Cardiotoxins (CTXs) from cobra venom show cytotoxicity toward several cell types. They cause systolic heart arrest and severe tissue necrosis. Their interaction with phospholipids is established but by itself fails to explain the specificity of these toxins; other component(s) of membrane must, therefore, intervene to direct them toward their target. We herein show, for the first time, that sulfated glycosaminoglycans, heparin, heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS), interact with CTX A3, a major component of Taiwan cobra venom, by use of affinity chromatography, circular dichroism, absorbance, and fluorescence intensity and anisotropy measurements. The relative strength of binding, determined by the NaCl concentration required to dissociate the CTX-glycosaminoglycan complex, varied as follows: heparin > DS > CS > HS. In physiological buffer (8 mM Na2HPO4, 2.7 mM KH2PO4, 138 mM NaCl, pH 7.4), however, only heparin and HS bound to CTX, with respective dissociation constants of 1.4 and 16 μM, while CS and DS failed to exhibit well defined binding behavior, as indicated by fluorescence measurements. We estimate that CTX makes 3–4 ionic contacts with heparin based on a salt-dependent binding study and that 40% of binding free energy is derived from purely electrostatic interactions under physiological conditions. Sulfated pentasaccharide may be sufficient to bind to CTX. We also found that heparin accentuates the penetration of CTX into phospholipid membranes as analyzed by Langmuir monolayer measurement. In view of these results we propose that heparin-like moieties of the cell surface may modulate the action of CTX.

Himatkumar V. Patel, Alka A. Vyas, Kavita A. Vyas, Yi-Shiuan Liu, Chien-Min Chiang, Lang-Ming Chi, and Wen-guey Wu‡

From the Department of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan 30043

Cardiotoxins (CTXs) from cobra venom are highly basic polypeptides consisting of 60–62 amino acid residues. They target several cell types, particularly cardiac myocytes (1, 2). Large doses of CTXs are injected into the victim, since more than 50% of cobra venom proteins are CTXs. Surprisingly, unlike the well known α-acetylcholine receptor for snake α-neurotoxins (3), a primary binding target for CTXs has not been identified. The membrane surface is believed to be the site of action, and binding of CTX with anionic and zwitterionic phospholipids is established (4–7). The toxins lyse a variety of cells (1, 2, 6, 8) and are also known as cytotoxins. Cardiotoxins are so named because they cause systolic heart arrest. They also cause severe tissue necrosis and local gangrene in humans (9).

The structure of CTXs has been established by x-ray and NMR spectroscopy (see, for instance, Refs. 10, 12–14). The toxins adopt an α-sheet three-finger loop structure held together by four disulfide bonds (for review, see Ref. 15). On the basis of distribution of charged and hydrophobic residues in CTX, we and others have proposed that CTX forms ion channels in cell membranes (6, 10). A CTX-induced ion-conducting pathway has indeed been detected in bullfrog atrial myocytes (16). A continuous hydrophobic domain, 34 Å long, capable of binding to and penetrating the membrane bilayers has been detected in the x-ray crystal structure of P-type CTXs. However, the presence of up to 12 cationic residues as in most CTXs controverts the suggestion, since charged residues are thermodynamically unfavorable in membrane bilayers.

The specific activity CTXs display toward different cells suggests that binding target(s) other than lipids must exist. Previous reports have ruled out carbohydrates as the membrane binding site, since CTXs display similar activity toward neuraminidase-treated erythrocytes or atrial muscle cells, the two established targets of CTXs (Refs. 1 and 2 and references therein). However, anionic carbohydrates abound at the membrane surface and in extracellular matrix and are unique to their location (17). Heparan sulfate proteoglycans, for example, are ubiquitous on the cell surface (18). We therefore reasoned that cell surface carbohydrates other than sialic acid may capture the basic CTXs and thus modulate their action.

Glycosaminoglycans (GAGs) are a family of linear, diverse, heterogeneous, and polyanionic polysaccharides widely distributed in the extracellular matrix of animal tissues. A variety of functions ranging from regulation of blood clotting to cell differentiation and proliferation and metastasis have been ascribed to them (for reviews, see Refs. 17–20). Numerous proteins have been shown to possess the ability to bind to GAGs specifically, the ligands including enzymes (lipoprotein lipase, phospholipase A2) (21–23), enzyme inhibitors (antithrombin), growth factors (fibroblast growth factors), and cell adhesion molecules (platelet and endothelial cell adhesion molecules), among others.

In continuation of the efforts of this laboratory to unravel the mode of action of CTXs (5–7, 12, 13) we report herein, by use of GAG affinity chromatography, CD, absorbance, and fluorescence intensity and anisotropy measurements, that GAGs bind to CTX with interesting specificity and induce conformational change in the toxin. We found that heparin and heparan sulfate (HS) bind to CTX with reasonable strength under physiological...
conditions and enhance the membrane-related activity of the toxin. The physical nature of the CTX-heparin interaction is also characterized by a salt-dependent binding study and analyzed by macromolecule-polyelectrolyte theory.

MATERIALS AND METHODS

Crude venom from Naja naja atra and various GAGs, heparin (porcine intestinal mucosal), heparan sulfate (bovine intestinal mucosal), chondroitin sulfate (from shark cartilage), dermatan sulfate (porcine intestinal mucosal), N-desulfated heparin (porcine mucosal), heparin disaccharides, low molecular mass heparin (homolytically depolymerized), and hyaluronan (bovine triache) were purchased from Sigma. Their average molecular weights (Mₐ) were 15,000, 7500, 50,000, 14,000, 15,000, 665, 3000, and 80,000, respectively. POPC (palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine) was purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals were of reagent grade.

Purification of CTX—CTX A3 was purified from crude venom by SP-Sephadex C-25 ion exchange chromatography and reverse phase HPLC as reported (5, 6). Established nomenclature was used to sort the chromatographic fractions (1, 6), and CTX A3 showing high hemolytic activity was pooled and subjected to purification by HPLC. The purity, analyzed by SDS-polyacrylamide gel electrophoresis and analytical reverse phase HPLC, was found to be higher than 99%. Protein concentration was determined by the Lowry method.

Acid Hydrolysis of Heparin—Heparin was subjected to acid hydrolysis as reported (24). Briefly, 4 mg of heparin was treated with 0.2 N HCl for 24 h at 100 °C to completely desulfate and depolymerize heparin. The solution was then neutralized (pH 7.4) with 10 N NaOH, and the derivative was reconstituted to 1 mM with distilled water.

GAG Affinity Chromatography—The relative binding strength of CTX A3 to various GAGs was evaluated by GAG affinity chromatography. For this purpose, HS, chondroitin sulfate (CS), and dermatan sulfate (DS) were covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) essentially following the procedure of Iverius (25). Heparin-Sepharose (HiTrap heparin) column was purchased from Pharmacia. The columns were equilibrated with 10 mM Tris, pH 7.5, containing 25 mM NaCl and CTX A3 (0.35 mg) was loaded onto each column. The columns were then washed with 10 mM Tris buffer until absorbance at 280 nm of the eluate returned to base line. A linear gradient of 0–1 M NaCl in 10 mM Tris, pH 7.5, was then applied for the elution of bound CTX.

Circular Dichroism Measurements—The GAG-induced structural change in CTX was monitored by CD spectroscopy. Spectra were recorded on AVIV 62A DS spectropolarimeter (Lakewood, NJ) as reported (13). The protein concentration was maintained at 20 μM so that absorbance of the solution was less than 2 from 190 to 260 nm. Typically, 20 μM CTX was titrated against GAG, and at each point, the solution was incubated for 3 min at room temperature before scanning. The spectra, averages of four repeats, were obtained by scanning from 260 to 190 nm. A 1-mm cell, bandwidth of 1 nm, and time constant of 1 s were used to collect data at 25°C, reported in millidegrees. GAG-induced structural perturbation was estimated by change in ellipticity at 195 and 215 nm due to an absorption problem, ellipticity ~195 nm could not be studied in the presence of high NaCl concentrations. Therefore, all CD experiments were performed in 20 mM sodium phosphate buffer, pH 7.4. Nevertheless, conformational change of CTX A3 in the presence of high salt was also studied in the presence of NaF (140 mM) to overcome the absorption problem at 195 nm. The degree of ellipticity change of CTX A3 in NaF salt was significantly reduced, but the overall trend of the change was found to be similar.

Fluorescence Measurements—The intrinsic fluorescence intensity of Tyr of CTX A3 was used to determine the binding constant of the toxin with GAGs, and its anisotropy was also studied to indicate the effect of binding on the physical property of the CTX-GAG complex. The excitation and emission wavelength were set at 285 and 318 nm, respectively, by using an SLM 4800 fluorescence spectrometer as reported (6). T-format measurement of fluorescence anisotropy (r) of CTX A3 upon GAG binding was performed by a macroprogram to obtain the fluorescence intensity (I) and gain (G) in the parallel and perpendicular modes. The fluorescence anisotropy values were then calculated using the equation

\[
r = (R_{90} - R_{45})/(R_{90} + R_{45})
\]

where \(R_{90} = G_{90}/G \) and \(R_{45} = G_{45}/G \) (26).

The dissociation constant, \(K_d\), was determined by nonlinear least squares fitting of data by assuming that a single independent, but equivalent, binding site is present in the studied GAGs using the equation,

\[
K_d = ([H] + [HC])C/[H][HC]
\]

where \([C]\), \([H]\), and \([HC]\) are the concentrations of CTX, heparin, and CTX-heparin complex, respectively. By using the fluorescence intensity, \(F\), as an indication of the fraction of binding, Equation 2 can be reformulated as the following three equations, which can be used to simulate the experimental fluorescence intensity of CTX A3 as a function of GAG concentration,

\[
F_S = F_0 + (F_S - F_0)X
\]

\[
X = (n[H] + [C]_0 + K_I Y)/(2[C]_0)
\]

\[
Y = ([n[H] + [C]_0 + K_I]^2 - 4n[C]_0[H]/K_I)^{1/2}
\]

where \(F_S\) is fluorescence intensity and \(F_0\) and \(F_S\) are intensities when CTX is free and at saturation, respectively. Complete (100%) binding was assumed to occur when the fluorescence intensity reached a plateau.

Absorbance Measurements—Change in turbidity of samples obtained during CD experiments performed in low salt buffer prompted us to study CTX-GAG interaction also by absorbance measurements. Typically, CTX A3 in 20 mM sodium phosphate buffer, pH 7.4, or phosphate-buffered saline (8 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, 138 mM NaCl, pH 7.4) was titrated against GAG, and the absorbance was measured at each point by using Beckman DU-70 spectrophotometer. Experiments were also performed in the reverse order by titrating CTX against constant GAG.

Phospholipid Monolayer—Monolayer experiments were performed on a Langmuir home-built minitrough (Joyce-Loebi Ltd.) as reported (7). Briefly, POPC dissolved in chloroform was deposited at the air-water interface to obtain a phospholipid monolayer of area about 60 m². The binding of CTX A3, in the presence and absence of heparin, to the monolayer was studied in PBS under lateral pressure of 18 mN/m and was evaluated from increase in area of the monolayer due to penetration of CTX or the CTX-heparin complex.

RESULTS

Conformational Change of CTX A3 as Revealed by Circular Dichroism Measurements—The interaction of GAGs with CTX A3 was monitored initially by CD spectroscopy, by titrating CTX A3 against GAGs in 20 mM phosphate buffer at pH 7.4. CTX A3 was chosen due to its abundance (~30%) in Taiwan cobra venom. Representative CD spectra of CTX A3, alone and in the presence of heparin, HS, CS, and DS, are shown in Fig. 1A. CTX A3 adopts predominantly β-sheet structure. It exhibits a characteristic positive band at 195 nm and a weak, broad negative band at ~215 nm as can be seen in Fig. 1. Upon the addition of GAGs, conformational change occurs in CTX A3 in two stages regardless of the type of GAG: initial decrease in ellipticity at 195 nm followed by recovery of the same ellipticity at higher GAG concentrations (Fig. 1B). Change in ellipticity around 215 nm follows a similar trend, although the sign is opposite (Fig. 1B).

The initial decrease and increase, respectively, in ellipticity at 195 and 215 nm indicate that CTX A3 undergoes structural perturbation, specifically reduction in β-sheet content (7, 13), in the presence of GAGs. It can be seen from Fig. 1B that different GAGs induce different extents of structural perturbation in CTX A3. At the effective concentration, defined here as the concentration required to cause maximum perturbation as detected by change in ellipticity, of heparin (~2 μM), the ellipticity falls from 12 to about 5 millidegrees whereas it decreases only to 9 millidegrees for the effective HS concentration (~4 μM). Furthermore, at higher than effective heparin and HS concentrations, ellipticity of CTX A3 reverts almost completely (Fig. 1B, ellipticity at 195 nm), whereas for excess CS and DS, ellipticity remains lower than that for free CTX A3, indicating that the two classes of GAGs (heparin/HS and CS/DS) bind differently.

Role of Sulfates and Carbohydrate Backbone—To gain in-
sight into the chemical nature of the binding, N-desulfated N-acetylated heparin, heparin disaccharides, and hydrolytically degraded heparin were studied. N-Desulfated heparin induced small conformational change in CTX A3 at an effective concentration ~20-fold higher than heparin (Fig. 2). Since \( M_r \) values of the two heparins are comparable, the reduction in binding ability can only be attributed to fewer available binding sites as a result of \( N \)-desulfation. This suggests that 2-N-SO_3 groups are crucial for binding.

To investigate the role of the carbohydrate backbone, the binding of mono-, di-, and trisulfated heparin disaccharides was studied. The titration curve of trisulfated disaccharide with CTX A3 is shown in Fig. 2; CTX A3 was found to be inert to all disaccharides, including the trisulfated derivative, up to 520 \( \mu M \) (data not shown for higher concentrations). Thus, the degree of sulfation or charge does not warrant binding without minimum saccharide length.

To rule out the involvement of other saccharides in natural GAGs, we studied the interaction of CTX A3 with chemically modified heparin. Acid hydrolysis of heparin effects first \( N \)-desulfation and then \( O \)-desulfation. Further hydrolysis at high temperature disintegrates the glycosidic linkage until finally a mixture of predominantly monosaccharides, to a lesser extent disaccharides, and a trace of pentasaccharides, lacking sulfates altogether, is formed. We performed titration of CTX A3 with heparin degraded in this manner. No significant change in

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**Fig. 1.** GAG-induced structural change in CTX A3 in 20 mM phosphate buffer at pH 7.4. **A**. representative CD spectra of CTX A3, alone and in the presence of GAGs at the indicated concentrations. **B**, ellipticity at 195 and 215 nm plotted as a function of the concentration of GAG. Hep, heparin.
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Comparison of the effect of heparin, heparin disaccharides, N-desulfated heparin, degraded heparin, and hyaluronan on CTX A3: ellipticity of CTX A3 at 195 nm plotted against GAG concentration. Trisulfated heparin disaccharide (open triangles), hyaluronan (filled triangles), N-desulfated heparin (open circles), and degraded heparin (open squares) had no significant effect on the ellipticity of CTX A3 even when in excess, whereas Hep (open diamonds) at 1.2 μM had a dramatic effect.

It can be concluded that sulfates as well as the backbone longer than disaccharides are required for binding. Hyaluronan, the GAG lacking sulfate, seemed an ideal candidate that would clarify the details by indicating whether an anionic carbohydrate backbone suffices or if the interaction is specific. As can be seen from Fig. 2, hyaluronan induces no change in β-sheet content of CTX even when in excess.

Modes of Binding—The reversibility of binding and regaining of ellipticity of CTX A3 at higher GAG concentrations seem to indicate that when supplied with more GAGs CTX can rearrange with the newly added GAG. The rearrangement of protein-GAG complex is reported by Bock et al. (27). They found that fluorescence anisotropy of dansyl-platelet factor-4 also increased initially and decreased subsequently at higher heparin concentrations. Acidic FGF-heparin complex also behaves similarly, as revealed by static and dynamic light scattering (28). These results are interpreted as change in size of the protein-heparin complex occurring upon binding.

To better understand the molecular rearrangement occurring during the binding process, we performed titration experiments in reverse order as that described earlier, i.e. varied CTX for fixed GAG concentration. Fig. 3A shows the profile of ellipticity of CTX A3 in the absence (control, open symbols) and presence (closed symbols) of heparin (2 μM, constant, effective concentration) versus concentration of CTX A3. It can be seen that upon the addition of up to 10–12 μM CTX A3 to heparin (CTX:heparin, 5–6:1), ellipticity rises steadily, to an extent comparable with free CTX (compare with control). Thus, initial binding causes minimal change in structure.

After 12 μM of CTX A3, however, further addition of toxin fails to cause an increase in ellipticity, which instead decreases until 15 μM (CTX:heparin, 7–8:1). The ellipticity here represents the combined effect of all binding states, because with heparin no longer in excess, the protein is forced to bind indiscriminately. The marked reduction in slope in this phase implies that maximum structural perturbation occurs. Beyond 18 μM, the ellipticity again rises sharply, and like that of free CTX A3 (compare with control), indicating that binding approaches saturation beyond 18 μM.

The absorbance at 320 nm was monitored for a similar titration. Interestingly, the absorbance or turbidity of the sample decreases slightly upon the addition of up to 12 μM CTX A3 to heparin (constant, 2 μM) (Fig. 3B). Further addition of 12–15 μM CTX, however, causes a sharp increase in absorbance, which remains constant for more CTX. An increase in absorbance, interpreted here as increase in size of the CTX-heparin complex, thus correlates well with conformational change detected by CD spectroscopy. It should be pointed out that turbidity of the sample does not affect ellipticity measurement, as demonstrated clearly by the steady rise in CD ellipticity above 20 μM CTX concentrations.

The three-phase profiles detected by CD and absorbance suggest that there exist two nonequivalent binding states and that the second binding state may be a manifestation of the aggregation of CTX molecules that heparin induces around saturation. The detected structural transition appears to be cooperative, since it is triggered by the last few CTX molecules binding to each GAG molecule; it occurs over a narrow concentration range, i.e., when the CTX:heparin ratio increases from 7 to 8. A schematic model to explain the suggested molecular rearrangement and aggregation of the CTX-GAG complex is described under “Discussion.” (see Fig. 9).

Binding under Physiological Conditions—To see whether the binding of CTX A3 with GAGs occurs under physiological conditions, we first determined the concentration of NaCl required to dissociate the protein bound to immobilized GAGs. Commercially available heparin-Sepharose was used, and CS, DS, and HS were coupled to CNBr-activated Sepharose essentially as reported (25). A solution of CTX A3 (0.35 mg) in 10 mM Tris buffer containing 25 mM NaCl was charged onto the GAG
Interestingly, both fluorescence intensity and anisotropy experiment is indeed due to the binding of CTX A3 with heparin. similarly, indicating that the fluorescence intensity enhancement is strong enough for CTX action. Due to an absorption problem in monitoring CD ellipticity in PBS at 195 nm, we used spectrophotometric and fluorescence measurements to monitor CTX-GAG interaction under physiological conditions.

Binding is only of biological significance if it occurs under physiological conditions. The strength reflected by NaCl elution, although seemingly unimpressive (comparable with hepatic lipase or myotoxin, Table I), is sufficiently high, nonetheless, to manifest physiological effect of the toxin. This is because the venom is injected in massive doses, ~180 mg/bite, into the prey (29). The local concentration of CTXs experienced by the tissue is therefore enormous, around submillimolar range. It is therefore important to quantitate binding of CTX to GAGs under physiological conditions to see whether the association is strong enough for CTX action. Due to an absorption problem in monitoring CD ellipticity in PBS at 195 nm, we used spectrophotometric and fluorescence measurements to monitor CTX-GAG interaction under physiological conditions.

Fig. 4 shows the profile of change in absorbance induced by GAGs, an indication of the aggregation state of the CTX-GAG complex, upon titration against CTX A3 at pH 7.4 in following buffers: 20 mM sodium phosphate (open squares); 10 mM Hepes, 150 mM NaCl (open triangles); and PBS (closed squares). A qualitatively similar trend is observed in these buffers, but effective concentrations and absorbance maxima vary significantly. High salt reduces extent of aggregation as indicated by lower absorbance. Thus, binding-induced aggregation of the CTX-heparin complex occurs under physiological conditions but is sensitive to experimental conditions.

Absorbance measurement in PBS reveals that interaction of CTX A3 is stronger with HS than with CS or DS, although the opposite is true in low salt buffer. The effective concentration of GAGs in PBS corresponds well with that detected by CD measurement, i.e. 2, 4, 2.3, and 0.6 μM, in the presence of low salt. Assuming that absorbance change occurs when binding approaches saturation, the effective concentration values reflect binding stoichiometry.

We tested the intrinsic fluorescence of Tyr of CTX A3 to study binding of CTX A3 with GAGs under physiological conditions. Titration of CTX A3 with heparin in PBS caused fluorescence intensity (Fig. 5A, open squares) to rise and later remain constant. Fluorescence anisotropy (Fig. 5B, open squares) behaves similarly, indicating that the fluorescence intensity enhancement is indeed due to the binding of CTX A3 with heparin. Interestingly, both fluorescence intensity and anisotropy exhibit apparent saturation around 1 μM and after that rise sharply again, followed by saturation at higher heparin concentrations. This implies again the existence of two binding states, the first one prevalent when the toxin is in excess. We determined the dissociation constant ($K_d$) and number of binding sites ($n$) by nonlinear least squares fit of the fluorescence data according to Equation 2. The first apparent saturation state detected for heparin concentration below 1 μM was ignored for calculation, since we attribute it to conformational change of the saturated CTX-heparin complex (Fig. 9). The nature of the initial binding is not established, but binding in this phase should be stronger than that in the second phase.

The curve of best fit is depicted in Fig. 5A (open squares). CTX A3 binds to heparin in PBS with $K_d$ = 1.4 μM and $n = 9$.

We also studied binding of CTX A3 with low molecular mass heparin (~3000 Da). The $K_d$ with low molecular mass heparin is estimated to be 2.25 μM, and $n = 1.6$. Thus, the binding constant compares well, regardless of the size of heparin, but the number of binding sites falls. After considering the mass of heparin and low mass heparin, it can be estimated that about 5.2–5.6 saccharides constitute an average binding unit of CTX A3. Since it is not likely that all saccharides are involved in the binding, sulfated pentasaccharide of heparin may be considered as an average binding unit.

Titration of CTX A3 with HS displayed similar, well defined binding behavior, although the change in fluorescence intensity (Fig. 6A, curve of best fit) and anisotropy (Fig. 6B) is smaller. HS binds to CTX A3 with $K_d = 16 μM$ and $n = 5$. In contrast, CS and DS caused no significant fluorescence intensity change, and no well defined binding curve was obtained in PBS. CS and DS may bind to CTX nonspecifically in the presence of low salt due to high electrostatic interaction. CTX A3 thus binds only to heparin and HS under physiological conditions. The binding to heparin is weaker than for FGF (0.47 μM) (30) but is stronger than for fibronectin (8.4 μM) (31).

**Ionic Strength Dependence of Heparin Binding to CTX—** Study of the salt dependence of binding of proteins with heparin is shown to clarify the nature of binding (30). We performed titration of CTX A3 against heparin in the presence of NaCl (75–300 mM, Fig. 5). Interestingly, the fluorescence anisotropy in the presence of 75 mM NaCl, for higher heparin concentrations decreased slightly (Fig. 5) rather than assuming a plateau. Heparin-dansyl-platelet factor 4 behaves similarly (27). We attribute this departure from well defined binding behavior to the higher aggregation that occurs in the presence of low salt. Fluorescence intensity, however, exhibited saturation (Fig. 5). The $K_d$ values obtained were 0.20, 1.42, 4.16, and 9.02 μM in the presence of 75, 150, 225, and 300 mM NaCl, respectively. The dissociation constant increased steadily, as expected.

### Table I

Comparison of NaCl concentration required to dissociate GAG-protein complex

| Protein a | Heparin | HS | DS | CS | Reference |
|-----------|---------|----|----|----|-----------|
| Cardiotoxin CTX A3 | 0.50 | 0.15 | 0.21 | 0.18 | Present work |
| Myotoxin | 0.50 | | | | 40 |
| Plasma lipoprotein | 0.26 | 0.09 | 0.15 | 0.08 | 41 |
| Fibronectin module III-13 | 0.43 | | | | 31 |
| tGF | 1.40–1.60 | | | | 42 |
| tGF b | 1.50–1.75 | 1.25–1.50 | 0.25–0.50 | 0.25–0.50 | 43 |
| Platelet factor 4 | 1.25–1.50 | | | | 44 |
| Interleukin 8 | 0.51 | | | | 45 |
| Leukocyte integrin Mac 1 | 0.40 | | | | 11 |
| Lipoprotein lipase | 0.88 | 0.57 | 0.46 | 0.32 | 21 |
| Hepatic lipase | 0.45 | 0.21 | 0.20 | No binding | 21 |

a GAGs were immobilized on matrix, and protein was eluted with NaCl.
b In this case, protein was immobilized on matrix, and GAGs were eluted with NaCl.
Fig. 7 depicts a plot of log $K_d$ versus log$[Na^+]$. The result is consistent with a prediction based on the theory of macromolecule-polyelectrolyte interaction (32, 33). Assuming that heparin behaves like a polyelectrolyte solution (34, 35), binding of CTX A3 to heparin in the presence of counterion ($Na^+$) would release a stoichiometric amount of $Na^+$ from the polymer according to the equation,

$$CTX^+ + heparin \rightarrow CTX$-heparin + $Z\Psi Na^+$ (Eq. 6)

where $Z$ is the number of purely ionic interactions between the two species, which results in displacement of $Z\Psi$-bound $Na^+$ from heparin, and $\Psi$ represents the fraction of $Na^+$ ions bound to heparin and is dependent on axial charge density of the polymer (36). A value of $\Psi = 0.8$ is reported for heparin (34, 35). The observed $K_d$ is thus related to the $K_d$ (nonionic) (Equation 7). This equation allows determination of $Z$ and $K_d$ (nonionic) if the dissociation constant in the presence of various concentrations of salt is known.

$$\log K_d = \log K_d (\text{nonionic}) + Z\Psi \log [Na^+] \quad (\text{Eq. 7})$$

Best fit of the salt-dependent $K_d$ values between CTX A3 and heparin yields a slope of $-2.7$. It gives an estimate of the purely ionic interactions involved in binding of CTX A3 to heparin; about 3.4 $Na^+$ ions are displaced when binding occurs. This value is slightly higher than for FGF ($-2.8$ $Na^+$) (30), indicating that higher electrostatic interaction is involved in binding of CTX A3 with heparin. Interestingly, the structure of co-crystal of bFGF with heparin hexasaccharide reveals that at least four ionic contacts are made (37). Thus, this treatment of binding data is not absolute, but it provides a fairly clear overall picture.

Assuming that at 1.0 M $NaCl$, all electrostatic interaction is neutralized and $\log K_d = \log K_d (\text{nonionic})$, then the $y$ intercept in the plot of $\log K_d$ versus $\log [NaCl]$ (Fig. 7) should be equal to $\log K_d (\text{nonionic})^0$ which was found to be 3.6. Therefore, $K_d (\text{nonionic})^0$ was 226 $\mu$M, whereas the observed apparent $K_d$ was 1.42 $\mu$M in the presence of 150 mM $NaCl$. It can be estimated by comparison of $\log K_d (\text{nonionic})^0$ ($-3.65$) with observed $\log K_d$ ($-5.85$) that $-40\%$ of binding free energy is derived from purely ionic interaction under physiological conditions at pH 7.4 and 25 °C. The salt dependence of heparin binding to bFGF, thrombin, and antithrombin (30, 34, 38) is also depicted in Fig. 7 to enable comparison with CTX. Similar treatment of binding of heparin with bFGF (30) indicates that nonionic interaction dictates the binding.

Heparin-induced Penetration of CTX into the Phospholipid Monolayer—Having shown that appreciable binding of CTX A3 occurs with heparin under physiological conditions, we then wished to examine how the binding ability translates into ultimate biological effect. We studied the effect of heparin on the activity of CTX on Langmuir phospholipid monolayer. CTXs are membrane active polypeptides: the membrane depolarization that follows exposure to toxin is believed to cause the eventual lysis of the affected cell, although the exact mechanism is unclear.

As can be seen in a representative trace of binding of CTX A3 to the POPC monolayer in PBS (Fig. 8A), the membrane surface area increases upon the addition of CTX. The increment in surface area is larger for higher concentrations of CTX, indicating that binding of CTX to phospholipid monolayers pro-

![Fig. 4. Interaction of CTX A3 with GAGs monitored by absorbance measurements (O.D.320). Shown is the titration of CTX A3 versus heparin (A), heparan sulfate (B), dermatan sulfate (C), and chondroitin sulfate (D) in different buffers. CTX A3 (constant, 20 $\mu$M) is shown in 20 mM phosphate buffer (open squares), in 10 mM Hepes buffer containing 150 mM NaCl (open triangles), and in PBS (close squares). Arrows indicate the effective concentration (concentration required to produce maximum change).](image-url)
duces the effect. We found that the addition of heparin markedly enhances the increase in surface area of Langmuir monolayers that CTX A3 causes (Fig. 8B). Heparin alone fails to cause any effect (Fig. 8A), confirming that the increase in surface area is due to binding of CTX A3 to heparin. The addition of preformed CTX-heparin complex to the monolayer caused a similar increment in surface area (Fig. 8C). Heparin thus enhances the penetration of CTX into the phospholipid monolayer.

DISCUSSION

We have herein demonstrated that CTX A3, a major component of Taiwan cobra venom, binds to heparin and HS under physiological conditions. In view of the results presented herein, the ubiquity of HS, the presence in HS of heparin-like moiety, and the fact that large doses of toxin are experienced by the injured tissue, we propose that heparin/HS may constitute a potential target for CTX action and that it may help the toxin exert its effect. The physiological ligands such as FGF have evolved to recognize heparin-like GAGs to exercise their biological function; this foreign toxin, on the other hand, may merely use the strategically positioned polysaccharide. Some aspects of the binding mode are uncovered: aggregation accompanies binding at a high CTX:GAG ratio, while aggregation fades as more GAG becomes available, until no aggregation can be detected. Fig. 9 is a schematic diagram summarizing a plausible model for the binding process. The polycationic CTX A3 can bind to polyanionic GAGs with reasonable strength under physiological conditions. A cooperative structural transition seems to occur at high CTX:GAG ratio as a result of collapse of the complex at saturation, which causes aggregation of the molecules. When more heparin becomes available, the toxin rearranges to occupy other sites in the newly added heparin, reducing the crowding or aggregation. This scenario is supported by preliminary electron microscopy data about the CTX-heparin complex. The complex exists as particles with a diameter of \( \sim 150 \) Å at saturation but is barely visible when more heparin is present. We also performed cross-linking experiments on the CTX-heparin complex (data not shown). Up to pentamers of CTX were clearly discernible by gel electrophoresis; heparin induces oligomerization of CTX. Interestingly, heparin also induces oligomerization of bFGF, which causes dimerization of FGF receptors. This sequence is necessary for transmission of the growth signal (39).

We estimate that a pentasaccharide of heparin may be sufficient to bind to CTX based on the number of binding sites determined by fluorescence measurements performed using heparin and low molecular mass heparin. Also, comparison of effective concentrations with the molecular weight of GAGs suggests that 4.6–5.5 saccharides are sufficient to bind to the CTX-heparin complex. 3

3 Y.-J. Chen, personal communication.
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The following observations made in our study point to an element of specificity greater than largely nonspecific electrostatic interaction in heparin-CTX interaction. First, only 40% of binding free energy is derived from purely ionic interaction as analyzed by the macromolecule-polyelectrolyte interaction theory. Second, the nature and extent of binding with various GAGs is different for the two classes of GAGs, i.e., heparin/HS and DS/CS. Third, DS, although more negative than HS, failed to bind in a well defined manner, according to fluorescence and DS/CS. Fourth, hyaluronan fails to bind. Fifth, there is a dramatic increase in the effective concentration of N-desulfated heparin relative to heparin, indicating that the 2-\(\text{N-SO}_3\) group is crucial for binding.

An important aspect of binding of CTX with heparin/HS is the implication to biological activity of CTX. Our data clearly demonstrate that binding to heparin increases penetration of CTX into phospholipid membranes. Although the detailed mechanism remains elusive, the structural transition of P-type CTX detected by the x-ray method\(^2\) may play a role. A decrease in the solubility of the CTX-heparin complex implies increased hydrophobicity of the complex, as does the enhancement of Tyr fluorescence intensity upon binding.

A high concentration of CTX accumulates in the affected tissue, and it is thus conceivable that heparin-like moieties of the cell surface, which abound in some heparan sulfate proteoglycans, may accentuate the membrane activity of the toxin. This can explain the severe tissue necrosis, a common symptom of cobra snake bite. The binding of several lipases, including phospholipase A\(\text{II}\) of cobra venom, to heparin has been demonstrated (40). Future study of the mechanism of conformational change of CTXs and its enhanced penetration into phospholipid membrane as a result of heparin binding may be useful in understanding the tripartite relationship between heparin/HS, CTXs, and phospholipids.

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