Change in Conformation with Reduction of \(\alpha\)-Helix Content Causes Loss of Neutrophil Binding Activity in Fully Cytotoxic Shiga Toxin 1

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Background: The role of Shiga toxins/neutrophils interactions in the pathogenesis of hemolytic uremic syndrome has been greatly debated.

Results: We show that limited toxin unfolding induces loss of neutrophil binding activity while maintaining toxicity.

Conclusion: The data indicate that Trp-203-related A chain moieties are recognized by neutrophils.

Significance: We gain new insights into the structure/function relationship of these well known toxins, explaining some conflicting results.

Shiga toxins (Stx) play an important role in the pathogenesis of hemolytic uremic syndrome, a life-threatening renal sequelae of human intestinal infection caused by specific Escherichia coli strains. Stx target a restricted subset of human endothelial cells that possess the globotriaosylceramide receptor, like that in renal glomeruli. The toxins, composed of five B chains and a single enzymatic A chain, by removing adenines from ribosomes and DNA, trigger apoptosis and the production of pro-inflammatory cytokines in target cells. Because bacteria are confined to the gut, the toxins move to the kidney through the circulation. Polymorphonuclear leukocytes (PMN) have been indicated as the carriers that “piggyback” shuttle toxins to the kidney. However, there is no consensus on this topic, because not all laboratories have been able to reproduce the Stx/PMN interaction. Here, we demonstrate that conformational changes of Shiga toxin 1, with reduction of \(\alpha\)-helix content and exposition to solvent of hydrophobic tryptophan residues, cause a loss of PMN binding activity. The partially unfolded toxin was found to express both enzymatic and globotriaosylceramide binding activities being fully active in intoxicating human endothelial cells; this suggests the presence of a distinct PMN-binding domain. By reviewing functional and structural data, we suggest that A chain moieties close to Trp-203 are recognized by PMN. Our findings could help explain the conflicting results regarding Stx/PMN interactions, especially as the groups reporting positive results obtained Stx by single-step affinity chromatography, which could have preserved the correct folding of Stx with respect to more complicated multi-step purification methods.

Hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in early childhood. Most cases arise from intestinal infections with Escherichia coli strains producing two main powerful toxins, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), and thus denominated Shiga toxin-producing E. coli. Approximately one-third of Shiga toxin-producing E. coli infections result in bloody diarrhea and abdominal cramps. HUS may further develop in one-tenth to one-fourth cases a week after the prodromal gastrointestinal phase. By this time, the noninvasive bacteria have already colonized the intestinal mucosa, producing the toxins that move from the intestine to the kidney. The damage to the endothelial cells lining the microvasculature of glomeruli is the end result of toxin action and the crucial point in the pathogenesis of HUS. The journey of the toxins from the gut to the kidney has been the object of great scrutiny and stimulated intense debate. The simplest hypothesis to explain the mode of delivery of Shiga toxins (Stx), i.e. the movement of free toxins in blood, is not evidence-based because Stx have never been detected in the plasma of HUS patients with concomitant detectable fecal toxin.

A breakthrough in this scenario was the discovery of the role of polymorphonuclear leukocytes (PMN) in shuttling the toxins. The authors gave convincing evidence of Stx1 binding to PMN: an immunohistological study, direct flow cytometric analysis with fluorescent toxin, Scatchard plot with iodinated Stx1, and calculation of the dissociation constant and of the number of binding sites on PMN. The lower affinity of Stx for PMN (\(K_d = 10^{-8}\) M) with respect to endothelial cells (\(K_d = 10^{-9}\)) is supported by the University of Bologna (RFO funds to M.B.) and Centro per la Cura e lo Studio della Sindrome Emolitico Uremica.

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2 The abbreviations used are: HUS, hemolytic uremic syndrome; Gb3, globotriaosylceramide; HUVEC, human umbilical vein endothelial cells; MCV, mean channel value of fluorescence; PMN, polymorphonuclear leukocytes; Stx, Shiga toxins; TCSPC, time-correlated single photon counting system.
Fluorescent Labeling of Stx1—Stx1 (100 µg) was conjugated with Alexa Fluor® 488 reactive fluorescent dye and purified by spin column according to the directions of the manufacturer (Molecular Probe, Invitrogen). The degree of labeling obtained was ~1 mol of Alexa Fluor® 488 dye/mol of toxin. No significant differences in conjugation were observed between different batches of toxin (batch 8, 1.113 mol of dye/mol of toxin; batch 9, 1.095 mol of dye/mol of toxin) regardless their binding activity on PMN (see below), suggesting similar toxin-dye interactions. The two fluorescent toxin batches (1 pmol) were also compared for their ability to interact with limiting amounts of immobilized receptor analog (globotriose-Fractogel) giving similar percentage of binding (batch 8, 75 ± 10%; batch 9, 71 ± 6%; mean ± S.D., n = 2). Finally, aliquots of Stx1-batch 9 before and after conjugation with Alexa Fluor® 488 were assayed on the Gb3-expressing (27) Raji cells (3 h incubation) cultured in RPMI 1640 medium (Lonza) containing antibiotics (60 units/ml penicillin, 60 µg/ml streptomycin; Cambrex) and supplemented with 4 mM 1-glutamine (Sigma) and 10% fetal bovine serum (Lonza). Protein synthesis assay as in Ref. 24 was inhibited to the same extent with fluorescent (IC50 = 0.48 pm, r = -0.99) or native (IC50 = 0.42 pm, r = -0.99) toxin, indicating that conjugation with dye did not affect Gb3 binding and enzymatic activities.

Binding of Native and Fluorescent Stx1 to PMN—To obtain endotoxin-free PMN (98% lobulated nuclei) from healthy donors, all the solutions used throughout the method (28) were sterile and prepared with endotoxin-free water. The binding experiments were performed in Eppendorf tubes precoated with PBS containing 1% BSA to avoid nonspecific loss of toxins (29). Freshly isolated endotoxin-free PMN (0.5 × 106) were immediately incubated with native or fluorescent Stx1 (50 nM) in 250 µl of PBS containing 1% BSA for 90 min at 37 °C, with occasional stirring by gentle inversion of the tube. The cells were spun down at 200 × g for 5 min and washed three times with 100 µl of the same buffer containing BSA at 37 °C.

Native Stx1 bound to PMN was detected by flow cytometry as previously described (10, 14). Briefly, PMN were incubated with appropriately diluted mouse monoclonal antibody against Stx1 in the presence of human serum to saturate Fc receptors. After incubation with FITC goat anti-mouse IgG, flow cytometric analysis was utilized to reveal the cell-bound fluorescence. The flow cytometer (FC500 Beckman Coulter) was set to acquire and gate events both by forward scatter versus 90° side scatter and by green fluorescence versus 90° side scatter. PMN were checked by staining with monoclonal antibodies to antigens associated to granulocytes (FITC-CD16 and FITC-CD65; Beckman Coulter). This setting resulted in a prompt analysis of both morphology and fluorescence, allowing a clear evaluation of control and positive samples. The mean channel value of fluorescence (MCV) of the cells was chosen as an objective parameter to measure the extent of binding of Stx1 to cells (10). The single values were calculated by subtracting the control MCV (range, 0.4–0.6), i.e. the MCV of cells incubated with primary and secondary antibodies in the absence of the toxin. The same values (MCV = 0.4–0.6) were obtained if anti-Stx1 mouse monoclonal antibodies were omitted in the assay in the presence of toxins and secondary antibodies. The assay had
Conformation of Stx1 and PMN Binding Activity

been previously validated by challenging Stx-positive PMN with a negative control antibody (14) and by comparing control subjects and HUS patients in a double blind fashion (10).

Fluorescent Stx1 bound to PMN was detected by direct flow cytometric analysis and expressed as MCV. The binding of fluorescent Stx1 in the presence of a 50-fold excess of unlabeled toxin (nonspecific binding) was subtracted in each experiment.

Adenine Release from DNA in Vitro—Adenine release from DNA was measured by using as substrate the 2251-bp [3H]DNA labeled in the purine ring of adenine obtained by PCR amplification of the 731–2981 region of the pBR322 plasmid (19). Enzymatic reactions were performed in 150 μl of 50 mM sodium acetate buffer (pH 4) containing 0.3 μg of substrate corresponding to 205.5 pmol of adenine having a specific radioactivity of 3000 dpm/pmol and 1 pmol of Stx1. After 40 min at 45 °C, DNA molecules were removed by passing the samples through Bond Elut® NH₂ columns as previously described (19), and the combined flow-through and washing was measured by liquid scintillation counting.

Endothelial Cell Cultures and Protein Synthesis—Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Janette A. M. Maier (University of Milan, Milan, Italy). HUVEC were cultured as previously described (20). Cells used in this study were all of early passages and were checked for the expression of von Willebrand factor by immunocytochemistry using a rabbit anti-human von Willebrand Factor (Dako, Milan, Italy) as primary antibody (30).

Spectroscopic Measurements—Circular dichroism spectra were obtained with a Jasco J-715 spectropolarimeter with 2-nm resolution. Fluorescence spectra were obtained on a Spex Fluorolog 111A spectrofluorimeter with right angle detection geometry. All of the spectroscopic experiments were carried out at 22 °C.

Time Domain Fluorescence Lifetimes—A time-correlated single photon counting system (TCSPC; IBH Consultants Ltd.) was used to study the time-resolved fluorescence of air-equilibrated solutions, excited at 278 nm with a Nano-LED source. Decay profiles were collected at 330 nm and analyzed using a multieponential function (Equation 1) and deconvolution of the instrumental response.

\[ I(t)_{330} = \sum a_i \times \exp(-t/\tau_i) \]  
(Eq. 1)

\[ f_i = (a_i \times \tau_i) / \sum (a_i \times \tau_i) \]  
(Eq. 2)

Equation 2 represents the fractional contribution of each decay time to the steady state intensity at 330 nm (31). The software package was from IBH Consultants Ltd. The time resolution limit after deconvolution was ~0.2 ns.

Frequency Resolved Decay Times—The fluorescence decay times (mean deviation of three independent measurements, ~5%) were evaluated with a Spex Fluorolog-τ system by Jobin-Yvon, which relies on the phase modulation technique. The excitation source (a xenon lamp) was modulated, at the selected excitation wavelength, in the 10–300 MHz range (with a time resolution down to 20 ps). To increase the signal intensity a broad band of the emitted light (wavelength > 305 nm, selected by a cut-off filter) was detected on a R928P Hamamatsu photomultiplier. The frequency domain intensity decays (phase angle and modulation versus frequency) were analyzed with the Globus Unlimited™ rev. 3 global analysis software (Ref. 32 and references therein).

Data Analysis—Differences in continuous variables were tested with t test after controlling the normality of their distribution. A value of p < 0.05 was considered statistically significant. Correlation between variables was assessed through calculation of the Pearson correlation coefficient.

RESULTS

We have purified Stx1 and Stx2 numerous times over the past years (1996–2009). Stx1 was routinely obtained by passing the bacterial lysate from E. coli C600 H19 through a receptor analog affinity chromatographic column (globiotriose-Fractogel) (25). The toxin was eluted with 6M guanidine HCl (pH 6.7), extensively dialyzed against PBS, and stored in small aliquots at −80 °C. We have obtained eight batches of Stx1, whose purity was assessed by visual inspection after SDS-PAGE followed by silver staining. The toxins had remarkable activity on a rabbit reticulocyte lysate protein synthesis system (IC₅₀ = 0.08 nm ± 0.04; mean ± S.D., n = 8), the gold standard assay employed in our laboratory to detect the enzymatic activity of Stx after proteolytic cleavage and reduction. Four of these batches were also assayed for the binding to PMN (MCV = 3.2 ± 0.4; mean ± S.D., n = 4) after the first demonstration of the phenomenon (6).

In 2009, we had some difficulty in preparing the new batch of Stx1, because only 0.3 mg of toxin were eluted from the affinity column in contrast to the higher amounts obtained in the previous preparations (1.58 mg ± 0.31, mean ± S.D., n = 8). This forced us to modify the purification procedure by introducing a concentration step with Centricon 3, performed after dialysis and a further freeze and thawing cycle. In this case, we made the unexpected observation that the new prepared Stx1 (named Stx1-batch 9 throughout the paper) was unable to bind to PMN (see below). This prompted us to perform a comparative analysis of the biological activity and of the physicochemical properties of the new batch with respect to the last yet available preparation (Stx1-batch 8), which was purified from the same E. coli strain using identical reagents and the same receptor analog affinity chromatographic column. The binding of the toxins to PMN was assayed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were treated with 50 nm toxins for 90 min at 37 °C in PBS containing 1% BSA, and after extensive washing with the same buffer, the leukocytes were incubated with a mouse monoclonal antibody to Stx1. The toxin bound to PMN was revealed by indirect and direct flow cytometric analysis. In the first case, human endotoxin-free PMN were t...
binding to PMN was confirmed (representative single histogram analysis in Fig. 1B) with toxins labeled with Alexa Fluor® 488 followed by direct cytofluorimetric analysis obtaining similar results (MCV = 8.0 ± 0.3 and 0.3 ± 0.1, respectively; p < 0.001). It should be noted that the fluorescent labeling did not impair the binding of Stx1 to Gb3-deficient cells such as PMN (11) nor to Raji cells (see “Experimental Procedures”) known to express Gb3 (27).

The two preparations of Stx1 were also compared for the ability to release adenine from [3H]DNA in vitro (19) as evidence of the integrity of their enzymatic active site (A chain). We did not observe any significant difference (p = 0.79) in the rate of depurination of DNA induced by Stx1-batches 8 and 9 (59.1 pmol of adenine ± 8.7 and 56.8 pmol of adenine ± 11.0, respectively; mean ± S.D., n = 3), according to previously reported values (21). Full activity of the toxins during the intoxication of target cells obviously requires the cooperation of both type of subunits. To check the functional cooperation between A and B chains, human endothelial cells were treated with toxins at different concentrations and, after 16 h incubation, the inhibitions of protein synthesis obtained with the two different batches of toxin were measured and compared. As shown in Fig. 2, the calculated IC₅₀ were very similar, indicating that both toxins were fully active in intoxicating endothelial cells. Moreover, the absolute IC₅₀ values are in line with those reported for other Stx1 preparations (20, 21). Consistently, the A and B chains of Stx1-batch 9 appeared undamaged as assessed by visual inspection of the image obtained after SDS-PAGE (Fig. 3) and by calculating their apparent molecular masses (A chain, 31.2 kDa; B chain, 9.8 kDa) with respect to standard proteins run in the same conditions. The values are similar to those obtained (Fig. 3) with Stx1-batch 8 (A chain, 30.0 kDa; B chain, 10.0 kDa). These results are in line with known estimates for the A chain (32 kDa), whereas in the case of B chain, they are 20%...
Conformation of Stx1 and PMN Binding Activity

To gain information on the conformational features of the Stx1 proteins, the two batches were examined by CD. This spectroscopic technique is a powerful method for determining protein conformation. Indeed in the far ultraviolet region (190–250 nm), the CD of a protein is dominated by the n, π* and π, π* electronic transitions of the amide groups and is influenced by the geometries of the polypeptide backbone. Each of the possible secondary structures, α-helix, β-sheet, and random coil, gives rise to a CD of characteristic shape and magnitude, so that the overall signal reflects the fractional contribution of each structure type, which can be determined by suitable algorithms (36). The CD spectra of the two preparations of Stx1 recorded in identical conditions are shown in Fig. 4. The spectrum of Stx1-batch 8 exhibits a negative minimum at 222 nm and a negative shoulder at ~210 nm, whereas the spectrum of the Stx1-batch 9 shows a much weaker negative band centered at 226 nm, and the signal becomes slightly positive ~210 nm. According to the CD spectral profiles of proteins of known structure (37), we can conclude that Stx1-batch 8 contains a significant fraction of α-helix structure, whereas Stx1-batch 9 reveals lower α-helix content and a major fraction of β-sheet structures. Thus, the CD spectra clearly indicate a conformational difference between the two proteins. This fact was confirmed by their intrinsic fluorescence. Tryptophan residues are the dominant fluorophores in proteins, and their emission properties reflect the average environment in which they are embedded. Emission spectra are generally structured and blue-shifted for Trp residues buried in apolar sites, whereas they are unstructured and red-shifted for Trp residues exposed to water (31). Stx1 possesses seven Trp residues, two of them in the A subunit and one in each of the five B subunits. Excitation at 295 nm of 1 μM Stx1 solutions led to the emission spectra shown in Fig. 5. Stx1-batch 8 exhibits a structured fluorescence peaked at 334 nm, whereas Stx1-batch 9 has unstructured emission with a maximum at 343 nm. These features indicate that the Trp average environment is less polar in the former sample than in the latter one. This result was also confirmed by the time-resolved fluorescence, which was measured by TCSPC (31), with excitation at 278 nm and detection of emission at 330 nm. Analysis of the decay profiles using a multiexponential function evidenced three τi components (with individual fractional intensities fi):

FIGURE 3. SDS-PAGE analysis of Stx1. Left panel, lane 1, Stx1-batch 8 (1 μg). Right panel, lane 1, molecular mass markers (112 ng/protein): bovine α-lactalbumin (14 kDa), bovine trypsinogen (24 kDa), bovine carbonic anhydrase (29 kDa), rabbit glyceraldehyde-3-phosphate (36 kDa); lane 2, Stx1-batch 9 (1.15 μg). After electrophoresis, the gels were silver-stained. Rl = distance of protein migration/distance of dye migration. The equations (Stx1-batch 8, y = 1.0136x + 4.949, r = −0.99; Stx1-batch 9, y = 0.7887x + 3.8461, r = −0.99) were obtained by plotting the mobility (Rf) of calibration proteins (see Fig. 3) versus log kDa. The Rf of A and B subunits of each batch allowed calculation of the molecular masses reported under “Results.”

FIGURE 4. Conformation of Stx1 examined by circular dichroism. The ellipticity of Stx1 (1 μM) in PBS at 22 °C in a 0.1-cm cell is shown. α, Stx1-batch 8; β, Stx1-batch 9.
and are no longer quenched in Stx1-batch 9. We also conclude residues, residing in hydrophobic environments of Stx1-batch 8 resolved fluorescence, we can conclude that some of the Trp components, similar in both Stx1 samples, points to classes of Trp environments of different average polarity. However, the component detected in both samples is below the apparatus 295 nm. The scatter of a Stx1-batch 9 sample using a frequency domain fluorometer with 10-ps resolution (31) (see “Experimental Procedures”). A biexponential decay kinetics with lifetimes \( \tau_1 = 2.2 \text{ ns} \) (17%), \( \tau_2 = 6.5 \text{ ns} \) (14%), and \( \tau_3 = 0.09 \text{ ns} \) (69%) in Stx1-batch 8 and \( \tau_1 = 1.8 \text{ ns} \) (20%), \( \tau_2 = 6.0 \text{ ns} \) (50%), and \( \tau_3 = 0.08 \text{ ns} \) (30%) in Stx1-batch 9. The presence of multiple lifetime components, similar in both Stx1 samples, points to classes of Trp environments of different average polarity. However, the \( \tau_3 \) component detected in both samples is below the apparatus resolution. To check for its meaning, we measured the emission decay of a Stx1-batch 9 sample using a frequency domain fluorometer with 10-ps resolution (31) (see “Experimental Procedures”). A biexponential decay kinetics with lifetimes \( \tau_1 = 1.5 \text{ ns} \) and \( \tau_2 = 6.4 \text{ ns} \) was ascertained. Thus, the nanosecond lifetime components of the emission were fairly well confirmed, but the ultrashort contribution was not detected. We conclude that in the TCSPC experiment, some excitation light was scattered by the large globular protein and reached the TCSPC photodetector, contributing to the overall signal. The scattering signal was more intense in the Stx1-batch 8 than in Stx1-batch 9 sample, indicating a difference in the shape of the two proteins. Thus, our interpretation of the emission behavior of the Stx1 samples will only refer to the \( \tau_1 \) and \( \tau_2 \) nanosecond components.

Focusing on the fractional intensities \( f_i \) (see “Experimental Procedures”), we notice that \( f_{\text{Trp}} \) relevant to the long-lived component, is relatively larger in the protein with red-shifted emission than in the protein with blue-shifted emission. Indeed, from a ratio \( f_2: f_1 \) of \( \sim 1 \) in Stx1-batch 8, we found a ratio 2.5 in Stx1-batch 9. This supports a difference in the conformation of the two proteins. In particular, from both steady state and time resolved fluorescence, we can conclude that some of the Trp residues, residing in hydrophobic environments of Stx1-batch 8 where they are strongly quenched, become exposed to solvent and are no longer quenched in Stx1-batch 9. We also conclude on the basis of the corresponding CD spectral profiles that the conformational change pertaining to Stx1-batch 9 involves the partial disruption of some \( \alpha \)-helix structures of the native protein.

To reproduce the partial denaturation of the toxin allowing the loss of PMN binding activity, Stx1-batch 8 samples were treated with different denaturating agents in mild conditions (Table 1). Microconcentration, chaotropic agents (guanidine, urea), heat treatment at neutral and acidic pH, or reduction by DTT did not result in substantial loss of PMN binding activity. Conversely, five freeze (\( -80 \text{ °C} \)) and thawing cycles significantly reduced (\( -40\% \)) the capacity of Stx1 to bind PMN, leaving unchanged the IC\(_{50}\) of the toxin on HUVEC translation (0.94 pm versus the IC\(_{50}\) shown in Fig. 2).

### DISCUSSION

Starting from a serendipitous observation, we have gained new insights into the structure/function relationship of the well known bacterial toxin Stx1. Spectroscopic and fluorescence measurements allowed us to demonstrate that conformational changes of the toxin, with reduction of \( \alpha \)-helix content and exposition to solvent of some of the hydrophobic Trp residues, cause loss of PMN binding activity. Interestingly, the partially unfolded toxin was found to be fully active in intoxicating human endothelial cells because both enzymatic (A chain) and Gb3 binding activities (B chains) were preserved. The data suggest that the PMN-binding domain on Stx1 is distinct from the Gb3-binding domain and from the active site of the toxin. The independence of the three activities was confirmed by the loss of PMN binding activity with preserved toxic activity in Stx1 subjected to freeze and thawing cycles.

Stx1 possesses seven tryptophan residues, one in the A1 fragment (Trp-203), one in the A2 fragment (Trp-277), and one in each of the five B subunits (Trp-34) (Fig. 6). To gain information about the Stx1 region recognized by PMN, it is important to identify the loosened \( \alpha \)-helices in proximity to or containing the tryptophan residue(s) exposed in the unfolded toxin. The crystal structure of Shiga toxin, differing from Stx1 by a single amino acid, is well known (23). By reviewing the structural data of the Trp containing moieties of the toxin, we ruled out Trp-34. The B chains are arranged in a pentamer forming a ring in which each subunit is folded into a single \( \alpha \)-helix (residues 34 – 46) containing Trp-34 and located within the ring (23). The A chain (A2 region) dips its C terminus into the center of the ring and forms an \( \alpha \)-helix (residues 279 – 286) encircled by

![FIGURE 5. Intrinsic fluorescence of Stx1. Fluorescence spectra of Stx1 (1 \( \mu M \)) in PBS at 22 °C are shown. a, Stx1-batch 8; b, Stx1-batch 9. Excitation was at 295 nm.](image-url)
the α-helices of the B subunits. On the outside the B pentamer, six strands belonging to each B subunit form antiparallel β-sheets. The conformation of the five B chain α-helices seems to be important for the overall structure and binding activity of the holotoxin, because Trp-34 is one of the amino acids involved in the interaction with Gb3 (4). Thus, it seems unlikely that the toxic activity would have been preserved after their unfolding. Indeed, site-directed mutagenesis studies showed that Trp-34 substitution with different amino acids reduced the cytotoxicity 2–100-fold, depending on the cell type (38, 39). In the present paper, we did not observe any reduction in the cytotoxic power of unfolded Stx1. Moreover, crystal structures of the B-pentamer bound to Gb3 trisaccharide analogs (39) revealed that Trp-34, located on the flat face of the B-pentamer opposite to the A chain (Fig. 6), was only fully exposed to the solvent in the complexes. However, exposure to water of five Trp residues of seven in the unfolded toxin would probably have led to a higher increase in the nonquenched long-lived components than our observations. Consistently, the A chain of the unfolded toxin, but not the B-chains, showed reduced silver affinity after SDS-PAGE. This might be explained by side chain modifications of amino acids involved in the interaction with silver ions occurring in the unfolded molecule. Taken together, present and previous observations point to the A chain of Stx1 as the unfolded region involved in the binding to PMN. This is consistent with the notion that Stx1 and the A chain of plant toxin ricin share a common receptor on PMN (24). It is worth noting that the A1 region of Stx1, endowed with the active site, and the A chain of the plant toxin show homology (23) with the same enzymatic mechanism of action, whereas the B chains of these toxins are quite dissimilar. Indeed, 149 structural equivalent residues have been found in the A chains of ricin and Stx1, and 23% of them are identical (23). These equivalent or identical residues are not present in the four-stranded sheet of Stx A subunit which lies on top of the B pentamer, constituting the border region between A2 and A1 fragments (Fig. 6). Excluding this region, the fold of the remaining part of the A1 fragment is very similar to the fold of the A chain of ricin (23). The nonconserved Trp-277 is not a good candidate for exposure to a hydrophilic environment upon α-helix unfolding because it belongs to the four-stranded sheet (A2 region). On this basis, we assumed that the region responsible for binding to PMN was located in the A1 region homologous to ricin A chain. Assuming that some crucial α-helix structures of this region are involved in binding to PMN, although not in the enzymatic activity, the following deductions could be made. The active site of Stx1 contains a cleft akin to that in the A chain of ricin (23, 40), containing seven invariant residues (Tyr-77, Val-78, Ser-112, Tyr-114, Glu-167, Ala-168, Arg-170, and Trp-203; numbers as in Shiga toxin and residues in α-helices in italics). These amino acids should have conserved the reciprocal spatial relationship required to fit the adenine residue during the preserved catalytic activity of the unfolded toxin. However, as shown in the crystallographic studies of ricin- or Stx-ligand complexes (41–43), few amino acids interact directly with adenine forming hydrogen bonds (Val-78, Ser-112, Tyr-114, and Arg-170; numbers as above). Thus, the unfolding of the α-helices containing the latter two amino acids (residues 114–121 and 152–170) would probably interfere with the enzymatic activity of the toxin. In contrast, Trp-203 seems to be a good candidate as the tryptophan responsible for the red shift of the spectrum from exposure to solvent in unfolded toxin: (i) it belongs to an α-helix (residues 203–212); (ii) it is not directly involved in the binding to adenine; and (iii) it is spatially close to the A2 fragment sequence containing Met-260, which blocks access to the active site in the holotoxin (Fig. 6). Thus, loosening of this helix would not reduce the enzymatic activity of the toxin. Alternatively, the unfolding of helices in spatial proximity to Trp-203 such as residues 194–201 and 229–236 might render the amino acid accessible to solvent without a direct unfolding of the α-helix (residues 203–212) itself. We suggest that the α-helix containing Trp-203 and/or the α-helices of the A1 region close to this residue are the moieties of Stx1 recognized by PMN.

The noncovalent change in the structure of a given protein is defined denaturation, a change that may alter the secondary, tertiary, or quaternary structure of the molecule. A variety of factors might induce these changes, such as increasing temperature, changes in pH, changes in dielectric constant, high ionic strength, and exposure to cross-linkers or to air-liquid interfaces. In the specific case reported in the present paper (Stx1-batch 9), the latter explanation appears more likely. In fact, during the receptor analog affinity chromatographic run, the toxin was eluted by the column at low concentration (~0.3 mg/ml), a condition known to favor surface denaturation. Moreover, the low concentrated protein was subjected to dialysis and to a freeze (~80 °C) and thawing cycle allowing air-liquid interactions that might have induced the unfolding of the protein. It should be noted that, among different agents/treatments capable of inducing protein unfolding, only repeated freeze and thawing cycles reproduced the modification of Stx1 responsible for the loss of PMN binding activity with preserved toxic activity (Table 1). This is consistent with the strong cor-
relation between the tendency of a protein to freeze denature and its tendency to surface denature (44).

Irrespective of structural predictions, these findings might help explain the conflicting results on PMN binding activity of Stx1 obtained by different groups. Clearly, to ensure preservation of the activity of a given toxin after purification to homogeneity, it is important to assay its enzymatic activity and/or its toxicity for target cells. In each of the papers dealing with the binding activity of Stx1 to PMN, these assays have been carefully performed. However, the data reported here demonstrate that it is possible to obtain fully cytoxic Stx1 endowed with its specific enzymatic activity, which is, however, completely unable to bind to PMN. It should be noted that the three different groups that gave positive (10–13) or partially positive (15) results on this topic have purified Stx by single-step affinity chromatography. In contrast, the three groups that were unable to reproduce Stx binding to PMN obtained the toxins by multistep purification methods (7, 8) or from commercial sources (7, 9). The methods employed, although sound and well known, contain numerous steps and chromatographic runs, which might have caused the partial conformational change inhibiting the binding of Stx1 to PMN.

In light of the present results, we suggest that any new positive or negative data on PMN/Stx interactions should be obtained using purified toxins assayed not only for enzymatic and toxic activity, but also for correct folding, by inspecting CD and/or fluorescence emission spectra and their maxima. This would better clarify the scenario on this still controversial topic.

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