Pore Formation by the *Escherichia coli* Hemolysin: Evidence for an Association-Dissociation Equilibrium of the Pore-Forming Aggregates

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Lipid bilayer experiments were performed in the presence of hemolysin of *Escherichia coli*. The toxin had a rather low activity in membranes formed of pure lipids, such as phosphatidylcholine or phosphatidylserine. In membranes from asolectin, a crude lipid mixture from soybean, hemolysin was able to increase the conductance by many orders of magnitude in a steep concentration-dependent fashion, which suggested that several hemolysin molecules could be involved in the conductive unit. Furthermore, the much higher toxin activity in asolectin membranes would be consistent with the assumption that this lipid contains a receptor needed for membrane activity of the toxin. The results of single-channel records showed that the membrane activity of hemolysin is due to the formation of ion-permeable channels with a single-channel conductance of about 500 pS in 0.15 M KCl. The hemolysin channel seemed to be formed by a toxin oligomer which showed an association-dissociation reaction and had a mean lifetime of about 2 s at small transmembrane voltages. The conductance of the hemolysin channels was only moderately dependent on the salt concentration in the aqueous phase. Zero-current membrane potential experiments showed that the hemolysin channel is cation selective. The mobility sequence of the cations in the channel was similar to their mobility sequence in the aqueous phase, which was consistent with the assumption that the hemolysin channel is wide and that the interior field strength is not very high. From the single-channel conductance, a lower limit of about 1.0 nm for the effective channel diameter could be estimated.

The membrane-active cytolsins (hemolysins) are extracellular toxic proteins which are produced by a large number of gram-positive and gram-negative bacteria (12). Cytolysin-secreting bacteria are frequently pathogenic, and the direct involvement of these extracellular proteins in pathogenesis has been demonstrated in some cases (15). Pore formation in the target cell membrane is a well-established mechanism for the alpha-toxin of *Staphylococcus aureus* and for members of the thiol-activated cytolsins synthesized by various gram-positive bacteria (8, 9).

The hemolysin of *Escherichia coli* is one of the best-studied cytolsins of gram-negative bacteria (11, 16, 20). The genetics of this toxin have been well characterized. Two genes, *hlyC* and *hlyA*, are required for the synthesis of the active hemolysin (17, 22). One of the two gene products, HlyA, is actively excreted with the help of a specific transport system consisting of the products of two other *hly* genes, *hlyB* and *hlyD* (29). The excreted HlyA protein is hemolytically inactive unless it is activated in the cytoplasm by HlyC. The activation mechanism which converts HlyA to the hemolytically active form, termed HlyA*, is still unknown. Recent data (28) showed that the active hemolysin is highly sensitive to phospholipase C and mechanical forces which may disrupt the structure of the toxin. It has therefore been suggested that hemolytically active HlyA* represents a complex which consists of HlyA and one or more phospholipid molecules noncovalently linked to the protein (28). Only the active HlyA*, but not HlyA, possesses pathogenic potential in vivo (J. Hacker, H. Hof, and W. Goebel, unpublished results). Synthesis of leukotrienes is induced in granulocytes, and these inflammatory substances are released together with histamine by sublytic doses of HlyA* specifically (and not by HlyA (25, 26))

Recent studies (8) have shown that *E. coli* hemolysin generates transmembrane pores 1.5 to 3.0 nm in diameter in erythrocyte membranes. It has been claimed (21) that pores of similar size are also formed by *E. coli* hemolysin in lipid bilayer membranes composed of pure phosphatidylcholine but that this pore formation is dependent on the presence of a transmembrane potential. Toxin monomers were proposed to be responsible for pore formation; i.e., the channel is formed by a single HlyA* molecule (21). Furthermore, it has been suggested that the channel represents a general diffusion pore similar to the bacterial porins (1, 24).

We now present lipid bilayer data that clearly indicate that bilayers formed from several pure phospholipids are relatively inactive targets for HlyA* and especially for HlyA. In artificial lipid bilayer membranes formed from asolectin (a crude mixture of soybean lipids), reversible pores are formed, but only by HlyA*. We describe an analysis of the unitary (single-channel) conductance changes which occur in these membranes exposed to HlyA*. We demonstrate that this protein forms defined channels in the in vitro system, with an average single-channel conductance of about 500 pS in 0.15 M KCl and a mean lifetime of about 2 s at 20 mV.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* 5K and the plasmids pANN202-812 (wild-type hemolysin HlyA*) and pANN202-812B (hemolytically inactive HlyA) used in this study for the isolation of hemolysin were described before (15a, 27).

**Isolation of active hemolysin (HlyA*) and inactive HlyA.**
Extracellular hemolysin (HlyA*) was isolated from the supernatants of an E. coli 5K(pANN202-812) culture (20 ml) grown at 37°C in double-concentrated yeast-tryptone broth (2×YT) to a density of 5 × 10^8 cells per ml. The cells were removed by centrifugation, and the supernatant was concentrated by ammonium sulfate precipitation (28). This preparation, when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, consisted of at least 90% HlyA protein (110 kilodaltons). The concentration of HlyA protein was determined by silver staining of stepwise dilutions of hemolysin and comparing the stain densities with those of known concentrations of β-galactosidase. Hemolytically inactive HlyA protein was isolated essentially by the same procedure with E. coli 5K(pANN202-812B). This plasmid determines defective HlyC, which is unable to activate HlyA to the hemolytically active form HlyA*. Hemolytic activity of the preparations was measured as described before (28).

Lipid bilayer experiments. The methods used for the black lipid bilayer experiments were described previously (4). The membranes were formed across circular holes (surface area, about 0.1 mm² for the single-channel experiments or 1 mm² for the macroscopic conductance and the selectivity measurements) in the thin wall of a Teflon chamber separating two aqueous compartments. Different lipids were applied as a 1% (wt/vol) solution in n-decane. The following lipids were used: diphytanoyl phosphatidylcholine, dioleoyl phosphatidylcholine, dioleoyl phosphatidylethanolamine, and phosphatidylserine (all obtained from Avanti Biochemicals, Birmingham, Ala.). Asolectin (lecithin type IIIs from soybeans; Sigma Chemical Co.) was also used. Bilayer formation was indicated when the membrane turned black in reflected light. Solvent-depleted membranes made by using the Montal-Mueller method were formed as described before (3) by the apposition of two monolayers from asolectin or diphytanoyl phosphatidylcholine. The small holes (diameter, approximately 0.1 mm) were pretreated with petrolatum or hexadecane (3). All salts were obtained from E. Merck AG (Darmstadt, Federal Republic of Germany) and were of analytical grade.

The current through the membranes was measured with two calomel electrodes connected in series with a voltage source and a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart or a tape recorder. For the macroscopic conductance measurements, the current amplifier was replaced by a Keithley electrometer (model 610C). After membrane formation, the toxin was added to the aqueous phase in concentrations between 100 and 1,000 ng/ml with stirring to allow equilibration. The zero-current membrane potentials were measured by using a Keithley 610C electrometer as the result of a salt gradient (the more dilute side was 50 mM) across a membrane in which between 10^6 and 10^7 pores were reconstituted (5). The membrane potential reached a steady state within 5 to 10 min after the addition of the concentrated salt solution to one side of the membrane.

RESULTS

Effect of hemolysin of the conductance of lipid bilayer membranes. Hemolysin interacts with membranes and binds to them (11). We performed conductance measurements with lipid bilayer membranes to study this interaction in more detail in an in vitro system. Membranes were formed from a variety of different pure lipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine and with asolectin, which is a mixture of many different lipids isolated from soybean. With pure lipids, only minor effects of the purified hemolysin on the membrane conductance were observed, and the specific membrane conductance increased for these lipids by, at most, a factor of 10 after the addition of 200 ng of hemolysin per ml (from 10⁻⁷ S/cm² to 10⁻⁵ S/cm²). Surprisingly, the addition of the same hemolysin concentration to black membranes made of asolectin–n-decane resulted in a much greater effect on the membrane conductance, and it increased by several orders of magnitude above that of the other membrane systems and those of asolectin membranes in the absence of hemolysin (from 10⁻⁷ S/cm² to 1.5 × 10⁻⁴ S/cm²). The difference between the effect of hemolysin on membranes made from the pure lipids and the effect on those made from asolectin may be explained by assuming that a component in the membranes facilitates either toxin binding or channel formation.

As described above, the addition of hemolysin to the aqueous phase resulted in a strong increase in the conductance of asolectin membranes. The conductance increase was not sudden, but it was a function of time after the addition of the hemolysin to membranes in the black state or of time after the blackening of the membranes if the toxin was present in the aqueous phase prior to membrane formation. Figure 1 shows experiments of the second type. Membranes were formed from asolectin–n-decane in solutions containing 0.15 M KCl and 200, 400, or 800 ng of hemolysin per ml with stirring to allow equilibration. At 2 to 5 min after formation, the membranes were in the black state and the increase in the membrane current was measured for 30 min. During this time the membrane conductances increased by more than two orders of magnitude. Only a small further increase (as compared with the initial one) occurred after that time. The same effect was observed irrespective of whether the hemolysin was added to one or both sides of the membranes. Furthermore, we observed a similar behavior of the membrane conductance when purified toxin or supernatants of cell cultures were added to the lipid bilayer membranes. Surprisingly, the supernatants of the cell cultures were found to be more active relative to the protein concentration than were the purified and lyophilized hemolysin. This result probably reflects a slow inactivation of the toxin. This inactivation was also observed with the supernatants of cell cultures. The activity of the supernatants was stable for about 2 to 3 days when the solutions were kept at 0°C. After that time, the activity decreased, and usually it was completely lost after 5 to 6 days. In general, it was very difficult to preserve the activity of the toxin for a longer time because freezing of the hemolysin-containing solutions resulted in a considerable loss of the lytic activity and in a decrease of the activity in lipid bilayer experiments.

The purified toxin (as obtained from the supernatants of cell cultures) probably contained traces of other proteins, including the outer membrane porins, towards which the lipid bilayer assay is very sensitive (1, 4, 7). The presence of porins was even more likely in the experiments with the supernatants because it has been shown that osmotic shock fluids and supernatants of cell cultures may contain porins (2). Control experiments were performed to check whether the membrane activity of the supernatants was caused by artifacts. In these experiments, supernatants of cell cultures lacking the hemolysin were added to the lipid bilayer membranes. No conductance increase or only an insignificant conductance increase was observed. The same was true when the supernatant of a strain was used which produced the inactive HlyA (Ludwig et al., in press). This result
suggested that the membrane activity of the hemolysin-containing solutions and of the purified toxin was not caused by an unspecific artifact. Furthermore, the pores formed by HlyA* and by bacterial porins have completely different properties (1, 24; see also below).

Figure 1 shows that small differences in the hemolysin concentration resulted in considerable variations of the specific membrane conductance. An increase in the hemolysin concentration by a factor of four resulted in a membrane conductance about 50- to 200-fold higher. This means that the relationship between hemolysin activity in membranes and toxin concentration in the aqueous phase was not linear. The dependence of the specific membrane conductance on the hemolysin concentration is shown in Fig. 2. The experimental points were measured about 20 min after membrane formation or after toxin addition. Although a considerable deviation of the experimental points was observed at a given hemolysin concentration, the data could be fitted to a straight line with a slope of approximately 3 on a double-logarithmic scale. This means that several hemolysin molecules could be involved in the formation of the conductive pathway in the membranes. Furthermore, we have to assume that an association-dissociation equilibrium existed between the oligomer responsible for membrane activity and the hemolysin monomers; otherwise, a linear relationship between membrane conductance and hemolysin concentration should have been observed. Figure 2 also shows that the influence of hemolysin on the membranes could only be studied in a rather narrow concentration range, between 100 and 1,000 ng/ml. At concentrations below 100 ng/ml, we frequently observed a membrane conductance which was only slightly greater than the specific conductance of bare membranes (i.e., the conductance in the absence of the toxin), indicating the formation of only a few pores (see below). At concentrations above 1 μg/ml, the membranes became unstable, presumably because of formation of a large number of pores.

**Single-channel analysis.** The addition of smaller concentrations of hemolysin (50 to 100 ng/ml) to asolectin−n-decane membranes of small surface area (0.1 mm²) allowed the resolution of stepwise conductance increases. Figure 3 shows a single-channel recording in the presence of hemolysin. The toxin was added 5 min after the membrane was in the black state. First, we observed only small steps, which frequently switched off again after a short time. Then much larger fluctuations in conductance appeared, which generally seemed to start from the small steps (Fig. 3, insert). These large fluctuations had a limited lifetime (mean lifetime, about 2 s) and usually decayed back to the small state. The occurrence of two types of pores caused by hemolysin can also be seen in Fig. 4, which shows a histogram of the conductance fluctuations observed under the conditions described for Fig. 3 (20-mV membrane potential).

For larger differences of the potential across the membranes, the histogram of the conductance fluctuations became more complicated because some additional conductance levels occurred. Figure 5 presents a single-channel recording measured at 100 mV. The initial on-step had the same single-channel conductance as the large steps at 20 mV (Fig. 5, arrows). However, the hemolysin channel switched to a substate of 170 pS that was not observed at 20 mV. These results suggested that the pores formed by the *E. coli* hemolysin did not form a rigid structure but underwent molecular changes as a result of time and applied membrane potential. The steep dependence of the conductance on the protein concentration seen in the multichannel experiments.

![Graph](http://iai.asm.org/)
(Fig. 2) is consistent with this assumption if it is assumed that the conductive unit (the hemolysin channel) is composed of several hemolysin monomers.

Single-channel experiments were also performed with salts containing ions other than K\(^+\) and Cl\(^-\). These experiments were done to study the ionic selectivity of the hemolysin channel. First, chloride was replaced by acetate (Table 1). This change had little, if any, influence on the conductance of the hemolysin channel. The influence of the cations on the single-channel conductance was more substantial (Table 1), which is consistent with the assumption that the hemolysin channel is cation selective. The ionic selectivity was Cs\(^+\) = Rb\(^+\) = K\(^+\) > Na\(^+\) > Li\(^+\), which means that the selectivity follows the mobility sequence of the ions in the aqueous phase. This selectivity sequence could also mean that the hemolysin channel is a wide channel which has inside only a small field strength and no small selectivity filter (i.e., no binding site), as is suggested by the fact that the large organic Tris cation could also penetrate the hemolysin channel. Table 1 shows also the average single-channel conductance, \(\lambda\), as a function of the KCl concentration in the aqueous phase. Surprisingly, we did not observe a 1:1 relationship between conductance and KCl concentration. Figure 6 shows a double-logarithmic plot of the conductance-versus-concentration curve. The slope of the straight line, approximately 0.5, suggested a complicated behavior of the hemolysin channel, which definitely does not contain a small selectivity filter (i.e., a binding site) for cations. Otherwise, we should have observed a linear relationship at small ion concentrations and saturation at large concentrations.

**Experiments with solvent-depleted membranes.** In a recent publication (21), the interaction of chromosomal hemolysin (8) with solvent-depleted membranes has been investigated. The results have suggested that the hemolysin forms long-lasting general diffusion pores similar to the porins of gram-negative bacteria (1). To test the possibility that \(n\)-decane used for membrane formation could influence the properties of the hemolysin channel, we performed single-channel experiments with solvent-depleted membranes formed by using the Montal-Mueller method (3). The single-channel conductances observed with solvent-depleted asolectin membranes were very similar, if not identical, to those given in Table 1 for solvent-containing membranes. This means that there did not exist a 1:1 relationship between bulk aqueous conductance and single-channel conductance. Furthermore, the channels had also in solvent-depleted membranes a limited lifetime at small transmembrane potentials.

**Zero-current membrane potentials.** Further information on the molecular structure of the hemolysin channel was obtained by zero-current membrane potential measurements. The experiments were performed as follows. After the incorporation of 100 to 1,000 hemolysin channels into the asolectin membranes, the salt concentration on one side of the membranes was raised 10-fold from 50 to 500 mM and the zero-current potential was measured 5 min after the gradient was established. For all four salts (KCl, LiCl, KCH\(_3\)COO, and Tris hydrochloride) used in these experiments, the more dilute side (50 mM) was always positive, which indicated preferential movement of the cations through the hemolysin channel; i.e., the channel is cation selective, as was already suggested from the single-channel recordings. Surprisingly, the zero-current membrane potential did not reach 59 mV, as is expected for an exclusively cation-selective channel. The
FIG. 3. Single-channel recording of hemolysin channels in the presence of 0.5 M KCl. The membrane was formed from asolectin–
$n$-decane. The applied voltage was 20 mV; the temperature was 25°C. The insert shows one of the channels on a faster time scale. The arrows
indicate the small conductance fluctuations described in the text.

FIG. 4. Histogram of the conductance fluctuations observed with asolectin–$n$-decane membranes in the presence of hemolysin of E. coli.
The average single-channel conductance of the right-side maximum was 0.99 nS for 145 events. The aqueous phase contained 0.5 M KCl and
100 ng of hemolysin per ml. The voltage was 20 mV, and the temperature was 25°C.
zero-current membrane potential for the salts mentioned above was between 38 mV (Tris hydrochloride) and 41 mV (LiCl). Analysis of this data by using the Goldman-Hodgkin-Katz equation (5) suggested that anions also could have a certain permeability through the channel because the ratio of the permeabilities $P_n$ for cations and $P_a$ for anions was approximately 9. On the other hand, the asymmetry potential was very similar for the four different salts, a result which is not expected for a general diffusion pore (1, 4, 5).

This suggested that the hemolysin channel could be ideally selective. This assumption is supported by the very small single-channel conductance in Tris hydrochloride and the fact that the channel had the same cation selectivity in this salt (Table 1). The properties of the channel appear to be controlled by negatively charged groups which repel co-ions and attract counterions at the pore mouth in such a way that only part of the full-bulk aqueous gradient drops across the channel (see Discussion).

**TABLE 1. Average single-channel conductance, $\bar{\Lambda}$, of the hemolysin channel for a variety of different salts and concentrations**

<table>
<thead>
<tr>
<th>Salt</th>
<th>$c_m$ (M)</th>
<th>$\bar{\Lambda}$ (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.01</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1,500</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3,900</td>
</tr>
<tr>
<td>LiCl</td>
<td>0.15</td>
<td>230</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.15</td>
<td>400</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.15</td>
<td>350</td>
</tr>
<tr>
<td>RbCl</td>
<td>0.15</td>
<td>550</td>
</tr>
<tr>
<td>CsCl</td>
<td>0.15</td>
<td>570</td>
</tr>
<tr>
<td>KCH₃COO (pH 7)</td>
<td>0.15</td>
<td>480</td>
</tr>
<tr>
<td>Tris hydrochloride</td>
<td>0.15</td>
<td>48</td>
</tr>
</tbody>
</table>

* The membranes were formed from asolectin-n-decane; the temperature was 25°C. The applied voltage was 20 mV. The pH was 6 unless otherwise indicated.

* $c_m$, Aqueous salt concentration.

* $\bar{\Lambda}$ was calculated as the average of at least 100 single events.

**DISCUSSION**

In this paper, we have demonstrated that hemolysin of *E. coli* forms ion-permeable channels in lipid bilayer membranes. The channels had a considerable single-channel conductance, comparable to that of the general diffusion porins of gram-negative bacteria (1). Nevertheless, some of the channel properties were different from those of the porins. The latter pores have a long lifetime and usually exhibit only a limited selectivity for ions, and the single-channel conductance is a linear function of the bulk aqueous conductance (1, 5). This was not the case for the hemolysin channel; the pores had a limited lifetime at 20 mV and their single-channel conductance-versus-concentration curve had a slope of about 0.5 to 0.6 on a double-logarithmic scale. Furthermore, the zero-current membrane potential was always 40 mV on the more dilute side irrespective of the salt, whereas in anion- or cation-selective general diffusion pores the asymmetry potential is dependent on the mobility of the ions in the aqueous phase (5). Therefore, we had to assume that either the surface potential of the membranes (about 20% of the lipid molecules in asolectin are negatively charged) or charges attached to the hemolysin channel influence its properties.
Negative charges either at the pore mouth or on the membrane surface result in substantial ionic strength-dependent surface potentials at the pore mouth which attract cations and repel anions. Accordingly, they influence both single-channel conductance and zero-current membrane potential. The influence of charges at the pore mouth is difficult to quantify. However, assuming that the charges on the membrane surface act as if they are uniformly smeared, the surface potential, $\phi$, can be estimated by using the following equations (6):

$$\phi = \frac{2RT}{F} \ln \left[ \frac{\sigma / \sigma_o + \sqrt{(\sigma / \sigma_o)^2 + 1}}{c} \right]$$

(1)

and

$$\sigma_o = \sqrt{8 \epsilon_0 \epsilon R T c}$$

(2)

where $\epsilon_0$ and $\epsilon$ ($\epsilon = 80$) are the absolute dielectric constant of vacuum and the relative dielectric constant of water, respectively, $c$ is the bulk aqueous salt concentration, $R$ is the gas constant, $T$ is the absolute temperature, and $F$ is the Faraday constant. $T$ and $F$ have the usual meanings. The concentration of the monovalent cations, $c_{o+}$, at the membrane-water interface is given by

$$c_{o+} = c \exp[-\phi F/(RT)]$$

(3)

The cation concentration $c_{o+}$ at the membrane-water interface, i.e., at the mouth of the pore, can now be used for the calculation of the effective conductance concentration curve. Table 2 gives the surface potential, $\phi$, assuming a surface charge density of one charge per 4 nm$^2$ [corresponding to 15% charged lipids and a total surface charge density of $\sigma = 4 \times 10^{-6}$ (A $\times$ s)/cm$^2$]; the surface concentration, $c_{o+}$, of potassium ions calculated by using equation 3; the experimentally measured single-channel conductance, $\lambda$; and the single-channel conductance, $\lambda^*$, corrected for the concentration increase of the cations at the membrane-water interface. The data shown in Table 2 demonstrate that an almost linear relationship is obtained between the calculated cation concentration at the membrane interface and the channel conductance under the conditions assumed above. This means that the channel properties are probably influenced by negative charges. On the other hand, when using this procedure it has to be kept in mind that the charge effects on the hemolysin channel are most likely caused by negatively charged groups at the pore mouth because the protein is so large that it creates its own sphere. This means that the ion concentration at the pore mouth may only be approximately covered by the calculations presented above.

### TABLE 2. Influence of surface charge on the concentration of cations $c_{o+}$ at the membrane-water interface

<table>
<thead>
<tr>
<th>$c$ (M)</th>
<th>$\phi$ (mV)</th>
<th>$c_{o+}$ (M)</th>
<th>$\lambda$ (pS)</th>
<th>$\lambda^*$ (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>-100</td>
<td>0.48</td>
<td>150</td>
<td>3.1</td>
</tr>
<tr>
<td>0.05</td>
<td>-62</td>
<td>0.56</td>
<td>370</td>
<td>33</td>
</tr>
<tr>
<td>0.15</td>
<td>-41</td>
<td>0.73</td>
<td>520</td>
<td>110</td>
</tr>
<tr>
<td>0.30</td>
<td>-30</td>
<td>0.97</td>
<td>720</td>
<td>220</td>
</tr>
<tr>
<td>0.50</td>
<td>-24</td>
<td>1.3</td>
<td>1,000</td>
<td>390</td>
</tr>
<tr>
<td>1.0</td>
<td>-17</td>
<td>2.0</td>
<td>1,500</td>
<td>770</td>
</tr>
<tr>
<td>3.0</td>
<td>-10</td>
<td>4.4</td>
<td>3,900</td>
<td>2,600</td>
</tr>
</tbody>
</table>

* The surface potential, $\phi$, was calculated by using equations 1 and 2 by assuming that the surface charge density is 1 elementary charge per 4-nm$^2$ surface (corresponding to a total charge of $4.0 \times 10^{-6}$ (A $\times$ s)/cm$^2$). The surface concentration, $c_{o+}$, of cations was calculated from equation 3 by using the corresponding values of the surface potential given in this table. $\lambda^*$ is the average single-channel conductance corrected for the increase in the cation concentration at the surface of the membranes caused by the negative surface charges.
The hemolysin channel had a limited lifetime, as Fig. 3 clearly shows. This means that the channel-forming unit is not a rigid structure similar to the alpha-toxin of Staphylococcus aureus (8, 9, 19) or to the porin pores of the outer membrane of gram-negative bacteria (1, 13) but undergoes molecular changes which lead to opening and closing of the pores. The mean lifetime of the hemolysin channel was about 2 s under the experimental conditions of Fig. 3 (20 mV). Under the assumption that a complex of several hemolysin molecules is responsible for the formation of the conductive unit, the mean lifetime of this complex is 2 s and its dissociation rate constant, $k_d$, is about 0.5 s$^{-1}$. The assumption of a pore-forming oligomer responsible for the hemolysin action seems to be justified by the steep conductance-concentration curve, which also suggested that several hemolysin molecules form the conductive unit and that an association-dissociation equilibrium existed between non-conductive monomers and conductive oligomers. Furthermore, it seems that the channel contains several substrates (compare Fig. 3 and 5).

In a recent publication by Menestrina et al. (21), the interaction of the hemolysin of E. coli with lipid bilayer membranes formed by using the Montal-Mueller method has been investigated in detail. The results differ considerably from those presented here. First, the lifetime of the hemolysin channel was much longer than the lifetime measured in this study. Second, no lipid specificity has been found by Menestrina et al. (21), whereas in our experimental approach the hemolysin was almost completely inactive in membranes composed of pure lipids and in defined lipid mixtures. Third, a basic difference exists in the conductance-concentration curve. In our system, we observed a steep curve with a slope of around 3 on the double-logarithmic scale (Fig. 2), whereas a linear dependence of the membrane conductance on the hemolysin concentration in the aqueous phase has been reported by Menestrina et al. (21). Fourth, another discrepancy consists in the dependence of the single-channel conductance on the bulk aqueous conductance. Menestrina et al. (21) published an almost linear relationship, while we found that the dependence of the single-channel conductance on the salt concentration had a slope of about 0.5 in a double-logarithmic plot (Fig. 6), which may indicate that fixed charges influenced the channel properties.

At present, we have no definite explanation for these conflicting results. They are definitely not caused by the use of two different methods for membrane formation, because we received the same results on solvent-depleted asolectin membranes (see above). The hemolysins used in the two studies are of different origin. Whereas the hemolysin used here derives from plasmid Hly152, that of Menestrina et al. (21) is encoded by the chromosome and its sequence has not yet been reported. On the other hand, previous data (14, 18) have shown that chromosomal and plasmid hemolysins exhibit high homology. It is therefore not very likely that the two hemolysins differ in such basic properties as recognition of membrane components, single-channel conductance, or the number of molecules involved in a single pore, because chromosomal hemolysin had in our membrane system a single-channel conductance similar to that of the hemolysin encoded by plasmid Hly152 (R. Benz, A. Ludwig, and W. Goebel, unpublished results). On the other hand, the chromosomal hemolysin had a considerably longer channel lifetime. We observed that the pore-forming activity of our hemolysin preparations was influenced by freeze-thawing. This procedure often resulted in the inactivation of the formation of specific hemolysin pores. All of our experiments were therefore performed with freshly prepared hemolysin which had been kept at 0°C. The observed pore-forming activity was definitely caused by HlyC-activated hemolysin (HlyA*), since no such activity was observed with similar concentrations of HlyA protein alone (i.e., synthesized and secreted in the absence of HlyC) or with a mutant hemolysin that was defective in the hydrophobic domain of HlyA. Neither HlyA alone nor the mutant hemolysin exhibited any lytic activity on erythrocytes (19a).

Our recent data (19a) with mutant hemolysins with site-specific mutations in the repeat domain of HlyA suggest that this region is essential for the recognition of a receptor on the erythrocyte membrane. Most of these mutant hemolysins require elevated Ca$^{2+}$ concentrations for hemolytic activity (19a). Surprisingly, these hemolysin mutants still exhibit a Ca$^{2+}$-independent pore-forming activity on asolectin membranes, similar to that of wild-type hemolysin. As a possible explanation for this result, one could assume that E. coli hemolysin may not require a specific receptor on the asolectin membrane. This is, however, not very likely, since pore formation on artificial lipid bilayer membranes appears to depend on the composition of the phospholipid and is, in our hands, much higher with asolectin from soybeans than with pure phospholipids such as diphytanoyl phosphatidylcholine. Alternatively, hemolysin may recognize a receptor molecule on the asolectin membrane which is different from that on the erythrocyte membrane or may represent only part of the erythrocyte receptor, the recognition of which does not require the repeat domains of HlyA and hence no Ca$^{2+}$ (19a). On the other hand, we cannot exclude the possibility that a lipid mixture facilitates pore formation.

The diameter of the hemolysin channel has been analyzed by other methods (8). In the electron microscopical analysis and in detergent solutions, it did not form stable structures similar to that of the alpha-toxin of Staphylococcus aureus (8, 9, 19). These results are consistent with our observations because the hemolysin channel did not form a rigid structure and fluctuated. Alternatively, the channel diameter was measured in lysis experiments with erythrocytes by using substances of different molecular weights (8). According to these measurements, hemolysin forms 1.5- to 3.0-nm-wide channels in the erythrocyte membrane because external dextran 4000 but not stachyose inhibited their lysis. Such a diameter has been found to be consistent with the result of artificial lipid bilayer membranes (21), assuming that the hemolysin channel forms a water-filled cylinder similar to the gram-negative bacterial porins (1, 23, 24). Our data suggest that such a calculation can be dangerous because of the high selectivity of the channel and because of the interference of charges with the channel conductance. If we want to give a rough estimate of the channel diameter, we have to correct for the negative surface potential and the fact that potassium ions, but not chloride ions, can penetrate the hemolysin channel. This rough estimate is only possible because the channel has obviously only a low field strength and because its selectivity followed the mobility sequence of the cations in the aqueous phase, which means that they move almost completely hydrated through the channel. Furthermore, the large organic Tris$^+$ cation can also penetrate the hemolysin channel at a rate which is similar to its mobility in the aqueous phase. At a bulk aqueous conductance of 0.15 M KCl, the concentration of the potassium ions at the mouth of the pore is probably close to 0.73 M (Table 2). By using the limiting molar conductivity, $G$, of potassium ions [$G = 73.5$ mS/(M cm)] (10), the diameter, $d$, of the hemolysin channel may be estimated as follows: $\Lambda^* = \frac{1}{2} \pi d^2 \mu \eta^2$.
where \( \hat{r} \) is the corrected single-channel conductance at 0.15 M KCl, \( r \) is the channel radius, and \( l \) is the length of the channel. Assuming that the length of the channel is approximately equal to the membrane thickness \( l = 6 \) nm, \( d \) is about 0.9 nm at 0.15 M. For other concentrations, \( d \) is about 1 nm. This channel diameter may be considered as a lower limit because of the assumptions used for its calculations. It is considerably smaller than the diameter calculated from lysis experiments (1.5 to 3 nm [8]). On the other hand, it is possible that the diameter calculated from lysis experiments is an overestimate, because the molecules used are not globular and may penetrate the channel in an extended form.

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