The adenylate cyclase (CyaA) secreted by Bordetella pertussis is a toxin that is able to enter eukaryotic cells and cause a dramatic increase in cAMP level. In addition, the toxin also exhibits an intrinsic hemolytic activity that is independent from the ATP cycling catalytic activity of the toxin. Both the cytotoxic and hemolytic activities are calcium-dependent. In this work, we have analyzed the calcium interacting properties of CyaA. We have shown that CyaA exposed to CaCl2 could retain membrane binding capability and hemolytic activity when it was further assayed in the presence of an excess of EGTA. Determination of the calcium content of CyaA exposed first to calcium and subsequently to EGTA indicated that some (3–5) calcium ions remained bound to the protein, suggesting the existence of Ca2+ binding sites of high affinity. Binding of Ca2+ to these sites might be necessary for both the membrane binding capability and the hemolytic activity of the toxin. In addition, CyaA possesses a large number (about 45) of low affinity (Kd = 0.5–0.8 mM) Ca2+ binding sites that are located in the C terminus of the toxin, between amino acids 1007 and 1706. This region mainly consists of about 45 repeated sequences of the type GXGXXDXX (where X represents any amino acid) that are characteristic of the RTX (Repeat in ToXin) bacterial protein family. Our data suggest that each one can bind one calcium ion. Circular dichroism spectroscopy analysis showed that calcium binding to the low affinity sites induces a large conformational change of CyaA, as revealed by an important increase in the content of α-helical structures. This conformational change might be directly involved in the Ca2+-dependent translocation of the catalytic domain of CyaA through the plasma membrane of target cells.

One of the essential virulence factors of the whooping cough agent, Bordetella pertussis, is the secreted adenylate cyclase toxin CyaA1 (1–4). It is a protein of 1706 residues that is converted to the active toxin by a post-translational palmitoylation of an internal lysine residue (5). This modification requires the product of the accessory cyaC gene (6). The toxin is a bifunctional protein. Its purified 177-kDa toxic form exhibits both invasive adenylate cyclase (cytotoxic) and hemolytic (pore-forming) activities (7–12). The adenylate cyclase (AC) catalytic domain of CyaA resides in the 400 amino-proximal residues and its enzymatic activity requires eukaryotic calmodulin (CaM) (13–16). The toxin delivers the AC domain directly across the plasma membrane of a variety of epithelial and immune effector cells and disrupts their physiological functions by uncontrolled synthesis of cAMP (11, 17–20). The C-terminal 1330 residues of CyaA are required for cell targeting and delivery of the AC-domain into cells (8, 10, 11) and can act independently as a hemolysin (12, 21, 22). Interestingly, no dedicated target cell binding domain could be identified within this hemolysin portion of CyaA. The structural integrity of CyaA appears to be essential for both delivery of the AC-domain into target cells and the pore-forming activity of the toxin.2 The C-terminal part of CyaA (residues 400–1706) exhibits several features common to all RTX (Repeat in ToXin) proteins (24). It contains four hydrophobic stretches (from residues 500–700) involved in formation of the cation-selective CyaA channels (25, 26), the target site for the post-translational modification of CyaA (lys residue 983, which is palmitoylated) (5), and 40–45 glycine and aspartate-rich nonapeptide repeats (from residues 1015 to 1613) of the prototype GXXG(D/N)DX(L/I/F)X (where X represents any amino acid) that are characteristic of the RTX bacterial protein family (14).

CyaA is a calcium-binding protein that undergoes conformational changes upon binding of calcium (18, 19). The repeat motifs of CyaA (between residues 1007 and 1612) are suspected to be involved in calcium binding, as it was shown that deletion of similar repeats abolished calcium binding to the α-hemolysin (HlyA) of Escherichia coli (27–29). Indeed the repeat motifs, GXXG(D/N)DX(L/I/F)X, were found, in the three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa, to constitute a new type of calcium-binding structure (30). The cytotoxic activity of CyaA is absolutely calcium-dependent; the translocation of the AC catalytic domain across target cell membranes occurs only at calcium concentrations above 0.1 mM. However, the calcium requirement of the hemolytic activity is still a matter of controversy (8, 12).

In this study, we have analyzed the calcium binding properties of CyaA and shown that CyaA harbors probably two classes of calcium binding sites of different affinities. Binding of calcium to a small number of high affinity binding sites might be
necessary for the hemolytic activity of the toxin. In addition, CyaA contains about 40–45 low affinity calcium binding sites, which are located in the repeat region of the toxin. Binding of calcium to these low affinity sites induces major structural rearrangements of CyaA that may be involved in delivery of the AC domain into target cells.

**MATERIALS AND METHODS**

Protein Analysis Techniques—SDS-PAGE analysis, determination of protein concentration, electrophoretic transfer of proteins to nitrocellulose membranes, and staining of electrotransferred proteins were performed according to standard protocols (31).

Construction of Plasmids—The in vitro DNA manipulations were performed according to standard protocols (31). When appropriate, the noncohesive ends of restriction fragments were blunt-ended before ligation with T4 DNA polymerase.3

Construction of in-frame deletions in the cyaA gene—Unless otherwise stated, pACT7 (32) was used for construction of in-frame deletions in the cyaA gene. The numbers that follow the symbol Δ in the names of the plasmids described below are the numbers of the first and the last codons of the deleted parts of the cyaA reading frame on the respective plasmids. pACTΔ1827–887 was generated by deletion of a 180-bp CiaI fragment; pACTΔ1622–779 was obtained by deletion of a 471-bp BglII fragment; pACTΔ1622–1489 resulted from the deletion of the 2601-bp NcoI–XhoI fragment; pACTΔ1400–1400 was provided by H. Sakamoto (pSP118), and it was generated by deletion of the 1443-bp XhoI–Smal fragment. pACTΔ1189–828 resulted from deletion of the 1920-bp EcoRV fragment of pACT7. Construction of pACT11–1006, pACT385–828, pACT385–1006, pACT385–1488, pACTI217, pDIA5240, was described previously (32, 33). pACT863, used for production of a polyepitope comprising the first 863 residues of CyaA, was generated by the replacement of the 2.6-kilobase EcoRI fragment of pACT7 by the KmΔ cassette KmO (34). pACT1007 was obtained by insertion of the 1.9-kilobase BstBI–XhoI fragment of pACT7 into the BstBI–PstI sites of pAHLI (33). This resulted in the interruption of the cyaA reading frame after the Glu1008 codon and in the addition of a linker coding for the nonapeptide VDSRGSPGT before the first in-frame stop codon. Construction of pACT3 for coexpression of the cyaA and cyaC genes from the same high copy number plasmid is described elsewhere (35).

Production and Purification of the CyaA-derived Proteins—Wild-type CyaA protein or its different truncated derivatives were produced in the presence or absence of the activating protein CyaC in E. coli strain XL-1 Blue (Stratagene) harboring the corresponding plasmid(s) as described previously (32). After sonication, the proteins were extracted from cell debris in 8 M urea, 50 mM Tris-HCl, pH 8.0, 0.2 mM CaCl2 and 0.1 mM phenylmethylsulfonyl fluoride, and 2 mM CaCl2. After centrifugation (20 min at 25,000 g), the supernatant was loaded on a phenyl-Sepharose column equilibrated in 50 mM Tris-HCl, pH 8.0, 2 mM EGTA and stored at 4°C, the supernatant was loaded on a second G25 column equilibrated in buffer A and 5 mM EDTA. The CyaA protein, recovered after this second G25 chromatography, was then loaded on a third G-25 column equilibrated in buffer A supplemented with 5 mM EDTA. The CyaA protein, recovered after this second G-25 chromatography, was then loaded on a third G-25 column equilibrated, again, in buffer A and 5 mM EDTA. Fractions of 0.5 ml were collected, and the protein content of each fraction was measured. The total calcium content of each fraction was determined by atomic absorption with an ARL 3580 atomic absorption spectrophotometer (performed by Dr. J.-L. Imbert, Analytical Chemistry laboratory of CNRS, Lyon, France). The amount of calcium bound per CyaA molecule was determined with 5 mM EDTA. The CyaA protein, recovered after this second G-25 chromatography, was then loaded on a third G-25 column equilibrated, again, in buffer A and 5 mM EDTA. Fractions of 0.5 ml were collected, and the protein content of each fraction was measured. The total calcium content of each fraction was determined by atomic absorption with an ARL 3580 atomic absorption spectrophotometer.
then deduced. To determine the calcium content of CyaA renatured in the absence of calcium, the procedure described above was repeated identically, except that the first Sephadex G-25 chromatography was carried out in buffer A containing 5 mM EDTA instead of 2 mM CaCl₂.

45Ca²⁺ Overlay—Equimolar amounts (4 units) of purified CyaA proteins were separated by SDS-PAGE (7.5% gel) and electrotransferred to nitrocellulose membrane. The proteins were probed for binding of 45Ca²⁺ ions as described by Bodhm et al. (28).

Calcium Binding Assay—Binding of calcium to CyaA, RD-CyaA, or ACT1007, was determined by ultrafiltration, as described previously (37). Briefly, 1-ml samples of a 13–17 μM solution of purified proteins in 25 mM Tris-HCl, pH 8.0, 0.1 mM NaCl were placed in the top compartment of Centricon-10 concentrators (molecular mass cut-off of 10 kDa). Twenty μl of a 2.5 mM CaCl₂ solution containing about 10⁶ cpm of 45CaCl₂ were added to the protein and thoroughly mixed by gentle vortexing. Twenty μl of this solution were taken out for counting of total radioactivity in a scintillation counter. The mixtures were then centrifuged at room temperature in a tabletop centrifuge for 30–40 s until 30–40 μl were filtered through. The ultrafiltrate was added back to the protein solution, which was again mixed and centrifuged as above (this second centrifugation was done to correct for a filtration membrane dead-volume of about 10 μl). Radioactivity of a 20-μl sample of the second filtrate, which contained the free calcium, was determined by scintillation counting. Then, 20 μl of a 5 mM unlabeled CaCl₂ solution were added to the top compartment, and the centrifugation procedure described above was repeated. Successive additions of unlabeled calcium were done to cover the range of desired total calcium concentrations (up to 3 mM). After each addition of cold ligand, the ratio, radioactivity in 20 μl of filtrate/radioactivity in 20 μl of top compartment (initial measurement), is equal to free calcium/total added calcium. The numbers of bound calcium ions/molecule of protein as a function of free calcium were then deduced.

Circular Dichroism Spectroscopy—Circular dichroism spectra were recorded on a J-500 Synchrotron CD6 dichrograph at 20°C with a 0.02-cm path length cylindrical suprasil quartz cell (Hellma). Circular dichroism intensity (ΔA) was calibrated with an aqueous solution of d-(-)-10-camphorsulfonic acid according to the method of Dornan and Maestre (38). Spectra were determined at protein concentrations of 0.1–0.2 mg/ml. Protein samples were equilibrated in 10 mM Tris-HCl, pH 8.0, by chromatography on prepacked Sephadex G-25 columns (PD-10, Pharmacia). Protein concentrations were calculated from the UV absorbance at 280 nm. Far UV region was scanned 5–10 times from 180 to 260 nm with 0.1-nm (high resolution) or 1.0-nm (low resolution) steps using an integration time of 2 s (0.02 cm cell). The left and right difference of mean residue molar circular dichroic extinction coefficients (Δε) is given in M⁻¹·cm⁻¹·deg⁻¹. Spectra were decomposed with the VARIable SElect thermodynamic method (39, 40), in a linear combination of 5 eigenvectors computed from 29 out of 32 standard proteins. The secondary structures of these reference proteins were computed with the DSSP program (23) from atomic coordinates of crystallized proteins. The proportion of pure canonical secondary structures were calculated from the coefficients of the eigenvectors.

RESULTS

Tight Binding of Calcium Ions to CyaA Is Critical for Hemolytic Activity of the Toxin—We have previously shown that the hemolytic activity of CyaA is strictly Ca²⁺-dependent (8). In contrast, Rogel et al. (12) reported that CyaA bound to erythrocytes and was hemolytic even in the presence of EGTA. However, their method of CyaA purification was different from ours in that they added Ca²⁺ during renaturation of the protein after urea extraction from the bacteria and we did not. Therefore, we have examined whether this difference could account for the calcium requirement of the hemolytic activity of the toxin. A recombinant CyaC-modified toxin was purified from E. coli by CaM-agarose chromatography. The purified toxin, eluted from CaM-agarose by 8 M urea, was renatured by chromatography on two parallel Sephadex G-25 columns equilibrated in buffer A and containing either 2 mM CaCl₂ or 2 mM EGTA. The two different preparations were then evaluated for their hemolytic and cytotoxic activities, and their membrane binding capability, either in the presence of 2 mM CaCl₂ or in the presence of 2 mM EGTA. As shown in Fig. 1, the toxin renatured in the presence of EGTA bound to erythrocytes and was hemolytic only when it was assayed in the presence of Ca²⁺. In contrast, the toxin renatured in the presence of Ca²⁺ was able to bind to erythrocyte membranes and lyse these cells even when it was assayed in the presence of EGTA. The amount of toxin bound to target cell membranes in the absence of calcium was about 30% of that bound in the presence of CaCl₂ (Fig. 1A). The hemolytic activity of the toxin measured in the presence of EGTA was much lower than that measured in the presence of 2 mM CaCl₂ and became apparent only after a long lag (Fig. 1B). In all cases, the cytotoxic activity (i.e., the ability to translocate the catalytic domain through the plasma membrane) of CyaA was dependent on exogenous calcium in the millimolar range (Fig. 1A), as reported previously (8, 10, 11, 18). The simplest interpretation of these results was that (i) when CyaA is refolded in the presence of Ca²⁺, some calcium ions become tightly bound to the protein and cannot be removed by the calcium chelator EGTA; (ii) calcium ions bound at these binding sites are required for membrane binding of CyaA.
and for lysis of the target cells.

To test this hypothesis, we attempted to measure the calcium content of the toxin renatured either in the presence or in the absence of Ca\textsuperscript{2+} and exposed subsequently to the calcium-chelator EDTA. The toxin, purified in the presence of 8 M urea and 2 mM EDTA, was renatured, as described above, by chromatography on Sephadex G-25 columns equilibrated in buffer A containing either 2 mM CaCl\textsubscript{2} or 5 mM EDTA. The renatured protein was then submitted to two subsequent chromatographies on G-25 columns equilibrated in buffer A supplemented with 5 mM EDTA. The calcium content of the proteins, recovered after the third G-25 column, was then analyzed by atomic absorption spectrophotometry. Between three and five calcium atoms were bound per CyaA molecule when the toxin had been renatured in the presence of 2 mM CaCl\textsubscript{2}. Less than 1 calcium ion was bound per CyaA molecule (from 0.6 to 0.8), when the toxin had been renatured in the presence of 5 mM EDTA. Unfortunately, we could not assay the membrane binding capacity and the hemolytic activity of the samples subjected to the atomic absorption analysis. In fact, at the high concentrations of toxin that were used in these experiments (4 mg/ml, 22 mM) in order to get a reliable measurement of the calcium content, CyaA had a tendency to aggregate, which diminished its biological activities substantially.

Altogether, however, these results suggest that when CyaA is exposed to calcium during its renaturation, it binds some calcium ions that could not be removed during a subsequent exposure of the toxin to EDTA or EGTA. These bound calcium ions are necessary and sufficient for the membrane binding capability and the hemolytic activity of CyaA, but insufficient for its cytotoxic activity.

Multiple Calcium Binding Sites Are located in the Asp-Gly-rich Repeat Region of CyaA—

We attempted to localize the calcium binding sites of the toxin by using a \textsuperscript{45}Ca\textsuperscript{2+} overlay technique. A set of in-frame deletions in the cyaA gene was generated on plasmid pACT7, which allows high level synthesis of the toxin (32). The truncated constructs (Fig. 2) were produced in E. coli and purified to over 80% homogeneity by a single-step CaM-agarose affinity chromatography (Fig. 3A). After separation by SDS-gel and transfer to nitrocellulose (Fig. 3C), the proteins were probed for calcium binding with \textsuperscript{45}Ca\textsuperscript{2+} (2 mM Ci/ml). The membrane was then washed, dried, and exposed for autoradiography. The autoradiogram shown was obtained after 3 days of exposure. C, Ponceau S staining of the membrane-bound proteins. The analyzed proteins are encoded by the following plasmids: lane 1, pCACT3; lane 2, pACT; lane 3, pACT\textsuperscript{C217}; lane 4, pACT\textsuperscript{1007}; lane 5, pACT863; lane 6, pACT\textsuperscript{1008–1490}; lane 7, pACT\textsuperscript{622–1489}; lane 8, pACT\textsuperscript{627–887}; lane 9, pACT\textsuperscript{622–779}; lane 10, pDIAS240.

FIG. 3. Localization of calcium binding sites of CyaA by \textsuperscript{45}Ca\textsuperscript{2+} overlay. A, SDS-PAGE analysis of the purified wild-type CyaA and of the truncated derivatives. B, \textsuperscript{45}Ca\textsuperscript{2+} overlay. The purified proteins were separated by SDS-PAGE (7.5% gel), electrotransferred to nitrocellulose membrane, and probed for calcium binding with 2 mM CaCl\textsubscript{2} solution containing \textsuperscript{45}Ca\textsuperscript{2+} (2 \mu Ci/ml). The membrane was then washed, dried, and exposed for autoradiography. The autoradiogram shown was obtained after 3 days of exposure. C, Ponceau S staining of the membrane-bound proteins. The analyzed proteins are encoded by the following plasmids: lane 1, pCACT3; lane 2, pACT; lane 3, pACT\textsuperscript{C217}; lane 4, pACT\textsuperscript{1007}; lane 5, pACT863; lane 6, pACT\textsuperscript{1008–1490}; lane 7, pACT\textsuperscript{622–1489}; lane 8, pACT\textsuperscript{627–887}; lane 9, pACT\textsuperscript{622–779}; lane 10, pDIAS240.

and for lysis of the target cells.

FIG. 4. Calcium binding properties of CyaA and RD-CyaA. Calcium binding to CyaA (13 \mu M, ●), RD-CyaA (17 \mu M, ○), and ACT1007 (15 \mu M, □) was determined by ultrafiltration, as described under "Materials and Methods." Similar data were obtained in three different experiments.
tions extending from residues 622 up to 1489 all bound $^{45}$Ca$^{2+}$ (Fig. 3B, lanes 6–9). These results indicate that the last 217 residues of CyaA (from residue 1489 to 1706) also contain Ca$^{2+}$ binding sites. It can therefore be concluded that the calcium binding sites of CyaA, detectable by this technique, are located in its last 700 residues. However, a reliable quantitation of calcium bound to the various proteins was hampered by differences in the efficiency of electrotransfer of the proteins to the membrane, as well as by possible differences in the efficiency of renaturation of the various truncated proteins.

Calcium Binding Properties of CyaA and of the Repeat Domain of CyaA in Solution—Ca$^{2+}$ binding to CyaA in solution was investigated by ultrafiltration using $^{45}$Ca$^{2+}$ as a tracer. As shown in Fig. 4, wild-type CyaA bound a large number of Ca$^{2+}$ ions (up to 50) with a low affinity (apparent $K_D > 0.3$ mM). A more precise stoichiometry and affinity of Ca$^{2+}$ binding to CyaA could not be determined accurately because the protein at the concentration used (13 $\mu$M, 2.5 mg/ml) had a propensity to aggregate (detectable by the appearance of turbidity) upon addition of calcium. Therefore, we investigated Ca$^{2+}$ binding to a deletion derivative of CyaA comprising the last 700 amino acids from the C terminus (residues 1007–1706, see Fig. 2). This protein, RD-CyaA, was expressed in E. coli and purified to homogeneity by calcium-dependent hydrophobic chromatography. RD-CyaA at 17 $\mu$M did not aggregate at CaCl$_2$ concentrations below 5 mM. However, addition of large excess of calcium (20–30 mM) still induced aggregation of the molecules. As shown in Fig. 4, RD-CyaA bound up to 45 calcium ions/molecule with an apparent $K_D$, deduced from the binding curve, of 0.6 mM ($> 0.1$ mM). These results suggest that each of the 40–45 aspartate/glycine-rich repeats of CyaA can bind one calcium ion. RD-CyaA repeatedly bound slightly less calcium than CyaA (Fig. 4). This difference might not be significant, due to the possible uncertainties in the determination of calcium binding to CyaA. Alternatively, it could indicate that additional calcium binding sites are present in the N-terminal 1007 amino acids of CyaA. Hence, we investigated Ca$^{2+}$ binding to ACT1007, the deletion derivative of CyaA that lacks the last 700 amino acids from the C terminus. As shown in Fig. 4, only background levels of calcium were bound to ACT1007 in the same conditions. This result shows that only few, if any, low affinity calcium binding sites are located in the first 1007 amino acids of CyaA.

Circular Dichroism of CyaA and RD-CyaA in Far UV—The far UV CD spectra of CyaA in the presence or in the absence of CaCl$_2$, are shown in Fig. 5A. Significant increase of the positive peak at 192 nm and decrease of the negative peak at 222 nm are observed upon addition of calcium to CyaA. These changes are indicative of an $\alpha$-helical formation and show that the $\alpha$-helical content of CyaA is enhanced upon calcium binding to the protein. The proportions of secondary structures obtained from the curve fitting of the CD spectra, following the procedure of Manavalan and Johnson (40), are given in Table I. Upon binding of calcium, the $\alpha$-helical content of the protein increased by about 5%, from 22% in the absence of added Ca$^{2+}$ to 27% in the presence of 2 mM Ca$^{2+}$. A small increase in the content of $\beta$-structures is also observed. The change of the mean residual molar $\Delta e$ at 220 nm as a function of added calcium is shown in Fig. 5C. The half-maximum transition occurred at CaCl$_2$ concentrations of 0.5–0.6 mM. The far UV CD spectra were similarly recorded for RD-CyaA, in the presence or in the absence of added CaCl$_2$. As shown in Fig. 5B, binding of calcium to the protein induces a large change of its CD spectrum, especially at 192 and 222 nm. This suggests that RD-CyaA undergoes major secondary structure modifications upon calcium binding. In the absence of added CaCl$_2$ (CaCl$_2 < 0.1$ mM), the secondary struc-

![Image](336x323 to 534x734)

**FIG. 5.** Far UV CD spectra of CyaA and RD-CyaA in the absence and in the presence of calcium. CD spectra of CyaA (A) and RD-CyaA (B) in the absence ($< 0.1$ mM CaCl$_2$, plain line) or in the presence of calcium (2 mM CaCl$_2$, dashed line), were recorded as described under "Materials and Methods." Each spectra represents the average of 5 scans, carried out at 20 °C between 180 and 260 nm, using 0.1-nm steps and an integration time of 2 s. C. calcium dependence of the mean differential residual molar circular dichroic extinction coefficient ($\Delta e$ in $\text{m}^{-1}\text{cm}^{-1}$) at 220 nm of CyaA (●) and of RD-CyaA (○). The error bars indicate the standard error mean of four independent titrations.

| Secondary structure composition | CyaA | RD-CyaA |
|--------------------------------|------|---------|
|                                | <0.1 mM Ca$^{2+}$ | 2 mM Ca$^{2+}$ | <0.1 mM Ca$^{2+}$ | 2 mM Ca$^{2+}$ |
| %                             |       |         |       |         |
| $\alpha$-Helix                 | 22    | 27      | 5     | 18      |
| $\beta$-Sheet                  | 26    | 29      | 27    | 35      |
| Turns                          | 25    | 26      | 28    | 19      |
| Other                          | 27    | 18      | 40    | 27      |
| Root mean square error         | 0.098 | 0.080   | 0.085 | 0.186   |

Deconvolution of spectra shown in Fig. 5 was performed using the VARIABLE SELECTION software, as described under "Materials and Methods."
binding of Ca$^{2+}$ to RD-CyaA is accompanied by a 13\% increase in the $\alpha$-helical content of the protein. Theoretically, at the equilibrium, this value would correspond to about 90 unordered residues of RD-CyaA (13\% of 700 residues), which acquired an $\alpha$-helical structure upon calcium binding. A similar number would be predicted for the full-length protein (5\% of 1706 residues, see above). When the acquisition of $\alpha$-helical structure of RD-CyaA was monitored as a function of added calcium (Fig. 5C), the half-maximal transition was observed at Ca$^{2+}$ concentrations of 0.9–1.1 mM. These results indicate that the C-terminal part of CyaA, which harbors the Asp-Gly rich repeats, undergoes a significant structural rearrangement upon binding of calcium.

**DISCUSSION**

The results presented here suggest that CyaA harbors two classes of calcium binding sites. The first class might consist of a small number (3–5) of high affinity Ca$^{2+}$ binding sites, whereas the second class consists of about 45 calcium binding sites of low affinity. Calcium binding to the high affinity sites appears to be critical for the membrane binding capability and the hemolytic activity of the toxin, while calcium binding to the low affinity sites could be involved in the cytotoxic activity of CyaA, that is, in the translocation of the catalytic domain through the membrane of the target cells.

The presence of high affinity calcium binding sites in CyaA is suggested by two sets of results. First, we have shown that CyaA, which had been renatured in the presence of calcium, exhibited membrane binding capability and hemolytic activity when it was further assayed in the presence of an excess of EGTA, whereas CyaA, which had been renatured in the absence of calcium, did not. These findings explain the apparent discrepancy between our previous data (8) and those of Rogel et al. (12). Indeed, Rogel et al. (12) observed a calcium-independent hemolytic activity of CyaA when it had been renatured in the presence of CaCl$_2$. In contrast, we showed an absolute calcium requirement for the hemolytic activity of the toxin that was renatured in the absence of calcium (8). Second, when CyaA had been renatured in the presence of calcium and subsequently exposed to the calcium chelator EDTA, 3–5 calcium ions remained bound to the protein, as determined by atomic absorption spectrophotometry. Altogether, these data suggest that (i) CyaA binds a few calcium ions that cannot be removed by addition of EGTA or EDTA without denaturation and (ii) these bound calcium ions are required for the membrane binding and hemolytic activities of the toxin.

A more precise determination of the number, and affinities of these putative high affinity calcium binding sites proved to be very difficult, essentially because CyaA had a tendency to aggregate at protein concentrations required to achieve reliable determinations of the bound calcium. Hence, direct measurements, on the same samples, of both the amount of calcium bound to the protein (assessed by atomic absorption) and the membrane binding capability and hemolytic activity of CyaA (measured in the presence of EGTA) could not be performed. However, our present data clearly indicate that there are only a small number of these potential high affinity calcium binding sites and that they should have a much higher affinity for calcium than EDTA or EGTA (that is, an equilibrium constant in the nanomolar range). At present, we do not know the localization of these potential high affinity calcium binding sites. It appears that they are not located in the last 700 residues of CyaA as the truncated derivative, RD-CyaA, did not exhibit tightly bound calcium.$^4$ However, one cannot exclude that the folding of the Asp-Gly-rich repeat region is somewhat different in RD-CyaA as compared with the full-length toxin. It is interesting to recall, that the $\alpha$-hemolysin of E. coli also exhibited a Ca$^{2+}$-independent hemolytic activity if it had been previously exposed to calcium ions (29). It will be interesting to determine whether HlyA also possesses high affinity calcium binding sites.

The second class of calcium binding sites of CyaA is represented by a large number (about 45) of low affinity sites, that bind Ca$^{2+}$ in the submillimolar range ($K_D = 0.5–0.8$ mM). These sites are located in the Asp-Gly rich repeat region of CyaA, at the C terminus. These repeats, the prototype of which is GGXG/N/DIDX(L/I/F)X, are common to several different proteins of the RTX family (24). Previous studies, carried out on E. coli $\alpha$-hemolysin, have shown that these repeated sequences could be involved in calcium binding (27–29). The recently reported three-dimensional structure of the alkaline protease of P. aeruginosa, which possesses a small number of such Asp-Gly-rich motifs, indicates that, indeed, these repeats constitute a novel type of calcium binding sites (30). The nonapeptide repeats are organized in a $\beta$-roll, which consists of two opposing sheets of paralleled $\beta$-strands. The $\beta$-strands comprise the last 5 amino acids of each nonapeptide sequence ([N/D]IDX(L/I/F)X) and are connected by loops derived from the first 4 amino acids of the repeated sequence (GGXG). Ca$^{2+}$ is hexacoordinated between two adjacent loops of the $\beta$-roll, and as a whole, each repeat binds one calcium ion. Among the RTX protein family, CyaA contains by far the largest number of these Asp-Gly rich motifs, 40–45, depending on the consensus criteria used for their computation (14). Our present results suggest that in solution, each repeat motif of CyaA is able to bind one calcium ion. However, in contrast to the tight Ca$^{2+}$ binding to the repeat motifs of alkaline protease (30), the majority of the repeat motifs of CyaA exhibited only low affinity for calcium. This is not unexpected as most of the repeat motifs of CyaA do not perfectly match the GGXGIDXLX consensus.

The secondary structure composition of RD-CyaA, as deduced from the CD spectroscopy studies, strongly suggests that the repeated sequences of CyaA are structurally organized, like those of P. aeruginosa alkaline protease, in a small $\beta$-strand followed by a connecting loop. The CD analysis indicates that the secondary structure of RD-CyaA consists, in the presence of calcium, of 35\% of $\beta$-structures. This is roughly the value one would expect if the first 5 amino acids of each nonapeptide repeats were in a $\beta$-strand conformation. The fact that the addition of Ca$^{2+}$ increases the $\beta$-sheet content of RD-CyaA indicates that binding of calcium to the repeat motifs stabilizes this secondary structure.

Interestingly, inspection of the amino acid sequence revealed a striking peculiarity of the repeat region of CyaA as compared with other RTX proteins (24). The nonapeptide repeats of CyaA are arranged in five groups of 8–10 repeats, separated by 20–30-residue-long unrelated sequences. This modular organization is unique to CyaA, as all of the other RTX proteins have only one group of 8–13 repeats. The “intervening” sequences consist essentially of short stretches of $\alpha$-helix-forming amino acids, that are interrupted by Gly and Pro residues. Our CD studies indicate a large increase in the $\alpha$-helix content of RD-CyaA upon Ca$^{2+}$ binding to the low affinity sites. We suggest that the intervening sequences, which bridge the subgroups of repeats, undergo a structural rearrangement as a consequence of Ca$^{2+}$ binding to CyaA, and shift from an extended conformation to an $\alpha$-helical one. Hewlett et al. (19) have previously observed, by electron microscopy, a conformational change of CyaA upon binding of calcium. The present study extends the previous observation and identifies the C-terminal part of CyaA as the main calcium-responsive domain.

$^4$ D. Ladant, unpublished results.
of the molecule at millimolar calcium concentrations. On the other hand, it is well established that millimolar concentrations of free Ca²⁺ are required for the cytotoxic activity of CyaA. It is thus tempting to speculate that the conformational change that occurs in the repeat region of CyaA upon calcium binding is directly involved in the Ca²⁺-dependent translocation of the catalytic domain of CyaA through the plasma membrane of target cells.

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REFERENCES
1. Weiss, A. A., and Hewlett, E. L. (1986) Annu. Rev. Microbiol. 40, 661–686
2. Masure, H. R., Shattuck, R. L., and Storm, D. R. (1987) Microbiol. Rev. 51, 60–65
3. Hanski, E. (1989) Trends Biochem. Sci. 14, 459–463
4. Mook, M., and Ullmann, A. (1993) Trends Microbiol. 1, 187–192
5. Hackett, M., Guo, L., Shabanowitz, J., Hunt, D. F., and Hewlett, E. L. (1994) Science 266, 433–435
6. Barry, E. M., Weiss, A. A., Ehrmann, I. E., Gray, M. C., Hewlett, E. L., and Goodwin, M. S. (1991) J. Bacteriol. 173, 720–726
7. Bellalou, J., Ladant, D., and Sakamoto, H. (1990) Infect. Immun. 58, 1195–1200
8. Bellalou, J., Sakamoto, H., Ladant, D., Geoffroy, C., and Ullmann, A. (1990) Infect. Immun. 58, 3242–3247
9. Gentile, F., Knipling, L. G., Sackett, D. L., and Wolff, J. (1990) J. Biol. Chem. 265, 10696–10692
10. Hewlett, E. L., Gordon, V. M., McCaffery, J. D., Sutherland, W. M., and Gray, M. C. (1989) J. Biol. Chem. 264, 19379–19384
11. Rogel, A., Schultz, J., Brownlie, R. M., Cooke, J. G., Parton, R., and Hanski, E. (1989) EMBO J. 8, 2755–2760
12. Rogel, A., Meller, R., and Hanski, E. (1991) J. Biol. Chem. 266, 3154–3161
13. Wolff, J., Cook, G. H., Goldhammer, A. R., and Berkowitz, S. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 77, 3841–3844
14. Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A., and Danchin, A. (1988) Mol. Microbiol. 2, 19–30
15. Glaser, P., Danchin, A., Ladant, D., Bárzu, O., and Ullmann, A. (1988) Tokai J. Exp. Clin. Med. 13, (suppl.), 239–252
16. Ladant, D., Michelson, S., Sarfati, R. S., Gilles, A.-M., Predelleanu, R., and Bárzu, O. (1989) J. Biol. Chem. 264, 4015–4020
17. Confer, D. L., and Eaton, J. W. (1982) Science 217, 948–950
18. Hanski, E., and Farfel, Z. (1985) J. Biol. Chem. 260, 5526–5532
19. Hewlett, E. L., Gray, L., Allietta, M., Ehrmann, I. E., Gordon, V. M., and Gray, M. C. (1991) J. Biol. Chem. 266, 17503–17508
20. Shattuck, R. L., and Storm, D. R. (1985) Biochemistry 24, 6323–6328
21. Sakamoto, H., Bellalou, J., Sebo, P., and Ladant, D. (1992) J. Biol. Chem. 267, 13598–13602
22. Ehrmann, I. E., Weiss, A. A., Goodwin, M. S., Gray, M. C., Barry, E., and Hewlett, E. L. (1992) FEBS Lett. 304, 51–56
23. Kabsch, W., and Sander, C. (1983) Biopolymers 22, 2577–2637
24. Welch, R. A. (1991) Mol. Microbiol. 5, 521–528
25. Benz, R., Maier, E., Ladant, D., Ullmann, A., and Sebo, P. (1994) J. Biol. Chem. 269, 27231–27239
26. Szabo, G., Gray, M. C., and Hewlett, E. L. (1994) J. Biol. Chem. 269, 22496–22499
27. Ludwig, A., Jarchau, T., Benz, R., and Goebel, W. (1988) Mol. & Gen. Genet. 214, 553–561
28. Boehm, D. F., Welch, R. A., and Snyder, I. S. (1990) Infect. Immun. 58, 1959–1964
29. Boehm, D. F., Welch, R. A., and Snyder, I. S. (1990) Infect. Immun. 58, 1951–1958
30. Baumann, U., Wu, S., Flaherty, K. M., and McKay, D. B. (1993) EMBO J. 12, 3357–3364
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Sebo, P., Glaser, P., Sakamoto, H., and Ullmann, A. (1991) Gene (Amst.) 104, 19–24
33. Sebo, P., and Ladant, D. (1993) Mol. Microbiol. 9, 1009–1010
34. Ladant, D., Glaser, P., and Ullmann, A. (1992) J. Biol. Chem. 267, 2244–2250
35. Betsou, F., Sebo, P., and Guiso, N. (1993) Infect. Immun. 61, 3583–3589
36. Ladant, D., Bérzin, C., Alonso, J.-M., Crenon, I., and Guiso, N. (1988) J. Biol. Chem. 263, 16264–16269
37. Ladant, D. (1995) J. Biol. Chem. 270, 3179–3185
38. Dorman, H. J., and Maestre, M. F. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 255–259
39. Hennessey, J. P., and Johnson, W. C. (1981) Biochemistry 20, 1085–1094
40. Manavalan, P., and Johnson, W. C. (1987) Anal. Biochem. 167, 76–85