Unidirectional Binding of Clostridial Collagenase to Triple Helical Substrates*

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Histotoxic clostridia produce collagenases responsible for extensive tissue destruction in gas gangrene. The C-terminal collagen-binding domain (CBD) of these enzymes is the minimal segment required to bind to collagen fibril. Collagen binding efficiency of CBD is more pronounced in the presence of Ca2+. We have shown that CBD can be functional to anchor growth factors in different tropocollagen analogues ((POG)103, ((GPOG)7PRG)3, and (PROXYL-G(POG)7)3, and (GPRG(POG)7C-PROXYL)3 (where PROXYL represents 2,2,5,5-tetramethyl-L-pyrrolidinyloxy), unambiguously demonstrated unidirectional binding of CBD to the tropocollagen analogues. Small angle x-ray scattering data revealed that CBD binds closer to a terminus for each of the five different tropocollagen analogues, which in conjunction with NMR titration studies, implies a binding mode where CBD binds to the C terminus of the triple helix.

Histotoxic clostridia produce collagenases that degrade collagen in connective tissue. Although the enzyme is assumed to be a causative agent for diseases like gas gangrene (1), it is beneficial to remove dead tissue from ulcers or burns and for nonsurgical treatment of Dupuytren’s disease (2, 3). For collagenases to hydrolyze tissue collagen, the enzymes must 1) anchor themselves onto an insoluble collagen fibril, which is a staggered array of tropocollagen and then 2) isolate a single triple helical molecule from the bundle and finally 3) unwind the triple helix to expose a scissile peptide bond. Clostridium histolyticum produces two classes of collagenases, which contain a catalytic domain belonging to the family M9B, followed by one or two copies of polycystic kidney disease domains and one or two copies of collagen-binding domains (CBD)2 (4).

Each CBD spans ~120 amino acid residues and binds specifically to insoluble collagen. CBD also binds to collagenous peptides with triple helical conformation but not to collagenous peptides that lack triple helix or to gelatin (denatured collagen), suggesting that the CBD-collagen interaction is conformation-specific (4, 5). Calcium ions enhance the binding at physiological concentration, and x-ray crystal structures of CBD have been solved in the presence and absence of calcium (6).

Since collagen fibrils constitute a major part of the extracellular matrix, bioactive molecules can be anchored with CBD for their prolonged effect. Nishi et al. (7) have demonstrated that growth factors fused to CBD remained at the sites of injection much longer than growth factors alone to induce extended cell proliferation. In order to gain an insight into the anchoring mechanism of CBD, we attempted to co-crystallize CBD and collagenous peptide without success. Also to better address the role of CBD in fibril disruption and transition states from insoluble substrate, solution studies of CBD with the triple helical collagenous peptide became necessary.

NMR titration methods were utilized to identify the collagen binding pocket on CBD. Since it has been shown that most peptidases bind to their substrate in one direction at their catalytic center (8, 9), there could be only one direction for the collagen triple helices at the binding site of CBD. On the other hand, CBD might allow bidirectional binding, since it is independent of the catalytic domain. To identify the binding direction, three different NMR titrations were performed with spin-labeled analogues of tropocollagen, where a nitrooxide spin label 2,2,5,5-tetramethyl-1-pyrrolidinylloxy (PROXYL) was attached to either the N or C terminus of the collagenous peptide. The nitrooxide moiety with an unpaired electron can cause enhancement in paramagnetic relaxation of NMR resonances of CBD via electron-nuclear dipolar coupling, thereby resulting in extreme line broadening of those resonances (10, 11). Furthermore, small angle x-ray scattering (SAXS) was utilized to obtain the three-dimensional structure of the CBD-collagenous peptide complex.

**The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–8.

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2 The abbreviations used are: CBD, collagen binding domain; SANS, small angle X-ray scattering; PROXYL, 2,2,5,5-tetramethyl-1-pyrrolidinylloxy; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
EXPERIMENTAL PROCEDURES

15N-Labeled Protein Production—A C-terminal CBD (Gly^{993–Lys^{1000}}) derived from the C. histolyticum class I collagenase (ColG) was expressed as a glutathione S-transferase fusion protein. The glutathione S-transferase tag was cleaved off by thrombin, and CBD was purified as described previously (4). Uniform 15N isotope labeling was achieved using Tanaka minimal medium containing 40 mM 15NH4Cl. The labeling efficiency was estimated to be 99.6% by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Peptides—(POG)10 was purchased from Peptide Institute, Inc. (Osaka, Japan). Other peptides were constructed manually based on the standard Fmoc (N-(9-fluorenylethoxycarbonyl)-based strategy on Rink-amide resins (Novabiochem, Darmstadt, Germany). N-terminal spin labeling was performed on the resin by treatment with 5 eq of 3-carboxy-PROXYL (Aldrich), 1-hydroxybenzotriazole, diisopropylcarbodiimide in N,N'-dimethylformamide at room temperature for 2 h. Peptide cleavage and deprotection was performed by treatment with a standard trifluoroacetic acid scavenger mixture (trifluoroacetic acid/m-cresol/thioanisole/water/triisopropylsilane = 82:5:5:2.5; v/v/v/v/v). Spin labeling at the C terminus was performed using 3-(2-iodoacetamido)-PROXYL (Sigma). Briefly, 10 molar excess of 3-(2-iodoacetamido)-PROXYL dissolved in ethanol was added to the same volume of 10 mg/ml peptide in 0.1 M Tris-HCl (pH 8.8), 5 mM EDTA. After reacting at room temperature for 1 h, the reaction was quenched by adding excess dithiothreitol. All peptides were purified by reverse-phase HPLC using a Cosmosil 5C18 AR-II column (Nacalai Tesque, Kyoto, Japan) and characterized by MALDI-TOF mass spectrometry. All of the measured masses agreed with the theoretical values.

Electron spin resonance spectra for the PROXYL-peptide solutions in 100 mM Na+, K+ phosphate-buffered saline (pH 7.4) containing 100 mM NaCl were recorded at 22 °C on an X-band electron spin resonance spectrometer JES-TE-1X (JEOL, Tokyo, Japan) under the following conditions: frequency, 9.4 GHz; microwave power, 5.0 milliwatts; modulation frequency, 100 kHz; modulation amplitude width, 0.1 mT; receiver gain, 100; response, 0.03 s; scanning time, 2 min; magnetic field 336 ± 5 milliteslas. Linear relationships between integral areas or spin concentrations and concentrations of PROXYL-peptide were found in the range of 20–200 μM.

Steady State Fluorescence Measurements—The tertiary fold of CBD with the addition of collagenous peptide was characterized by its fluorescence spectra measured at 25 °C on a Hitachi F-2500 fluorimeter (Tokyo, Japan) with excitation and emission bandwidths at 2.5 and 10 nm, respectively. Measurements were carried out with the excitation wavelength of 280 nm to detect the contribution of both tyrosine and tryptophan residues. The emissions were monitored between 300 and 450 nm. Protein with concentration of 25 μg/ml in 10 mM Tris-HCl at pH 7.5 containing 100 mM NaCl and 1 mM CaCl2 was used. The concentration of ([POG]10)_3 was 63 μg/ml.

CD Spectroscopy—The triple helical conformation and the stability of the collagenous peptides were verified using CD spectroscopy. CD spectra were recorded with a J-820 CD spectropolarimeter (JASCO Co., Hachioji, Japan) equipped with a Peltier thermo controller, using a 0.5-mm quartz cuvette and connected to a data station for signal averaging. All peptide samples were dissolved in water (1 mg/ml) and stored at 4 °C for 24 h. The spectra are reported in terms of ellipticity units/molar peptide residues [θ]_{225}. Thermostability of the triple helix was monitored by the [θ]_{225} values of each peptide with increasing temperature at the rate of 0.3 °C/min.

NMR Spectroscopy—NMR experiments were performed at 25 ± 0.5 °C on a Bruker 700-MHz spectrometer equipped with a Cryoprobe™. The concentration of the protein used was 1.0 mM in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 20 mM CaCl2. A highly concentrated stock solution of the collagenous peptides (4 mM) was prepared and equilibrated at 4 °C for 24 h. The concentrated stock solution facilitated the addition of small volumes of peptide(s) on the course of titration; thus, any volume changes were very small and did not affect the intensity measurements of the cross-peaks in the 1H–15N HSQC spectrum. All aliquots of collagenous peptide(s) were added to the protein and equilibrated for 5 min before acquiring 1H–15N HSQC spectra. The pH of the NMR samples was monitored during the titration, and there was no significant change in the pH at the end of each titration (within ±0.2 units). Changes in the average amide chemical shifts (Δδ) were calculated using Equation 1,

$$\Delta \delta = \sqrt{(\Delta \delta^H/2 + 0.2(\Delta \delta^{15N})^2)}$$

(Eq. 1)

where ΔδH and Δδ15N are the amide proton and amide nitrogen chemical shift differences between the free and bound state of the protein (12). NMR chemical shift changes were fit using KaleidaGraph 4.0 (Synergy Software) to a simple bimolecular binding model through a nonlinear regression analysis using Equation 2,

$$\Delta \delta_{obs} = \Delta \delta_{max}((K_d + [P]_0 + [L]_0) - \sqrt{(K_d + [P]_0 + [L]_0)^2 - 4[L]_0[P]_0})/2[P]_0$$

(Eq. 2)

where Δδ_{obs} is the observed chemical shift, Δδ_{max} is the maximum chemical shift, [P]_0 is the total concentration of the protein, [L]_0 is the total concentration of collagenous peptide, and K_d is the dissociation constant of the complex.

Small Angle X-ray Scattering Experiments—The SAXS data were collected on solutions of CBD, various collagenous peptides, and the CBD-collagenous peptide complexes in 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 20 mM CaCl2 at the XOR beamline of sector 12-ID at the Advanced Photon Source (APS) in the Argonne National Laboratory. The main advantage of x-ray scattering is that it can be carried out in solution in nearly physiological conditions (13). All of the samples and buffers were filtered through a 0.22 μm filter prior to data collection. To minimize the radiation damage during data collection, the samples were continuously oscillated through a 1.5-mm-wide quartz capillary at a flow rate of 7 μl/s. During exposure, the beam was focused to a size of 0.6 mm wide by 0.2 mm high. The energy of the x-ray beam was 12 keV, and the flux was 3 × 10^{12} (photons/s). The average exposure time was 1 s. Ten SAXS exposures were collected for each sample with a charge-coupled device x-ray detector at a camera length of 780
The optical configuration used allows a range from $0.02$ to $0.6 \text{ Å}^{-1}$ to be measured, where $Q = (4\pi \sin(\theta)/\lambda)$ is the magnitude of the scattering vector, $\theta$ is the scattering angle, and $\lambda = 1 \text{ Å}$ is the wavelength of the x-ray. All scattering data were acquired at a sample temperature of $10^\circ \text{C}$. The scattering patterns were circularly averaged with a C program. For further analysis, the program IGOR Pro 5.5 A (WaveMetrics) was used. The scattering profiles of the protein were obtained after subtracting the buffer profiles. The reduced scattering data were plotted as scattering intensity $I(Q)$ versus $Q$. The radius of gyration, $R_g$, was obtained from the Guinier approximation by linear least squares fitting in the $QR_g^2 < 1$ region, where the forward scattering intensity $I(0)$ is proportional to the molecular weight of the protein complex (14). An indirect Fourier transformation of $I(Q)$ data using GNOM (15) gave the particle distribution function $P(r)$ in the real space (Fig. 5). The point on the $x$ axis where $P(r)$ reaches zero represents the maximum diameter $D_{\text{max}}$ averaged in all orientations. The molecular envelopes were constructed for all of the samples based on the SAXS data after ab initio calculations with the program GASBOR (16). In GASBOR, simulated annealing minimization of randomly distributed dummy atom models represents the protein structure after being tested for the best fit to the $I(Q)$ scattering data. The atomic models are represented as a compact interconnected configuration of beads inside a sphere with diameter $D_{\text{max}}$ that fits the experimental data $I_{\exp}(s)$ to minimize discrepancy. Atomic models were fit into ab initio envelopes with the program SUBCOMB (17).

Docking Model—The Protein Data Bank data used to generate the CBD-collagenous peptide complex are from entries...
NQD and K6F for CBD and collagenous peptide, respectively. To obtain the complex model, the soft docking algorithm BiGGER (18) was used. CBD-collagenous peptide models generated were filtered using NMR titration data, and the highest scoring model that satisfied NMR, SAXS, and mutation results was chosen.

RESULTS AND DISCUSSION

CBD Retained Its Tertiary Structure upon Binding to Collagenous Peptides—Fluorescence measurements were carried out to monitor the tertiary structural changes on CBD upon binding to the collagenous peptide. The native spectrum of CBD has only one-emission maxima at 317 nm. This comes from solvent-exposed tyrosine residues with the tryptophan being buried inside the core (no emission maxima at 350 nm) (6). The emission spectrum of CBD-collagenous peptide complex was identical to the native CBD spectrum, indicating that the tertiary structure of CBD does not change upon binding to tropocollagen (supplemental Fig. 1).

Characterization of Tropocollagen Binding Interface from $^1$H-$^{15}$N HSQC Titration—$^1$H-$^{15}$N HSQC titration is a sensitive technique to study protein-protein interactions in solution (19, 20). To identify the residues on CBD that bind to collagen, NMR titration experiments were performed where $^{15}$N-labeled CBD was titrated with a triple helical collagenous peptide, ((POG)$_{10}$)$_3$. ((POG)$_{10}$)$_3$ retained triple helical conformation at 25 ± 0.5 °C (21–23). Resonances of all nonprolyl residues of CBD except Lys$^{996}$, Glu$^{925}$, Asp$^{926}$, Lys$^{937}$, Leu$^{958}$, Arg$^{967}$, Ile$^{968}$, and Tyr$^{970}$ have been unambiguously assigned using various multidimensional NMR experiments (24). Resonances of those residues exhibited diminished and broadened signals in all of the spectra and thus could not be assigned. $^1$H-$^{15}$N HSQC titration of CBD with the collagenous peptide followed intermediate exchange kinetics on the NMR time scale, which facilitated the assignment of NMR chemical shifts of the ((POG)$_{10}$)$_3$-bound state by starting with complete assignments of the free protein and tracking small shifts in resonances at each step of titration. $^1$H-$^{15}$N HSQC spectra were collected and analyzed for ((POG)$_{10}$)$_3$/CBD molar ratios ranging from 0.02:1 to 1.7:1. No significant change was observed in the $^1$H-$^{15}$N HSQC spectra acquired at ((POG)$_{10}$)$_3$/CBD molar ratios below 0.07:1. At higher concentration of ((POG)$_{10}$)$_3$, two distinct phenomena occurred. At a ratio of 0.23:1 ((POG)$_{10}$)$_3$/CBD, cross-peaks belonging to five residues, Ser$^{928}$, Thr$^{975}$, Gly$^{951}$, Lys$^{956}$, and

![Figure 2. Relative affinity of CBD for collagenous peptides.](image-url)
Tyr<sup>996</sup>, disappeared completely from the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum, as seen from the superposition of a region of the two-dimensional NMR spectra of CBD and CBD-collagenous peptide complex (Fig. 1A). Amide resonances of these residues in CBD must be in the close proximity of collagenous peptide upon binding. The intensity decrease of the cross-peaks could be due to chemical exchange between amide resonances of CBD and collagenous peptide on the NMR timescale (Fig. 1B). The amide resonances that disappeared during the course of titration were neighbors to the “hot spot” side chains recognized by mutation studies (6). NMR chemical shifts of few resonances were perturbed when the ratios of ((POG)<sub>10</sub>)<sub>3</sub>/CBD were increased from 0.1:1 to 0.7:1. Changes in the chemical shifts occur due to the environmental changes on the protein surface as a result of interaction with ligand (11). The amide resonances that shift can be seen in superposition of a region of the two-dimensional <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectra of CBD during the titration (Fig. 1C). Chemical shift displacement for each residue was calculated from the chemical shift changes of both nuclei <sup>1</sup>H and <sup>15</sup>N using Equation 1. Amide resonances in CBD that exhibited prominent chemical shift perturbation (Δδ greater than 0.15 ppm) include Leu<sup>924</sup>, Thr<sup>957</sup>, Gln<sup>972</sup>, Asp<sup>974</sup>, Leu<sup>991</sup>, and Val<sup>993</sup> (Fig. 1D). These residues are located in close proximity to the five amide resonances that broadened and disappeared. Binding curves for residues in CBD (Fig. 2, filled circle) that show significant chemical shift changes upon the addition of increasing amounts of ((POG)<sub>10</sub>)<sub>3</sub> were obtained using Equation 2. As seen from Fig. 2, binding of ((POG)<sub>10</sub>)<sub>3</sub> to CBD saturated around a 1:1 molar ratio, indicating 1:1 stoichiometry of the complex. An average value of 57 μM was obtained for $K_d$ (supplemental Table 1) calculated for the six residues showing a large total chemical shift change. This signifies a moderate binding between CBD and collagenous peptide. The binding constant measured is consistent with the value determined from surface plasmon resonance (57 μM) (6).

The NMR titration study presented here mapped the tropocollagen binding cleft in solution for the first time (Fig. 3). Residues Leu<sup>924</sup>, Ser<sup>928</sup>, Arg<sup>929</sup>, Thr<sup>957</sup>, Tyr<sup>970</sup>, Gln<sup>972</sup>, Leu<sup>992</sup>, Tyr<sup>994</sup>, Lys<sup>995</sup>, and Tyr<sup>996</sup> of CBD shape the binding cleft. Side chains of these surface residues are within 3 Å of 11 amide groups whose resonances either line-broaden or shift in NMR titration. Mutations of the side chains to alanine were performed for all of the residues except Leu<sup>924</sup>, Ser<sup>928</sup>, Thr<sup>957</sup>, and Lys<sup>995</sup>, and those mutations lowered collagen binding affinity of CBD (6). Gly<sup>925</sup> and Asp<sup>926</sup> may also be involved in binding, but their resonances could not be unambiguously assigned, since they exhibited weakened and broadened signals in all of the spectra collected.

Unidirectional Binding of Collagenous Peptide on CBD—To identify the orientation of a tropocollagen on CBD, a collagenous peptide was modified to accommodate a nitroxyl group in the N terminus, (PROXYL-G(POG)-PRG)<sub>3</sub> (supplemental Fig. 2). The PROXYL group being paramagnetic enhances the relaxation rate of the NMR resonances of the protein under study via electron-nuclei dipolar coupling (10). An arginine residue was placed in the C-terminal triplet to increase solubility while stabilizing the triple helix to a similar extent as a hydroxyproline residue (25). The spin-labeled collagenous peptide is stable and retained the triple helical conformation under our experimental conditions as evident from CD spectra (sup-
NMR titrations with this spin-labeled analogue were carried out at the molar ratios described earlier. Five residues broadened, and six residues underwent chemical shift perturbation just as observed upon ((POG)_{10})_{3}-CBD complex titration. In addition, the amide resonance corresponding to Val^{973} appreciably line-broadened and vanished from the $^1$H-$^1$N HSQC spectrum at a 0.33:1 PROXYL-(G(POG)_{7})-CBD ratio (Fig. 4, A and B). Resonances of no other residues were perturbed due to the PROXYL group even at higher (PROXYL-(G(POG)_{7})_{3}) CBD ratios. In a three-dimensional space, Val^{973} is located at an edge of the $\beta$-sandwich fold at the opposite end of Ca^{2+}-binding site (Fig. 4F). This in turn implied that the N terminus of the tropocollagen is pointed away from the Ca^{2+}-binding site. To further confirm the direction of the tropocollagen, a shorter peptide, (PROXYL-(G(POG)_{7})_{3}) was synthesized and titrated with $^1$H-$^1$N-labeled CBD. Again, five residues extensively broadened, and six residues underwent chemical shift perturbation, as seen in the previous two titrations. In addition to Val^{973}, amide resonances corresponding to Gly^{975}, Ser^{979}, and Gly^{998} were also extensively line-broadened and vanished from (GPRG(POG)_{7}C-carbamidomethyl)_{3}-CBD (cyan) at a ratio of 1:1. In the absence of spin label, amide resonances of Ser^{906}, Arg^{929}, Ser^{997}, and Gly^{998} are not line-broadened.
Collagen Triple Helix on Binding Domain

also that the distance between the N terminus and CBD became shorter with \((\text{PROXYL-}G(\text{POG})_7)\)_3.

To further illustrate that the C terminus of the tropocollagen is directed toward the \(\text{Ca}^{2+}\)-binding site in CBD, another spin-labeled collagenous peptide, \((\text{GPRG})(\text{POG})_7C\)-PROXYL)\(_3\) was synthesized, where the PROXYL spin label was attached to a cysteine residue placed at the C terminus (supplemental Fig. 2). An arginine residue at the third position was made to increase the solubility of the peptide. NMR titrations with this peptide were repeated in a similar manner as with \((\text{POG})_{10}\)_3. On the titrations were repeated. The titration results with these peptides were nearly identical with those of \((\text{POG})_{10}\)_3. On the other hand, Val\(_{973}\) that disappeared due to the PROXYL group also remained unchanged at a 1:1 \((\text{GPRG})(\text{POG})_7C\)-carbamidomethyl)_3/CBD ratio (Fig. 4I). Results of these NMR titrations collectively demonstrate that all six tropocollagen analogues influenced the amide resonances of CBD in nearly identical fashion. Line broadening of additional residues was due to the presence of PROXYL. When the PROXYL group was reduced by ascorbic acid (the \((\text{PROXYL-}G(\text{POG})_7)\)_3-CBD complex, Val\(_{973}\) peak emerged (supplemental Fig. 5A), further confirming that the amide resonance of Val\(_{973}\) was line-broadened by the PROXYL moiety. When the \((\text{PROXYL-}G(\text{POG})_7)\)_3-CBD complex was reduced with ascorbic acid, amide resonances corresponding to Val\(_{973}\), Gly\(_{975}\), and Ser\(_{979}\) reappeared (supplemental Fig. 5B). Similarly, amide resonances for Ser\(_{906}\), Arg\(_{929}\), Ser\(_{997}\), and Gly\(_{998}\) emerged in the \((\text{GPRG})(\text{POG})_7C\)-PROXYL)\(_3\)-CBD complex when PROXYL was reduced with ascorbic acid (supplemental Fig. 5C).

Binding curves for the six residues (Fig. 2) in CBD that show significant chemical shift changes with increasing amounts of different collagenous peptides were obtained using Equation 2. Slopes of the binding curves for all six residues in CBD upon titration(s) with six different collagenous peptides were similar and consistent with moderate binding affinity (average \(K_d\) ~52–57 \(\mu\)M) (supplemental Table 1).

The PROXYL group is known to induce line broadening of nuclear resonances in a distance-dependent manner (26). Since more residues are line-broadened with PROXYL at the C terminus than at the N terminus (Fig. 4F), CBD plausibly binds closer to the C terminus of the tropocollagen analogue. Spin quantization of electron spin resonance spectra in the (\(\text{GPRG})(\text{POG})_7C\)-PROXYL)\(_3\) is 2.2 times larger than that of \((\text{PROXYL-}G(\text{POG})_7\beta\_\text{PRG})_3\) and \((\text{PROXYL-}G(\text{POG})_7\gamma\_\text{PRG})_3\), which could be due to a more dynamic nature of the PROXYL moiety attached to cysteine at the C terminus (supplemental Fig. 6). More residues line-broadened upon NMR titration with \((\text{GPRG})(\text{POG})_7C\)-PROXYL)\(_3\) might also be accredited to the dynamics of the PROXYL moiety. The experiment, however, clearly demonstrated unidirectional binding of collagenous peptide on CBD.

Earlier binding studies using extensive mutagenesis of surface residues postulated a Tyr-rich interface for CBD-collagen binding (6). Three binding modes were proposed, in which two modes oriented collagenous peptide perpendicular to a Tyr-rich \(\beta\)-sheet region in CBD and the third mode placed it diagonally. Two tropocollagen binding modes that predicted it to bind perpendicular to the \(\beta\)-sheets of CBD are consistent with the NMR titration studies done with various spin-labeled collagenous peptides. Residues that were extensively line-broadened due to collagen binding and proximity of the spin label are

\begin{table}
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\caption{R\(_{\text{g}}\) and P(r) values}
\begin{tabular}{lcc}
\hline
& CBD & Collagen-peptide & CBD-collagen peptide complex \\
R\(_{\text{g}}\)(Å) & 19.68 ± 0.01 & 18.68 ± 0.03 & 22.55 ± 0.02 \\
R\(_{\text{g}}\)(Å) (SAXS reciprocal space) & 19.73 ± 0.01 & 18.74 ± 0.01 & 22.62 ± 0.04 \\
Diameter at P(r) = 0(Å) (SAXS) & 93.33 & 80.27 & 93.34 \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{SAXS data of CBD (a), CBD-(G(POG))\(_7\)PRG)\(_3\) complex (b), and (G(POG))\(_7\)PRG)\(_3\) (c). A, the scattering profile where the intensity, I(Q) is plotted against scattering vector Q. B, pair-distance distribution function P(r) in the real space obtained using GNOM (15).}
\end{figure}
in accordance with the perpendicular binding mode. The binding mode that placed tropocollagen interacting diagonally with CBD can be eliminated, since no residues that could account for the diagonal binding mode are line-broadened with the PROXYL moiety at either terminus. The tropocollagen binding surface in CBD is narrowed to a 10-Å-wide and 25-Å-long cleft (Fig. 3). The width of the binding cleft in CBD matches with the diameter of the triple helix (27) (supplemental Fig. 7), and its length could accommodate ((POG)3)3.

Asymmetric CBD-Collagen Complex—To further confirm the solution structure of the CBD-collagenous peptide complex, SAXS studies were conducted. Using SAXS and an *ab initio* shape determination procedure, the three-dimensional structure of the macromolecule can be constructed. Linear Guinier plots from SAXS data for CBD, (G(POG)7PRG)3, and CBD-(G(POG)7PRG)3 complex (Fig. 5A) yielded the radius of gyration \( R_g \) values (Table 1). The pair-distance distribution function \( P(r) \) and the maximum diameter \( D_{max} \) averaged in all orientations were computed (Fig. 5B and Table 1). The \( R_g \) values computed from \( P(r) \) are in excellent agreement with those obtained from Guiner approximation (Table 1). The three-dimensional molecular envelopes constructed for CBD and the triple helical collagen peptide from SAXS data after *ab initio* calculation and simulated annealing minimization (for details, see “Experimental Procedures”) are in excellent agreement with the overall dimensions and shape of the crystal structure (Fig. 6, A and B). The overall shape of the tropocollagen analogue in solution is “8” or “∞”, which could be due to the more dynamic nature of collagen peptide at the ends as evident from its high B-factors in crystal structure (27). The three-dimensional SAXS envelope calculated for CBD-(G(POG)7PRG)3 showed asymmetric binding (Fig. 6C). Three-dimensional structures calculated by SAXS for four other CBD-collagenous peptide complexes also confirmed the asymmetric binding mode (supplemental Fig. 8). Combining NMR titration and SAXS data, CBD plausibly binds closer to the C terminus of the collagenous peptide. It is interesting to note that the scattering density of the collagenous peptide changes to a rod shape upon binding to CBD. Changes in dynamics of the collagenous peptide due to binding could influence the appearance of the SAXS envelopes (Fig. 6, B and C). If the collagenous peptide is placed in accordance with its orientation determined by NMR titration studies, CBD binds to C-terminal (POG)3 repeats (i.e. the sixth to eighth POG repeats in (POG)3) (Fig. 7). The dynamics of collagen does not appear to dictate CBD binding. However the non-triple helical nature of the tropocollagen at the carboxyl end (27, 28) might lure CBD toward the C terminus. The distance from the N-terminal spin label of (PROXYL-G(POG)7PRG)3 to Val973 in the CBD would be ~37 Å, and the distances from the N terminus of (PROXYL-G(POG)7PRG)3 to Val973, Gly975, and Ser979 are about 29 Å. Whereas Ser906, Arg929, Ser997, and Gly998 are about 15 Å from the C-terminal spin label of (GPRG(POG)7-C-PROXYL)3. Paramagnetic relaxation is usually observed in distances ranging between 15 and 24 Å (29); however, in our case, when the PROXYL is attached in the main chain at the N terminus, three PROXYL moieties are clustered together (supplemental Fig. 2), and long range relaxation effects might be pronounced. The SAXS envelope calculated for the (PROXYL-G(POG)7PRG)3-CBD complex shows an additional strong density at the N terminus, indicating that the PROXYL moieties at the N terminus are clustered (supplemental Fig. 8B). There is no such strong scattering density when the PROXYL moiety is attached to the side chain (supplemental Fig. 8C).

CBD binds unidirectionally to the C-terminal region of collagenous peptides. However, whether CBD binds only at the C-terminal region of tropocollagen remains uncertain. Though CBD targets the least ordered region of the collagenous peptides, CBD could also target partly unwound regions even in the middle of a tropocollagen. The catalytic domain is situated at the N terminus of CBD. Bacterial collagenolysis may involve optimal orientation of the catalytic domain with respect to tropocollagen. Furthermore, the collagenolysis could initiate from partly unwound regions in tropocollagen. Since the complexes of CBD and various col-
lagenous peptides are structurally homogeneous, co-crystals are probably attainable.

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