The Calcium-binding C-terminal Domain of Escherichia coli α-Hemolysin Is a Major Determinant in the Surface-active Properties of the Protein*

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α-Hemolysin (HlyA) from Escherichia coli is a protein toxin (1024 amino acids) that targets eukaryotic cell membranes, causing loss of the permeability barrier. HlyA consists of two main regions, an N-terminal domain rich in amphipathic helices, and a C-terminal Ca2+-binding domain containing a Gly-rich Asp-rich nonapeptide repeated in tandem 11–17 times. The latter is called the RTX domain and gives its name to the RTX protein family. It had been commonly assumed that membrane interaction occurred mainly if not exclusively through the amphipathic helix domain. However, we have cloned and expressed the C-terminal region of HlyA, containing the RTX domain plus a few stabilizing sequences, and found that it is a potent surface-active molecule. The isolated domain binds Ca2+ with about the same affinity (apparent Kd ≈150 μM) as the parent protein HlyA, and Ca2+ binding induces in turn a more compact folding with an increased proportion of β-sheet structure. Both with and without Ca2+ the C-terminal region of HlyA can interact with lipid monolayers spread at an air-water interface. However, the C-terminal domain by itself is devoid of membrane lytic properties. The present results can be interpreted in the light of our previous studies that involved in receptor binding a peptide in the C-terminal region of HlyA. We had also shown experimentally the distinction between reversible membrane adsorption and irreversible lytic insertion of the toxin. In this context, the present data allow us to propose that both major domains of HlyA are directly involved in membrane-toxin interaction, the nonapeptide repeat, calcium-binding RTX domain being responsible for the early stages of HlyA docking to the target membrane.

α-Hemolysin (HlyA)3 is a toxin protein (107 kDa) secreted by some pathogenic strains of Escherichia coli. HlyA targets the plasma membrane of eukaryotic host cells, where it becomes permanently inserted and causes breakdown of the membrane permeability barrier (for reviews, see Refs. 1–4). The secondary structure of HlyA shows two clearly distinguishable regions, the N-terminal domain, characterized by nine amphipathic helices, presumed to interact with the hydrophobic matrix of the host membrane (5), and the C-terminal domain, whose main feature is the presence of 11–17 nonapeptides repeated in tandem, of consensus sequence GGXGXDUX (X, any amino acid; U, large hydrophobic amino acid) (1, 6). The latter region is characteristic of a protein family known as the RTX family (3) and binds one Ca2+ ion per nonapeptide (1–3, 6).

HlyA interaction with the target membrane is a complex process, of which many aspects are still poorly understood. The process includes two main steps, namely reversible binding (adsorption) and irreversible insertion (7). Binding occurs probably via specific receptors (8–10), although the latter are not essential, since the toxin has also a lytic effect, at higher concentrations, on pure lipid liposomes (9). A recent report (11) cast doubt on our previous observation that α-glycophorin acted as a HlyA receptor in human red blood cells (9). However, in their studies Valeva et al. (11) used rabbit erythrocytes, which lack α-glycophorin (12), instead of human ones. As a consequence their observation of non-saturable hemolysis by HlyA in rabbit red blood cells actually confirms the proposed role of α-glycophorin as a receptor.

We had also shown that a short sequence in the C-terminal domain (aa 914–936) was the main HlyA region recognizing the α-glycophorin receptor (10), and earlier reports suggested that the nonapeptide repeat, calcium-binding region was involved in the early stages of receptor/membrane binding by HlyA (13, 14). It was therefore decided to clone and express the nonapeptide repeat region, to study its properties in the absence of the amphipathic helix domain. According to the crystal structures of two other (non-lytic) RTX proteins, namely Pseudomonas aeruginosa alkaline protease (AprA) (15, 16) and Serratia marcescens metalloprotease (PrtA) (17, 18), the repetitive sequences form a parallel β-roll structure, to which Ca2+ ions bind. The first six residues of this sequence motif form a loop and the last three form a short β-strand. Each thalene-1,2,3-trisulfonic acid; DPX, p-xylene bis(pyridinium bromide); LUV, large unilamellar vesicles.
Ca\(^{2+}\) ion binds a pair of these loops. Although the role and mechanism of the repetitive sequences remains to be fully understood, analysis of the secretion of mutant proteins of HlyA (19) as well as the characterization of various fragments from the adenylate cyclase toxin (CyaA) (20) suggest that the repetitive sequences are not required for secretion but are essential for activity. Bauche et al. (20) have also shown that, in CyaA, the adjacent polypeptide sequences are essential for the folding and calcium responsiveness of the RTX module.

With respect to protein folding it can be anticipated that the right-handed \(\beta\)-2-solenoid (\(\beta\)-roll) adopted by the repeats upon Ca\(^{2+}\) binding is not able to fold correctly by itself. It can probably accommodate a rather variable number of nonapeptides, but a number of hydrogen bonds remain unsatisfied at each end regardless of its length. In solved structures of proteins containing this motif (Protein Data Bank codes: 1SAT, 1K7I, and 1KAP) the \(\beta\)-roll region edges are hydrogen-bonded to strands of adjacent \(\beta\)-sandwich. With the aim of identifying residues belonging to the putative flanking \(\beta\)-region we used the PHD PredictProtein Server. The N-terminal end of the protein is predicted to contain predominantly \(\alpha\)-helical structure. The N-terminal end of our construction was chosen to lie at the C-terminal end of the \(\alpha\)-helical region, i.e. the start of the segment predicted as \(\beta\)-strand that precedes the nonapeptide repeats. An additional region is predicted as \(\beta\)-strand from residues following the \(\beta\)-roll up to residue 963. Three helices are identified in the C-terminal end of the protein that were decided to be left integral in the construction to increase the polypeptide stability (Fig. 1). In consequence, a DNA segment expressing amino acids 602–1024 of HlyA was cloned. A very similar polypeptide was used in the study by Hyland et al. (5).

In a recent report (21) we described the insertion of HlyA into lipid monolayers extended at a lipid-water interface and showed that such insertion was not necessarily coupled to membrane lysis. In that work we implicitly assumed that the protein region interacting with the lipid monolayer was the N-terminal, amphiphatic helix region. However, our studies with the isolated calcium-binding domain reveal that this part of the protein may be the one that is (reversibly) adsorbing to the membrane in the early stages of HlyA-membrane interaction. This would lead to a novel interpretation of the role assigned to the nonapeptide repeat domain of RTX toxins.

**EXPERIMENTAL PROCEDURES**

*Materials—* Egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were from Lipid Products (South Nutfield, UK). Cholesterol was supplied by Avanti Polar Lipids (Alabaster, AL). ANTS (8-amino-1,3,6-naphthalene-1,2,3-trisulfonic acid) and DPX (p-xylene bis(pyridinium bromide)) were from Molecular Probes (Eugene, OR). \(^{45}\text{Ca}\) in the form of \(\text{CaCl}_2\) was obtained from Amersham Biosciences (Uppsala, Sweden). Horse erythrocytes were purchased from Microlab (Madrid, Spain).

**Construction of HlyA\(\Delta N601)—** The mutant HlyA\(\Delta N601\) was obtained by PCR amplification of the \(hlyA\) fragment including the DNA sequence corresponding to aa 602–1024, using as primers two oligonucleotides that contain in their sequences Ndel (A) and BamHI (B) restriction sites (in boldface), respectively (A, 5'-AGATATACATATGGTCGGTAATAACCAGTA-3' \((T_m = 61.3 \degree C)\) and B, 5’-GACGATCCTTAGCTGTATGGCTAGGTTATTGAGTTCCGTCATAACTAAA-3' \((T_m > 75 \degree C)\)).

The fragment obtained was sequenced and the sequence was found to be the expected one. The fragment was then inserted in the expression vector pET3a, so that \(hlyA\) was expressed from the T7 polymerase promoter.

**Purification of Wild-type and Mutant HlyA\(\Delta N601\) Proteins—** Wild-type HlyA was purified as described previously by Ostołaza et al. (22). The HlyA\(\Delta N601\) purification protocol was achieved by extracting the protein from inclusion bodies. HlyA\(\Delta N601\) was expressed in *E. coli* BL21 C41(DE3). Bacteria were grown in Luria-Bertani liquid medium with 0.1 mg/ml ampicillin, at 37 \degree C, to \(A_{600} = 0.6–0.8\). 1 mm isopropyl \(\beta\)-d-thiogalactopyranoside was then added, and expression was allowed to occur for 3 h. Cells were harvested by centrifugation (6000 \times g, 10 min, 4 \degree C). The cell pellet was resuspended in “TES buffer” (10 mM EDTA, 10% (v/v) sucrose, 50 mM Tris, pH 7.0), and stored at −20 \degree C overnight. The cells were thawed at 37 \degree C and treated with lysozyme (0.5 mg/ml), 2.5 mM benzamidine and 0.1 mM PMSF in “lysis buffer” (1 mM dithiothreitol, 0.1 mM EDTA, 0.5% (v/v) Triton X-100, 50 mM Tris, pH 7.0). Cell lysis was allowed to occur for 45 min in an ice bath, followed by 25 sonication cycles (15 s sonication at 12 \mu m amplitude + 25 s rest) in a Soniprep 150 MSE probe sonicator. The suspension was centrifuged at 18,000 \times g (20 min, 4 \degree C), and HlyA\(\Delta N601\) inclusion bodies, recovered in the pellet, were resuspended in “TU buffer” (6 M urea, 50 mM Tris, pH = 7.0). The sample in TU buffer was then applied to a DEAE-Sepharose ion exchange column (GE Healthcare, Uppsala, Sweden). The protein was eluted in a stepwise 0–250 mM NaCl gradient in the same TU buffer. The protein eluted at \(\approx 50\) mM NaCl. Flow rate was 1
ml/min; 5-ml fractions were collected. The purified protein was stored at −20 °C in 50 mM NaCl, 6 mM urea, 50 mM Tris-HCl, pH 7.0. HlyAΔN601 was identified in SDS-PAGE gels by Western blotting with rabbit anti-HlyA polyclonal antibodies.

Binding of 45Ca—The amount of Ca2+ bound to the proteins was measured by isotopic dilution of 45Ca in the form of calcium chloride, as described previously by Ostolaza et al. (23). Protein (230 nM) was incubated with increasing 45CaCl2 concentrations for 15 min at room temperature; total volume was 1 ml. Free and bound Ca2+ were separated by filtration through cellulose mixed-ester filters (Millipore). Nonspecific binding was estimated by adding an excess (20-fold) non-radioactive Ca2+.

Intrinsic Fluorescence Measurements—The intrinsic fluorescence of HlyAΔN601 was used to detect conformational changes promoted by calcium binding. Fluorescence was measured in a SLM Aminco 8100 spectrofluorometer. The excitation wavelength was 295 nm, and emission was scanned between 310 and 400 nm. Slits were 4 nm; 1-ml cuvettes with a 1-cm optical path were used. Protein concentration was 230 nM; fluorescence was measured after equilibrating (5 min) for each calcium concentration. The proteins were dialyzed, when required, against TC buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.0) or TC + 6 mM urea buffer in the presence of 0.1 mM EGTA at 4 °C, overnight, and then against EGTA-free buffers, at 4 °C, for 2 h.

Infrared Spectroscopy Measurements—The protein solution was concentrated to 5 mg/ml using Millipore filters (30,000 molecular weight cutoff). To remove all the urea present in the sample, the concentrated protein was dialyzed against TC buffer at 4 °C for 6 h. The dialyzed proteins were centrifuged at 15,000 × g for 30 min at 4 °C to avoid protein aggregates. Protein aliquots (0.06 ml) were dried in a Savant evaporator. Finally, they were resuspended in the appropriate volume of D2O and the infrared spectra were collected. The spectra were recorded in a Bruker Tensor 27 (Bruker Optik GmbH, Ettlingen, Germany) spectrometer equipped with a liquid nitrogen-refrigerated mercury-cadmium-telluride detector. Samples were measured using a demountable Peltier liquid cell (Biotools Inc.) with evacuated calcium fluoride 50-mm diameter BioCell windows (Biotools), 25 μm optical path. 143 interferograms/min for each, background and sample, were collected at 2 cm−1 resolution and averaged after each minute. Opus 5.0 software from Bruker Optics was used for data acquisition. Data treatment and band decomposition of the original amide I have been described elsewhere (24, 25).

Circular Dichroism—CD spectra were recorded in a J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan) using quartz cuvettes 1 mm optical path. The protein (15 μM) was suspended in 20 mM Tris-HCl, 50 mM NaCl, pH 7.0. Spectra were recorded in the 205–250 nm range.

Surface Pressure Measurements—Surface pressure experiments were carried out with a MicroTrough S system from Kibron (Helsinki, Finland) at 25 °C, under constant stirring. The aqueous phase consisted of 1.0 ml 20 mM Tris-HCl, 150 mM NaCl, pH 7.0. The lipid, dissolved in chloroform/methanol (2:1), was gently spread over the surface until the desired initial surface pressure was attained. The protein was injected with a micropipette through a hole connected to the subphase. The increment in surface pressure versus time was recorded until a stable signal was obtained. The plots show one of two closely similar independent measurements.

Hemolysis Assay—A standard red blood cell suspension was used, obtained by diluting the erythrocytes with saline so that 37.5 μl of the mixture in 3 ml distilled water gave an absorbance of 0.6 at 412 nm. Equal volumes of the standard suspension of washed horse erythrocytes were added to serial 2-fold dilutions of proteins in buffer (150 mM NaCl, 10 mM CaCl2, 20 mM Tris-HCl, pH 7.0). The assay was carried out in microtitre plates; cell and toxin mixtures were incubated at room temperature for 3 h, then centrifuged in microtitre plates in a Jouan centrifuge at 4000 × g, 10 min, 4 °C. The supernatants (100 μl) were diluted in 1 ml of distilled water, and the absorbance was read at 412 nm. The blank (zero hemolysis) consisted of a mixture of equal volumes of buffer and erythrocytes.

Large Unilamellar Vesicles—Large unilamellar vesicles (LUV) made of egg phosphatidylcholine:egg phosphatidyethanolamine:cholesterol (2:1:1) were prepared by extrusion through polycarbonate filters (Nuclepore, Pleasanton, CA), pore size 0.1 μm. Buffer was 150 mM NaCl, 20 mM Tris-HCl, pH 7.0. The diameter of the resulting vesicles was of ~100 nm, according to quasi-elastic light scattering measurements.

Release of Liposomal Contents—Leakage of vesicular aqueous contents was assayed according to Ellens et al. (26), with the fluorescent probe ANTS and its quencher DPX entrapped in the liposomes. LUV were prepared in 70 mM NaCl, 12.5 mM ANTS, 45 mM DPX, 20 mM Tris-HCl, pH 7.0. Non-entrapped probes were removed passing the LUV through a Sephadex G-75 column, eluted with 150 mM NaCl, 20 mM Tris-HCl, pH 7.0. The proteins (HlyA and HlyAΔN601) at different concentrations were mixed with 100 μM lipid in a total volume of 1 ml, with continuous stirring, at 25 °C. Buffer was as above, with the addition of 10 mM CaCl2. ANTS fluorescence was recorded continuously (λex = 355 nm; λem = 520 nm). When leakage reached equilibrium, Triton X-100 was added (final concentration 0.1% w/v) to induce 100% release. Percent release was computed as follows: % release = (Ff − Fp)/(F100 − Fp) × 100, where Ff, F100, and Fp were the fluorescence intensity values observed after protein addition, after Triton X-100, and before any addition, respectively.

RESULTS

Purification of HlyAΔN601—A DNA segment coding for the C-terminal domain of HlyA (aa residues 602–1024) was amplified by PCR as indicated under “Experimental Procedures” and cloned in the expression vector pET3a, under the T7 polymerase promoter. E. coli strain B121 C41(DE3) allowed highest expression of the protein among three B121(DE3) strains tested. Optimum yields (38 mg/l) were obtained after 3 h post-isopropyl β-D-thiogalactopyranoside induction at 37 °C. The protein accumulated in the bacterial cells in the form of inclusion bodies. The latter were solubilized in a buffer containing 6 mM urea (22) and loaded onto an ion exchange DEAE-Sepharose column, from which the protein was eluted with a 0–250 mM NaCl gradient. HlyAΔN601 eluted at 50 mM NaCl with a high degree of purity. Purification was followed by the presence in
The Calcium-binding C-terminal Domain of E. coli HlyA

**FIGURE 2. SDS-PAGE of crude and purified HlyΔΔN601.** A, Coomassie Blue stain. Lanes: 1, molecular weight markers; 2, crude extract; 3, purified protein as eluted from the DEAE-Sepharose column. B, lane 1, molecular weight markers, silver stain; lane 2, purified protein as eluted from the DEAE-Sepharose column, silver stain; lane 3, purified protein as eluted from the DEAE-Sepharose column, Western blot with rabbit anti-HlyA antibodies.

The gels of a band of molecular mass ~46 kDa that was recognized by anti-HlyA antibodies (Fig. 2). The protein was stable for months after storage at -20 °C in 6 M urea, just as the parent molecule HlyA (27).

**Calcium Binding**—Calcium binding by HlyΔΔN601 was directly assayed using 45Ca. After incubating the protein with the radioactive isotope, an excess cold isotope was added to remove non-specifically bound calcium. The results in Fig. 3 show that HlyΔΔN601 binds about 12 Ca2+ ions/molecule, with a sigmoidal binding isotherm and an apparent $K_{0.5} \approx 150 \mu M$ Ca2+. The number of bound Ca2+ ions corresponded very well with the predicted number of canonical nonapeptide repeats in the protein, suggesting that, under these conditions, all nonapeptides were accessible to the solvent. This was in contrast to the situation when native HlyA was used (23) when only 4 Ca2+/protein molecule were bound, and the apparent $K_{0.5}$ was ~100 $\mu M$ Ca2+. Ca2+ binding by the whole protein is also shown in Fig. 3 (inset). These results indicated that, in the whole protein, most of the nonapeptide repeats were not easily accessible to the solvent, in agreement with our proposed hypothesis (23).

Previous studies from this laboratory (23, 27) had shown that Ca2+ binding induced significant changes in the HlyA intrinsic fluorescence. The C-terminal fragment HlyΔΔN601 contains only one (W914) of the four Trp residues in the native protein. Trp-914 is located immediately after the nonapeptide repeat domain (Fig. 1). Fluorescence emission spectra of HlyΔΔN601, arising essentially from the intrinsic fluorescence of Trp-914, showed a very large increase (almost 3-fold) in fluorescence intensity in the presence of Ca2+ (Fig. 4). The effect was dose-dependent, and the binding isotherm was again sigmoidal (see Fig. 3), its apparent $K_{0.5}$ being ~50 $\mu M$ Ca2+. The latter figure was clearly lower than the one shown in Fig. 3, when binding was directly measured. Thus Trp-914 was sensing in particular the binding of Ca2+ to sites with a somewhat higher affinity than the average. The possibility that some Trp residues in native HlyA could be more sensitive to Ca2+ in the solvent than others had already been put forward by Ostolaza et al. (23).

The Ca2+-induced increase in fluorescence intensity (Fig. 4) was not accompanied by any significant shift in the maximum wavelength of fluorescence emission. Fluorescence intensity increments are often accompanied by a blue shift of the emission maxima when the fluorophore environment becomes less polar. Both increased intensity and blue shift were observed for native HlyA (23). The absence of emission band shift in HlyΔΔN601 indicates that the Ca2+ effect cannot be simply interpreted in terms of Trp-914 sensing a less polar environment.

As its parent molecule, HlyΔΔN601 was best stored in 6 M urea, so that urea had to be removed before performing any tests of biological activity (or IR spectroscopy, see below). Thus it was important to test whether different procedures of urea removal had an influence on the protein properties. Fluores-
cence changes caused by Ca\(^{2+}\) binding were chosen as a convenient and sensitive activity to test. In the experiments shown in Figs. 3 and 4, the protein had been dialyzed against a calcium-free buffer. For comparison, fluorescence spectra were also recorded under conditions when (i) the protein had been dialyzed against calcium-free buffer containing 6 M urea, then urea had been diluted out in the fluorescence spectroscopy cuvette (typically 10 \(\mu\)l of the protein suspension were added to 1 ml urea-free, calcium-free buffer), or (ii) a non-dialyzed fraction eluted from the DEAE-Sepharose column had been directly diluted in urea-free, calcium-free buffer in the cuvette. As seen in Fig. 5, all three procedures gave essentially similar fluorescence changes in response to Ca\(^{2+}\) addition. These results confirmed that the protein could be stored in 6 M urea and then dialyzed without impairment of its Ca\(^{2+}\)-binding properties and presumably of its tertiary structure.

**IR and CD Spectroscopy Structural Studies**—The observation that HlyAΔN601 could be dialyzed without apparent loss of structure/properties opened the way to IR structural studies. IR spectroscopy is a useful tool in the study of protein structure in solution (24, 25), but urea interferes markedly with the main protein amide absorption region, the so-called amide I band (1600–1700 cm\(^{-1}\)). Dialysis under the conditions given in the Methods section provided urea-free protein suspensions. The whole HlyA exists in solution in equilibrium between the monomer and aggregated forms, and the aggregates appear to be a form of storage rather than an expression of denaturation (27). Centrifugation of urea-free HlyAΔN601 provided a supernatant essentially free from protein aggregates. At the concentration used in our studies (≈2 mg/ml), aggregation was slow enough to assert that the IR samples consisted essentially of monomeric HlyAΔN601. All buffers for IR spectroscopy contained D\(_2\)O instead of H\(_2\)O.

The amide I region of IR spectra of HlyAΔN601 in the presence and absence of Ca\(^{2+}\) is shown in Fig. 6. The different spectral lineshapes revealed clear effects of Ca\(^{2+}\) on the protein secondary structure. The spectra were decomposed as described elsewhere (24, 25) to obtain the various bands corresponding to the different secondary structure elements. The component bands are also shown in Fig. 6. The positions, assignments, and percent areas for each band component are given in Table 1. The predominant structure was the \(\beta\)-sheet, and its proportion increased with Ca\(^{2+}\). This is in agreement with the structural role of Ca\(^{2+}\) in the \(\beta\)-roll structure that has been proposed for the nonapeptide repeat domain on the basis of x-ray data from a homologous domain in a P. aeruginosa alkaline protease (15), with the \(\beta\)-sheet hydrophobic side chains facing the protein core and stabilizing the structure.

Another clear effect of Ca\(^{2+}\) was to decrease the proportion of secondary structure involved in nonspecific intermolecular contacts (bands at 1684 cm\(^{-1}\) and 1613–1615 cm\(^{-1}\)), probably as a result of the protein becoming more compact and stable in the presence of the cation.

Circular dichroism was used as an alternative method to study calcium-induced changes in HlyAΔN601 secondary structure. In the absence of calcium (20 mM Tris, 50 mM NaCl), spectra could not be recorded below 205 nm, due to the high absorbance of the sample. The minimum was however located below 205 nm, indicating the prevalence of unordered confor-
mations. Addition of 10 mM Ca²⁺ caused obvious changes in the spectrum, now showing a minimum at 218 nm, typical of a β-sheet structure (data not shown). Thus CD results confirm the above IR data.

Surface and Membrane Properties—We have recently shown (21) that native HlyA is a surface-active molecule, which adsorbs readily at the air-water interface, decreasing the water surface tension as measured in a Langmuir balance. Surface activity of HlyA was attributed mainly to the presence of amphipathic helices at the N-terminal domain of the protein (aa 177–411). Surprisingly, however, the C-terminal fragment HlyA_N601 alone was also surface-active (Fig. 7). Adsorption was concentration-dependent, with the maximum surface pressure (∆π) increasing and the t₁/₂ of adsorption decreasing with protein concentration. The data shown in Fig. 7 were obtained in the absence and presence of Ca²⁺; data obtained in the presence of Ca²⁺ were qualitatively similar, but the observed increases in ∆π were smaller. The data shown in Fig. 8 were obtained in the absence (■) or presence (□) of 10 mM Ca²⁺.

When the average contribution of an amino acid residue to the maximum observed ∆π was computed in the presence of Ca²⁺, values of 0.023 (HlyA) and 0.033 (HlyA_N601) mN/m × residue were obtained. This suggests that, contrary to our previous assumption, the C-terminal region of the protein is a
major contributor to the overall surface effect of HlyA at the air-water interface.

Native HlyA was able to insert into lipid monolayers extended at an air-water interface at an initial surface pressure \( \pi_0 \approx 25 \text{ mN/m} \), i.e. above the maximum \( \Delta \pi \) caused by adsorption of pure HlyA at an air-water interface (21). HlyAΔN601 could also insert into lipid monolayers at initial \( \pi \) above the values reached by the protein alone at the air-water interface, i.e. 20 and 14 mN/m, respectively, in the absence and presence of Ca\(^{2+}\). Experiments were performed with monolayers composed of either pure phosphatidylcholine or of phosphatidylcholine/phosphatidylethanolamine/cholesterol (2:1:1, molar ratio) with essentially similar results. A summary of the monolayer insertion data can be seen in Fig. 9. A value of \( \Delta \pi = 0 \), i.e. no insertion is detected above 32 mN/m in the absence of Ca\(^{2+}\) or above 26–28 mN/m in the presence of Ca\(^{2+}\). This is in contrast with the corresponding values of 34.6 and 37.3 mN/m, respectively, in the absence and presence of Ca\(^{2+}\), for native HlyA (21). The average \( \pi \) for cell membranes is estimated at \( \sim 30 \text{ mN/m} \) (28). This implies that, particularly under lytic conditions, i.e. in the presence of Ca\(^{2+}\), HlyAΔN601 would not be able to insert into cell membranes. This is confirmed by the complete lack of lytic activity of HlyAΔN601 either on erythrocytes or on liposomes (Fig. 10). Also significant in this context is the observation that, after incubating HlyAΔN601 with liposomes and removing the unbound protein by centrifugation, no membrane-bound protein was recovered (data not shown).

**DISCUSSION**

HlyA is a large protein (1064 aa) in which primary and secondary structure observations and predictions reveal a clear two-part structure, with an N-terminal region rich in amphipathic \( \alpha \)-helices, and a C-terminal part characterized by the nonapeptide-repeat, Ca\(^{2+}\)-binding domain, in which \( \beta \)-structure predominates. Conventional wisdom has associated up to now the amphipathic helix domain, but not the repeat domain, to the various stages of HlyA interaction with membranes. However, the results in this paper suggest that this consensus view must be modified.

The role of the calcium-binding domain in HlyA and homologous RTX toxins remains largely unknown, despite the fact that Ca\(^{2+}\) is essential for toxin activity (23, 29). Various reports have linked the RTX domain to target membrane binding (13, 14). In addition we had found that the sequence 914–936, located very close to that domain, inte-
acted specifically with α-glycophorin, an HlyA receptor in human erythrocytes (9, 10).

This prompted us to clone and express the calcium-binding domain of HlyA, together with several preceding and following sequences that would stabilize the domain. HlyAΔN601 can be obtained in good yields and shares with the parent protein the interesting ability of being stable in 6 M urea at -20 °C. When isolated from the N-terminal domain, the calcium-binding region maintains indeed its calcium-binding capacity, in fact virtually all Ca\(^{2+}\) ions (∼12) can be exchanged with Ca\(^{2+}\) in the solvent (Fig. 3), when only 3–4 could be exchanged in native HlyA (23). Interestingly Soloaga et al. (27) had found that, under partially denaturing conditions, i.e. 3 M urea, HlyA could also bind 12 Ca\(^{2+}\) ions. Bauche et al. (20) have also observed the binding of ∼30 Ca\(^{2+}\) ions to the isolated RTX domain of Bordetella pertussis adenylate cyclase toxin, which contains about as many canonical nonapeptides. The single Trp residue (Trp914) left in our construct was very sensitive to Ca\(^{2+}\) binding (Fig. 4). Ca\(^{2+}\) binding increased the proportion of β-structure in HlyAΔN601 according to IR spectroscopy (Fig. 6 and Table 1), as expected from the x-ray structures of homologous RTX domains (15–18) showing that Ca\(^{2+}\) stabilizes the β-roll structure. By increasing the proportion of regular structures in the domain, Ca\(^{2+}\) also made the truncated toxin more stable, as seen from the decrease in nonspecific aggregation bands in IR spectra. A similar effect of Ca\(^{2+}\) on the secondary structure of the nodulation protein NodO from Rhizobium leguminosarum, also a protein belonging to the RTX family, was described by Dalla Serra et al. (30).

All the above data are interesting in characterizing the isolated C-terminal region of HlyA, and they are in agreement with the recent observations by Bauche et al. (20) with the B. pertussis homologue toxin. A more novel and remarkable observation, however, is the fact that HlyAΔN601 is a potent surface-active agent. In the presence of Ca\(^{2+}\), when the domain is fully structured, it reduces the buffer surface tension by up to 14 mn/m. The whole HlyA does so by 24 mn/m (21).

Although the surface-active properties of the various domains in a given protein are not necessarily additive, the data in Fig. 8 clearly indicate that the C-terminal region of HlyA is surface-active and that it contributes significantly to the surface-active properties of the whole protein. When the average per residue surface activity is computed, a value of 0.033 mn/m × residue is obtained for HlyAΔN601, versus 0.023 mn/m × residue for the whole protein, i.e. the isolated C-terminal domain is a more potent surfactant than the whole HlyA. This value places HlyAΔN601 among well known surface-active proteins, e.g. equinatoxin II (0.039 mn/m × residue), or apolipoprotein (0.042 mn/m × residue), see Table 3 in (21). This, together with the location of a major receptor-binding sequence in this region (10), points to the Ca\(^{2+}\)-binding domain as the part of the protein making early contact with the target membrane.

Also interesting and illustrative is the observation that HlyAΔN601 can become inserted into lipid monolayers, in the presence of Ca\(^{2+}\), but only when the latter are extended at initial surface pressures up to 26–28 mn/m, i.e. below the average surface pressure thought to exist in cell membranes, of about 30 mn/m. This makes the isolated RTX domain unable to insert in membranes, thus devoid of lytic activity by itself (Fig. 10). Native HlyA can become inserted into monolayers at initial surface pressures up to 37 mn/m (21), thus it can become inserted in membranes.

The fact that HlyAΔN601 cannot penetrate the membrane bilayer does not preclude a role for the RTX domain in membrane binding. Various possibilities may be considered: (i) in the native protein, changes in tertiary structure cause the surface activity of the Ca\(^{2+}\)-binding domain to increase; (ii) the fatty acyl residue at Lys-690, which is lacking in our construct, increases the domain surface activity; note in this respect that the precursor protein proHlyA, which lacks two fatty acyl residues present in mature HlyA, has also an impaired ability to penetrate lipid monolayers (21), or (iii) considerable fluctuations in surface pressure are known to exist in cell membranes, around the average value of 30 mn/m, that may allow the reversible insertion of the Ca\(^{2+}\)-binding domain. Those fluctuations would be at the origin of the channel-forming activity detected for the RTX domain of the B. pertussis protein in supported asolectin bilayers (20). In any case it would be unlikely that a remarkable surface activity as shown by HlyAΔN601 would not be involved in the membrane targeting of the toxin.

These observations suggest that a re-interpretation of previous data, including our own, has to be considered. In a recent report (21) we described the surface-active properties of HlyA, and these properties were attributed, at least implicitly, to the amphipathic helix region of the protein, in agreement with the common consensus. We also observed that HlyA binding to lipids was not necessarily coupled to membrane lysis. This can be explained in the light of the present results. In view of the previously established implication of a C-terminal peptide in receptor binding (10) and of the newly observed surface-active properties, it can be proposed that the C-terminal region of HlyA is directly involved in the early, reversible stages of HlyA docking at the target membrane. We had previously been able to dissect the (reversible) binding from the (irreversible) lytic insertion of the toxin (7). Reversible binding took place even in the absence of Ca\(^{2+}\). This can also be understood in the light of the present observations, since HlyAΔN601 insertion in lipid monolayers (Fig. 9) occurs as well in the absence of Ca\(^{2+}\). Thus we propose that both the N-terminal, amphipathic helix domain and the C-terminal, calcium-binding domain of HlyA are directed but differently involved in toxin-membrane interaction, the latter being responsible for the early reversible steps of adsorption and the former for the late stages involving irreversible interaction with the membrane hydrophobic matrix and subsequent loss of the permeability barrier.

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