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Dynamic properties of membrane proteins: Reversible insertion into membrane vesicles of a colicin E1 channel-forming peptide

(protein dynamics/intervesicle peptide transfer)

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ABSTRACT **The binding of colicin E1 and its COOH-terminal channel-forming peptides to artificial membrane vesicles has an optimum at acidic pH values. The M_r 18,000 thermolytic peptide inserted into membrane vesicles at pH 4.0 has a limited accessibility to exogenous protease. It is converted by trypsin cleavage after Lys-381 and Lys-382 to a lower M_r 14,000 peptide. However, when the pH of a vesicle suspension to which peptide has been bound at pH 4.0 is shifted to 6.0, the accessibility to protease increased greatly. This was shown (*i*) by the large decrease in the amount of M_r 14,000 or M_r 18,000 peptide after the pH 4 → 6 shift and treatment with trypsin or Pronase, consistent with (*ii*) a previously observed decrease in membrane-bound radiolabeled peptide after protease treatment. (*iii*) When a photoactivable nitrene-generating phospholipid probe was used to label the colicin peptide inserted into the bilayer, the extent of labeling decreased by a factor of 3 when the pH was shifted from 4.0 to 6.5. (*iv*) Colicin peptide added to vesicles at pH 4.0 can "hop" to other vesicles if the pH and ionic strength of donor vesicles are successively increased. It is proposed that deprotonation of acidic residues in contact with the hydrophobic bilayer or the membrane surface destabilizes the inserted channel and causes it to be extruded from the membrane. The pH-dependent extrusion of the inserted colicin channel provides an example of dynamic properties of an intrinsic membrane protein.**

The bactericidal molecule colicin E1 exerts its lethal effect by a one-hit mechanism (1) through formation of an ion channel in the cytoplasmic membrane, which is conductive enough (2, 3) to overcome the ability of the cell to polarize itself by protein pumping. The channel-forming activity has been localized in a COOH-terminal domain of the molecule (4). Either the colicin or COOH-terminal peptides can be used as model systems for polypeptide insertion and channel formation in artificial membranes (5). The process of protein insertion into membranes is generally viewed as a unidirectional process, with a large equilibrium constant favoring insertion of a hydrophobic protein or amphipathic channel into and across the membrane bilayer. Relatively little attention has been paid to the dynamic or motional properties of inserted proteins, which are generally described as static structures.

Demonstration of reversible insertion of a protein into a membrane provides documentation of the existence of dynamic properties of the inserted protein. Such reversibility has been shown for cytochrome b_5 (6–10), which has one prominent hydrophobic domain that can either span the membrane bilayer or cross only half-way and loop back. The latter conformation has been proposed to be the transferable form that can undergo intermembrane transfer between phospholipid membrane vesicles (7, 8). The nontransferable form is stabilized in small vesicles (10).

Binding and insertion of the colicin channel into membrane vesicles *in vitro* occurs with an acidic pH optimum near 4.0 (11, 12). The acidic pH dependence of binding and activity was ascribed to pH effects on the colicin protein or peptide since the use of vesicles consisting of phosphatidylethanolamine, phosphatidylcholine, and cardiolipin (70:25:5) instead of asolectin did not change the pH dependence (11). Reversible insertion of the colicin channel into the asolectin vesicles was indicated by a decrease in the amount of radiolabeled colicin or channel peptide remaining in the vesicles after trypsin treatment if the pH was raised before proteolysis (11). The present work documents that the colicin channel peptide inserted into the bilayer of membrane vesicles can be reversibly extruded from the membrane when the pH is raised and that the extruded peptide can undergo intermembrane transfer. A preliminary report on this work has been published (13).

MATERIALS AND METHODS

Preparation of Colicin E1 and Its COOH-Terminal Peptide. Colicin E1 was prepared as described (12) and the M_r 18,000 COOH-terminal thermolytic peptide was prepared as in ref. 14.

Vesicle Preparation. Artificial membrane vesicles were formed from asolectin (Associated Concentrates, Woodside, NY; ref. 15) or phosphatidylethanolamine/phosphatidylglycerol (70:30, wt/wt), by the procedure described in ref. 16. Dioleoyl phosphatidylethanolamine and phosphatidylglycerol were purchased from Avanti Polar Lipids.

Colicin-Induced Cl^- Efflux from Membrane Vesicles. Channel-forming activity was determined by protein or peptide-mediated Cl^- efflux from large mostly unilamellar vesicles (12, 16). Vesicles containing 0.1 M KCl, 10 mM dimethylglutaric acid, 1 mM $CaCl_2$ (pH 5.0) were diluted to 0.1 mg/ml in 0.1 M choline nitrate/0.1 M $NaNO_3$/10 mM dimethylglutaric acid/2 mM $CaCl_2$ (20 ml total vol) at the desired pH. Potassium diffusion potentials of −135 mV were established by addition of valinomycin (final concentration, 15 nM) from a methanolic stock solution. Colicin E1 (≈3 ng/ml) was added at pH 4. The Cl^- efflux was measured with a Cl^--sensitive electrode (Orion model 94-17B) and a double-junction reference electrode (Orion model 90-02).

Extraction of Colicin Peptide Inserted into Vesicles. After enzyme digestion, the reaction was terminated by addition of $CaCl_2$ (final concentration, 20 mM) to precipitate the membranes together with the incorporated peptide. The pellet was sedimented (10 min in microcentrifuge), the sediment was dissolved in $CHCl_3$/MeOH (2:1, vol/vol) to a final lipid

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concentration of 20 mg/ml, and 1/2 vol of 15% trichloroacetic acid was added. The protein precipitate was recovered from the interphase layer between the organic and aqueous solution, 1 vol of ethyl ether was added to extract the remaining trichloroacetic acid, and the peptide was dried under vacuum for 3 min.

Purification of M_r 14,000 Thermolytic Peptide by Reverse-Phase HPLC. The protein extracted from lipid was dissolved in 70% formic acid at a concentration of 0.5 mg/ml, and injected into a C-18 reverse-phase column. H_2O and acetonitrile, with 0.1% trifluoroacetic acid, were used to elute as follows: [0–5 min, 100% H_2O, linear gradient (0.7 ml/min) to 50% acetonitrile after 10 min, and then to 100% after 35 min. The M_r 14,000 peptide, the main peak at the position corresponding to 80% acetonitrile, was collected, lyophilized, and analyzed for purity by sodium dodecyl sulfate (SDS)/PAGE.

Peptide Sequencing. Peptides were sequenced in an Applied Biosystems model 470A microsequencer or a Beckman 890C sequencer according to the manufacturer's instructions.

Colicin Binding to Vesicles. Colicin was incubated at various pH values with vesicles containing photoreactive radiolabeled phosphatidylcholine (17); all operations were under red light (25-W bulb). Illumination with near-UV light then caused covalent binding of label to protein inserted into the vesicle membrane. The radiolabeled phospholipid photoaffinity probe 1-myristoyl-2-[12-(4-azido-2-nitrophenyl)amino]-dodecanoyl-*sn*-glycero-3-[^{14}C]phosphocholine was mixed as described in ref. 18 with asolectin in chloroform, dried, and resuspended at 2.8 mg/ml in 100 mM KCl/5 mM dimethylglutaric acid/0.5 mM $CaCl_2$, pH 5.0. Vesicles were formed by sonicating the lipid in a bath-type sonicator to clarity, followed by twice freezing and thawing. The vesicles (0.1 ml) were diluted 1:5 into 100 mM KNO_3/10 mM dimethylglutaric acid/0.5 mM $Ca(NO_3)_2$, at the desired pH, 10 μg of colicin was added, protein incubated for 10 min, and illuminated for 15 min with long wavelength UV light (366 nm) using a model UVGL gel illuminator (Ultraviolet Light Products, San Gabriel, CA). The protein was transferred to an Eppendorf tube and sedimented (10 min in Microfuge) after the addition of 50% trichloroacetic acid (final concentration, 5%) while Vortex mixing, and it was subsequently incubated on ice for 10 min. The centrifuge pellet was neutralized with Tris base, and the noncovalently bound label was separated from the protein by SDS/PAGE. After identification of the bands by Coomassie blue stain and quantitation by densitometry, the bands were cut out and transferred to a scintillation vial.

RESULTS

Membrane Topography of the Channel Peptide at pH 4.0 Probed by Protease Accessibility. The COOH-terminal peptide that exhibits voltage-dependent channel activity when added to membrane vesicles or planar bilayers at acidic pH values (5, 12) could be extracted from the vesicles as a single component with a mobility slightly less than M_r 18,000 (Fig. 1 *Upper*, lane E), similar to the mobility of the starting peptide material (lane B). The peptide incorporated into vesicles at pH 4.0 was cleaved by trypsin to a smaller peptide with M_r slightly below 14,000 (lane G). The residual peptide left in the membranes after trypsin treatment was extracted and purified by reverse-phase HPLC as a single M_r 14,000 band on the gel (Fig. 2, lanes B–D).

The NH_2-terminal amino acid sequence of the extracted tryptic peptide revealed the tryptic cleavage site to be after Lys-381 and Lys-382 (Table 1). There are 16 other lysine residues and 1 arginine in the 140 amino acids between these trypsin sites and the COOH terminus. However, it appears that none of these 17 residues is sufficiently exposed on the exterior of the vesicles to allow trypsin accessibility, although the possibility of cleavage at Lys-510 and Lys-512 of

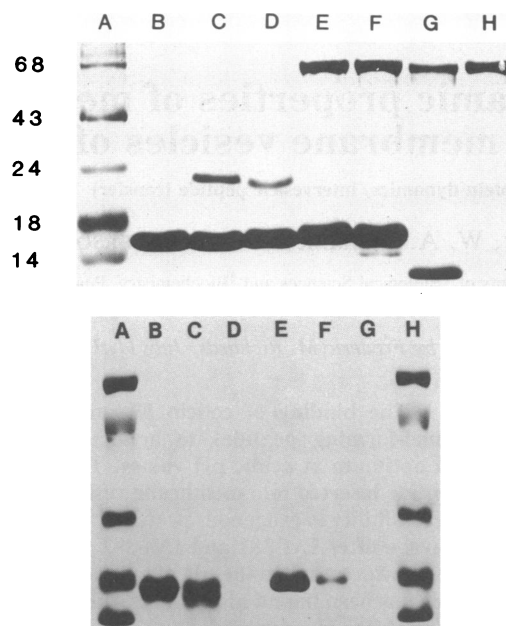

FIG. 1. Extrusion of the colicin E1 channel peptide from the membrane by a pH shift. (*Upper*) Detection by increased accessibility to trypsin. Lanes: A, M_r standards ($\times 10^{-3}$) (bovine serum albumin, ovalbumin, trypsinogen, β-lactoglobulin, lysozyme); B, M_r 18,000 COOH-terminal thermolytic peptide; C, thermolytic peptide mixed with trypsin (Sigma T-1005) [trypsin/peptide (1:5, wt/wt), pH 4.0, 22°C–24°C, 4 hr]; D, as in lane C except 100 mM sodium dimethylglutaric acid (at pH 11.6) added to raise the pH to 6.0; E, peptide incubated with liposomes (0.5 ml) at pH 4.0 for 4 hr; F, peptide incubated with liposomes (0.5 ml) for 30 min at pH 4 before pH was raised to 6.0 and incubated for 10 min; G, as in lane E, pH 4.0, trypsin was added after 30 min [trypsin/peptide (1:5, wt/wt)]; H, pH shifted from 4.0 to 6.0, as in lane F, and then trypsin (1:5, wt/wt) was added. M_r 18,000 peptide (60 μg) was added to all lanes. The extra band at M_r 23,000 in lanes C and D arises from trypsin, which is not seen in lanes G and H because trypsin is not recovered when membranes are precipitated with Ca^{2+}. The extra band in lanes E–H arises from bovine serum albumin added after Ca^{2+} precipitation to assay efficiency of protein recovery. (*Lower*) Detection by increased accessibility to Pronase E (Sigma XXV, from *S. griseus*). Lanes: A and H, M_r standards; E, M_r 18,000 thermolytic peptide (30 μg); B–D, peptide incubated with Pronase E [pronase/peptide (1:6, 5:6, 25:6, wt/wt), pH 4.0, 22°C–24°C, 10 min]; F and G, incubated at pH 4.0 for 30 min, then pH changed to 6.0, and Pronase added (1:6, 5:6, wt/wt, for lanes F and G). Phenylmethylsulfonyl fluoride (1 mM) was added after Pronase incubations and before extraction.

10–12 residues from the COOH terminus cannot be excluded. The COOH terminus is accessible to exogenous protease, as demonstrated by the small decrease (ΔM_r = 1000–2000) in molecular weight caused by treatment with carboxypeptidase Y (Fig. 3). Thus, 130–140 residues of the COOH-terminal region, extending from Ile-383 at least to Lys-510, are inserted into the membrane or sufficiently close to the membrane surface that they are inaccessible to the exogenous protease. The protease acts on inserted colicin peptide exposed on the outer surface of the vesicle, and not the internal membrane face, since the vesicles are known to be impermeable to the added protease. The latter point was checked by showing that vesicles loaded with Cl^- as a test solute retained this solute when incubated with trypsin for 4 hr (data not shown).

Extrusion of the Channel Peptide from the Membrane by a pH Shift (4.0 → 6.0). When trypsin was added to a suspension of membrane vesicles whose pH was shifted to 6.0 after initial binding and insertion of the colicin peptide at pH 4.0, the extent of trypsinolysis was increased greatly so that the peptide was completely digested or digested to very small

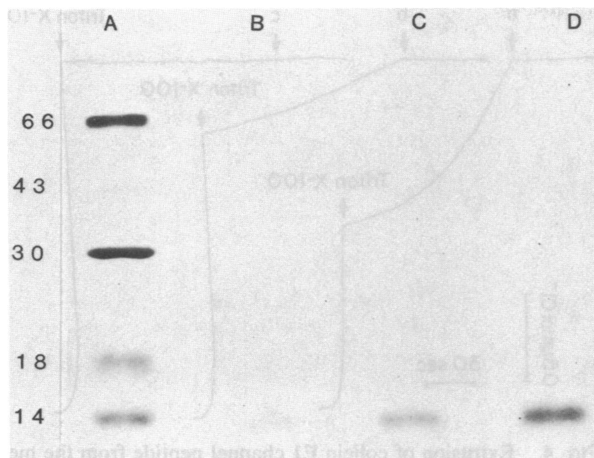

FIG. 2. Purification by HPLC of M_r 14,000 peptide (as in Fig. 1, lanes C) resulting from tryptic cleavage at pH 4.0 of the M_r 18,000 thermolytic peptide. The thermolytic peptide (0.5 mg) was incorporated into liposomes (4 ml, pH 4.0, 20 mM dimethylglutaric acid/100 mM $NaNO_3$). Trypsin (0.1 mg) was added to liposomes 0.5 hr after addition of peptide and incubated for 4 hr at 22°C–24°C before the M_r 14,000 cleavage product was extracted. Lanes: A, protein M_r standards ($\times 10^{-3}$); B–D, loaded with 5, 10, and 20 μl of the M_r 14,000 peptide.

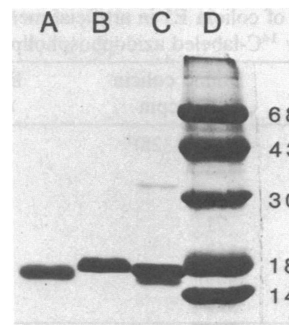

FIG. 3. Cleavage by carboxypeptidase Y of thermolytic peptide incorporated into liposomes. Lane A, thermolytic peptide (0.5 mg) was incubated with liposomes (4 ml, 100 mM $NaNO_3$/20 mM dimethylglutaric acid, pH 4.0) for 30 min, carboxypeptidase Y (0.1 mg) was added and incubated for 3.5 hr at 22°C–24°C, at which point the reaction was terminated by addition of 80 μl of $Ca(NO_3)_2$ (final concentration, 20 mM) to precipitate the vesicles. The peptide was extracted from the liposomes as described. Lane B, as in lane A without carboxypeptidase Y treatment. Lane C, as in lane A, but without liposomes. Lane D, protein M_r standards ($\times 10^{-3}$).

peptides (Fig. 1 *Upper*, lane H). Similar results were obtained (11) by using the change in accessibility of radiolabeled colicin peptide to Pronase E, a nonspecific relatively pH-independent protease: An increase in accessibility of inserted colicin peptide to Pronase E dependent on the pH 4 → 6 shift could also be documented by SDS/PAGE (Fig. 1 *Lower*, lanes F and G). The soluble peptide in the absence of membranes was insensitive to trypsin (Fig. 1 *Upper*, lanes C and D) or Pronase E (data not shown) at pH 4 and 6. The specific activity of trypsin is reduced at pH 4 relative to pH 6 but, as noted above, at pH 4 trypsin can cleave the membrane-bound M_r 18,000 peptide to a M_r 14,000 species, and it can cleave M_r 57,000 colicin E1 in solution to the M_r 20,000 peptide or in the membrane to the M_r 14,000 peptide (data not shown). Thus, (*i*) trypsin is active at pH 4 toward soluble protein and membrane-bound peptide; (*ii*) the peptide in solution is extremely resistant to protease, as already known since it was prepared as a thermolysin- or trypsin-resistant peptide (4); (*iii*) the conformation of the protease accessible region of the membrane-bound peptide is different from its solution conformation at pH 4, since the peptide in the membrane can be further cleaved by protease to M_r 14,000; (*iv*) the increased sensitivity to protease of the membrane-bound peptide is a consequence of a change in conformation and/or accessibility. It was concluded that the pH shift caused an increased accessibility of the incorporated peptide to added protease for the following reasons: (*i*) it occurs for both trypsin and Pronase; (*ii*) the channel peptide must be mostly embedded in the membrane at pH 4 to account for its effective channel activity; (*iii*) by using radiolabeled peptide, >2/3 of the peptide mass was removed from the bilayer by Pronase E treatment after the pH shift (11); (*iv*) the change in phospholipid photoaffinity probe labeling and the intermembrane transfer of the peptide upon the pH 4 → 6 shift, documented below, show that the peptide is extruded from the bilayer.

A pH-dependent extrusion from the membrane bilayer of the inserted channel peptide was implied by a decrease in peptide labeling by ^{14}C-labeled phosphatidylcholine containing a photoreactive azido group at the end of one of the acyl chains, through which it is believed to preferentially react with protein and lipid groups near the center of the membrane bilayer (17). The amount of radiolabel associated with the colicin extracted from the membrane vesicles increased with decreasing pH of incubation with the vesicles (Table 2), indicating that at acidic pH values there was a greater extent of colicin channel insertion into the center of the membrane bilayer, consistent with previous studies on colicin E1 binding to membranes (11), as well as the binding of diphtheria toxin (18), tetanus toxin (20), and colicin E3 (21).

Upon incubating the samples at pH 4 and then raising the pH to 5.6 or 6.5 prior to illumination, there was less ^{14}C label associated with the protein than with samples illuminated while at pH 4, yet more than in samples incubated only at pH 6. The decrease in labeling was found with lipid made of asolectin or a phosphatidylethanolamine/phosphatidylglycerol ratio of 70:30. The similar results obtained with the latter two lipids, whose pK values are <<4 (22), indicate that the pH effects involve protonatable groups on the protein. The maximum time for colicin extrusion from the membrane bilayer, as determined in these experiments, is on the order

Table 1. NH_2-terminal sequence of colicin peptide remaining in membrane vesicles after trypsin proteolysis

	Cycle									
Sequence	1	2	3	4	5	6	7	8	9	10
Measured*	I/K	G/I	N/G	V/N	N/V	E/N	A/E	L/A	A/L	A/A ....
Predicted†	I	G	N	V	N	E	A	L	A	A ....

*Two amino acids (designated by the single-letter code) were found in each cycle, indicating that proteolysis occurred after both K-381 and K-382. Although 10 cycles of the amino acid sequenator output are shown (residues 383–392), 25 cycles were determined that matched the sequence predicted by the nucleotide sequence of Yamada *et al.* (19).

†Given the first few residues, the amino acid sequence predicted from the nucleotide sequence determination of Yamada *et al.* (19) is shown (residues 383–392).

Table 2. Labeling of colicin E1 in artificial membrane vesicles as a function of pH by ^{14}C-labeled azidophospholipid*

pH	Bound colicin E1, cpm	Binding, % of that at pH 4
4.0	999 (1328)†	100
4.8	364	37
5.6	158	16
6.5	71	7
4 → 5.6‡	612	62
4 → 6.0	(400)†	30
4 → 6.5	316	32

*1-Myristoyl-2[12-(4-azido-2-nitrophenyl)amino]dodecanoyl-*sn*-glycero-3-[^{14}C]phosphocholine; procedure as described in *Materials and Methods* except 47 μl of asolectin (100 mg/ml in $CHCl_3$) and 62 μl of probe were mixed into a dram vial and dried under N_2 gas and a vacuum pump for another 30–40 min. The lipid was dissolved in 1.6 ml of 100 mM $NaNO_3$/10 mM dimethylglutaric acid/0.5 mM $Ca(NO_3)_2$, pH 4.0, and 0.4 ml of the vesicles was mixed with 0.6 ml buffer, and after 5 min colicin E1 (20 μg) was added and incubated for 30 min.

†Liposomes made with phosphatidylethanolamine/phosphatidylglycerol (70:30, wt/wt).

‡pH was raised by addition of KOH after incubation for 10 min at pH 4; samples were incubated for 15 min at elevated pH before illumination for 10 or 15 min.

of 10–20 min, the sum of the times of incubation at pH 6.0 after the pH shift and the period of illumination by UV light for photoaffinity labeling.

Extrusion of the Channel-Forming Colicin Peptide and Intermembrane Transfer. Cl^- efflux is observed at a typical rate of 3–5 × 10^4 Cl^- per channel per sec (16) when colicin peptide (4 ng/ml) was added to KCl-loaded vesicles (Fig. 4, b). Triton detergent was added at the end of each run to release all Cl^- in the vesicles. No such efflux was seen when the peptide was added to KNO_3-loaded vesicles (data not shown). Intermembrane transfer caused by a pH shift was inferred from the following experiment: when colicin peptide was added to the KNO_3-loaded vesicles at pH 4.0, the pH of these vesicles was shifted to 6.0, and the ionic strength was increased from 0.1 M to 0.6 M, these vesicles provided a source of free peptide that induced Cl^- efflux from a second vesicle population loaded with Cl^- (Fig. 4, a; summary in Fig. 5). However, if the pH of the KNO_3-loaded peptide-containing vesicle suspension was not increased from 4 to 6, or the ionic strength was not increased to 0.6 M, then the vesicle suspension could not serve as a source of active free peptide for the Cl^--loaded vesicles (Fig. 4, c).

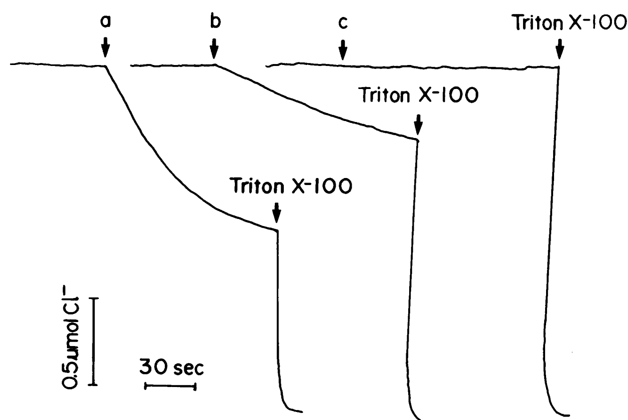

FIG. 4. Extrusion of colicin E1 channel peptide from the membrane by a pH shift. Demonstration by transfer of inserted peptide from NO_3^--loaded to Cl^- vesicles. Cl^- efflux was measured to assay channel-forming activity. Vesicles (100 μl) loaded with 100 mM KCl were diluted from 20 ml of 133 mM Na_2SO_4 (pH 4.0), 10^{-8} M valinomycin was added, and then free peptide or peptide previously inserted into NO_3^--loaded vesicles was added to those loaded with Cl^-. a, Peptide bound to vesicles at pH 4, pH shifted to 6, ionic strength raised, and small aliquot added to Cl^--loaded vesicles at pH 4, as follows: the peptide (0.16 ml, 0.78 mg/ml) was first incubated with the NO_3^--loaded vesicles (1 ml, pH 4.0, 30 min), 100 mM dimethylglutaric acid (Na^+ salt, pH 11.6) was added to raise the pH to 6, 4 M $NaNO_3$ was added after 10 min to increase the salt concentration to 0.6 M, the sample was incubated for 20 min, and 30 μl (final concentration, 120 ng/ml) was added to the Cl^--loaded vesicles (20 ml). b, Free peptide added to Cl^--loaded vesicles: M_r 18,000 peptide (10 μl, final concentration, 3.9 ng/ml) was added to Cl^--loaded vesicles. c, Peptide bound to vesicles at pH 4, which were then added to Cl^--loaded vesicles without shifting the pH to 6 or increasing ionic strength. Arrow, addition of sample; Triton X-100 (final concentration, 0.1%) added at second arrow to disrupt the liposomes and release remaining entrapped Cl^-. From the amount of peptide added, and the relative slopes of the recorded traces in a and b, it was calculated that 15% of the peptide was released from the NO_3^--loaded vesicles.

DISCUSSION

Topography of the Colicin Channel Peptide in Membrane Vesicles at pH 4.0. The binding and activity of the colicin peptide is optimum near pH 4.0. At this pH, the COOH terminus was found to be exposed on the *cis* side of the membrane, consistent with a previous report using membrane bilayers (3). In addition, the only detectable trypsin sites were within 30–35 residues of the NH_2 terminus of the thermolytic peptide, as judged by the change in M_r from 18,000 to 14,000 after trypsin treatment of the incorporated peptide. The finding that the NH_2-terminal residue of the residual tryptic peptide left in the membranes is Lys-382 or -383 was consistent with the M_r 14,000 value determined by SDS/PAGE. Thus, most of all of the remainder of the colicin COOH-terminal domain, 130–140 residues, including 1 arginine and 16 lysine residues, are either inserted into the membrane or bound tightly enough to it that they are not accessible to trypsin. Similar results were obtained after exposure of incorporated peptide to Pronase E. It has been proposed that the COOH-terminal peptide of minimum size may start at residue 429 or even further toward the COOH terminus (23). If so, there would be 46 residues upstream (extending to Lys-382), including 1 arginine and 7 lysine residues, that are bound closely enough to the membrane surface that they are inaccessible to trypsin, even though they are not needed for the structure of the active channel.

pH Dependence and Reversibility of Binding. The number of colicin molecules inserted into lipid at acidic pH values may reflect the fraction of the protein population in which a critical number of acidic amino acids in the channel-forming domain have been protonated. Once inserted, an anchor portion of the protein containing no charged amino acids (the hydrophobic segment extending from amino acid 474 to 508 in E1) may remain inserted regardless of pH. The 4.0 → 6.0 pH shift presumably affects only aspartic acid, glutamic acid, and possibly histidine residues located either within the membrane, and accessible through the channel lumen, and/or exposed at the outer membrane surface. The increase in net negative charge of the channel peptide in the low dielectric medium, or of the exposed segments of the channel at the membrane surface in the presence of the net negative membrane surface charge, would be sufficiently destabilizing to cause extrusion from the membrane. The experiments on protease accessibility, protease cleavage of membrane-bound radiolabeled peptide (11), phospholipid probe labeling, and intervesicle transfer indicate that the pH shift causes extrusion of a significant fraction of the peptide mass to the vesicle surface. After the shift from pH 4.0 to pH 6.0 or 6.5 the peptide is (*i*) bound electrostatically at the membrane surface from which it can be released by high ionic strength

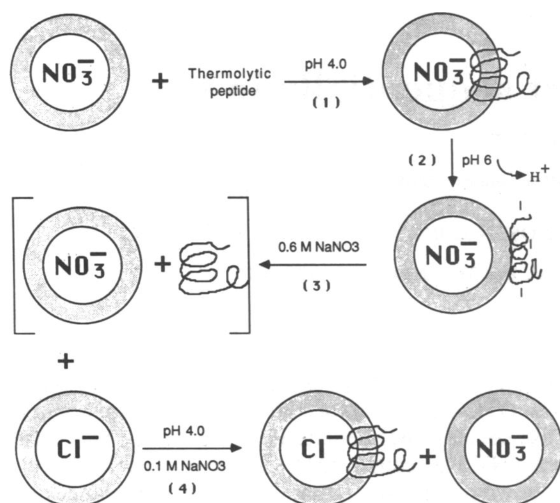

FIG. 5. Schematic diagram for the extrusion from the membrane of the channel peptide by the pH shift. The steps in the extrusion process are numbered. (*1*) Channel inserted into membrane of NO_3^--loaded vesicles at pH 4.0. (*2*) Deprotonation of protein carboxylic residues upon the pH shift from 4 to 6; destabilization of inserted state due to charges inserted into bilayer or to charge repulsion at the membrane surface; the hydrophobic anchor region, residues 474–508, may remain inserted. (*3*) Release by high ionic strength of channel peptide adsorbed to the membrane surface. (*4*) NO_3^--loaded vesicles, to which colicin peptide was added at pH 4.0, the pH then shifted from 4 to 6, and the ionic strength increased to 0.6 M, acting as a source of free peptide when added to Cl^--loaded vesicles.

(Fig. 5), and (*ii*) sufficiently exposed at the membrane surface that many trypsin cleavage sites become accessible; (*iii*) 1/3–1/2 of the sites initially accessible at pH 4.0 to the phospholipid probe located near the center of the bilayer can still be labeled.

Comparison with Cytochrome b_5. It is of interest that cytochrome b_5, the only other molecule for which intermembrane transfer using artificial membrane vesicles has been well documented, is similar in size (molecular weight, 15,223) to the colicin channel peptide and also has a 35-residue hydrophobic domain near the COOH terminus. At present, there is no evidence for two binding states of the colicin channel peptide analogous to the transferable and nontransferable forms inferred for cytochrome b_5, although this is not excluded by present data. The other structural possibility suggested by studies of the porin channel (24) is that the hydrophobic "anchor domain," residues 474–508, spans the membrane twice in the β-sheet conformation (25).

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