Brominated Phospholipids as a Tool for Monitoring the Membrane Insertion of Colicin A†

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ABSTRACT: The intrinsic fluorescence of the colicin A thermolytic fragment does not change after insertion into normal phospholipid vesicles and is thus an unsuitable probe for monitoring the membrane insertion process. In this paper, we report the results of studies on the quenching of this fluorescence by brominated dioleoylphosphatidylglycerol (Br-DOPG) vesicles. Bromine atoms located at the midpoint of the phospholipid acyl chain quench the tryptophan fluorescence, indicating contact between fluorophores of the protein and the bilayer's hydrophobic core. Addition of Br-DOPG vesicles to a protein solution quenches the tryptophan fluorescence in a time-dependent manner. This quenching can be fitted to a single-exponential function, and thus interpreted as a one-step process. This allows calculation of an apparent rate constant of protein insertion into the membrane. Parameters known to affect the insertion of the thermolytic fragment into phospholipid monolayers or vesicles (pH and negative charge density) also affect the rate constant in comparable ways. In addition to the information gained concerning membrane exposure in the steady state, this approach provides the first real-time method for measuring the insertion of colicin into membranes. It is highly quantitative and can be used on all versions of the protein, e.g., full size, proteolytic fragments, and mutants. Brominated lipids provide experimental conditions identical to normal lipids and allow for great flexibility in protein/lipid ratios and concentrations. The kinetic analysis shows clearly the existence of a two-step process involving a rapid adsorption of the protein to the lipid surface followed by a slow insertion.

In recent years, a new approach applied to the determination of membrane protein topology has been the use of brominated phospholipids (Leto et al., 1980; East & Lee, 1982; Markello et al., 1985). Bromine atoms can be easily incorporated into the unsaturated fatty acid acyl chain of phospholipids, and the characteristics of the resulting phospholipid are very similar to those of the unsaturated precursor (East & Lee, 1982). Bromine atoms are known to quench the intrinsic tryptophan fluorescence of proteins. The mechanism of this quenching is probably collisional (East & Lee, 1982; McIntosh & Holloway, 1987; Yeager & Feigenson, 1990), although a distance-dependent quenching mechanism has recently been proposed (Bolen & Holloway, 1990). The introduction of bromine atoms at different positions along the fatty acid acyl chain of the phospholipid allows for the determination of the depth at which the fluorophore group of the protein is located within the bilayer (McIntosh & Holloway, 1987). All the reported measurements up to now have been done under steady-state conditions, and the extent of quenching enabled the study of quenching constants (De Kroon et al., 1990), the depth of the fluorophore within the membrane (Markello et al., 1985; De Kroon et al., 1990), lipid affinity (East & Lee, 1982), or solubilization (De Foresta et al., 1989).

We have used the quenching of the intrinsic fluorescence of colicin A by brominated dioleoylphosphatidylglycerol vesicles to monitor both the tryptophan membrane exposure and the crucial membrane insertion step of this toxin. Colicin A is a plasmid-encoded protein produced by *Citrobacter freundii*, which is active against certain strains of *Escherichia coli* (Lazdunski et al., 1988). The mechanism of action in vivo comprises three steps. After it binds to the receptor, the colicin A molecule is translocated across the outer membrane with the intervention of a set of proteins, tolQ, tolR, tolA, and tolB. Once the colicin A has reached the periplasmic space, it inserts into the inner membrane and forms a voltage-gated pore which depolarizes the cell and kills it. Each step is performed by one of three different domains in the protein. The N-terminal domain is responsible for protein translocation, the central domain is the receptor-binding domain, and the C-terminal domain forms the pore (Pattus et al., 1990).

This C-terminal domain can be obtained from the whole colicin A molecule by thermolysin proteolysis, and it is thus called the thermolytic fragment of colicin A. It has been crystallized and its structure determined by X-ray crystallography. On the basis of the three-dimensional structure of this fragment, a model for the insertion has been proposed (Parker et al., 1989, 1991) in which the compact soluble structure, upon interaction with the phospholipid bilayer, unfolds into the so-called "umbrella model". We report in this paper that the quenching of colicin A thermolytic fragment intrinsic fluorescence by Br-DOPG vesicles allows us to follow quantitatively in real time the membrane insertion of this protein. It is revealed to be a slow kinetic process definable in terms of a first-order reaction model. An apparent kinetic constant can be calculated which quantifies the insertion rate. This constant, k, shows pH and negative charge density dependence identical to that observed using completely different experimental approaches (Pattus et al., 1983; Collarini et al., 1987; Massotte et al., 1989). The results obtained confirm the umbrella model for the spontaneous insertion of colicin A thermolytic fragment into vesicles, and the occurrence during this process of a "molten globule" intermediate.

MATERIALS AND METHODS

Dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylcholine (DOPC) were purchased from Sigma. Bro-

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mine and analytical-grade solvents (chloroform, ethanol, and acetic acid) were from Merck. The silica gel used was Kieselgel 60 (Fluka) with a particle size range of 0.04–0.063 mm (230–400 mesh ASTM). Tris(hydroxymethyl)aminomethane (Tris), reagent grade, was obtained from Sigma.

Cell Growth and Purification. Colicin A was purified from *Citrobacter freundii* strain CA31 and was converted into its thermolytic fragment as described previously (Cavard & Lazdunski, 1979; Tucker et al., 1986). The C-terminal peptide was purified by filtration on a Sephadex G50 column, dialyzed against water, and lyophilized. The lyophilized peptide was disolved in the desired buffer, and the solution was spun in an Eppendorf centrifuge for 15 min to remove suspended particles and nonsolubilized material. The thermolytic fragment concentration was measured from the absorbance at 280 nm using the molar absorption coefficient ($\epsilon_{280} = 2.43 \times 10^4$ M^{-1} cm^{-1}) determined previously (Massotte et al., 1989).

Synthesis of Brominated Phospholipids. The brominated phospholipid was synthesized as already reported (East & Lee, 1982). Briefly, to 100 mg of DOPG (or DOPC) in chloroform at −20 °C was added 40 μL of bromine, and the reaction was allowed to proceed for 30 min. Excess bromine was eliminated by placing the reaction mixture on a silicic acid column preequilibrated with chloroform and eluting with 250 mL of chloroform and 250 mL of 10% (v/v) methanol in chloroform. Subsequent elution with 250 mL of 50% methanol in chloroform released the brominated phospholipid. Fractions were collected, and thin-layer chromatography identified those containing the brominated phospholipid. In all the lipid-containing fractions, only one spot was present. Fractions containing brominated phospholipid were evaporated in a rotary evaporator. The evaporated phospholipid was resuspended in chloroform/methanol (1/1 v/v) and stored under nitrogen at −80 °C.

Preparation of Vesicles. Br-DOPG or Br-DOPC were taken from the stock solution and evaporated in a rotary evaporator. To remove any trace of solvent, the evaporated film of lipid was placed overnight under vacuum (water pump). To the film of lipid was added the buffer solution (Tris–acetate, 50 mM at the required pH) to yield a final lipid concentration of 10 mg/mL, and the milky suspension was thoroughly vortexed. Small unilamellar vesicles were formed by bath sonication at room temperature until the suspension was clear, which took approximately 15 min. Liposomes with high Br-DOPG content only formed clear solutions after extended periods (more than 30 min) or by brief probe sonication (60 s). The sonicated vesicles were centrifuged in an Eppendorf centrifuge for 30 min at 4 °C, and the supernatant containing the small unilamellar vesicles was collected for use. Lipid concentration was assessed by the method of Bartlett (1959).

Binding of Colicin A to Vesicles. The extent of binding of the thermolytic peptide to Br-DOPG vesicles was determined by the decrease in fluorescence of the protein–vesicle mixture after centrifuging the suspension in a Beckman TL-100 tabletop ultracentrifuge, using a TLA-100.2 fixed-angle rotor at 100 000 rpm for 1 h, at 30 °C. Control experiments with radiolabeled lipid showed that under these conditions (435000*g*), more than 97% of the total lipid pelleted.

Fluorescence Measurements. Fluorescence spectra were recorded at 30 °C by an SLM 8000 spectrofluorometer operating in the ratio mode with spectral bandwidths of 4 nm for both excitation and emission. A Cornu pseudodepolarizor (Steeg and Reuter, Giessen, Germany) was placed in the excitation beam to reduce polarization anomalies (Marriot et al., 1990). Emission spectra were automatically corrected by factors supplied by SLM Inc. The light-scattering effect of the vesicles was reduced, if not eliminated, by exciting the sample with vertically polarized light at 280 nm, and measuring horizontally polarized emitted light. The scattered light is thus highly vertically polarized, and its contribution to the horizontally polarized light reaching the photomultiplier tube was minimal. The inner filter effect has been minimized by using 0.5-cm path-length cuvettes and by keeping the optical density at the excitation wavelength of the protein samples used below 0.03. The Raman scatter contribution was removed by subtraction of appropriate blanks.

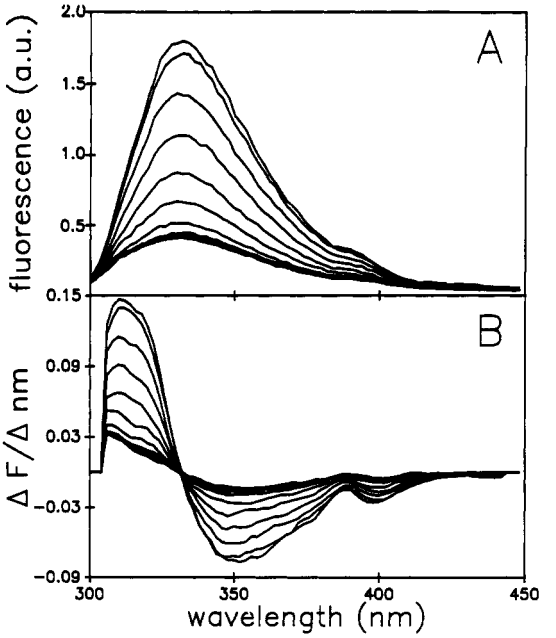

FIGURE 1: (A) Corrected tryptophan fluorescence emission spectra of colicin A thermolytic fragment in the absence (top spectrum) and in the presence of increasing amounts of Br-DOPG vesicles. Protein concentration was 20 μg/mL, in 50 mM Tris–acetate buffer, pH 5, at 30 °C. (B) First derivative of the spectra shown above. The isosbestic point appears at 332 nm. The small peak at 396 nm is an artifact due to a Woods anomaly in the emission monochromator.

RESULTS

Quenching of the Intrinsic Fluorescence of the Pore-Forming Domain of Colicin A by Br-DOPG Vesicles. Figure 1A shows the fluorescence emission spectra of the thermolytic fragment of colicin A in the absence and in the presence of increasing amounts of Br-DOPG vesicles, at pH 5. The brominated phospholipid vesicles strongly quench the intrinsic fluorescence of the pore-forming domain of the colicin A molecule. The observed residual fluorescence may be due either to a fraction of the tryptophans which is inaccessible to the core of the membrane or to inefficiency of the bromine atom as a quencher. The second possibility seems to be correct for two reasons: first, that addition of potassium iodide (a water-soluble quencher of tryptophan) to the lipid–protein mixture does not induce further quenching (unpublished observation); second, that the quenching efficiency for the system indole-2,3-dibromobutane has been reported to be 0.82 (Bolen & Holloway, 1990), in complete agreement with our observations. It can be seen that no scattering contribution appears in the different spectra, even at the highest vesicle concentration, where an increase in sample turbidity is readily observed.

Figure 1B shows the first derivative of the spectra in Figure 1A. The presence of the isobestic point at 332 nm indicates

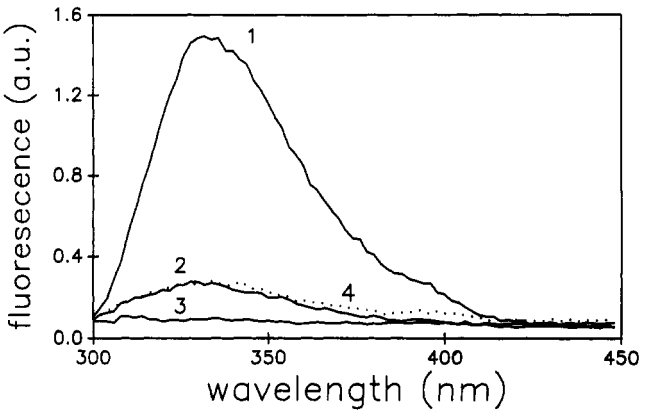

FIGURE 2: Binding of colicin A thermolytic fragment to Br-DOPG vesicles in 50 mM Tris–acetate buffer pH 5, 30 °C. (1) Tryptophan fluorescence emission spectrum of colicin A thermolytic fragment (20 μg/mL). (2) After addition of Br-DOPG vesicles at a final lipid to protein molar ratio (R_i) value of 450. (3) Spectrum of the supernatant after ultracentrifugation (see Materials and Methods). (4) Spectrum of the pellet resuspended in its own supernatant (dotted line).

that the wavelength of maximum fluorescence emission has not changed in the presence of Br-DOPG vesicles. This is evidence that the overall polarity of the microenvironment surrounding the three tryptophans in the pore-forming domain of colicin A has not changed upon addition of Br-DOPG. Hence, the bromolipids behave exactly like all the lipids and lipid mixtures previously employed. The X-ray structure of colicin A thermolytic peptide (Parker et al., 1989, 1991) shows that the three tryptophans in the soluble form of the protein are located in the hydrophobic core of the molecule, in an apolar environment, while after insertion in the phospholipid bilayer they remain in a hydrophobic environment, as shown by fluorescence quenching experiments with both hydrophilic and hydrophobic quenchers (Lakey et al., 1991).

Binding of the Pore-Forming Domain of Colicin A to Br-DOPG Vesicles. In order to measure the degree of binding of the pore-forming domain of colicin A to Br-DOPG vesicles at pH 5, fluorescence emission spectra were taken before and after addition of the vesicles. The lipid–protein mixture was ultracentrifuged as described under Materials and Methods, and the spectrum of the supernatant was measured to detect any fluorescence not associated with the lipid. Figure 2 shows the results. Spectrum 1 corresponds to the protein in solution. A typical tryptophan emission fluorescence spectrum is observed with an emission maximum at 332 nm. Spectrum 2 was taken 1 h after addition of brominated vesicles, to allow the sample to equilibrate. The fluorescence emission is almost totally quenched by the bromolipid. Spectrum 3 was measured after ultracentrifugation in conditions which pelleted more than 97% of the vesicles (see Materials and Methods). The spectrum is flat, and corresponds to the buffer base line. This means that at pH 5 all of the protein is associated with the vesicles. Resuspension of the vesicle pellet with its own supernatant gave spectrum 4 which is identical to the spectrum 2 taken before the ultracentrifugation. These results were reproduced at lipid to protein molar ratios ranging between 65 and 524 (data not shown).

Insertion of the Pore-Forming Domain of Colicin A into Br-DOPG Vesicles. The quenching of the intrinsic fluorescence of the pore-forming domain of colicin A can be followed kinetically (Figure 3, solid line). Addition of Br-DOPG vesicles to a protein suspension resulted in an exponential decrease of the fluorescence emission that follows first-order kinetics.

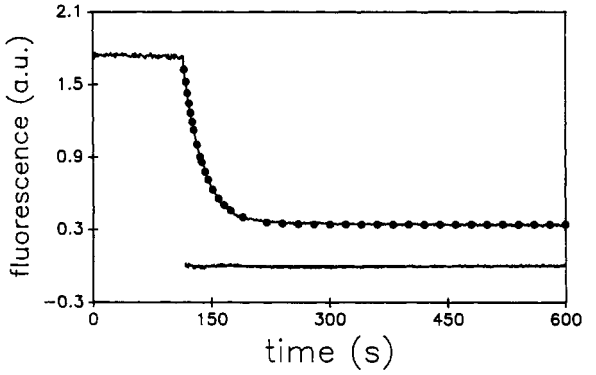

FIGURE 3: Kinetics of the quenching of the intrinsic fluorescence of colicin A thermolytic fragment by Br-DOPG vesicles. To 400 μL of the protein, 20 μg/mL in 50 mM Tris–acetate buffer, pH 5, 30 °C, was added 15 μL of a 10 mg/mL vesicle suspension in the same buffer (R_i = 450), and the tryptophan emission fluorescence was recorded as a function of time. The experimental curve (solid line) is plotted together with the simulated curve (dotted line), obtained by introduction of the calculated k value into the expression: $F = F_\infty + (F_0 - F_\infty)e^{-kt}$. The lower line shows the difference between the observed and theoretical values.

At time $t = 0$, all the protein is noninserted, and the fluorescence we observe is F_0, whereas at time = ∞ all the protein is inserted and $F = F_\infty$. At any time, the observed fluorescence is proportional to the fraction of noninserted colicin:

$$F(t) = F_\infty + (F_0 - F_\infty)e^{-kt} \quad (1)$$

Thus, if we plot ln $\{[F(t) - F_\infty]/(F_0 - F_\infty)\}$ versus time, we obtain a straight line whose slope is $-k$. This observed k is the sum of the rates of insertion and deinsertion of the protein. In our experimental conditions, 100% of the protein is bound to the vesicles as indicated by ultracentrifugation experiments. Hence, the present analysis considers the rate of deinsertion to be very small, and the apparent rate constant for the insertion of the pore-forming domain of colicin A into Br-DOPG vesicles can be readily calculated. As a control, we constructed a theoretical curve in which the calculated value for k was introduced into eq 1. The result is presented in Figure 3 (dotted line). The degree of fitting (Figure 3, bottom) between experimental and theoretical values shows that this kinetic model is sufficient to analyze the present data.

Effect of Lipid and Protein Concentrations on the Insertion Rate. In order to gain some insight into the kinetic model, the effect of lipid and protein concentrations was examined. The proposed model only takes into account two protein states: The fluorescent noninserted state and the nonfluorescent inserted state. The lipid/protein ratio is high enough to assume that the free lipid concentration is constant during the initial steps of the process. Under these conditions, we can consider the insertion of the pore-forming domain of colicin A as a pseudo-first-order process.

If we maintain the protein concentration at a constant value and vary the lipid concentration, we observe that the apparent kinetic constant for the insertion process increases when increasing the lipid concentration in a linear fashion (Figure 4). This result supports a model involving a biomolecular reaction between the protein and the vesicles, since at a constant protein concentration, k depends linearly on lipid concentration.

Conversely, if we maintain the lipid concentration constant and change the protein concentration, it is found that k decreases when the protein concentration increases, in contradiction to a biomolecular reaction (Figure 5). This can be

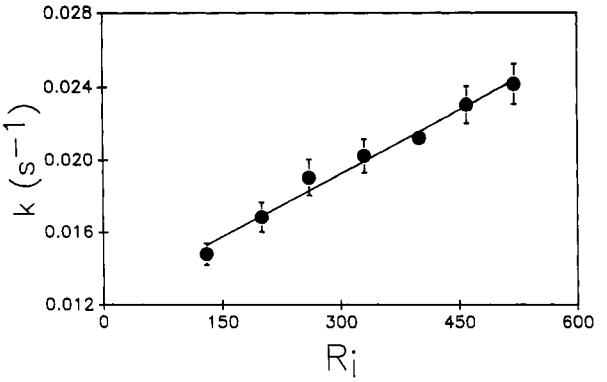

FIGURE 4: Dependence of the kinetic rate constant on the lipid to protein ratio. To 400 μL of colicin A thermolytic fragment (20 μg/mL in 50 mM Tris–acetate buffer, pH 5, 30 °C) were added different amounts of Br-DOPG vesicles, and the kinetic rate constant was calculated. Each point is the mean of at least three experiments. Bars are standard deviations.

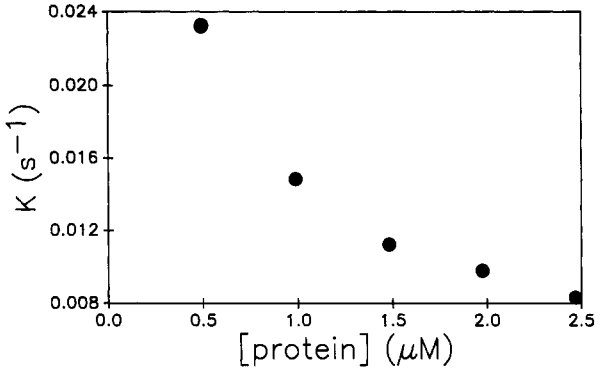

FIGURE 5: Dependence of the kinetic rate constant on protein concentration. To 400 μL of colicin A thermolytic fragment (different concentrations in 50 mM Tris–acetate buffer, pH 5, 30 °C) was added 15 μL of 10 mg/mL Br-DOPG vesicle suspension in the same buffer, and the kinetic rate constant was calculated. Each point is the mean of at least three experiments.

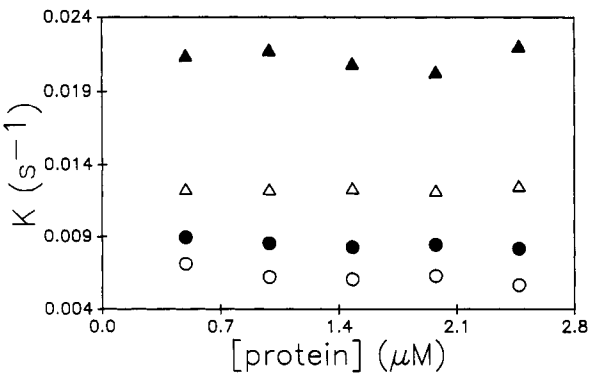

FIGURE 6: Dependence of the kinetic rate constant on protein concentration, at given R_i values. To 400 μL of colicin A thermolytic fragment (different concentrations in 50 mM Tris–acetate buffer, pH 5, 30 °C) was added the required amount of a 10 mg/mL Br-DOPG vesicle suspension in the same buffer to obtain a given R_i value, and the kinetic rate constant was calculated. (▲) R_i = 394; (△) R_i = 192; (●) R_i = 96; (○) R_i = 48.

Table I: Dependence of the Kinetic Rate Constant on pH[a]

pH	F_0	F_∞	% RF	k (×10⁵ s⁻¹)
4.0	489.5 ± 4.7	80.4 ± 2.2	16.4	11234.5 ± 1998.2
4.2	520.0 ± 6.1	84.7 ± 2.5	16.3	12176.5 ± 408.0
4.5	497.7 ± 10.9	72.4 ± 2.2	14.5	8916.3 ± 537.5
4.8	538.5 ± 10.2	75.5 ± 5.6	14.0	8484.3 ± 500.7
5.0	560.2 ± 34.6	83.0 ± 6.7	14.8	1748.5 ± 38.2
5.4	620.9 ± 24.7	89.7 ± 4.3	14.5	308.5 ± 16.8
6.0	686.7 ± 20.0	84.8 ± 11.2	12.3	23.3 ± 6.8

[a] The integral of the tryptophan emission fluorescence spectrum of 400 μL of colicin A thermolytic fragment (20 μg/mL in Tris–acetate buffer, different pH, 30 °C) is the F_0 value; 15 μL of a 10 mg/mL Br-DOPG vesicle suspension in the same buffer was added, and the kinetic rate constant was calculated. After completion of the quenching process, the tryptophan fluorescence emission was recorded, and allowed for the calculation of F_∞. The ratio F_∞/F_0 gives the percentage of residual fluorescence (% RF) at each pH tested. Each value is the mean of four experiments. Standard deviations are shown.

thought of as a result of protein competition for lipid surface. In both cases, the insertion rate is proportional to R_i (R_i = [lipid]/[protein], and k is directly proportional either to [lipid] or to 1/[protein]). This means that at a given R_i, the insertion rate constant is the same, regardless of the free protein concentration. In order to test this hypothesis, we measured k at given R_i values, and different protein concentrations (at every protein concentration, the correct amount of lipid was added in order to keep R_i constant). The results are represented in Figure 6. At every R_i value tested, the insertion rate is independent of protein concentration, and the plot k vs R_i gives the same straight line as shown in Figure 4. These results demonstrate that the insertion process depends neither on the lipid nor on the protein bulk concentration. Instead, it is a clear function of the lipid to protein ratio R_i and therefore the effective two-dimensional surface concentrations of both protein and lipids.

Effect of Negative Charge Density on Insertion into Br-DOPG Vesicles. In order to confirm the use of Br-DOPG vesicles as a suitable system, we must compare its results with previous data. One of the parameters that affect the insertion of colicin A into bilayers is the negative charge density (Pattus et al., 1983; Massotte et al., 1989; Lakey et al., 1991). We changed the negative charge density by forming vesicles with Br-DOPG + Br-DOPC at different molar ratios, but maintained the lipid to protein ratio (R_i = 400). The calculated rate constant decreases when the molar fraction of Br-DOPC increases. The relationship is linear. For insertion to occur, high negative charge density is necessary. The rate of the process is greatly affected by even small amounts of neutral lipids. In 100% Br-DOPG vesicles, k is 0.020 ± 0.0005 s⁻¹. At 0.25 Br-DOPC molar fraction, the calculated k is approximately half of the value in 100% Br-DOPG, while at 0.5 molar ratio Br-DOPC, by extrapolation of the experimental points, k is near zero. These results agree with those obtained by Massotte et al. (1989) using DMPC/DMPG mixtures.

Effect of pH on Insertion into Br-DOPG Vesicles. The insertion of the pore-forming domain of colicin A into lipid monolayers (Pattus et al., 1983) or planar lipid bilayers (Collarini et al., 1987) in pH-dependent. Thus, insertion into Br-DOPG vesicles was studied at different pH values (Table I). As the fluorescence quantum yield of tryptophan is pH-dependent, we measured the tryptophan fluorescence emission spectrum before the addition of Br-DOPG vesicles and after addition of the lipids, when the sample had reached equilibrium. The integrals of the spectra gave us the F_0 and F_∞ values for each sample, at each different pH. The ratio F_∞/F_0 is the residual fluorescence, and gives an estimation of the degree of insertion. At every pH tested, the residual fluorescence was 12–16% of the initial value, and, therefore, we can conclude that at every pH value, all the protein had bound to the vesicles.

At pH values above 5.4, the rate of insertion is very low (or, conversely, the deinsertion rate is high). Below pH 5.4, the rate constant starts to increase as the pH is lowered and levels off at pH 4.2. The apparent midpoint for the transition

between slow and fast insertion is at pH 4.8, slightly lower than the value reported in lipid monolayers (Pattus et al., 1983). The pH dependence of the full-length colicin A insertion process is practically identical to that of the thermolytic fragment (data not shown).

DISCUSSION

In order to exert their lethal activity, bacterial toxins must interact with the phospholipid bilayer of the target organism, either to form a pore in it or to traverse it and reach the intracellular target (Pattus et al., 1990; Schwarz et al., 1987; Menestrina et al., 1989; Koehler & Collier, 1991; Demel et al., 1991; Sekharam et al., 1991; Jiang & London, 1990). The nature of this interaction seems to be common to many of these toxins (Parker et al., 1990), and in some cases, a detailed kinetic analysis has been carried out in order to elucidate the mechanism of this process (Schwarz et al., 1987; Menestrina et al., 1989; Sekharam et al., 1991). Thus, alamethicin insertion into small unilamellar DOPC vesicles (Schwarz et al., 1987) follows a single-exponential time course which can be formally described as a one-step association/dissociation process, with separate rate constants. The interaction of melittin with small unilamellar DMPC vesicles (Sekharam et al., 1991) follows a similar time course. In some cases, such as the interaction of tetanus toxin with small unilamellar vesicles (Menestrina et al., 1989), the process is more complicated, and has to be described by the use of two-exponential components.

Liposomes made of brominated phospholipids are an efficient system for the study of these kinds of lipid–protein interactions and were essential in the case of colicin A, the fluorescence of which was not affected upon binding unbrominated phospholipids (Lakey et al., 1991). The effect of bromine addition to DOPC has been extensively studied, and might be extrapolated to DOPG: The motional properties of bilayers of DOPC and Br-DOPC are similar, the transition temperature for Br-DOPC occurs below 5 °C, the perturbation of the membrane caused by the bromine atoms is minimal because lipid affinities and enzyme activities are preserved (East & Lee, 1982), and vesicles can be made of 100% bromolipid. Quenching by bromolipid occurs over a short distance and is mainly static in nature (East & Lee, 1982; Markello et al., 1985), and the residence time of the lipid in a certain position of the bilayer is very long when compared with the fluorescence lifetime of the excited fluorophore (Yeager & Feigenson, 1990). The intrinsic fluorescence of the colicin A thermolytic fragment is quenched by Br-DOPG vesicles (Figure 1A). However, the maximum emission wavelength is constant (Figure 1B), indicating that the polarity of the fluorophore environment has not changed, as already reported (Lakey et al., 1991). The correlation between the effects of pH and surface charge on the insertion kinetics with those on steady-state binding measured by other methods (Massotte et al., 1989; Lakey et al., 1991) further demonstrates the validity of this system.

The intrinsic fluorescence quenching of the protein by the brominated vesicles follows a single-exponential time course, as shown in Figure 3. In the case of alamethicin and tetanus toxin, the proposed mechanism of interaction is (Schwarz et al., 1987)

$$P + \text{lipid} \leftrightarrow P_{as.} \leftrightarrow P_{ins}$$

A similar mechanism for the mode of action of phospholipase A_2 [for a review, see Jain et al. (1989)] has been proposed.

The first step is a bimolecular reaction and leads to a protein population in close contact with the vesicle surface. It is generally fast with, as an upper limit, a diffusion-controlled step. This associated ($P_{as.}$) species, after equilibration with the local conditions at the surface of the vesicle, undergoes a transition to an insertion-competent state (conformational change) and yields the inserted form of the molecule, P_{ins}. This second process should follow first-order kinetics. With alamethicin, the two steps are comparably fast (Schwartz et al., 1987) while with phospholipase A_2, on negatively charged vesicles, the transition to the catalytically productive inserted state is 10^6-fold slower than the first step (Jain et al., 1988, 1989). The same scheme can be applied, as a first approximation, to the case of colicin A thermolytic fragment insertion into Br-DOPG vesicles. Analysis of the influence of lipid and protein concentrations on the kinetics should indicate which steps are rate-limiting for colicin A.

Experiments were carried out in a range of lipid to protein molar ratios between 65 and 524, where complete binding of the protein to the vesicles occurs (see Results). The reaction rate constant was found to be strictly dependent upon R_i and not simply on lipid or protein concentration. If one assumes that the rate-limiting process is a "bimolecular" reaction in the sense that a protein molecule must collide with a lipid vesicle for insertion to occur, the reaction rate, at constant lipid concentration, would be expected to remain constant with increasing protein concentration. This was not the case; k decreased when the protein concentration was increased. Exactly the same result was observed in the binding of melittin to lipid vesicles (Sekharam et al., 1991). This could be explained as a competition either in the binding or in the insertion process. In the first case, the protein molecules that bind first to the vesicle might shield charges on the lipid headgroups and/or induce changes in the bilayer which make successive binding events less probable. However, if this were true, the kinetics should not follow a first-order rate because the increasing amount of associated colicin molecules with time would affect the kinetic constant. The strong fit between experimental and theoretical kinetics using a single kinetic constant (Figure 3) demonstrates that this is not the case. The alternative explanation for this result is an effect of competition on the transition from the associated to the inserted conformation. In this case, the insertion step is rate-limiting, all the protein is already adsorbed to the surface of the vesicle, and the unfolding process is interfered with by the presence of neighboring protein molecules, consequently slowing the process (see later). The direct correlation between k and R_i (the inverse of the total protein surface concentration) strongly suggests such an effect.

At a given R_i value, the reaction rate is independent of protein and lipid concentrations, or in other words, dilution of both component species does not affect the rate constant. This again implies that we are not observing the bimolecular collision between lipids and proteins in solution.

There must, however, be a protein–lipid encounter process which behaves as a bimolecular reaction. It is probable that, as in the case of melittin (Sekharam et al., 1991) or phospholipase A_2 (Ghomashchi et al., 1991), the protein–vesicle encounter time is too fast to be detected by our experimental system. Hence, upon addition of the vesicles to the protein solution, association of the pore-forming domain of colicin A with the vesicles is, without stopped-flow-type measurements, apparently instantaneous. Electrostatic interactions might play an essential role in this process (Parker et al., 1989). This event is thus "invisible" under our experimental conditions for

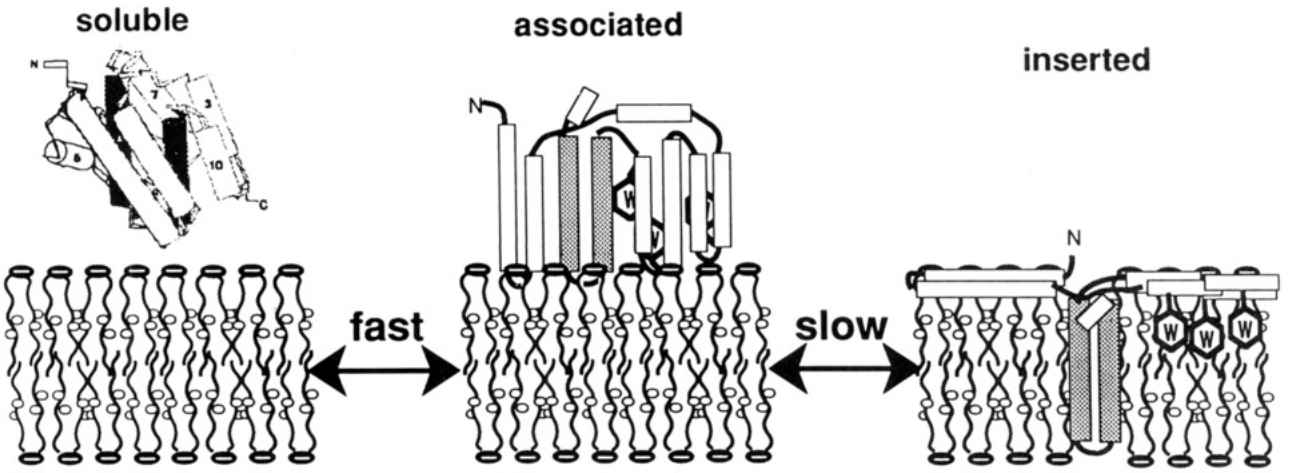

FIGURE 7: Model for the spontaneous insertion of the colicin A thermolytic fragment into phospholipid bilayers [adapted from Parker et al., (1989, 1991)]. Electrostatic interaction of the pore-forming domain of colicin A with the negatively charged lipids (left). This process is fast. The local acidic pH induces the formation of the "molten globular state" which accumulates at the interface (middle). The structure opens as an "umbrella", leading to the insertion of the hydrophobic hairpin into the lipid bilayer (right). The tryptophan residues become accessible to the bromine atoms located in the middle of the fatty acyl chain.

two reasons: first, no quenching will occur in this encounter state; second, the insertion process is the rate-limiting step. Once the protein is associated with the vesicle, the local environment at the surface of the vesicle promotes the transition from the native to the insertion competent state, which is then quenched upon insertion. This is the "visible" process whose reaction rate we have calculated.

The process can then be described as

$$[\text{colicin} + \text{vesicles}] \overset{\text{very fast}}{\leftrightarrow} [\text{associated colicin}] \overset{\text{slow}}{\leftrightarrow} [\text{inserted colicin}]$$

The first step has not been measured here, but appears to be completed within the mixing time of the solution (6–10 s). Once the protein has "landed" on the surface of the vesicle, the transition to an insertion-competent state may take place, and it is either this process or the insertion itself which is rate-limiting and thus measured by the quenching which takes place during the insertion. This model is essentially identical to the one proposed from the X-ray structure by Parker et al. (1989, 1991) (Figure 7).

Negatively charged lipids and acidic pH are required for colicin A insertion into membranes. The role of the negatively charged lipids has been postulated to promote electrostatic interaction with positively charged residues on the colicin A pore-forming domain (Figure 7, left). In a recent study (van der Goot et al., 1991), we have shown that the colicin A pore-forming domain undergoes a transition to the "molten globule" state at acidic pH, the main characteristics of which are conservation of secondary structure, absence of tertiary structure, and water inaccessibility to the core of the molecule (the tryptophan emission wavelength remains unchanged) (Dolgikh et al., 1981). The "molten globule" has been observed as an early intermediate in protein folding and was suggested as a possible model for the membrane insertion-competent state of proteins (Bychkova et al., 1988).

The kinetic scheme and the results presented in this study indicate that an intermediate accumulates at the membrane surface. The key role of pH and negatively charged lipids on the kinetics suggests that this intermediate is related to the molten globular state. Negatively charged lipids are known to lower the surface pH with respect to the bulk pH (Winiski et al., 1986). This ΔpH is due to the presence of the electrostatic potential which is formed at the surface of the vesicle. When this ΔpH is taken into account, an excellent correlation can be found between the increase of the rate constant k and the proportion of colicin A adopting the characteristics of the molten globule state in solution (van der Goot et al., 1991). Increasing amounts of neutral phospholipid decrease both the ΔpH and the insertion rate constant. Therefore, it may be concluded that the presence of negative charges on the surface of the vesicle promotes the transition from the native protein to the molten globule state and, hence, the insertion rate. The tryptophans remain inaccessible within this insertion-competent state and therefore should not be quenched by the bromine atoms within the core of the bilayer (Figure 7). This state can be identified with the colicin E1 insertion-competent intermediate described by Merrill et al. (1990). Similar effects have been observed with tetanus toxin (Menestrina et al., 1989), anthrax toxin (Koehler & Collier, 1991), diphtheria toxin (Demel et al., 1991), and exotoxin A (Jiang & London, 1990).

The observed protein competititon for lipid surface during the insertion process (k increases with R_i) could be explained in light of this model. The molten globule state concentration is highly dependent on the physicochemical properties of the lipid/water interface (local pH, surface charge density, etc.). At low R_i, the high protein surface concentration may diminish the capacity of the interface to induce the molten globular state competent for membrane insertion.

In conclusion, the use of brominated phospholipids has permitted the study of the previously unknown kinetics of colicin insertion into vesicles. It reveals that the insertion is a slow process which occurs after a fast binding to the lipid surface. The insertion is dependent upon the number of lipid molecules per protein, pH, and negative charge density. The highly quantitative kinetic data have enabled comparison of the insertion process with data concerning the formation of the molten globule state in solution. Stopped-flow energy-transfer experiments, currently underway, support the above model and will be the subject of a subsequent paper on the rapid kinetics of colicin A insertion.

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