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Interactions of Colicin A Domains with Phospholipid Monolayers and Liposomes: Relevance to the Mechanism of Action†

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ABSTRACT: The colicin A polypeptide chain (592 amino acid residues) contains three domains which are linearly organized and participate in the sequential steps involved in colicin action. We have compared the penetrating ability in phospholipid monolayers and the ability to promote vesicle fusion at acidic pH of colicin A and of protein derivatives containing various combinations of its domains. The NH_2-terminal domain (171 amino acid residues), required for translocation across the outer membrane, has little affinity for dilauroylphosphatidylglycerol (DLPG) monolayers at all pHs tested. The central domain has a pH-dependent affinity, although lower than that of the entire colicin A. The COOH-terminal domain contains a high-affinity lipid binding site, but in addition an electrostatic interaction is required as a first step in the process of penetration into negatively charged DLPG films. In contrast to the constructs containing the ionophoric domain, the NH_2-terminal domain alone has no fusogenic activity for liposomes. These results are discussed with regard to the mechanism of entry and action of colicin A in sensitive cells. Our results suggest the existence of a pH-dependent interaction between the receptor binding domain (amino acid residues 172–388) and the pore-forming domain of colicin A (amino acid residues 389–592).

Colicin A is a 592-residue bactericidal protein (Morlon et al., 1983), known to exert its lethal effect through an ability to depolarize the cytoplasmic membrane of sensitive *Escherichia coli* cells (Schein et al., 1978). Studies of the properties of this colicin in artificial membranes have led to the conclusion that depolarization occurs as a result of formation by the colicin of a nonspecific ion channel in the inner membrane (Schein et al., 1978; Konisky, 1982; Pattus et al., 1983a). Colicin A first interacts with its specific receptor at the cell surface (Cavard & Lazdunski, 1981), is then translocated across the outer membrane, and finally reaches the cytoplasmic membrane to insert its pore-forming domain (Lazdunski et al., 1988). In this process, the colicin A polypeptide chain must switch from a water-soluble conformation to one that is more stable in the membrane. The domains associated with the three steps defined above are organized in three distinct regions of the polypeptide chain (Baty et al., 1988). The COOH-terminal domain which carries the ionophoric activity (Martinez et al., 1983) has been crystallized (Tucker et al., 1986), its three-dimensional structure at 6.0 Å is resolved (Tucker, unpublished result), and the 2.7-Å resolution should become available in the near future.

The mechanics of colicin uptake are poorly understood. This is a difficult problem since the number of colicin molecules entering a cell is below the level of detection and a single colicin molecule is theoretically sufficient to kill a sensitive cell. Since translocation through membranes occurs during uptake, membrane lipids may play a role in one or more of the steps described above.

Earlier studies on the interaction of colicin A with lipid model membranes showed that its affinity for lipid increases drastically at acidic pH (Pattus et al., 1983b). Moreover, colicin A was found to promote efficient fusion of lipid vesicles at acidic pH. Fusion was observed not only with pore-forming colicins (A,E1) but also with colicins that contain nuclease activities (E2,E3) (Pattus et al., 1985a). The appearance of a lipid binding site in colicin E3 at acidic pH has been demonstrated (Escyuer et al., 1986) as well as an acidic pH requirement for insertion of colicin E1 into artificial membrane vesicles (Davidson et al., 1985).

Such a requirement resembles the pH dependence in vitro for the actions of diphteria toxin (Sandvig & Olsnes, 1980),

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tetanus toxin (Boquet & Duflot, 1982), and the fusion with target membranes of enveloped animal viruses (Skehel et al., 1982; White & Helenius, 1980). The COOH-terminal colicin A peptide, although bearing a lipid-binding domain, was unable to promote vesicle fusion (Pattus et al., 1985a). These results imply that a lipid binding site, located within the first two-thirds of the colicin sequence, is unmasked at acidic pH. This lipid binding site may play an important role in the translocation step of colicins to the inside of the target cell.

Since we have now isolated and purified the various domains of colicin A using recombinant genetic techniques (Baty et al., 1987a,b; Knibiehler, personal communication), we can investigate their specific ability to insert into phospholipid monolayers, to fuse lipid vesicles, and to form ion channels in planar bilayers at neutral and acid pH. The advantage of the monolayer technique is that we can vary at will the "quality of the interface" (Verger & Pattus, 1982), enabling a detailed study of protein–lipid interactions.

The results presented here confirm, as suggested previously, that there is a pH-dependent association between the COOH-terminal domain of colicin A carrying the pore-forming peptide and another domain of the molecule which affects both the pore sensitivity to membrane potential (Collarini et al., 1987) and the fusion activity of the protein. They also demonstrate that, although the major determinant for the insertion into membrane at low pH is located in the pore-forming domain, there is a lipid binding site in the central domain of the molecule. The NH_2-terminal domain, although it is required for translocation across the outer membrane, has very little affinity for lipids, and even this low affinity is not pH dependent. Interaction between the central and the COOH-terminal domain seems to regulate the expression of the fusion activity of colicin A.

Materials and Methods

Materials. Asolectin (phosphatidylcholine type II-S) from soybean and *E. coli* phospholipid (phosphatidylethanolamine type IX) were Sigma products. These phospholipids are total lipid extracts from soybean and *E. coli*, respectively. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine rhodamine β-sulfonyl)phosphatidylethanolamine (N-Rho-PE) were purchased from Avanti Biochemicals, Birmingham, AL.

Proteins. Colicin A, its thermolysin fragment, and the various mutant proteins were purified as previously described (Baty et al., 1987a,b; Tucker et al., 1986). The genetic constructions and the techniques used for oligonucleotide-directed mutagenesis have been reported (Baty et al., 1987a,b; Knibiehler, personal communication).

Monolayers. Dilauroylphosphatidylglycerol (DLPG) was kindly provided by Dr. J. F. Tocanne. It was dissolved in a chloroform–methanol (2:1) mixture at a concentration of 1 mg/mL and then deposited gently on the air–water interface with a microsyringe in order to obtain a lipid monolayer.

All precautions required to avoid contamination by tensioactive impurities have been described elsewhere (Pattus et al., 1979). Surface pressure (π) was measured with a platinum plate according to the Wilhelmy method in a KSV 2000 (Helsinki) apparatus.

The technique used to measure surface pressure changes has been previously described (Pattus et al., 1983b). The buffer used was 50 mM Tris–acetate, 100 mM NaCl, and 1 mM $CaCl_2$.

Fusion Assay by Resonance Energy Transfer. Fusion was monitored by resonance energy transfer as described by Struck et al. (1981). Phospholipid vesicles (1 mg/mL phospholipid) were prepared by detergent dialysis in 10 mM Tris-acetate buffer, pH 7.4, containing 0.1 M NaCl as previously described (Pattus et al. 1983b). This leads to a homogeneous population of single-walled vesicles (350-Å mean diameter). A volume of 2.9 mL of unlabeled phospholipid vesicles was mixed with 0.2 mL of vesicles containing 2% w/w each of N-NBD-PE and N-Rho-PE. This mixture was excited at 450 nm. At this surface concentration of fluorescent probe, there is an efficient energy transfer between the donor N-NBD-PE and the acceptor N-Rho-PE leading to a quenching of the fluorescence at 530 nm. Upon a decrease in the surface density of the probe (e.g., by fusion) this efficiency of transfer decreases. As a result, the emission of the donor at 530 nm increases while the emission of the acceptor at 590 nm decreases. The comparison of the fluorescence ratios 590 nm/530 nm before and after the interaction of the liposomes with the proteins, at a concentration of 2×10^{-7} M, gives the value of the fusogenic activity (Pattus et al., 1985a). The values of the fusogenic activities of AT1 (containing the NH_2-terminal 1–172 amino acids of colicin A) and the thermolysin fragment (containing the COOH-terminal domain of col A; Tucker et al., 1986), which correspond to 50% of the activity of colicin A at pH 4.2, are considered as unspecific fusogenic activity.

Results

Protein Constructs Used in Studies on the Interaction with Phospholipid Monolayers. All proteins used in this study, except the thermolysin fragment, were obtained by constructing deletions with naturally occuring or site-directed mutagenesis engineered restriction sites present in the *col A* gene. These constructions were previously described (Baty et al., 1987a,b; Knibiehler, unpublished result). The preparation and purification of the thermolysin fragment has also been previously reported (Tucker et al., 1986). The polypeptide chains of these proteins are shown in Figure 1. The protein AT1 contains the NH_2-terminal domain, whereas AJ is the stable form resulting from degradation of the COOH end of the C4 protein which originally had 542 amino acids (Baty et al., 1987a), and contains the NH_2-terminal plus the central domains. The protein BD2 has a small deletion of 15 amino acids in the translocation domain (NH_2 terminal) while the protein AR1 contains the central and the pore-forming domains but lacks most of the translocation region. The thermolysin fragment and the protein BE1 both contain the pore-forming domain, in addition, BE1 contains residues 1–30 of Col A. The hydrophobic moment plot (Eisenberg et al., 1984) which indicates the location of putative membrane-seeking helices in the colicin A polypeptide chain is also presented in Figure 1.

Colicin Penetration into Phospholipid Monolayers. The ability of colicin A and its derivatives to penetrate into a lipid layer can be easily measured by injecting the proteins beneath a phospholipid monolayer film and measuring the increase in surface pressure at constant surface area (Verger & Pattus, 1982; Pattus et al., 1983b). Typical pressure increase kinetics obtained with AR1, as example, are shown in Figure 2. The lower the initial pressure of the film (initial lipid packing), the higher the increase in the surface pressure due to protein insertion in the lipid layer. Plots of the surface pressure increase as function of initial surface pressure are generally linear. One can extrapolate from such plots to determine the pressure above which the protein is no longer able to penetrate into the lipid film (i.e., the critical pressure).

Six representative plots are shown in Figure 3. Measurements were carried out both at pH 4.0 and at pH 8.0. The AT1 protein, which contains only the putative translocation

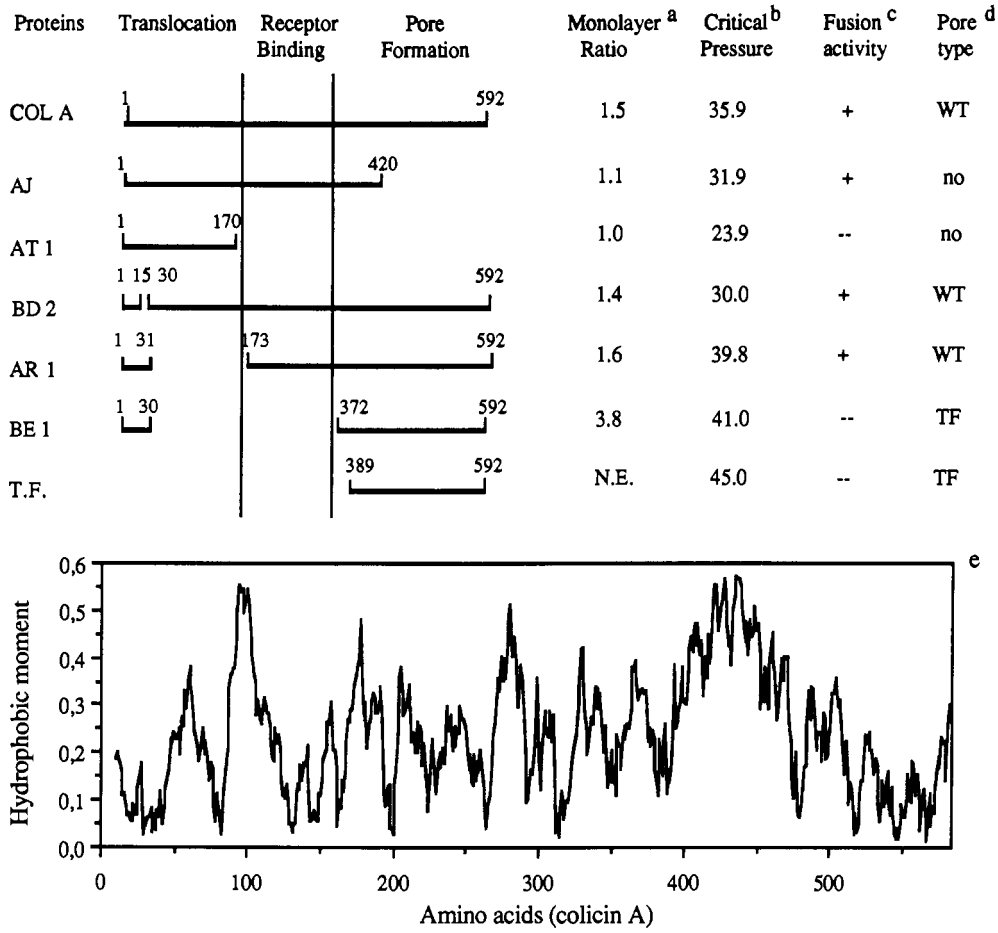

FIGURE 1: Proteins derived from colicin A. The various polypeptide regions contained in the protein derivatives used in this study are indicated as well as the ratio of the surface pressure increase at pH 4.0 to that at pH 8.0, the critical pressure at pH 4.0, the fusion activity, and the pore type. Footnotes: (a) The monolayer ratio is the ratio of increase in surface pressure between pH 4.0 and pH 8.0. The initial surface pressure in all cases was 16 mN m^{-1} and the protein concentration was 2×10^{-8} M. N.E. means not established; no ratio could be determined for the thermolysin fragment due to the fact that no surface pressure increase was detectable at pH 8.0. (b) Critical pressures at pH 4.0. (c) Fusion activities at pH 4.2. (d) WT stands for wild type and means that there is an equilibrium between an acidic and a basic form of the channel, and TF stands for thermolysin fragment and means that there is only the acidic form of the channel which is characteirstic of this peptide (Collarini et al., 1987). The AJ and AT1 derivatives present no pore type because of the fact that they do not bear the pore-forming domain. (e) The hydrophobic moment plot for the colicin A polypeptide chain (window size: 19 amino acids) was calculated according to Eisenberg et al., (1984).

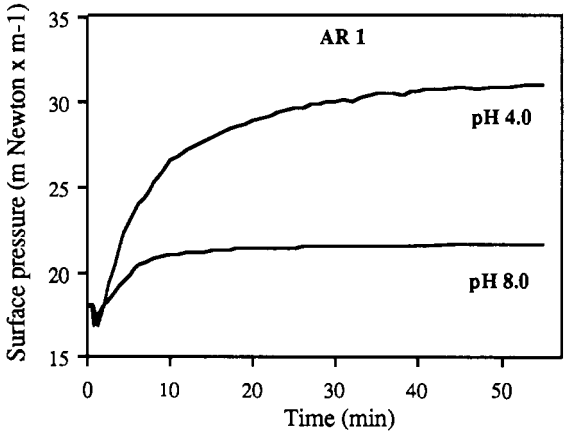

FIGURE 2: pH dependence of surface pressure increase. The protein AR1 (2×10^{-8} M) was injected in the subphase buffer at 25 °C beneath DLPG monolayers at pH 4 and at pH 8.

domain, had low and pH-independent penetration ability. Adding the receptor domain to the translocation domain (AJ mutant) slightly increases the penetration ability at slightly alkaline pH and conferred sensitivity to pH which was, however, not as marked as that for native colicin A. Mutant AR1 which does not contain the translocation domain and mutant BD2, which lacks residues 15–30, behaved like colicin A.

For the thermolysin fragment, we could not detect any significant interaction with the phospholipid monolayer at pH 8.0 even at very low initial surface pressure (12 mN m^{-1}) in the routinely used buffer which contains 100 mM NaCl. In contrast, this fragment was able to penetrate the films when NaCl was omitted. Therefore, electrostatic interactions appear to be very important for penetration of the thermolysin fragment into these monolayers. With the protein BE1, bearing the pore-forming domain and the first 30 amino acids, good penetration of the films at pH 4.0 was observed although penetrating ability was weaker than that of colicin A at pH 8.0.

Fusogenic Activity. The ability of colicin A to fuse phospholipid vesicles at acidic pH has previously been demonstrated by electron microscopy and resonance energy transfer (Pattus et al., 1985a). The results suggested that the glycine-rich first 70 NH_2-terminal amino acids and the COOH-terminal end of colicin A are involved in the fusion activity of the protein. The pH dependence of the fusogenic activity of AT1,AR1,AJ, and colicin A is shown in Figure 4. The translocation domain (AT1) by itself is almost unable to induce vesicle fusion at acidic pH, and its level could be considered as the base line of fusogenic activity because the results are the same as that obtained with bovine serum albumin in the same conditions,

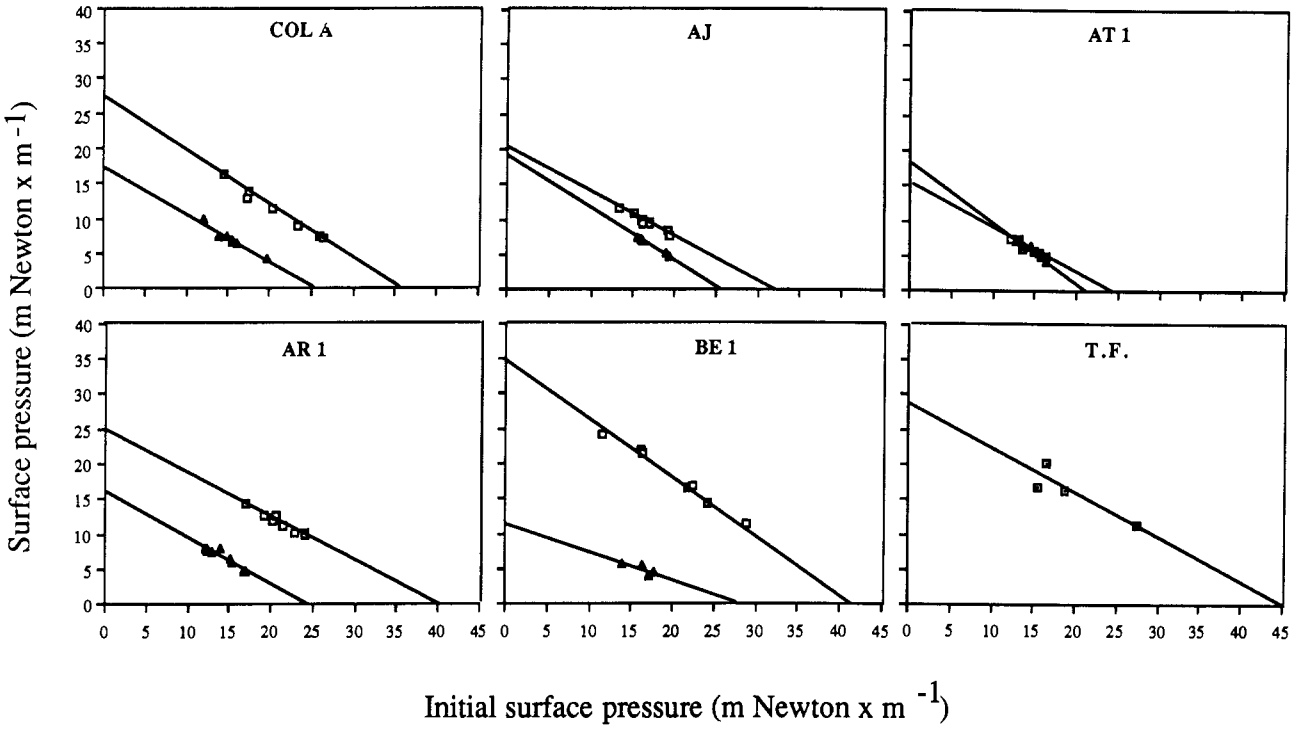

FIGURE 3: Dependence of surface pressure increase versus initial surface pressure. Proteins were injected below the lipid surface (DLPG) to a final concentration of 2×10^{-8} M at pH 8 (▲) and at pH 4 (□). Each point represents the final surface pressure reached at the plateau (see Figure 2) for each initial surface pressure.

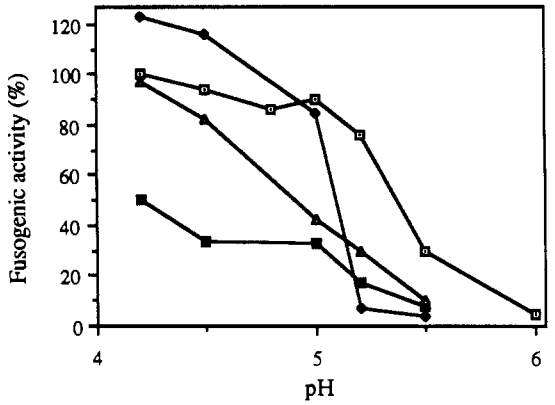

FIGURE 4: Fusogenic activity of colicin A and three derivatives versus pH. The fusion of lipid vesicles promoted by the four proteins was assayed by resonance energy transfer as previously described (Pattus et al., 1985a): colicin A (□), AR1 (◆), AT1 (■), and AJ (▲). The 100% value of the fusogenic activity was that obtained with colicin A; the control without protein was used as blank and was stable for 3–4 h.

whereas the last two-thirds of the polypeptide chain behaves like native colicin A (Figure 4). With the protein AJ, which lacks the COOH-terminal domain, we did not observe the sharp pH-dependent transition in the fusogenic activity observed with colicin A or AR1 (Figure 4). In contrast, there was a slow pH-dependent increase in fusogenic activity between pHs 5.5 and 4.2.

The fusion activities of the various protein constructs at pH 4.2 are presented in Figure 1 as well as the properties of the ion channels that they formed. From this table it can be concluded that it is the receptor binding domain which is essential for the fusion activity and not the translocation domain. However, the properties of AJ and the COOH-terminal mutants indicate that something else is required. Some answers may be obtained by comparing the fusion and the pore activities of the various constructs (Figure 1). These mutants can be classified into three groups according to their channel-forming activity: (i) those which are identical with colicin A and which show a pH-dependent equilibrium between two forms of the channel, (ii) those which are identical with the thermolysin peptide and do not show these equilibrium, and (iii) those devoid of channel activity. The equilibrium between the two channel states which is displayed by colicin A was suggested to be due to a pH-dependent association between two domains (Collarini et al., 1987). It is possible that the disruption of this interaction between the two domains at acid pH coincides with the sharp transition in the fusion activity.

Discussion

Many lines of evidence indicate that the colicin A polypeptide chain should expose hydrophobic regions while crossing the cell envelope to reach the cytoplasmic membrane where it forms a voltage-dependent channel. This polypeptide chain has some unusual features which may be related to its ability to expose these hydrophobic regions. First, it does not contain any disulfide bridge although it contains 592 amino acid residues, and despite this fact, it it released to the extracellular medium (Baty et al., 1987b): in contrast, most secreted bacterial proteins contain disulfide bridges and are very resistant to proteolysis. Colicin A, like other colicins, is very sensitive to proteolysis, and under certain conditions it is even cleaved by a protease exposed at the outer surface of producing cells (Cavard et al., 1982). A second unusual property is its ability to form stable oligomers under various conditions. Stable dimers can be formed by nascent cytoplasmic colicin A, and the dimeric form is even conserved in the presence of SDS at 20 °C (Knibiehler & Lazdunski, 1987).

Colicin A tetramers are spontaneously formed at very acidic pH (below pH 4). These tetramers may be formed by tail-to-tail hydrophobic interactions since in the presence of an excess of lipid they can be dissociated into monomers complexed to 20–30 lipid molecules (Cavard et al., 1988), indicating the exposure of a high-affinity lipid binding site.

The analysis of the pH dependence of circular dichroism of colicin A and of its COOH-terminal domain has indicated

that a sharp transition occurs between pH 4 and pH 3. This transition is very much reduced for the COOH-terminal domain in the presence of a nonionic detergent (Carvard et al., 1988). The penetration of colicin A into lipid monolayers and liposomes has also been found to be regulated by groups with a pK of about 5.5 (Pattus et al., 1983b, 1985b).

All of these results suggest that protonation of one or more amino acid residues in the molecule causes a conformational change accompanied by an increase in hydrophobicity. Such a conclusion has also been reached for colicin E1 (Davidson et al., 1985).

The results presented here indicate that colicin A contains two pH-dependent lipid binding sites, one located in the COOH-terminal pore-forming domain and one in the receptor binding region. Rather surprisingly, the NH_2-terminal domain seems to contributes very weakly to the interaction of colicin A with lipids, although it is thought to be involved in the translocation (Baty et al., 1988).

The COOH-terminal lipid binding site which is shared by all colicins with pore forming activity is responsible for the insertion of the channel into the membrane. According to our results on the penetration of phospholipids monolayers by the thermolysin fragment at slightly alkaline pH, the COOH-terminal domain of colicin A binds weakly to lipid membranes, its affinity being much lower than that of the entire colicin molecule. In contrast, at acidic pH this domain apparently contains most of the driving force to insert the protein into the lipid bilayer, since it does so as readily as the native protein.

According to the interaction properties of the deletion mutants of colicin A with phospholipid monolayers, a lipid binding site should be localized between residues 173 and 420 of the polypeptide chain. A lipid binding site, located in the central region, was also shown to the present in colicin E3 (Escuyer et al., 1986). This may be a common feature of colicins regardless of their killing activity (channel forming or nuclease), suggesting that this domain may be involved in an event occurring before colicins reach their target inside the cell. This event may involve membrane fusion although at present we have only obtained in vitro evidences and we cannot assign a physiological relevance to the fusogenic activity of colicin A. We have observed that colicins E2 and E3 which do not contain a pore-forming domain are also fusogenic (Pattus et al., 1985a), but their fusogenic activities are effective at a more acid pH (4.0–4.5) than colicin A (Pattus, unpublished result).

We have experimental evidence that pH-dependent conformational changes not only occur within each domain but may also alter the interactions between domains. This is suggested by the fact that colicin A, but not its COOH-terminal fragment, undergoes a pH-dependent transition between an "acidic" and a "basic" form of the pore with an apparent pK of 5.3. The two forms of the pore differ by their switching voltage but not by the channel size (Collarini et al., 1987). We have thus previously proposed the existence of a pH-dependent association between the COOH-terminal domain carrying the lumen of the pore and another domain of the molecule which affects the sensitivity of the pore to membrane potential (Collarini et al., 1987). The results presented in this study indicate that although the major determinant of fusion activity is located in the central domain of colicin A, this activity is affected by disruption of the interaction between these domains as shown in Figure 4. Some residual interaction may be retained at pH 4.0 as evidenced by the lack of full expression of the "penetrating capacity" of colicin A, AR1, and BD2 as compared to that of BE1 and the thermolysin fragment. Alternatively, the presence of two lipid binding sites within the polypeptide chain may add more constraint to the insertion within the lipid monolayer.

To conclude, our results suggest that colicin A contains two lipid binding sites. The COOH-terminal site is associated with the pore-forming activity while the central site may exist in all colicins regardless of their mode of action. The NH_2-terminal domain does not contain a lipid binding site. At present, it is not clear whether the fusion activity is really involved into the translocation of colicins across the cell envelope. However, we believe that binding of the central domain of colicins to membrane lipids may occur during their pathway of entry into sensitive cells.

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Interactions of Triglycerides with Phospholipids: Incorporation into the Bilayer Structure and Formation of Emulsions†

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ABSTRACT: Interactions of carbonyl ^{13}C-enriched triacylglycerols (TG) with phospholipid bilayers [egg phosphatidylcholine (PC), dipalmitoylphosphatidylcholine (DPPC), and an ether-linked phosphatidylcholine] were studied by ^{13}C NMR spectroscopy. Up to 3 mol % triolein (TO) or tripalmitin (TP) was incorporated into DPPC vesicles by cosonication of the TG and DPPC at ~50 °C. NMR studies were carried out in a temperature range (30–50 °C) in which pure TO is a liquid whereas pure TP is a solid. In spectra of DPPC vesicles with TG at 40–50 °C, both TO and TP had narrow carbonyl resonances, indicative of rapid motions, and chemical shifts indicative of H bonding of the TG carbonyls with solvent (H_2O) at the aqueous interfaces of the vesicle bilayer. Below the phase transition temperature of the DPPC/TG vesicles (~36 °C), most phospholipid peaks broadened markedly. In DPPC vesicles with TP, the TP carbonyl peaks broadened beyond detection below the transition, whereas in vesicles with TO, the TO carbonyl peaks showed little change in line width or chemical shift and no change in the integrated intensity. Thus, in the gel phase, TP solidified with DPPC, whereas TO was fluid and remained oriented at the aqueous interfaces. Egg PC vesicles incorporated up to 2 mol % TP at 35 °C; the TP carbonyl peaks had line-width and chemical shift values similar to those for TP (or TO) in liquid-crystalline DPPC. TO incorporated into ether-linked PC had properties very similar to TO in ester-linked PC. Thus, long-chain TG with different bulk phase properties have similar properties when present as a surface component in liquid-crystalline ester- or ether-linked PC. These properties (extent of solubility in the PC surface, conformation, solvent accessibility, and molecular mobility) may be important for enzymatic hydrolysis and protein-mediated transfer of TG. In gel-phase DPPC, the molecular mobility of the TG depends on the nature of the TG acyl chains. In the DPPC/TG mixtures studied, attempts to incorporate TG in excess of the bilayer solubility resulted in production of emulsion particles. The significance of these results for TG metabolism is discussed.

Triglycerides (triacylglycerols, TG)[1] are neutral lipids which partition primarily into phases made up of other nonpolar or weakly polar lipids. However, even the biologically important long-chain TG, which are extremely water insoluble, exhibit interfacial properties. Thus, they orient at air–water interfaces with the polar glyceryl portion interacting with water, both in the pure form and in mixtures with phospholipids (Desnuelle et al., 1951; Smaby & Brockman, 1987). In small, unilamellar egg phosphatidylcholine (PC) vesicles, triolein (TO) has a finite solubility and a preferred orientation with the carbonyl groups at the aqueous interface (Hamilton & Small, 1981). Similar results were obtained with triolein in multilamellar egg PC (Gorrissen et al., 1982). These findings raise the interesting possibility that intracellular or lipoprotein TG may not be completely segregated into separate phases but may be present in small proportions intercalated in phospholipid bilayers of biological membranes and in the phospholipid-rich surface monolayers of plasma lipoproteins. Such interfacial TG may be the species which interact with lipolytic enzymes and carrier proteins (Smaby & Brockman, 1987; Hamilton & Small, 1981).

Previous studies with egg PC/TO vesicles used ^{13}C NMR spectroscopy and ^{13}C enrichment of the TG carbonyl carbons to monitor the small amounts of interfacial TG (Hamilton & Small, 1981). The method permitted precise determination of the amount of vesicle-solubilized TG and clearly demonstrated the interfacial nature of this TG. Because of the acyl chain complexity in biological TG and phospholipids, it is imperative to examine other model systems in order to judge the generality of these results. The present study uses similar procedures to determine the solubility and orientation of a long-chain saturated triglyceride, tripalmitin (TP), in egg PC vesicles. The effect of phospholipid acyl chain fluidity on

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[1] Abbreviations: TG, triacylglyerol; PC, phosphatidylcholine; TO, triolein; TP, tripalmitin; DPPC, dipalmitoylphosphatidylcholine; T_1, spin-lattice relaxation time; NOE, nuclear Overhauser enhancement.

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