upon binding to sphingomyelin-containing lipid membranes as deduced from the far UV-CD spectrum. However, we cannot rule out that changes of the secondary structure of various parts of eiseniapore may compensate each other and, thus, become invisible in the spectrum. In contrast, we found moderate alterations of tryptophan fluorescence which may indicate some reorientation of tertiary and/or quaternary structure. A rearrangement of tertiary structure of membrane active proteins without a significant change in secondary structure has been observed for the pore-forming bacterial toxins colicin A and aerolysin (39–41).

Our results on the interaction of eiseniapore with sphingomyelin have important implications for understanding the immune response of earthworms. First, we propose that a self-killing activity of eiseniapore may be prevented by an altered molecular structure of sphingolipids. Of course, an efficient mechanism would consider the absence of those lipids from respective target membranes of annelids. Although no comprehensive lipid analysis of various earthworm tissues is available, apparently sphingolipids represent only a minor component of annelid membranes (42–44) and, more specifically, of Eisenia fetida (45). For instance, Okamura et al. (46) found that the earthworm nervous system does not contain sphingomyelin. Second, earthworm parasites, i.e. ciliates, cestodes, and nematodes, do not survive in the coelomic fluid. Remarkably, sphingolipids are typical lipids for those parasites (47–51). Third, our analysis suggests a strategy that could answer whether eiseniapore receptors different from sphingolipids exist. For example, cells such as bacteria, sensitive to whole coelomic fluid (3, 52) but lacking sphingolipids, are interesting biological systems for those studies. Finally, we emphasize that the general conclusion on distinct sphingolipids as receptors for hemolysins of annelids and other invertebrates is awaiting the confirmation by future studies using hemolysins from other species (see also Refs. 53–57).

Acknowledgments—We thank Prof. Dr. Gregor Damaschun and Dr. Dietrich Zirwer (Max-Delbrück-Center, Berlin-Buch) for assistance in CD measurements and Rudolf Erdemann for helpful discussion.

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J. Biol. Chem. 1997, 272:20884-20892.
doi: 10.1074/jbc.272.33.20884

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