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Formation of Ion Channels by Colicin B in Planar Lipid Bilayers

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Summary. The gene for the antibacterial peptide colicin B was cloned and transformed into a host background where it was constitutively overexpressed. The purified gene product was biologically active and formed voltage-dependent, ion-conducting channels in planar phospholipid bilayers composed of asolectin. Colicin B channels exhibited two distinct unitary conductance levels, and a slight preference for Na^+ over Cl^-. Kinetic analysis of the voltage-driven opening and closing of colicin channels revealed the existence of at least two conducting states and two nonconducting states of the protein. Both the ion selectivity and the kinetics of colicin B channels were highly dependent on pH. Excess colicin protein was readily removed from the system by perfusing the bilayer, but open channels could be washed out only after they were allowed to close. A monospecific polyclonal antiserum generated against electrophoretically purified colicin B eliminated both the biological and in vitro activity of the protein. Membrane-associated channels, whether open or closed, remained functionally unaffected by the presence of the antiserum. Taken together, our results suggest that the voltage-independent binding of colicin B to the membrane is the rate-limiting step for the formation of ion channels, and that this process is accompanied by a major conformational rearrangement of the protein.

Key Words Colicin B · planar bilayer · ion channel · polyclonal antibody

Introduction

Colicin B is one of a group of bactericidal proteins known to form ion-conducting channels in membranes [18, 35, 38]. The lethal action of these toxins results from the ability of the ion channels to depolarize the cytoplasmic membrane of the target cell, thereby short circuiting the cell's energy metabolism [41]. The channel-forming regions of colicins A [29] and E1 [13, 15, 26] have been shown to lie at the C-terminal ends of these proteins, and to consist of no more than 200 amino acid residues. Colicins are plasmid encoded, and complete nucleotide and amino acid sequences have been determined for colicins E1 [14, 55], A [32], and Ib [28, 49], as well as colicin B [42]. The C-terminal regions of these proteins are highly homologous. Approximately 13% of the amino acids are identical in all four colicins, and 33% are identical in at least three of the proteins. The similarity of colicin A and B is even more striking, with 57% identity between the C-terminal regions of these two proteins, and an additional 19% of the amino acids being homologous at identical positions. Mutations of the genes encoding these proteins have been readily generated [4, 26, 44, 45]. In addition, these colicins are regulated by the SOS response in that DNA damaging treatments such as ultraviolet irradiation or exposure to mitomycin C result in the derepression of the colicin genes [50], and they are manufactured by the host bacteria in large amounts and can be readily purified. The colicins thus constitute a well-defined and easily manipulated system in which to study a number of biologically important protein functions, including membrane assembly, voltage-dependent channel gating, and ion translocation.

After specific interaction of a colicin with its cognate receptor, the molecule traverses the outer membrane by an unknown mechanism. In order to form an active ion-conducting channel, the aqueous colicin molecule must be inserted through the membrane to form a pore structure. In artificial membrane systems this assembly process occurs spontaneously, without mediation by any other membrane-bound or aqueous protein. The mechanisms underlying these membrane-protein interactions are believed to be important to the assembly of many proteins which span the membrane [54]. Conversion of a globular, water soluble protein to an integral membrane protein is expected to involve several steps and considerable conformational rearrangement. Using electrical methods such as those employed in the present study, the number of functional ion-conducting channels can be monitored directly. Distinctions about the steps that make up the process can be drawn from analysis of the kinetics of the appearance and disappearance of open channels in response to experimental interventions.

In the present study, the structural (*cba*) and immunity (*cbi*) genes for colicin B were cloned and expressed in a variety of host backgrounds. The maximal expression of colicin B in a *lexA* mutant strain, where it was the major protein species produced, facilitated its purification. The purified product was used to generate a polyclonal monospecific mouse antiserum and was shown to be active in lipid bilayers, where it displayed unique characteristics. The effects of pH, perfusion, and the monospecific antiserum on the kinetic behavior of the voltage-dependent channels formed by colicin B were examined. Each of the various colicins exhibits quite distinctive behavior in its interactions with lipid bilayer membranes. Because this variation must ultimately be associated with the small differences in their respective primary structures, these studies will contribute toward our understanding of the molecular basis of the function of membrane proteins.

Materials and Methods

Bacterial Strains and Plasmids

The colicin B-producing strain R2.1/Vλ [21] was the source of the pColB-K260 plasmid from which the *cba* and *cbi* structural genes were cloned. The plasmid vector used in the cloning experiments was pBR328 [48]. The recombinant plasmid pCLB1 contains a 4.65 kilobase (kb) *PstI* fragment from pColB-K260 harboring the *cba* and *cbi* genes cloned into the *PstI* site of pBR328. *E. coli* K-12 strains used in this study included HB101 [8], AB1157 (*thr-1 leuB6 thi-1 supE44 galK2 ara-14 xyl-5 mtl-1* Δ[*gpt-proA*]*62 hisG4 lacY1 argE3 rpsL31 tsx33*) [34] and its SOS-defective derivatives DM49 (*lexA3*) [23], GC3217 (*lacY*$^+$ *ilv-ts sulA211 recA441*) [33], DM1187 (as GC3217 except *argE*$^+$ *lexA51*) (33), and DM1420 (as DM1187 except *recA*$^+$ *his*$^+$) [33].

Media and Chemicals

Bacterial strains were routinely grown in Luria-Bertani (LB) broth [30] or nutrient broth (Difco, Detroit, MI) with shaking at 37°C. Agar plates and soft agar overlays contained 1.5 and 0.6% agar (Difco), respectively. Antibiotics were used at the following concentrations (in μg/ml): ampicillin, 25; chloramphenicol, 30; tetracycline, 10; and mitomycin C, 0.4. Molecular biology reagents, including restriction endonucleases and T4 DNA ligase, were from Promega Biotec (Madison, WI) or United States Biochemicals (Cleveland, OH).

Molecular Genetic Techniques

pColB-K260 plasmid DNA was prepared from R2.1/Vλ cells by the procedure of Hansen and Olsen [22]. Recombinant and vector plasmids were purified as described by Birnboim and Doly [5]. All other molecular genetic techniques, including restriction enzyme digestion, DNA ligation, transformation, and agarose gel electrophoresis, were performed as described [27].

Table. Production of colicin B in SOS mutant strains

Strain	SOS genotype	Colicin production[a]	
		Uninduced	Induced
AB1157	wild type	128	16,384
GC3217	*sulA recA441*	256	>32,768
DM49	*lexA3*[b]	8	16
DM1187	*sulA recA441 lexA51*	>32,768	>32,768
DM1420	*sulA lexA51*	>32,768	>32,768

[a] Each strain harboring pCLB1 was grown with or without the inducing agent mitomycin C and cleared sonicates were prepared. Colicin titers are given as the reciprocal of the last dilution that gave a detectable zone of killing when a 1-μl sample of each sonicate dilution was spotted on a lawn of the sensitive strain AB1157.

[b] The *lexA3* mutation results in a LexA repressor protein with greater than normal repressing activity.

Overproduction and Purification of Colicin B

The expression of the colicin B structural gene (*cba*), like those of several other colicins, has been shown to be mediated by the SOS response [42], and therefore under the control of the *lexA* repressor gene. Expression was thus maximized by introducing pCLB1 into mutant strains defective in LexA function (Table). Colicin B titers were derived by assaying cleared sonicates for killing activity in a spot dilution bioassay. Strains harboring pCLB1 were grown to late logarithmic phase in the presence or absence of mitomycin C. The cells were broken by sonication and cell debris was removed by centrifugation. The cleared sonicates were twofold serially diluted to 2^{-15} and 1-μl samples of each dilution was spotted on a lawn of the sensitive strain AB1157. Colicin B production was mitomycin C-inducible in the wild-type parental background and repressed in DM49, harboring the super-repressor allele *lexA3*. In *lexA51* mutants (devoid of LexA activity), production was constitutively high. Therefore, DM1187 (pCLB1) was used for purification purposes.

The techniques of Pressler et al. [38] and Braun and Maas [9] were modified to produce purified colicin B. DM1187 (pCLB1) was grown overnight in LB broth containing tetracycline. Cells were harvested and washed once with 1 mM EDTA in 10 mM Tris-HCl (pH 8). The cell pellet was suspended in 10 ml of 50 mM phosphate buffer containing 100 μg/ml DNase. After the cells were sonically disrupted at 0°C and unbroken cells removed by centrifugation, the supernatant was centrifuged at 100,000 × *g* for 1 hr to pellet the membranes and insoluble material. The resulting supernatant was applied to a DEAE-Sepharose CL-6B (Pharmacia, Piscataway, NJ) column and chromatographed as described [38]. Colicin B was identified by spotting 0.5-μl of each protein-containing fraction onto a lawn of sensitive cells (AB1157). Active fractions were pooled and chromatographed on a Sephacryl S-200 (Pharmacia) column (2.5 × 100 cm) equilibrated with 50 mM phosphate buffer (pH 7.1), 0.02% sodium

azide. Colicin B was eluted using the same buffer, tested for activity on AB1157, examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [2], and was shown to migrate as a single 58-kDa band, regardless of solubilization temperature prior to electrophoresis. Colicin B preparations were very stable when stored at −20°C in phosphate buffer with or without glycerol to 30%. Thawed aqueous samples were stable at 0–4°C for several weeks when stored in plastic containers. Activity was rapidly lost when samples were stored in glass containers. Repeated freezing and thawing also destroyed activity.

Preparation and Characterization of Anti-Colicin B Antiserum

Colicin B was purified by preparative gel electrophoresis after solubilization at 25°C in buffer containing 12.5% glycerol, 1.25% SDS, 1.25% 2-mercaptoethanol, 0.005% bromophenol blue, and 0.25 M Tris-HCl (pH 6.8). This preparation was subjected to SDS-PAGE using a 12% separating gel as described [2]. The 58-kDa colicin B protein band was visualized by treatment of the gel with 4 M sodium acetate [24]. The gel band containing colicin B was excised, finely macerated in 1 ml of saline, mixed with an equal volume of Freund's complete adjuvant and injected intraperitoneally into Balb C-Nu mice. Nineteen days later, the mice were boosted intraperitoneally with no adjuvant. One week after the boost, sera were collected from the mice and pooled.

The specificity of the murine immune serum was examined by Western immunoblot analysis. Solubilized bacterial cells or purified colicin B were electrophoresed on a 12% SDS-PAGE gel and subjected to immunoblotting as described [3] using a 1 : 1000 dilution of the antiserum. After incubation with horseradish peroxidase-conjugated goat anti-mouse IgG, the blot was developed using 4-chloro-1-naphthol as the chromagen.

The antiserum to colicin B was assayed for its neutralizing ability in two separate inhibition assays. In a simple plate test, approximately 50 μg of partially purified colicin B were applied to the surface of an LB broth plate. A filter disc saturated with undiluted anti-colicin B antiserum was placed atop the area of the plate containing the colicin and the plate was incubated 1 hr at 37°C. An agar overlay containing the colicin B-sensitive strain AB1157 was applied to the plate prior to incubation overnight at 37°C. The second assay consisted of mixing twofold dilutions of the antiserum with a constant quantity of colicin B. The tubes were incubated for 1 hr at 37°C, and 5-μl samples were spotted onto a lawn of AB1157. After overnight incubation, the plate was scored for colicin activity.

Membrane Conductance Measurements

Planar phospholipid bilayer membranes of the solvent-free type [31] were formed across apertures in Teflon septi as previously described [13]. The volume of aqueous solution bathing each side of the membrane was either 1.5 or 5 ml. For single-channel measurements, apertures 50–75 μm in diameter were used; for currents mediated by macroscopic ensembles consisting of 10^2–10^4 channels, the apertures were 250–300 μm in diameter.

Electrical contact with the two aqueous compartments was established by means of a single pair of miniature calomel electrodes, one of which also served as the reference electrode for pH measurements. Voltage-clamp conditions were established by means by a Burr Brown 3523L operational amplifier configured as a current-to-voltage converter. The output of this amplifier, which was proportional to transmembrane electric current, was electronically filtered by an eight-pole, low-pass, Bessel filter (Frequency Devices, Haverhill, MA) and monitored by an oscilloscope and a chart recorder. The aqueous compartments were magnetically stirred, so that only a few seconds were required for complete mixing in the bulk phase. All measurements were made at room temperature.

After a membrane had been formed, small aliquots of aqueous stock solutions of purified colicin B protein were added to one compartment, defined as the *cis* side of the membrane. The final concentration of colicin B generally ranged from 25–300 ng protein/ml of aqueous solution. Electrical potentials applied to the membrane are reported as the voltage of the *trans* compartment relative to the *cis* compartment. Currents corresponding to the flow of positive charges from *cis* to *trans* compartments are shown as upward deflections in all figures. Conductance is defined as the current per unit applied voltage and is given in units of pS. Only membranes exhibiting a high resistance ($>10^8$ Ω-cm) and low level of noise were considered suitable for the introduction of protein. The pH in the *cis* compartment was monitored by means of a miniature glass electrode (Model 407B, Microelectrodes, Londonderry, NH) and a small, battery-powered pH meter. Perfusion of the aqueous compartments was accomplished using a peristaltic pump. Typically, the rate of perfusion was 3 ml/min.

Bovine serum albumin, essentially globulin free (Sigma Chemical, St. Louis, MO) was used to stabilize colicin-antibody interaction; that of lesser purity had adverse effects upon planar bilayer stability and colicin B activity. The lipid used was asolectin type IV-S (Sigma) and was washed in acetone. Salts and solvents were of reagent grade and used without further purification. Conductivity grade water (18 MΩ-cm) was used for all solutions. The primary electrolyte in the bathing solutions was NaCl. These solutions also contained 3 mM $CaCl_2$ and 3 mM of each of the buffer compounds appropriate for the pH range to be encountered. The buffers used were glutaric acid, 2-(N-morpholino) ethanesulfonic acid (MES), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), and 1,3-*bis*[tris(hydroxymethyl)methylamino]propane (*bis*-tris propane). The chloride and sodium activities of all solutions used in selectivity experiments were measured using ion-selective electrodes (Orion Research, Cambridge, MA).

Results

Cloning and Expression of the Colicin B Structural Gene

Cleavage of the pColB-K260 plasmid with the enzyme *Pst*I resulted in seven DNA fragments ranging in size from 2 to 7 kb. These fragments were ligated into the *Pst*I site in the ampicillin resistance gene of pBR328, transformed into HB101, and the transformants were screened for colicin production by replica plating on a colicin B-sensitive bacterial lawn. All colicin B-producing transformants contained the same 4.65 kb *Pst*I fragment (Fig. 1). The colicin B structural (*cba*) and immunity (*cbi*) genes were localized on this fragment by Tn*5* mutagenesis (*data not shown*) and by comparison of the restriction

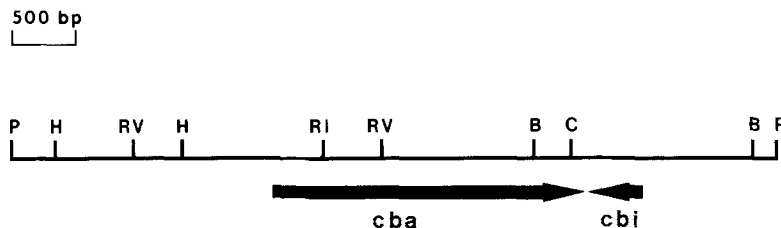


Fig. 1. Physical map of the cloned region of pCLB1 containing the colicin B structural gene, *cba*, and immunity gene, *cbi*. DNA fragments generated by restriction of pColB-K260 with *Pst*I were ligated to the *Pst*I site of pBR328 and transformants screened for production of colicin. The genes were localized on the basis of results obtained by transposon (Tn*5*) mutagenesis (*data not shown*). Arrows denote the direction of transcription. Abbreviations: *B*, *Bst*EII; *C*, *Cla*I; *H*, *Hpa*I; *P*, *Pst*I; *RI*, *Eco*RI; and *RV*, *Eco*RV

map with the similar genetic region from the ColBM plasmid pF166 [42, 43]. The recombinant plasmid carrying this 4.65 kb *Pst*I fragment was designated pCLB1.

When pCLB1 was introduced into SOS mutant strains carrying the *lexA51* mutation, colicin B was constitutively produced at very high levels (Table). In strain DM1187(pCLB1), colicin B represented a major protein species in whole-cell preparations and cytoplasmic fractions, and its overexpression appeared to have no detrimental effect on the growth of the cells. The colicin B obtained from this mutant was highly active and amenable to purification by conventional means. The molecular weight of colicin B as determined by SDS-PAGE was 58 kDa, in agreement with previous reports [38]. The 58-kDa product retained bioactivity after electrophoresis.

Colicin B Produces a Voltage-Dependent Conductance in Planar Lipid Bilayers

The effect of adding colicin B to the aqueous solution bathing one side of an asolectin membrane is shown in Fig. 2. Following addition of protein at point *A*, a voltage of −50 mV was applied. After a delay of several minutes, the current flowing across the membrane began to increase slowly. Applied potentials as small as −20 mV were found to be sufficient to induce an increase in conductance. When the polarity of the applied voltage was reversed, the direction of the current also reversed. Initially, the magnitude of this current was equal to that flowing in the opposite direction immediately before the polarity reversal. Subsequently, the current declined with time. The current did not decay to zero within the interval shown in Fig. 2, but in other experiments the current clearly continued to approach zero slowly as the membrane remained clamped at positive potentials. Because of these slow components in the decay of the current, its time course was not well fitted by a single exponential. When a negative voltage was restored, the instantaneous reversal of current through the small residual conductance was followed by a rapid phase of conductance increase. Following this rapid phase, the conductance continued to increase at a rate comparable to that originally observed. The conductance activated during the rapid phase of the current rise was more than the amount which had previously turned off in response to application of the positive potential. In subsequent reversals of applied voltage, this general pattern of current flow was repeated. The overall effect was a steady increase in the total conductance of the membrane.

The slow phase of the conductance increase continued indefinitely so long as a negative potential was maintained. While the rate of increase in current was sometimes observed to decline with time, no steady-state level of ion flow was ever attained. Therefore, the dependence of steady-state conductance upon voltage, protein concentration, pH, ionic strength or other parameters of interest could not be determined. Furthermore, the absolute rate of conductance increase was variable from membrane to membrane, and from one protein addition to any subsequent addition to the same membrane.

Properties of Single Channels Formed by Colicin B

The pattern of current flow just described arises from the opening and closing of discrete ion-conducting channels formed by colicin B. In Fig. 3, a record obtained at 100 times the sensitivity of that in Fig. 2 is shown. While the changes in current in response to the applied voltage can be seen to consist of stepwise single-channel events, the time

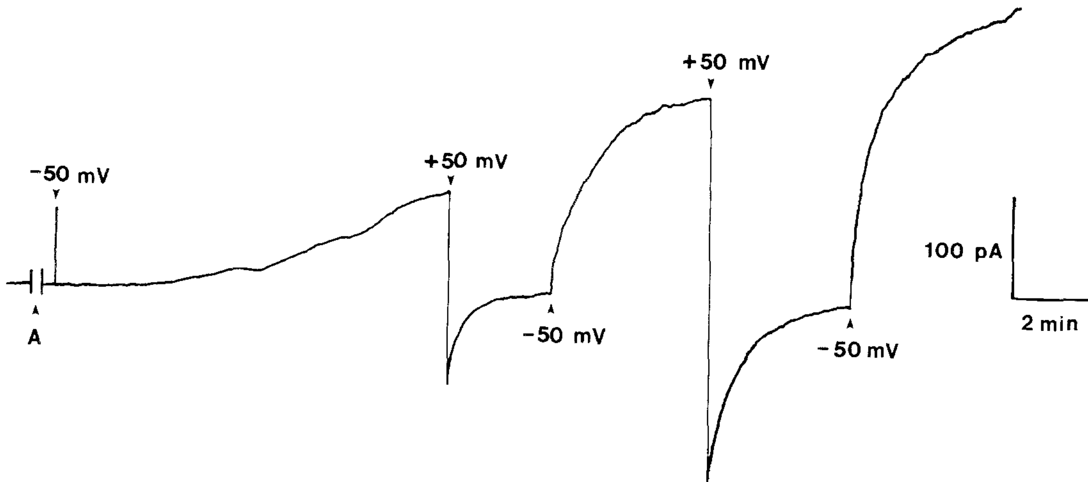


Fig. 2. Voltage-dependent conductance induced by colicin B in planar bilayers. A large asolectin membrane was bathed in solutions containing 1 M NaCl, 3 mM $CaCl_2$, 3 mM glutaric acid, pH 5.0. At point *A*, colicin B was added to a final concentration of 25 ng/ml, while the membrane voltage remained clamped at 0 mV. The amount of current which flowed when a potential of −50 mV was applied to the membrane was extremely small, indicating that the initial membrane resistance was very high. After some delay, the current began to increase. When the voltage was reversed to +50 mV, the current instantaneously reversed direction and then began to decay towards zero. Upon restoration of a −50 mV potential, the reversal of direction of current through the small residual conductance was followed by a biphasic increase in conductance. Repeated voltage reversals produced similar patterns of incomplete decays and biphasic recoveries, superimposed upon a steadily increasing level of total membrane conductance. The position of the horizontal calibration bar corresponds to zero current

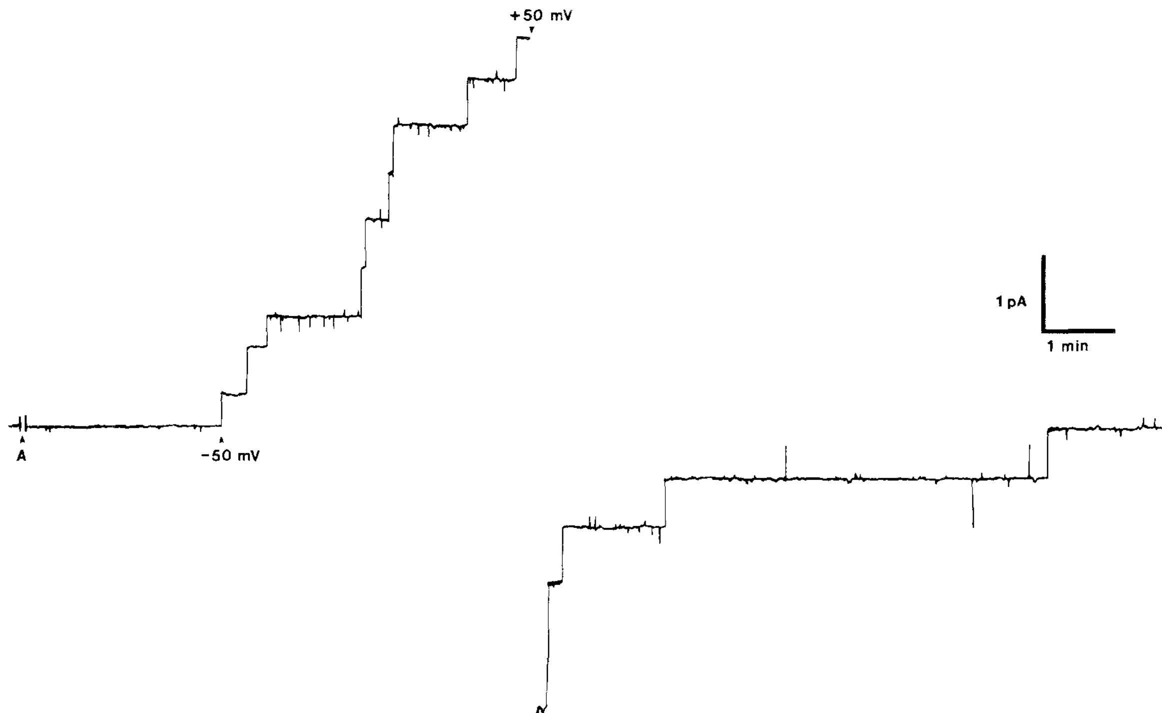


Fig. 3. Single-channel properties of colicin B. A small asolectin membrane was bathed in solutions containing 1 M NaCl, 3 mM $CaCl_2$, 3 mM glutaric acid, pH 5.0. Colicin B was added at point *A* to a concentration of 5 ng/ml. Application of −50 mV induced a stepwise increase in membrane current. When the voltage was reversed, the channels closed in a stepwise manner. The closing events of the first three channels could not be resolved on this time scale. The last two channels remained open until the membrane broke at a point several minutes beyond the time shown. The opening and closing behavior closely parallels the macroscopic behavior. The position of the horizontal calibration bar corresponds to zero current

course of the openings and closings closely paralleled the behavior observed in macroscopic ensembles of channels. The size of the current steps is not uniform, however. Note in particular that the third channel to open in Fig. 3 carried a distinctly smaller amount of current than any of the others. In Fig. 4, conductance data from a total of 123 channel closings observed in 26 membranes, and from 89 openings observed in 22 membranes are presented as frequency histograms. Two statistically distinct populations of channel sizes are evident in both distributions (χ^2 test, $P < 0.01$). The average conductances of the higher conductance populations were 13.3 ± 1.5 pS (n = 85) for channel closings at +50 mV and 12.9 ± 0.9 pS (n = 48) for channel openings at −50 mV. For the lower conductance populations the corresponding values were 7.1 ± 1.7 pS (n = 38) at +50 mV and 7.2 ± 1.6 pS (n = 41) at −50 mV. All values are given as mean ± SD.

The differences in mean conductance for openings at −50 mV *versus* closings at +50 mV are not significant for either of the two channel types. Paired comparisons of the total conductance of groups of several open channels before and after reversal of the voltage revealed an average deviation of less than 2%. This behavior would be expected on the basis of the macroscopic responses to voltage reversals. On the other hand, there is a clear disparity between opening and closing events in the relative proportions of the two channel types. If the opening and closing data represented the same population of channels, these results would be *prima facie* evidence that the two unitary conductances represent different states of the same channel. Because of a bias in the sampling of single-channel events, however, our findings are also consistent with two independent channel populations which differ in kinetics as well as conductance. In experiments of this type, it is easier to resolve the first few channels to open and the last few to close. When the voltage was reversed in the experiment shown in Fig. 3, for example, three channels closed too quickly to resolve the individual steps. The last two channels remained open several minutes beyond the time shown, at which time the membrane broke. Channel openings often showed the opposite tendency. The average time between openings became smaller with each successive event. These trends can be seen even more clearly in the macroscopic behavior shown in Fig. 2. The initial increase in conductance was concave-up in appearance, and its decay on voltage reversal was concave-down. Thus, the limitations of our data collection method tend to bias the sampling of channel openings towards faster events and that of channel closings towards slower events. Examining the time distribution of large and small channels within the samples themselves provides more direct evidence for kinetic differences. Within each separate experimental record, the resolvable single-channel openings or closings were divided equally into early and late categories according to the order of their occurrence. In 64% of the records of channel openings, there were more small channels among the early events than among the late events. Closings of small channels occurred predominately among early events in 87% of the records. This coarse categorization was necessary because of the variable number of channels in the records. Because of the crudeness of the measure and the inherent bias of the samples, the significance levels of these tests are not high (0.30 and 0.10, respectively). The results are nevertheless suggestive of kinetic differences. In those few experiments in which all the individual openings and closings of a distinct group of channels could be resolved, the proportion of low and high conductance channels always remained constant. We thus have no direct evidence to support the idea that the two conductance levels represent different states of the same channel.

Ion Selectivity of Colicin B-Induced Conductance

In order to determine the extent to which colicin B channels discriminate between Na^+ and Cl^-, colicin was added to a chamber containing 0.1 mM NaCl in the *cis* compartment and 1.0 mM NaCl in the *trans* compartment. The potential at which no current passed through a macroscopic ensemble of open colicin B channels was then determined. The zero current potential (ZCP) was −27.7 ± 1.1 mV (n = 11) at pH 5, −39.0 ± 0.8 mV (n = 13) at pH 6, and −42.3 ± 0.6 mV (n = 3) at pH 7. The ratio of Na^+ conductance to Cl^- conductance (g_{Na}/g_{Cl}) can be calculated according to:

$$g_{Na}/g_{Cl} = (ZCP - E_{Cl})/(E_{Na} - ZCP) \quad (1)$$

using values for the equilibrium potentials of the test solutions, E_{Na} and E_{Cl}, which were experimentally determined using ion-selective electrodes. This conductance ratio was found to be 2.6 : 1 at pH 5, 4.8 : 1 at pH 6, and 6.5 : 1 at pH 7. The intrinsic selectivity of the protein may be somewhat obscured in these measurements by the acidic lipids which make up approximately 20% of the total lipid in asolectin. Fixed negative charges on the surface of a membrane tend to increase the concentration of cations and decrease that of anions at the membrane-water interface. Therefore, the channel itself

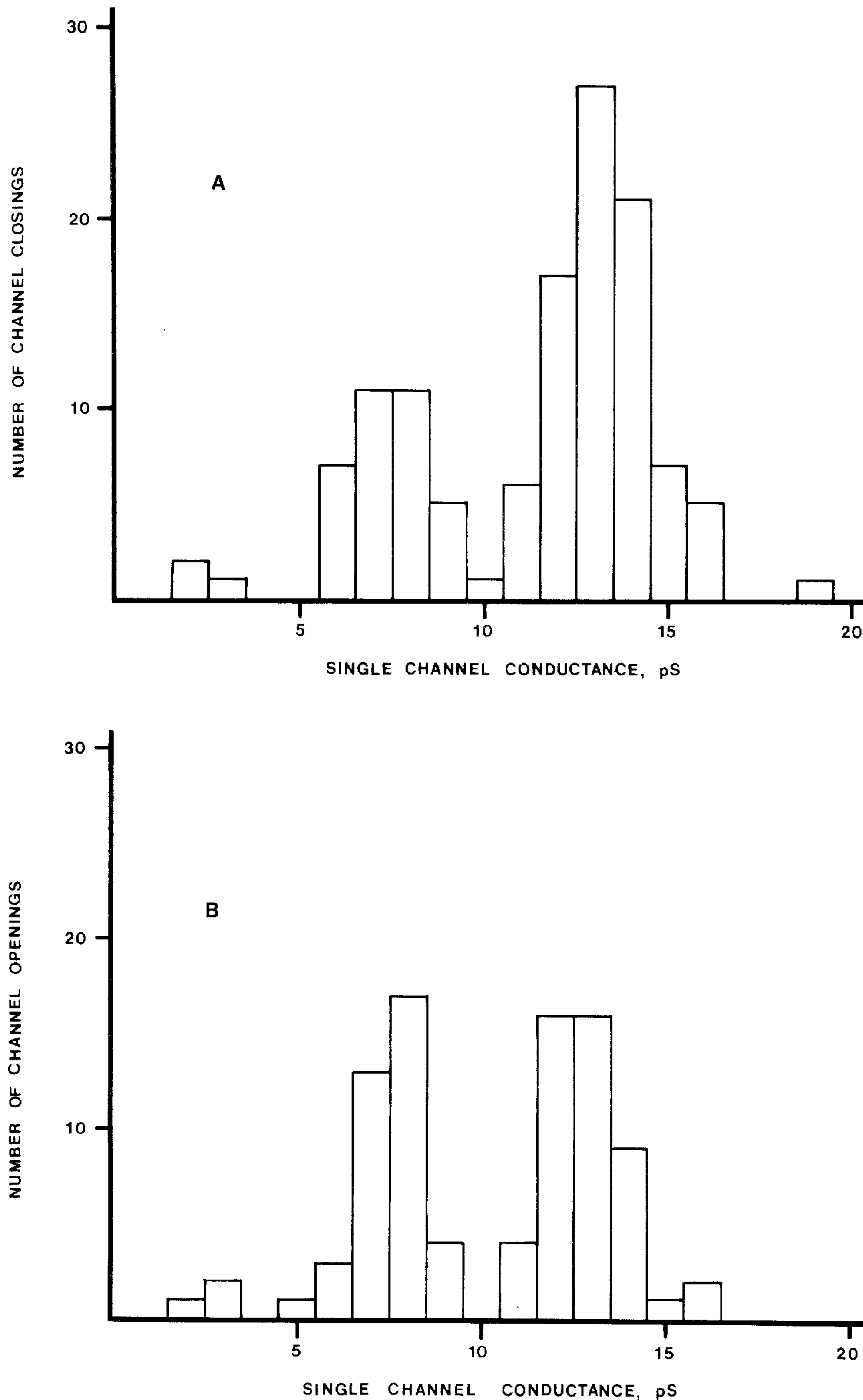


Fig. 4. Frequency distribution of the unitary conductance of channel opening and closing events. All measurements were taken from small asolectin membranes bathed in 1 M NaCl, 3 mM $CaCl_2$, 3 mM glutaric acid, pH 5.0. (*A*) Histogram of 123 channel closings observed at +50 mV in 26 membranes (*B*) Histogram of 89 channel openings observed at −50 mV in 22 membranes. *See* text for description

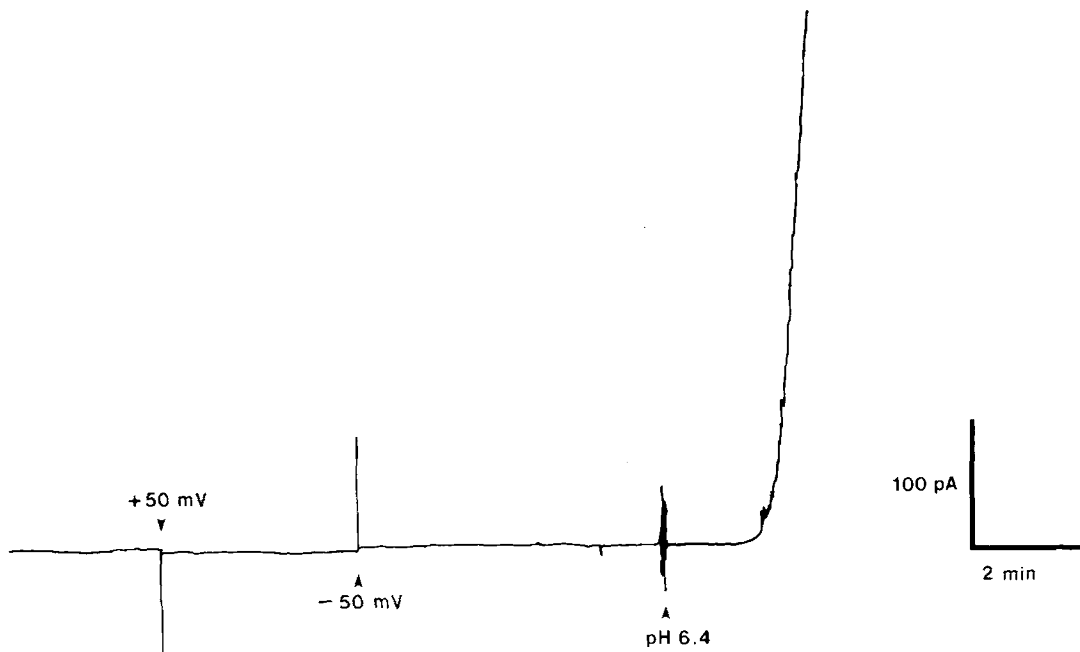


Fig. 5. Effects of pH on the channel opening kinetics of colicin B. An asolectin membrane was bathed in solution containing 1 M NaCl, 3 mM $CaCl_2$, 3 mM glutaric acid, 3 mM MES and 3 mM HEPES, pH 6.9. Colicin B had been added to a concentration of 1500 ng/ml several minutes prior to the time shown, and the conductance increase induced by application of −50 mV was extremely slow. At the time indicated, an aliquot of 1 M HCl was added to the *cis* compartment to lower the pH 6.4. The disturbance in the current tracing is an artifact caused by the addition. The rate of conductance increase was greatly accelerated by the addition of acid. The position of the horizontal calibration bar corresponds to zero current. Colicin B had been stored for two weeks at −10°C

is likely to be less cation-selective than data obtained in asolectin membranes would seem to indicate.

Effects of pH on the Kinetics of Voltage-Dependent Opening and Closing of Colicin B Channels

The rate of channel formation by colicin B was highly dependent on pH. As shown in Fig. 5, very little channel activity could be observed at pH 7, but addition of a small amount of acid to the compartment containing the protein greatly accelerated channel opening. Over the course of a few months of frozen storage, the activity of colicin B at higher pH values gradually increased. Eventually, the initial rates of channel opening became pH indifferent. While storage in glycerol retarded this process, colicin B purified by SDS-PAGE was completely pH insensitive (*data not shown*). No changes in other pH-dependent properties accompanied the disinhibition of channel formation.

The effect of pH on the rate of channel closing is shown in Fig. 6. In both panels, colicin B was added at the points indicated by the letter *A*. In response to application of a potential of −50 mV, slow, continuous increases in membrane conductance were observed both at pH 4 (Fig. 6*A*) and at pH 7 (Fig. 6*B*). At pH 4, the conductance fell very rapidly to zero when the direction of the potential was reversed. When the voltage was reversed at pH 7, the conductance decreased very slowly. In the pH range between 4 and 7, the closing kinetics were a complex mixture of fast and slow components (*see* Fig. 2). With increasing pH, the fast components became slower and less prominent, and the very slow components became more prominent.

When negative potentials were restored, a population of rapidly activated channels was observed at all pH values between 4 and 7. Such biphasic opening kinetics is also characteristic of colicins A, Ia, and Ib. Colicin B differs from these other proteins in that the number of rapidly activated channels was always greater than the number which previously had been closed by positive voltages. Furthermore, the size of this excess increased with the length of time positive voltages were applied (*data not shown*). A different situation was observed at pH 9, as illustrated in Fig. 7. The initial slow phase of channel opening was similar to that at lower pH, and the behavior of the channel closings

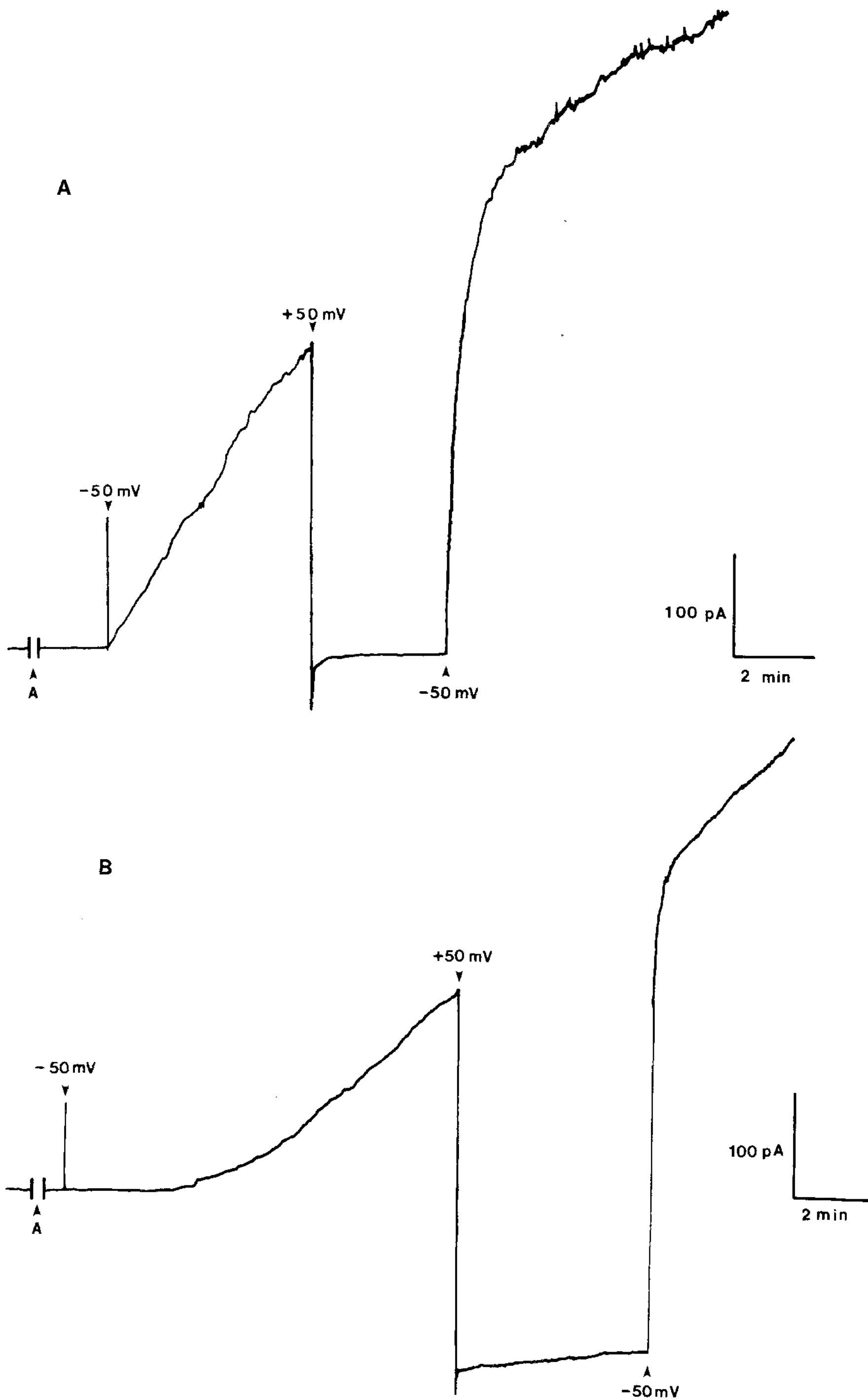


Fig. 6. Effects of pH on closing kinetics of colicin B channels. (*A*) pH 4.0.(*B*) pH 7.0. Colicin B was added to a concentration of 150 ng/ml at point *A* and potentials applied as indicated. The decay of colicin conductance was rapid and nearly complete at pH 4.0, but very slow at 7.0. Asolectin membranes were bathed in 1 M NaCl, 3 mM $CaCl_2$ and 3 mM glutaric acid (*A*) or HEPES (*B*). The position of the horizontal calibration bar corresponds to zero current

at positive voltages was virtually identical to that observed at pH 7. Following restoration of negative voltages, however, no rapid phase of channel opening was seen. Instead, the slow, continuous phase simply resumed. At pH 9, channels which previously had been open and subsequently closed were kinetically indistinguishable from channels which had never been open.

Effects of Perfusion on Colicin Channel Kinetics

In order to examine the reversibility of the interaction of colicin B with planar membranes, we attempted to wash the colicin out of the membrane by perfusing the chamber with protein-free buffer. The results of such an experiment are shown in Fig. 8.

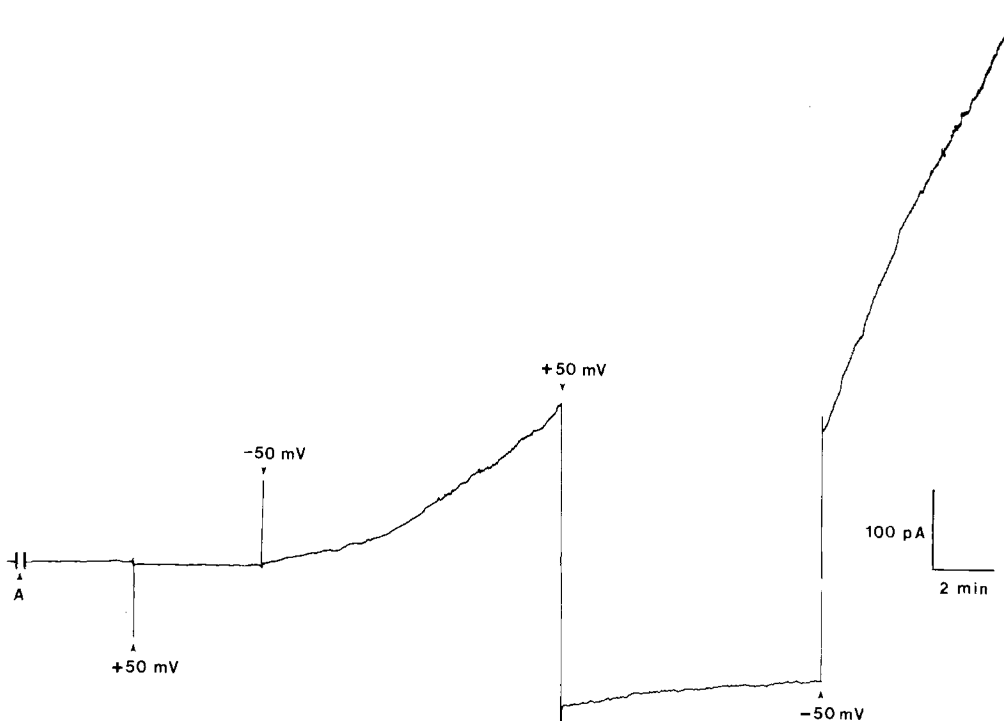


Fig. 7. Kinetics of colicin B channels at pH 9.0. An asolectin membrane was bathed in solutions containing 1 M NaCl, 3 mM $CaCl_2$, 3 mM *bis*-tris propane, pH 9.0. Colicin B was added to a concentration of 300 ng/ml at point *A*, and potentials applied as indicated. The responses to the initial applications of −50 and +50 mV were virtually identical in behavior to those observed at pH 7.0. Following the second application of −50 mV, however, the rapid phase of channel opening was absent. The position of the horizontal calibration bar corresponds to zero current

The protocol of applied voltage is represented in the lower tracing, while membrane current in shown in the upper tracing. Colicin B was added to an asolectin membrane bathed in 200 mM NaCl, pH 7, at point *A*. The subsequently applied series of potential changes elicited a pattern of responses in the current tracing similar to that previously described. Although "slow" phase of the conductance increase appears to be fairly rapid in this experiment, an even more rapid component and an instantaneous jump in current can be discerned whenever −50 mV is applied. The *cis* chamber was then perfused with colicin-free buffer while the membrane was clamped at 0 mV. The total volume of perfusate amounted to six times the compartment volume. When −50 mV was applied to the membrane following perfusion, the slow, continuous phase of conductance increase was completely absent. Instead, the instantaneous jump of current flowing through the extant open channels was followed by the rapid and complete opening of a distinct population of channels, after which the current remained constant. Upon reversal of the voltage, the conductance began to decay, as it had before perfusion. When the negative potential was restored, however, the number of rapidly activated channels was substantially smaller than the number which had just been turned off at +50 mV. From these results, it appears that the kinetic precursors of the channels which were opened slowly by negative membrane potentials must have been rapidly interconvertible with aqueous colicin B. Likewise, when open channels were turned off by positive voltages, they were slowly lost to the aqueous phase following perfusion. On the other hand, once channels had been opened, they could not be removed from the membrane so long as they were kept open.

While a marked reduction in the rate of conductance increase could consistently be achieved following a 3-min perfusion, the complete elimination of the slow phase was not always as immediate as in the record shown. In these cases, when negative voltages were applied following perfusion, the conductance continued to rise slowly instead of reaching a constant level. The rate of this residual conductance increase generally tended to decline with time, and a steady state was often reached eventually. When perfused membranes were broken and reformed, the observed rates of channel opening were comparable to the residual activity before the

Fig. 8. Effects of perfusion on the kinetics of colicin B. An asolectin membrane was bathed in 200 mM NaCl, 3 mM $CaCl_2$, 3 mM HEPES, pH 7.0. The potential applied to the membrane is indicated in the lower tracing and membrane current is shown in the upper tracing. Colicin B was added to a concentration of 150 ng/ml at point *A*. Application of positive and negative voltages induced a typical pattern of channel openings and closings. The *cis* compartment was then perfused with a total of six volumes of colicin-free buffer. Following perfusion, only the rapid phase of channel opening was present, after which the current maintained a constant level. Only a portion of the channels which were induced to close at +50 mV could be reopened at −50 mV. The position of the horizontal calibration bar corresponds to zero current

breakage and not to the rate before perfusion. Perfusion was effective at pH 5 as well as pH 7, and in 1 M NaCl instead of 200 mM NaCl. At pH 9, the effects of perfusion, while still apparent, were distinctly slower and less consistent than at lower pH.

Antiserum to Colicin B

A murine polyclonal antiserum was generated against gel-purified 58-kDa colicin B protein, and Western blot analysis (Fig. 9) revealed that the 58-kDa protein was the only antigen detected when total cellular proteins of DM1187(pCLB1) or column-purified colicin B were probed with the immune serum. The host strain, DM1187(pBR328) showed no reactivity with the antiserum, and preimmune serum did not react with colicin B (*data not shown*).

Neutralization of colicin B was demonstrated by the inhibition of cell lysis surrounding an antiserum-impregnated disc applied to a colicin-treated lawn of sensitive bacteria. The neutralization titer of the antiserum was such that a 1 : 20 dilution inhibited the lytic effects of 0.5 μg of colicin B. No colicin neutralization activity was observed in preimmune control antiserum.

Effects of Antiserum on Colicin B Activity in Planar Bilayers

The effects of the monospecific antiserum on colicin B activity in planar bilayers was examined in order to determine the step in the channel-forming process at which antibody binding might interfere with channel formation. All experiments with antiserum were performed at pH 7 in 200 mM NaCl. The antiserum and preimmune serum alike produced a small, nonselective and voltage-independent increase in bilayer conductance when added to one aqueous compartment at a 1 : 150 dilution. This leak conductance slowly increased with time and eventually led to membrane instability. The duration of the experiments, therefore, was limited to the initial period when these nonspecific effects were negligible, usually at least 60 min. At higher serum con-

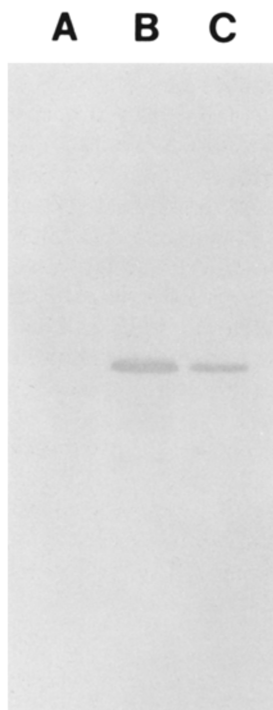


Fig. 9. Immunoblot reactivity of colicin B-specific antiserum. Purified colicin B and cells carrying vector or pCLB1 were solubilized and subjected to electrophoresis and immunoblotting as described in Materials and Methods. Lanes: *A*, DM1187(pBR328); *B*, DM1187(pCLB1); *C*, purified colicin B (6 μg)

centrations, this effect became more troublesome. The channel-forming ability of colicin B in planar bilayers was eliminated by preincubation of the protein in a 1:4 dilution of the antiserum. In these experiments, colicin at concentrations of up to 25 μg/ml was incubated in dilute serum. A 20-μl aliquot of the incubation mixture was then added to a bilayer chamber containing 1.5 ml of solution. The final serum dilution was then 1:300, and the colicin concentration approximately 330 ng/ml. No more than a few minutes time was required to inactivate the colicin, and incubation with preimmune serum had no effect.

When the preincubation was performed in 1:300 serum instead of 1:4 serum, normal colicin B activity was observed in planar bilayers. Addition of BSA to the solutions at a concentration of 1% restored the blocking activity of the antiserum. Under these conditions, not only was the preincubated colicin inactivated, but further additions of untreated colicin to the *cis* compartment failed to induce any conductance increase in the planar membrane. In contrast, the addition of colicin B to the opposite compartment induced conductance increases which behaved in a completely typical fashion. BSA alone had no effect either on the planar bilayers or on the behavior of the colicin-induced conductance. Preimmune serum did not exhibit blocking activity.

From these results, it is clear that the antiserum prevented aqueous colicin B protein from forming channels in planar membranes, but had no effect when the two agents were placed on the opposite sides of the membranes. Two sets of experiments were carried out to assess the interaction of antiserum with membrane-associated colicin B when both were present on the same side of membrane. First, a planar membrane containing open colicin B channels was perfused to eliminate the remaining aqueous protein. Addition of antiserum at a 1:150 dilution had no effect on the number or behavior of the channels remaining in the membrane. Finally, a planar membrane was preincubated for 15 min with colicin B while being clamped to +50 mV so that no channels would be opened. Antiserum was then added, and after an additional 15-min period, −50 mV was applied to the membrane. The response was identical to that of perfused membranes such as that shown in Fig. 8. A distinct population of rapidly activated channels was observed, but the slow phase was completely absent. Thus, while the antiserum is a potent inactivator of aqueous colicin B, it appears to have no effect on membrane channels whether opened or closed.

Discussion

The data presented here show that the behavior of colicin B in planar lipid bilayers is similar to that of each of the other channel-forming colicins. Of this group, colicin E1 has been the most extensively studied in planar bilayers [6, 13, 15, 18, 26, 39, 40, 47], but there are also published descriptions of the behavior of colicins A [16, 36, 41], Ia [12] and Ib [51].

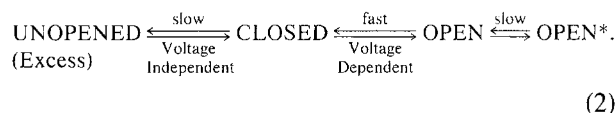
The point of greatest similarity among the various colicins is their cation *versus* anion selectivity. Values reported for the zero current potentials of the various colicins in 10-fold gradients of NaCl all agree within a few millivolts, both at pH 5 and pH 6 [12, 13, 39, 41]. By contrast, the single-channel conductances of the colicins are scattered throughout a 10-fold range. The single-channel conductance of approximately 13 pS reported here for colicin B compares with values of 4 pS for colicin A [16, 36], 20 pS for colicin E1 [13], and 45 pS for Ia [12] obtained under the same conditions. Reports of heterogeneity in channel conductance have been widespread. Although the speculation that such occurrences may represent multiple conductance

states of an individual channel is attractive, we have no evidence from the present study to distinguish this possibility from the existence of separate populations of channels with different properties.

The properties of colicin B in planar bilayers have been examined previously by Pressler et al. [38]. The planar membranes used in that study contained decane, whereas those used in the present study and in all previously published studies of the channel-forming colicins were of the solvent-free type. While none of the earlier publications cite the reason for this choice, unpublished observations in our laboratory have indicated that both colicin E1 and colicin Ia are several orders of magnitude less active in decane-containing membranes. More recently, Slatin et al. [47] have examined the effects of adding decane to initially solvent-free membranes containing open colicin E1 channels. They reported that the properties of the open channels were relatively unaltered by the decane. However, when channels were turned off by positive voltages, they could not be turned on again. This behavior was attributed to the greatly increased thickness of the decane-containing membranes. Comparison of these results with those of Pressler et al. [38] and those of the present study suggests that similar phenomena may occur in the case of colicin B. The values reported for the single-channel conductance of colicin B in the presence and absence of decane are in close agreement. However, much higher voltages and protein concentrations were found to be necessary to open colicin B channels in decane-containing membranes. The amount of decane dissolved in such films, and therefore their thicknesses, decreases with increasing voltage [52]. The voltage dependence of the channel opening rate in decane films may therefore be at least partly attributable to an electrostrictive compensation of the membrane-thickening effect of decane.

Pressler et al. [38] also presented extensive data concerning the dependence of the single-channel conductance of colicin B upon the nature and concentration of the current-carrying cation. However, colicin B, like all other channel forming colicins, is not ideally selective for cations. Because an appreciable but variable fraction of the current through the channels is expected to be carried by Cl^-, results of such experiments cannot be interpreted in terms of the structure of a cation selectivity site within the channel lumen. Further, it should be emphasized that the single-channel conductance is not a good indication of the diameter of the channel lumen. Raymond et al. [39] have shown that very large cations and anions can pass through the colicin E1 channel, even though small ions pass through quite slowly. In this same study, both colicin A and colicin Ia were found to pass large ions more readily than colicin E1. In NaCl solution, however, the single-channel conductance of colicin A is less than one-fourth that of colicin E1, and that of colicin Ib more than twice that of E1. In the case of gramicidin, Andersen and Durkin [1] have shown that chemical modification of a single amino acid side chain can reduce the single-channel conductance of this channel by an order of magnitude, even though the side chain is entirely outside the channel lumen. The steric topology of the pore can be explored much more effectively through studies of the permeation and blockage of the channel by large test ions [39].

The kinetics of channel formation by colicin B are particularly interesting because of the wide variation in behavior exhibited by this protein over the range of pH examined in this study. The general features of colicin B kinetics are consistent with a sequential four state model:



(2)

A characteristic feature of all colicin-planar bilayer systems is that channels continue to open as long as negative voltages are applied; a steady state is never reached. This is the direct result of the fact that the unopened to closed transition is intrinsically so slow that unopened channels must be present in large excess if the forward reaction is to proceed at an observable rate. Thus, our experimental system relies on mass action to provide the driving force for channel formation. That membrane potential also provides a driving force for channel formation by colicin proteins is unequivocal. This voltage dependence must *a priori* reside in processes which occur while the protein is at least partially inserted into the membrane, since the membrane electric field does not extend into the aqueous phase. While the initial voltage-independent membrane binding need not be either strong or kinetically observable, it appears to be the rate-limiting step in the formation of channels by colicin B.

The initial rate of the forward reaction for opening channels is slow, but the concave-up appearance of the records suggests the existence of more than one nonconducting state. More direct evidence for a second nonconducting state comes from the observation that some channels can be closed and reopened by changes in voltage more rapidly than the rest. In terms of the model, application of positive voltages causes all channels in the open state to enter the closed state. When negative voltages are

restored, the process is reversed: channels in the closed state rapidly enter the open state. An important feature of colicin B behavior is that the closed state continues to be filled from the unopened state at the same rate, regardless of whether the channels are able to go on to the open state. As can be seen from the records in Figs. 2 and 6, all the channels which have accumulated in the closed state during application of positive voltages are rapidly dumped into the open state by negative voltages. After the closed state is emptied, channels continue to trickle from the unopened state into the open state through the nearly empty closed state. The total conductance of the membrane at this point is the same as if the trickle had been allowed to continue uninterrupted. The unopened to closed transition is therefore both rate limiting and voltage independent.

Even if the voltage-dependent open-to-closed transition is driven with voltages of up to +100 mV, the closing kinetics are biphasic at pH 5. This behavior necessitates the inclusion of a second conducting state in the model. The slow phase of channel closing corresponds to the transition from the open* state to the closed state by way of the nearly empty open state. The proportion of channels reaching the second conducting state is dependent upon the pH.

When the pH is raised to 9, the features we have associated with the filling and emptying of the closed state of colicin B disappear. In terms of our four-state sequential model, there are two conditions under which a middle state will appear to vanish. If the transition from the closed state to the open state becomes much slower than the unopened to closed step, the first two states become kinetically indistinguishable. Alternatively, if the free energy of the closed state becomes very high, its occupancy becomes insignificant. In effect, the second state becomes part of the energy barrier between the first and third states. These alternatives could in principle be distinguished by examining the transitional behavior between pH 7 and pH 9. The channel kinetics of colicin E1 at pH 7 is similar to that of colicin B at pH 9. Interestingly, several C-terminal proteolytic fragments of colicin E1 have been shown to exhibit biphasic opening kinetics at pH 7 [40]. This finding implies that removal of the N-terminal residues stabilizes a previously unobservable closed state in this colicin.

The data presented here suggest that colicins in general are characterized by at least two conducting and two nonconducting conformational states. A simple sequential model is sufficient to achieve consistency with the level of experimental detail which is presently available. In order to distinguish the sequential model from more complicated branched or cyclic schemes, a complete kinetic analysis must be undertaken. As we previously had indicated, the difficulty in accomplishing this is that the first step in the sequence is too slow to allow study of its approach to the steady state. Important mechanistic distinctions can, however, be made on the basis of experiments using perfusion and antisera.

Perfusion of the aqueous compartment containing colicin B removed all unopened channels from the system. The unopened state must, therefore, correspond to the aqueous protein itself or a membrane-bound pool which is rapidly interconvertible with it. Since open channels must correspond to membrane-spanning structures, we would not expect them to be removed from the membrane by perfusion, and this expectation was borne out by our experimental findings. A direct kinetic path between the open and unopened state is therefore disallowed. On the other hand, the results of Fig. 7 indicate that once channels are driven into the closed state by voltage, they are able to leave the membrane. While perfusion removes unopened channels very quickly, the loss of closed channels is slower than either the rate of channel closing or that of channel opening. This slow process corresponds to the transition from the closed state to the unopened state. The rate-limiting unopened to closed transition thus corresponds to the strong but reversible binding of colicin B to the membrane. This behavior is in sharp contrast to that of colicins A and E1, which bind to the membrane so quickly and in such excess that the process appears to be irreversible.

Experiments using mouse antiserum were important adjuncts to those involving perfusion. Like perfusion, the action of the antiserum was to functionally eliminate the pool of unopened channel precursors without directly affecting channels in either the open or closed states. These results imply that antiserum abolished colicin activity not by interfering with ion transport directly, but by stabilizing an inactive form of the protein, probably by sterically inhibiting direct interaction of the colicin with the bilayer. The amount of antibody bound by a particular form of the protein, and thus the degree of stabilization of that form, will depend upon the number of recognizable epitopes exposed to the aqueous environment. We would not, in general, expect a specific monoclonal antibody to produce this kind of stabilization effect. Since the closed and open states are neither functionally inactivated nor stabilized by antiserum, at least some of the epitopes recognized by the antibodies appear to be disrupted or sequestered by the conformational changes which accompany the transition to the closed state. Together with the perfusion experiments, these

results suggest that the strong binding of colicin B to membranes is associated with a major conformational rearrangement. Pattus et al. [37] came to similar conclusions about the binding of colicin A. From measurements of surface pressure, these investigators concluded that when this protein binds to lipid monolayers a substantial part of the colicin A molecule is inserted at least deep enough to displace the head groups of the lipid. For such a large area of an aqueous protein molecule to come into direct contact with the hydrophobic interior of the membrane, the binding process must involve a substantial conformational rearrangement.

All channel-forming colicins possess an uninterrupted stretch of hydrophobic residues near the C-terminus. Cleveland et al. [15] have speculated that this sequence forms an alpha-helical hairpin structure which serves to tether the aqueous colicin molecule to the membrane. Results of studies of the binding of colicin E1 using circular dichroism [5] and pronase digestion [13] were consistent with the involvement of the hydrophobic stretch. These findings did not, however, bear on the question of whether the sequence acts as a tether, or is instead part of a larger region of the protein which contacts the membrane interior. If the protein were tethered, binding would be accompanied by a minimal disruption of the membrane, in contrast to the findings for colicin A. If the large aqueous domain of a tethered protein retained a conformational structure similar to the original aqueous protein, one would expect polyclonal antibodies raised against the aqueous protein to recognize epitopes on the membrane-bound protein. This does not seem to be the case for colicin B, and similar experiments carried out using colicin E1 and colicin A could help clarify the nature of the membrane-binding processes of these proteins.

Acidic pH has been considered to be a general requirement for the various in vitro actions of the colicins [13, 17, 32, 37, 41], as well as diphtheria [19, 20, 25] and tetanus toxins [7], and enveloped animal viruses [53]. We have found the inhibition of colicin B by high pH to be labile. Since the inhibition is lost upon storage or electrophoretic purification, we think it unlikely that the cause is chemical modification of specific residues. Proteolytic cleavage seems also to be ruled out by the fact that loss of inhibition is not accompanied by a change in electrophoretic mobility on SDS gels. It remains possible that the protein is inhibited by a small molecular weight modulator which is labile in storage, and removed but not detected by electrophoresis. This potential modulator is not the colicin B immunity protein also encoded on pCLB1, since at 20 kDa it should be readily observable under the electrophoretic conditions utilized in these experiments. We favor the view that the inhibited colicin represents a less stable form of the protein, and that the mildly denaturing conditions in frozen storage accelerate the slow but inevitable conversion of colicin B to its stable, uninhibited conformation. It is not clear whether the pH-sensitive protein represents the native conformation or whether it is artifactually produced during the purification procedure. The complete removal of the inhibition by SDS-PAGE is likely an effect of reversible denaturation by SDS. Incubation of colicin E1 with SDS has been found to dramatically reduce the pH dependence of this protein (F.S. Cohen, *personal communication*).

Other properties of the colicins in planar bilayers are also affected by pH, and individual colicins differ in their behavior. The ion selectivities of all colicins so far studied are pH dependent. The channel closing kinetics of colicins E1 [13, 15, 40], A [16] and B (present results) have been shown to be influenced by pH. By contrast, the single-channel conductance of colicin E1 has been reported to be independent of pH [17, 39], while distinct dependencies on pH have been observed for colicins A [16] and B [38]. A pH-dependent shift in the voltage dependence of colicin A has been reported [16], but none has been reported for any other colicin. Significantly, whether or not colicin B is in its inhibited form has no influence on any of the other pH-dependent characteristics.

It seems to us unlikely that any of the pH-sensitive properties are controlled by the localized influence of distinct titratable residues as suggested by Cramer and his associates [10, 17]. The observation that the selectivity of colicin E1 in certain lipids depends not only on pH but on the pH history of the protein [11] supports this view. Rather, behavior of the protein appears to be altered by global effects of its overall degree of protonation. According to this global influence hypothesis, changes in pH produce alterations in the distribution of fixed charges and mobile counterions associated with the protein. As a result, the free energies of both the stable and transitional conformational states of the protein may be shifted. If, as much of the currently available information suggests, the conformational changes accompanying channel formation involve substantial rearrangements of the entire protein, it must be allowed that ionic conditions in widely separated locales of the protein molecule may contribute to its overall conformational stability. Such long range influences on conformational transitions are evident in the studies of C-terminal peptide fragments of colicin E1 by Raymond et al. [40]. These investigators found that removal of successively longer portions of the N-terminus reduced the pH

dependence of channel opening and closing kinetics, even though this portion of the molecule is not directly involved in forming the structure of the channel. Since all residues contribute to some extent to the stability of a particular conformational state, such influences are not limited to the ionizable groups. The effects of amino acid substitution at the 468 position of colicin E1 studied by Shiver et al. [46] illustrate this principle. These investigators examined the role of Glu-468 in the pH requirement by changing this residue to Leu, Ser, Gln, and Lys through site-directed mutagenesis. The leucine form of the protein did exhibit lessened dependence on pH, but in the other mutants this property appeared relatively unaltered. Thus, while residue 468 does seem to be involved in membrane binding, its protonation is clearly not a pivotal event. Since Glu-468 is conserved in colicin B as well as A, E1, Ia and Ib, our observations confirm the conclusion that this residue does not confer a requirement for acidic pH on the channel-forming action of the colicins. In the most recent study from this same laboratory, a Glu residue was placed in the C-terminal hydrophobic sequence by site-directed mutagenesis [44]. Although the rate of channel formation was reduced by this substitution, the extent of binding at high pH was increased. The stabilization of a bound but closed state of the protein relative to the aqueous form was attributed to a partial unfolding of the hydrophobic C-terminus. Such behavior is similar to the spontaneous aging process we report here for colicin B.

Differences in conformational states are likely to exist not only between different colicins, but also between the in vitro and physiological environments in which their action is observed. Colicins are bactericidal under conditions in which the proteins are unlikely to encounter an acidic compartment while being taken up by bacteria [18]. In the physiological situation, where colicins are bound to an outer membrane receptor and extend into the periplasmic space, the conformational state of these proteins is likely to be different than that assumed in vitro. It has previously been suggested that this conformational condition may shift the effective pK of a critical acidic group [10, 17]. Alternatively, the conformational transitions necessary for membrane binding and insertion may be rendered pH indifferent by the preliminary, but global, rearrangements which accompany receptor binding and translocation.

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