Heparin Binding Stabilizes the Membrane-bound Form of Cobra Cardiotoxin*

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It has been shown previously that the long chain fragments of heparin bind to the β-sheet cationic belt of the three-finger cobra cardiotoxin (or cytotoxin, CTX) and hence enhance its penetration into phospholipid monolayer under physiological ionic conditions. By taking lysophosphatidylcholine (LPC) micelles as a membrane model, we have shown by 1H NMR study that the binding of heparin-derived hexasaccharide (Hep-6) to CTX at the β-strand region can induce conformational changes of CTX near its membrane binding loops and promote the binding activity of CTX toward LPC. The Fourier-transform infrared spectra and NMR nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; FTIR, Fourier transform infrared.

Cobra cardiotoxins (or cytotoxins, CTXs) are highly basic, three-fingered polypeptides with the capability of inducing direct lytic effect on many cell types, including cardiac myocytes (1, 2). The available x-ray and NMR structures of CTXs are similar with a short antiparallel double-stranded β-sheet region connected by loop I and with a long antiparallel triple-stranded β-sheet connected by loop II and III. Two distinct types of CTXs, i.e. P- and S-type, can be distinguished by the presence of Pro30 and Ser28, respectively, near the tip of second finger (loop II) based on its binding activity toward neutral phospholipid bilayers and micelles (3). P-type CTXs, including the major CTX A3 from Taiwan cobra (Naja atra) venom, have been shown to induce a reversible formation of an extra outwardly rectifying conductance in bullfrog atrial myocytes (4) and also redistribute themselves between penetrating and peripheral binding states in phosphatidylcholine (PC) dispersion under favorable condition (5). Despite of the apparent perturbing effect of CTX toward phospholipid membranes, it is not clear how CTXs might act specifically toward different cell types.

Recently, it was suggested that the sulfated polysaccharides such as glycosaminoglycans (GAGs), which are abundant in the extracellular matrix of most animal cells, are potential targets for CTX action (6–8). Heparin not only binds to homologous CTXs, with different specificity, but also promotes its penetration into phospholipid monolayer under physiological ionic conditions (6). Analysis of binding of 10 CTX homologues to heparin reveals a new heparin-binding structural motif by involving several positive residues at the concave surface of the three-finger toxin (7). The positive residues flanking the three-finger loops seem to form a positive cradle for binding to long chain heparin. It is, therefore, interesting to learn how carbohydrate-polypeptide interaction can promote the membrane binding activity of CTXs.

The understanding of heparin-induced conformational change of GAG-binding protein is an essential factor to understand the biological function of GAGs. For instance, significant progresses have recently been achieved to understand the role of GAGs in regulating blood clotting and cell proliferation at molecular level. The x-ray structure of the fibroblast growth factor (FGF), FGF receptor (FGFR), and heparin ternary complex provides a structural basis for the crucial role of heparan sulfate in FGF signaling (9, 10). Heparin molecule not only binds to the FGF/FGFR asymmetricaly and links two FGF ligands into dimer that bridges between two receptor chains, but also induces conformational changes of receptor within the FGF-FGFR complex by involving possibly the cis-trans isomerization of Pro292 in the linker region (10). The structural information also explains reasonably well why an octasaccharide is the minimal heparin length required to support FGF physiological activity, whereas shorter heparin fragments causes no significant enhanced, or even inhibitory, effect (11).

Considering CTX as a GAG-binding protein (12), we have used fluorescence, Fourier transform infrared (FTIR), and NMR spectroscopy to investigate the binding of hexasaccharide fragment of heparin and lysophosphatidylcholine (LPC) micelles to CTX and to understand how heparin binding to CTX can promote protein-membrane interaction via a novel carbohydrate-induced conformational change. It is demonstrated for the first time that the binding of two chemically different molecules, heparin and phospholipid, at different sites is capable of producing a similar conformational change of CTX. It is...
also suggested that similar mechanism involving tripartite inter-
actions of heparin, protein, and lipid molecules may be op-
erative near the extracellular matrix of cell membranes.

EXPERIMENTAL PROCEDURES

Sample Preparation—CTX A3 from N. atraea (Sigma) were purified by
SP-Sephadex C-25 ion exchange column chromatography followed by high
performance liquid chromatography on a reverse-phase C-18 (10-
nm) column as described previously (3). Heparin-derived disaccharide
(Hep-2) and hexasaccharide (Hep-6) were prepared by depolymerizing
porcine intestinal heparin (100 mg, Sigma H-0930) by Flavobacterium
heparinase I (40 mU, Sigma H-2519) in a 1-ml reaction buffer (13). A
P-10 gel filtration column and a Sephadex G-15 gel filtration column
were used for further purifying and final desalting, respectively. The
LPC, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine, used in this
work was obtained commercially (Avanti Polar Lipids, Alabaster, AL).
High molecular weight heparin (Hep-HMW) from porcine intestinal
mucosa (150 mg, Sigma) and chondroitin sulfate A (CS) from bovine
trachea (15 mg, consisting of 70% chondroitin 4-sulfate (CS4) and 30%
chondroitin 6-sulfate (CS6)) were used for fluorescence binding measure-
ments obtained from Sigma.

NMR Spectroscopy and Chemical Shift Variation Analysis—The
NMR samples were prepared by dissolving CTX A3 (3.4 mg) in 500 ml
of 90% D2O, 10% D2O mixture with 10 mM phosphate buffer to reach
final concentration of 1 mM. The desired amount of LPC- or heparin-
derived oligosaccharides was added into the sample for the study of
heparin- and LPC-induced conformational change. The pH of the sam-
ple was adjusted to 6.0 ± 0.1 by titrating with NaOH and HCl.

Two-dimensional NOESY experiments (14) with 150-ms mixing time
were recorded on a Bruker DRX600 spectrometer equipped with a
5-mm triple resonance probe. Water suppression was achieved by
pulsed field gradient with the 3-9-19 WATERGATE sequence. The
NMR experiments for the sample of CTX A3-heparin-derived polysac-
charide mixture and CTX A3-LPC mixture were performed at 27 and
45 °C, respectively. Higher temperature was used for CTX A3-LPC
mixture because of the broader NMR line width of the sample. All
spectra were acquired at 150-MHz proton frequency of either 0-MHz
or 15-MHz under low salt condition and reported in the following
sections. These observations suggest that both heparin and LPC mole-

ules participate in the CTX-LPC interaction under physiological condi-
tions (4). A nonproteglycan (such as CS) is used for further purifying and final desalting, respectively. The deduced interaction constant, Kd, was
simulated by nonlinear least squares fitting of data according to Equation 1.

\[
K_d = \frac{[P]_0 - [P]_n} {[P]_n} \frac{[L]_n - [P]_n} {[P]_n} \frac{[L]_n}{[P]_n}
\]

(Eq. 1)

[PL] represents the total concentration of CTX A3 and LPC, respectively.
[PL] represents the total concentration of lipid-protein complexes, which was
estimated by the change of fluorescence intensity. The n value represents
the number of lipid molecules to constitute a CTX A3 binding site.

FTIR Measurement—Infrared spectra were collected at ambient tem-
perature by using a Bomem DA8.3 FTIR system with an MCT detector
as described previously (15). The desired CTX samples in the presence
and absence of the studied ligands were initially ultrasonically buffer-
ning condition (10 mM phosphate in H2O, pH 7.4). The same
amount of deuterated water (D2O, 99.9%, Cambridge Isotope Labora-
tory Inc.) was then added to exchange labile protons and kept for 3 h.
The procedure repeated two times. The sample was finally placed in a
sealed IR cell consisting of two CaF2 windows separated by a 25-μm
Teflon spacer. All spectra were recorded at a resolution of 2 cm
1 for 500

scans and Fourier-transformed after an application of triangular apo-
dization function.

To determine the contribution of each of the amide I and side chain
components to the total signal intensity, spectra analysis was per-
formed using the program GRAMS/386 as described (17). Deconvoluted
spectra were fitted from 1710 cm
1 to 1550 cm
1 with Lorentzian band
profiles. Initial band positions were taken directly from the second
derivative of spectra, which were calculated analytically with the OM-
NIC 5.0 software to determine the initial wavenumber of each vibra-
tional mode. None of the spectra was subjected to smoothing before
analysis. A nonsloping line was used for estimating the signal
intensity of the desired components.

RESULTS

Heparin-enhanced CTX Binding to LPC Micelles as Reflected by Tyr Fluorescence—We have demonstrated previously that
Hep-HMW accentuates the penetration of CTX into phospho-
lipid membranes, as analyzed by Monolayer measurement under physiological salt condition (6). A similar heparin-
enhanced binding of CTX A3 toward LPC micelles can also be
monitored by Tyr intrinsic fluorescence intensity change of
CTX A3 under 150 mM NaCl and 10 mM phosphate buffer at pH 7.4
(Fig. 1A, closed triangles). The deduced binding isotherms
indicate clearly the dramatic effect of heparin on the CTX-LPC
interaction (compare closed triangles with closed squares in the
inset panel). In contrast, other polyanionic GAGs such as
CS produces similar fluorescence intensity enhancement as the
control (bottom traces with closed circles and squares). The
result is consistent with a previous observation that GAGs bind
to CTX with different specificity (8) and suggests further that
heparin-enhanced CTX binding to LPC micelles is also specific.

We should point out that the binding of Hep-HMW to CTX A3
produces significant aggregation, as evidenced by the in-
crease of turbidity of the studied samples (6). It prevents the
usage of other spectroscopic techniques such as high resolution
NMR to understand at the molecular level how carbohydrate-
polypeptide interaction can promote the membrane binding
activity of CTXs. The aggregation of the studied complex can be
reduced if depolymerized heparins such as heparin-derived
oligosaccharides are used (20). Despite of the concurrent reduc-
tion of heparin-enhanced CTX binding to LPC micelles, the
presence of Hep-6 can still enhance CTX-LPC interaction un-
der physiological salt condition (Fig. 1B). Because we have
demonstrated previously that sulfated pentasaccharide may be
sufficient to bind to CTX (9), the study of CTX-LPC interaction
in the presence of Hep-6 can then provide a useful system to
understand the tripartite interaction of heparin, phospholipid,
and CTX molecules. As we will show below, the weak effect of
Hep-6 in promoting the CTX-LPC interaction under physiolog-
ical salt condition can be enhanced if lower salt concentrations
(10 mM phosphate buffer at pH 7.4) are used. The enhancement
under low salt condition without the complication of significant
sample aggregation allows us to use Hep-6-CTX-LPC sample as
mimics of heparin-CTX-membrane interaction under physi-o-
logical salt condition. Detailed spectroscopic studies involving
fluorescence, NMR, and FTIR techniques are therefore carried
out under low salt conditions and reported in the following
sections.

As shown in Fig. 1C, the binding of CTX A3 toward to LPC
micelles can be significantly perturbed in the presence of hepar-
in-derived oligosaccharides. The presence of either Hep-6 or
Hep-2 changes the intrinsic Tyr fluorescence of CTX A3. The
Tyr fluorescence of CTX A3 in Hep-6 is also found to increase in
dose-dependent manner as LPC concentration increases.
These observations suggest that both heparin and LPC mole-
ules can bind to CTX A3 equally well and perturb its con-
formation. Assuming a two-state model, the fitting of fluorescence intensity of the titration experiment allows the determination of their apparent Kd and n values. The upper binding isotherm
trace (Fig. 1C, closed circles) suggests that Hep-6 can enhance the binding of CTX A3 toward LPC micelles by decreasing both the apparent $K_d$ (0.18 M) and $n$ (1101113) values.

The binding of CTX A3 toward LPC micelles as determined by Tyr intrinsic fluorescence intensity change of CTX A3 (Fig. 3C, bottom trace (fluorescence intensity) with closed squares) suggests a binding stoichiometry ($n$) of 4/15 lipid molecules per CTX A3 with dissociation constant ($K_d$) of 4/15 M (inset panel of Fig. 1C, middle trace (binding isotherm)). Similar binding study of other P-type CTX such as CT II from Naja oxiana snake venom toward perdeuterated 12:0 dodecyl phosphocholine (DPC) micelles was performed using 1H NMR spectroscopy previously (18). Their result indicates that approximately two molecules of toxin are bound to one micelle consisting 66 molecules of DPC at 30° C and no significant change of the aggregation number of DPC molecules per micelle in the presence and absence of CT II. Because there are about 100 lipid molecules/LPC micelle (19), it is estimated that approximately 2–3 molecules of CTX A3 are present in 1 LPC micelle.

For the bottom (isotherm) trace (closed triangles, Fig. 1C), it is not possible to quantitate the effect of Hep-2 on the apparent $K_d$ and $n$ values of CTX A3-LPC interaction, as the fluorescence enhancement has not reached saturation level at the highest studied lipid concentration range. However, the fact that it could not reach saturation state at similar concentration range indicates that Hep-2 may have an inhibitory effect on the binding of CTX A3 to LPC micelles. The inhibitory effect of Hep-2 becomes clear if we arbitrarily normalize the highest observed fluorescence intensity as 90% saturation (curve of closed triangles in the inset panel of Fig. 1A). For 50% of CTX A3 to become membrane-bound, the LPC concentration must be raised by approximately 2 times in the presence of Hep-2, but only half of LPC is needed in the presence of Hep-6.

**NMR Chemical Shift Perturbation Caused by Heparin—**

After establishing that Hep-6, as in the case of long chain heparin, can also enhance the binding of CTX to LPC micelles, we carried out two-dimensional NMR experiments to monitor the effects of both Hep-2 and Hep-6 on the $\alpha$H and NH chemical shift of CTX A3 molecule (Fig. 2). The overall effects of the two studied heparin molecules on the $\text{H}_1$ chemical shift of CTX A3...
molecule are similar. The most significant perturbation effects are detected near Cys 38 NH, indicating that both molecules share a similar binding site near the region. Structural analysis of Hep-2/H18528 CTX A3 complex based on the NOE buildup curve of the bound conformation of Hep-2 shows that Hep-2 binds to the convex side of the CTX molecule near the rigid core region of Cys 38 (20). The determined Hep-2 binding site is placed remotely as well as on the side opposite to the concave side of the three-finger loops, which was proposed previously for long chain heparin binding (7).

The disclosed two distinct heparin binding sites, one revealed by the NMR study of Hep-2 and the other suggested by comparing the binding strength of 10 CTX homologues, are consistent with properties of CTX-heparin interaction reported so far. First, Hep-6 enhances the interaction between CTX and LPC micelles, whereas Hep-2 inhibits the same. Second, a significant amount of aggregation of CTX A3 has been taken place during the binding of Hep-6 with CTX A3 at or above an equimolar concentration of these two, but no such aggregation has been observed for Hep-2 to CTX A3 irrespective of the wide range of molar concentrations used. Third, although the chemical shift perturbation of CTX A3 caused by Hep-2 and Hep-6 are qualitatively similar, there is still a difference quantitatively. This can best be seen in the bottom panels of Fig. 3A, showing the calculated differential chemical shift perturbations based on the data obtained from Hep-6-CTX A3 and Hep-2-CTX A3 mixtures.

Fig. 3A also shows a pictorial representation of those NH positions (solid spheres) in CTX A3 with significant chemical shift variations. The additional chemical shift variations between Hep-6/H18528 CTX A3 and Hep-2/H18528 CTX A3 mixtures are distributed not only on the three connecting loops but also extend toward the rigid core of its...
β-strands. The chemical shift perturbation near cationic β-strand cradle provides spectroscopic evidence for the heparin binding site predicted previously, based on the binding strength of 10 CTX homologues with long chain heparin. However, the most dramatic chemical shift perturbations, i.e. down-field shift detected at Leu9 NH and Val34 NH and up-field shift at Met26 NH, are unexpectedly found to be located near the tip of loop I and loop II. Because similar residues have been reported to exhibit most significant chemical shift perturbations by studying the binding of other CTX homologues such as CT II and toxin γ toward perdeuterated DPC micelles (18, 21), it led us to perform similar NMR binding experiments on CTX-LPC mixtures.

NMR Chemical Shift Perturbation Caused by LPC Micelles—The effect of LPC binding to CTX molecule as monitored by the 1H NMR chemical shift variation was studied at protein/lipid ratio of 25:1 at 45 °C (Fig. 3B). Higher temperature was used for this study because of the broader NMR line width and the apparent higher viscosity of the sample. The binding of LPC toward CTX A3 can indeed produce significant variation of the 1H NMR chemical shift for both αH and NH located near the tip of all three loops as shown in the bottom panels of Fig. 3B. It should be pointed out that most of the hydrophobic residues near the tips of the loops are located on the convex side of the CTX molecule. The intermolecular NOEs observed between LPC fatty acyl chain and CTX A3 molecule suggest further that the amino acid residues in the convex side of the loop region are involved in lipid-CTX interaction (data not shown). The results reconfirm that all three hydrophobic loops (I, II, and III) of the P-type CTXs are involved in its binding to phospholipid dispersions (18, 21) and the convex side of CTX A3 molecule with hydrophobic residues is in contact with the membrane surface.

To facilitate the comparison between the effect of Hep-6 and LPC micelles on CTX A3, an analysis of the chemical shift perturbation similar to Hep-6 was also performed for CTX A3 and CTX A3-LPC mixtures (Fig. 3B). Dark solid spheres show those proton positions observed to be similarly perturbed by the binding of either Hep-6 carbohydrate molecules or LPC lipid micelles. They are mainly located near the tips of the loops and consist of ~50% of those protons exhibiting significant chemical shift perturbations as a result of Hep-6 and LPC binding. Those regions perturbed only by Hep-6, but not by LPC micelles, can be seen to be located near the β-strand region rather than in the tips of the loops. The result is consistent with a view that Hep-6 binds to CTX A3 near the β-strand region and induces a conformational change near the tip of the loop.

Because the chemical shift perturbations of Leu9 NH, Met26 NH, and Val34 NH as a result of LPC or Hep-6 binding are most significant among all amino acid residues, it provides a reliable parameter for performing NMR titration experiment on CTX A3 against varying LPC concentration. As shown in Fig. 4, all the indicated chemical shift variations follow a similar concentration dependent manner and reach their saturation states at similar LPC/CTX molar ratio of 25/1 (Fig. 4). Because the reported resonance positions distributed over all three loops, it appears that all the three loops constitute a LPC binding site with the exchange rate faster than the inverse of the reported chemical shift difference, i.e. >> ms.

Heparin-enhanced CTX Binding to LPC Micelles as Reflected by NMR—Chemical shift is sensitive to steric property of protein and reflects the secondary and tertiary structure in general. The binding of CTXs, including toxin γ, CT II, and A3, to phospholipid all are consistently lead to a dramatic down-field shift detected at Leu9 NH and Val34 NH and up-field shift at Met26 NH. The shifts seem to be a represented character when CTX changes to a membrane binding conformation. Similar shifts, both magnitude and sign, were also found in the case of Hep-6 binding. Hence, it is rational to suggest that binding of Hep-6 has the capability to induce CTX to favor its membrane binding conformation. The suggestion is also consistent with fluorescence binding measurement that Hep-6 can enhance the CTX membrane binding activity. Therefore, it is interesting to see whether similar heparin-enhanced CTX binding can also be monitored by NMR, especially via the chemical shift parameters.

To address this question, we used 1H NMR to study the binding of Hep-6 to CTX-LPC micelles. Samples of 1/10 molar ratio of CTX-LPC micelles were prepared, and then 0.1 or 0.2 mM Hep-6 was added to examine the effect of Hep-6 binding on the chemical shift of CTX A3. In general, the chemical shift of all NH and αH of CTX A3 changes as and when more CTX molecules interact with LPC (data not shown). For instance, if we assume the chemical shift variation of Leu9 NH, Met26 NH, and Val34 NH in the saturation state (1:50 molar ratio as shown in Fig. 4) as 100% binding, Hep-6 can enhance the binding of CTX to LPC micelle from 40–50% to 90% based on the Hep-6 induced additional chemical shift variation (Fig. 5). The result strongly suggests that Hep-6 can indeed induce conformational change of CTX in favor of its micelle binding activity to allow more CTX molecules bound to phospholipid membranes.

NOE Effect—By monitoring chemical shift variations at Leu9, Met26, and Val34, we demonstrated that long chain heparin such as Hep-6 can induce conformational change near the three-finger connecting loop region to favor its membrane binding form. It will be interesting if the exact conformational change responsible for the observed chemical shift variation of the two CTX-Hep-6 and CTX-LPC complexes can be determined. Specific conformational change should be detectable if one can determine the NOE of the proton pairs involved in the binding. However, previous efforts in identifying DPC-induced
conformational change of CT II and toxin γ based on NOE approach were found not to be very fruitful (18, 21). There is also no detectable difference in the NOE peaks for amino acid positions of CTX near the tip of the three-finger loops where chemical shift variation showing significant structural perturbation. As a matter of fact, the NMR structures determined based on distance geometric constraint show a similar CTX structure in the presence and absence of phospholipid micelles (18). Although no significant conformational change was observed, it is still worthwhile to measure the exact change of NOE intensities in the presence of LPC or Hep-6.

As shown in Table I, the most significant variations of the NOE intensities as a result of either Hep-6 or LPC binding were found to be located mainly in the β-strand region. For instance, the long range NOE between Lys\(^{12}\) NH and Asn\(^{4}\) αH located in the first β-strand of CTX molecule can be seen to increase significantly in the presence of Hep-6 (Fig. 6A) or LPC (Fig. 6B) as compared with CTX by itself (upper panels of Fig. 6). In the same figure, the NOE cross-peaks between a more rigid proton pairs of Asn\(^{4}\) NH and Asn\(^{4}\) αH are also shown to remain roughly the same.

Table I documents the NOEs identified to show over 50% change of its relative intensity upon binding to Hep-6 or LPC. Their resonance positions are located mainly in the double-stranded β-sheet of the loop I and also the triple-stranded β-sheet of loop II and III. Those proton pairs exhibit decreased NOE intensity are located at the interfacial region between the β-strand and its connecting loop, i.e., Tyr\(^{11}\) αH/Lys\(^{5}\) NH, Asn\(^{4}\) αH/Tyr\(^{11}\) αH, and Phe\(^{25}\) αH/Lys\(^{30}\) NH. In contrast, most, if not all, other proton pairs showing enhanced NOEs are located within the β-strand of well defined hydrogen-bond pattern. Although some of the observed NOEs can be different by as much as 2–3-fold, the overall static structure of the CTX A3 molecule should remain the same because the NOE is known to depend strongly on the distance between protons pair (1/R\(^6\)). Therefore, the observed NOE effect most likely reflects a delicate change in the dynamics or stability of the β-sheet structure. This is interesting if one considers that the phospholipid-binding site is located near the tip of the loop, but the observed NOE changes spread all the way to the rigid core of the molecule. It suggests that the structural stability and/or dynamics of the β-sheet depend on the exact conformation of the segments connecting these strands. It should be stressed that the spectral perturbation observed in this study is relatively small, future relaxation studies should therefore be performed to test the proposed hypothesis.

**FTIR Experiment**—Infrared amide I band being assigned to the secondary structure of the β-band and the changes in band components can be attributed to the variations in β-sheet and β-turn cross-interaction. For instance, by monitoring the intensity change of two β-bands at 1620 cm\(^{-1}\) and 1633 cm\(^{-1}\) for lentil lectin protein, the structural stability of its β-sheet structure was found to be perturbed under different pH condition (22). In fact, the binding of negatively charged dimyristoylphosphatidylglycerol to CTX has already been studied by FTIR, and the results show a structural transition in its β-structure (17).

Fig. 7 shows the second derivative spectra in the amide I' region (−1700−1600 cm\(^{-1}\), C=O) of CTX A3 in the presence and absence of the designated Hep-6 and/or LPC molecule. It reveals the presence of at least seven component bands in the amide I' region, including the characteristic band at ~1620 and 1631 cm\(^{-1}\) of the amide groups in a β-sheet conformation. Additionally, the high frequency band around 1677 cm\(^{-1}\) has been assigned to the “in phase” vibration of neighboring peptide C=O groups in an antiparallel β-sheet conformation. As shown in the figure, the addition of Hep-6 and/or LPC induces a significant change of the vibration band of CTX A3 at 1631 and 1677 cm\(^{-1}\).

Decomposition of the original amide I' band based on the identified band position into its constituents allows quantification of the integrated intensity (Fig. 8). The relative integrated intensity of the peak near 1631 cm\(^{-1}\) increases slightly from 19.5% to 20.5% upon Hep-6 binding. However, its intensity becomes more significant (up to 23.2%) in the presence of both Hep-6 and LPC. The other noticeable change is the decrease of the signal intensity near 1620 cm\(^{-1}\) from 13.5% to 10.0%. A similar change of these two β-band signal intensities is already known for the other β-sheet protein of lentil lectin to indicate the change of its structural stability as a result of conformational change in its connecting loop (22).

**DISCUSSION**

CTX is a slightly curved molecule with the N and C termini located in the concave side. Most of the hydrophobic residues near the membrane binding loops are located on the convex side.

### Table I

Relative NOE intensities of selected cross-peaks in the absence or presence of Hep-6 or LPC

| Proton pairs          | Relative intensity of native CTX A3 | Relative intensity of CTX A3 · Hep-6 (2/1) | Relative intensity of CTX A3 · LPC (1/10) |
|-----------------------|-------------------------------------|------------------------------------------|------------------------------------------|
| Tyr\(^{11}\) αH/Lys\(^{5}\) NH | 0.22                               | 0.09 (−59%)                               | 0.10 (−55%)                               |
| Asn\(^{4}\) αH/Tyr\(^{11}\) αH  | 0.19                               | 0.05 (−74%)                               | 0.06 (−68%)                               |
| Asn\(^{4}\) αH/Lys\(^{12}\) NH  | 0.04                               | 0.10 (+150%)                              | 0.10 (+150%)                              |
| Cys\(^{35}\) αH/Ile\(^{39}\) NH  | 0.07                               | 0.12 (+71%)                               | 0.10 (+43%)                               |
| Lys\(^{35}\) αH/Gly\(^{27}\) NH  | 0.02                               | 0.09 (+450%)                              | 0.04 (+200%)                              |
| Cys\(^{35}\) αH/Tyr\(^{22}\) NH  | 0.06                               | 0.20 (+233%)                              | 0.10 (+67%)                               |
| Phe\(^{25}\) NH/Lys\(^{30}\) NH  | 0.16                               | 0.10 (−38%)                               | 0.07 (−56%)                               |
| Phe\(^{25}\) NH/Tyr\(^{31}\) αH  | 0.10                               | 0.15 (+50%)                               | 0.11 (+10%)                               |
The chemical shift perturbation of side chain protons also provided evidences for the binding site of PC membrane is located on the convex side (21). Actually, the intermolecular NOEs observed between LPC fatty acyl chain and CTX A3 molecule suggest further that the amino acid residues in the convex side of the loop region are involved in lipid-CTX interaction. It explains why the chemical shift perturbation caused by LPC is more localized near the tip. In contrast, Hep-6 is a hydrophilic molecule and interacts with CTX A3 mainly through charge-charge interaction. Because the positively charged residues are mainly located near the interface between the connecting loop and the β-strand core at concave side, the observed effect of Hep-6 on CTX A3 is in good agreement with the binding mode of long chain heparin on CTXs as proposed previously (Fig. 9).

In this spectroscopic investigation of the tripartite interac-

FIG. 6. The NOESY cross-peaks of the NH of Lys¹² and the αH of Asn¹ in the absence (upper panel) and presence (lower panel) of Hep-6 at 27 °C (A) or LPC at 45 °C (B). Despite the similar NOE intensity for the cross-peak of the Asn¹ NH and Asn¹ αH, those of Lys¹² NH and Asn¹ αH increased significantly in the presence of added ligands.

FIG. 7. Second derivative infrared spectra in the amide I region of CTX A3 (1.2% w/v) in 10 mM phosphate buffer. When compared with the FTIR spectra of native CTX A3, significant variation of the 1630 and 1677 cm⁻¹ bands can be readily observed by adding Hep-6 at 1/1, LPC at 1/50, and Hep-6/LPC at 1/1/50 toxin to ligand molar ratio.

FIG. 8. The infrared amide I band contour with the best fitted individual component for CTX A3 in phosphate buffer (A), in Hep-6 (B), in LPC (C), and in Hep-6-LPC mixture (D). All the band shapes were fitted by Lorentzian shapes. Solid lines indicate the characteristic β-strand, and dotted lines indicate other secondary structures.

FIG. 9. Schematic diagram of the proposed model to explain the tripartite interactions of heparin (Hep-6 and Hep-2), CTX A3, and PC molecules near the surface of membranes.
tion among CTX A3, LPC, and Hep-6, it is demonstrated for the first time that the binding of two chemically different molecules, i.e., Hep-6 and LPC, at different sites produce a similar conformational change of CTX A3. By using LPC micelles as a model, we confirm in this spectroscopic investigation that Hep-6 can indeed enhance the interaction of CTX with phospholipid membranes. Surprisingly, a shorter chain of Hep-2 inhibits such an interaction. When Hep-2 binds to the convex side of the CTX molecule (20) and the long chain heparin binds to the concave side (7), it is most reasonable to suggest that Hep-6 have two binding sites at both convex and concave side of CTX A3 molecule. The convex binding site appears to be more specific and higher affinity for Hep-6 and located in the positively charged β-sheet cradle of CTX molecule, as suggested previously. Otherwise, the convex binding site provides a weaker interaction with Hep-6. The proposed molecular interactions explain clearly the reason for the membrane activity that exhibited by Hep-2 and Hep-6 though it is chemically similar (Fig. 9). It is suggested that the binding of long chain heparin at the convex site stabilize the membrane binding loops of CTX A3 and the binding of Hep-2 at the convex site cause steric effect with PC headgroup of phospholipid membranes.

It is interesting that, despite the fact that binding sites of LPC micelles and Hep-6 are not the same, the chemical shift and NOEs within CTX A3 molecule are similarly perturbed by the bindings of these entities. Because all the chemical shift perturbations are located near the tip of the loop as a result of LPC binding, the observed change of the NOEs of CTX A3 molecule within the β-strand most likely reflect a cross-interaction between the connecting loop and the β-strand. Such a cross-interaction has recently been observed in FTIR investigation of lentil lectin, based on the pH titration of the charged residues also in the connecting loop of the β-sheet (22). Our FTIR investigation of the interaction of LPC micelles with CTX A3 is also consistent with NMR NOE results, as judged by the change of the relative intensity of the 1620 and 1631 cm$^{-1}$ vibration bands for the amide groups in the β-sheet conformation.

The binding of long chain heparin such as Hep-6 to CTX A3 produces conformational change similar to the binding of LPC micelles. Based on the structural model proposed in Fig. 9, one of the likely reasons for the hydrophilic Hep-6 and hydrophobic LPC molecule to produce similar conformational change of CTX A3 is the stabilization of the conformation near the tip of the loop. Therefore, the stabilization of the loop conformation can be induced either by hydrophilic interaction between LPC and CTX molecules through the three loops directly or by hydrophilic charge-charge interaction between Hep-6 and CTX molecules through the delicate cross-interaction between β-strands and loops. Furthermore, a bound structural water molecule has been identified in all studied P-type (Pro30 consisting) CTXs (15). We are currently testing the hypothesis that the binding of Hep-6 near the region can further stabilize the bound water and thus the loop conformation suitable for membrane binding.

Finally, many of the functionally important proteins in the extracellular matrix such as the cell adhesion molecules and receptors involved in cellular immune recognition consist of structural modules with β-sheet structure. Because GAG molecules are also abundant in the region, it is likely that the interaction of GAG molecule with proteins with β-sheet structure may undergo conformational change as we demonstrated here for GAG-CTX interaction. Such a conformational change can therefore enhance oligomerization of nearby protein molecules or protein-membrane interaction via the suggested conformational change. Tripartite interactions of heparin, protein, and lipid molecules may then be operative near the extracellular matrix of cell membranes.

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