Nanomechanics of Hemichannel Conformations

CONNEXIN FLEXIBILITY UNDERLYING CHANNEL OPENING AND CLOSING*

Fei Liu†, Fernando Terán Arce†, Srinivasan Ramachandran†, and Ratnesh Lal‡

From the Neuroscience Research Institute, University of California, Santa Barbara, California 93106

Gap junctional hemichannels mediate cell-extracellular communication. A hemichannel is made of six connexin (Cx) subunits; each connexin has four transmembrane domains, two extracellular loops, and cytoplasmic amino- and carboxyl-terminals (CTs). The extracellular domains are arranged differently at non-junctional and junctional (gap junction) regions, although very little is known about their flexibility and conformational energetics. The cytoplasmic tail differs considerably in size and amino acid sequence for different connexins and is predicted to be involved in the channel open and closed conformations. For large connexins, such as Cx43, the CT makes large cytoplasmic fuzz visible under electron microscopy. If this CT domain controls channel permeability by physical occlusion of the pore mouth, movement of this portion could open or close the channel. We used atomic force microscopy-based single molecule spectroscopy with antibody-modified atomic force microscopy tips and connexin mimetic peptide modified tips to examine the flexibility of extracellular loop and CT domains and to estimate the energetics of their movements. Antibody to the CT portion closer to the membrane stretches the tail to a shorter length, and the antibody to CT tail stretches the tail to a longer length. The stretch length and the energy required for stretching the various portions of the carboxyl tail support the ball and chain model for hemichannel conformational changes.

Gap junctions are intercellular channels that directly couple two apposing cells. They allow diffusion-driven intercellular transfer of ions and small molecules (<1000 Da), synchronize electrical activity, and regulate metabolic homeostasis, cell growth, and differentiation (1–3). Structurally, a gap junction is made of two hemichannels (connexons) aligned head-to-head across an extracellular “gap,” which is roughly cylindrical with a central ion-permeable pore (4–9).

Each connexon comprises six connexin proteins, and each connexin has cytoplasmic amino and carboxyl termini, four membrane-spanning regions, two extracellular loops (ELs), and a single cytoplasmic loop. There is very limited variation in the sequences of two disulfide-linked extracellular loops, which are crucially positioned for the docking of the connexons to form gap junctions (3, 6). Docking between connexons involves interactions between the extracellular loops (7, 10). The details of how the extracellular loops are arranged and dock are currently unresolved. Current models based on three-dimensional structural studies suggest that hemichannel docking involves multivalent interactions of subunits between hemichannels. In addition, hemichannels are also present in the non-junctional regions wherein their extracellular loops with many hydrophobic amino acids would require a different conformation to shield the hydrophobic amino acids from the aqueous environment (11).

The opening and closing of hemichannels, in response to both membrane potential and chemical signals, are being examined extensively, and several models, based on structural information from x-ray diffraction and electron microscopy, have been proposed for gap junction opening and closing. In the early model by Unwin and Zampighi (12, 13), they proposed that the channel is closed by differential rotations of apposing hemichannels thus narrowing the pore diameter. But, little is known about non-junctional hemichannels present in the free margins of the plasma membrane. Sosinsky and coworkers have reported calcium-induced extracellular conformational changes in Cx26 hemichannels using atomic force microscopy (AFM) (8). Recently, Thimm et al. (14) have proposed calcium-dependent folding/refolding of extracellular loops leading to altered pore mouth opening or closing. They obtained three-dimensional conformations of the open and closed Cx43 hemichannels reconstituted in lipid bilayer and have provided an estimate of conformational energetics (14). Such information is missing about the channel gating from the cytoplasmic side. For both hemichannel and gap junctional gating, especially for connexins with a large cytoplasmic tail such as Cx43, Delmar and coworkers (15, 16) proposed the “particle-receptor” hypothesis similar to the “ball and chain” model of potassium channels. According to this, the long cytoplasmic tail “chain” folds into a “ball” (gating particle) and binds to the cytoplasmic loop domain (a possible receptor) and leads to channel closure (16, 17). However, little structural or mechanistic data are available to support this model.

AFM-based single molecule force spectroscopy is being used as an effective approach to define protein folding and unfolding and three-dimensional conformations. The binding of ligands immobilized on AFM tips to their receptors adsorbed on a surface is examined by applying a force to the receptor-ligand complex until the bond breaks at a measurable unbinding force.

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† These authors contributed equally to this work.

‡ To whom correspondence should be addressed: Neuroscience Research Institute, University of California, 5126 BioSci II Bldg., Santa Barbara, CA 93106, Tel: 805-893-2350; Fax: 805-893-2005; E-mail: lal@niris.ucsb.edu.

§ The abbreviations used are: EL, extracellular loop; AFM, atomic force microscopy; CT, carboxyl terminal; PBS, phosphate-buffered saline; PEG, polyethylene glycol; NHS, N-hydroxysuccinimide; N, newton(s); pN, piconewton(s); FJC, Freely-Joint-Chain; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine.

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(18–20). Such an approach has been applied to investigate the binding potentials of receptor-ligand pairs involved in cell adhesion (21), polysaccharide elasticity (22), DNA mechanics (23), and the function of molecular motors (24).

In the present study, using single molecule force spectroscopy, we examined the interactions between individual anti-Cx43 antibody and the carboxyl-terminal (CT) region of full-length Cx43 hemichannels reconstituted in supported lipid bilayer as well as between connexin mimetic peptides to the Cx43 extracellular loop and reconstituted Cx43 hemichannels. Antibodies to specific connexin epitopes have been used to provide some qualitative details about the hemichannel structure and subunit interactions (14, 25). Previously, connexin mimetic peptides, short synthetic peptides to specific sequences in connexins EL and cytoplasmic loop domains, have been used to block the functional gap junction coupling in a variety of systems (26, 27).

Our results provide distinct signatures of the extracellular and cytoplasmic sides of hemichannels reconstituted in bilayer: mimetic peptide recognizes the EL loops, whereas anti-CT antibodies recognize the CT domain. Antibody binding to the CT domain displays typical single interactions and molecular stretches. The extended molecule stretch is consistent with the elongated random coil nature of the Cx43 CT domain and is sensitive to calcium. The extent of stretching the various portions of the carboxyl tail suggests the ball and chain mechanism for hemichannel conformational changes.

ExPERIMENTAL PROCEDURES

Hemichannel Isolation and Purification—Wild-type BICR-M1RK (Marshall) cell line expressing Cx43 was grown as described previously (28). Connexons were isolated as described (14, 29) with modification. The entire procedure was described previously (28). Connexons were isolated as

M1Rk (Marshall) cell line expressing Cx43 was grown as

to anti-CT360–382 (Chemicon) and 1 mg of phosphatidylcholine/ml. Connexons were eluted with buffer S containing 5 g/ml at room temperature. Samples were rinsed with PBS for at least five times. They were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1 g/ml, ZyMaxTm, Invitrogen) for 2 h at room temperature (33). Samples were then washed again with PBS. Fluorescence images were captured using an Olympus IX71 inverted microscope with 100× oil lens (numerical aperture, 1.45). Immunofluorescence images were collected with red channel filter set (Ex 482/Em 536 nm) for bilayers and with green channel filter set (Ex 525/Em 585 nm) for connexons. Bilayers reconstituted with connexons but without any antibody incubation, bilayers reconstituted with connexons incubated with secondary antibodies directly, and bilayers without connexon reconstitution but incubated with both primary and secondary antibodies were also imaged as controls.

Preparation of Functionalized AFM Tips—Mouse anti-Cx43 monoclonal antibody against the cytoplasmic domain CT252–270 (Chemicon), connexin mimetic peptide GAP26 (VCYDKSF-PISHVR) and scrambled GAP26 (PSFDSRHCIVKYV) (Sigma-Genosys, Haverhill, UK) were used to functionalize the AFM tips as described (34) with slight modification. Briefly, standard 200-μm-long Si3N4 tips (Veeco, Santa Barbara, CA) and 450-μm-long silicon tips (Applied NanoStructures, Santa Clara, CA) were cleaned with chloroform three times and dried with N2. Tips were then washed with ethanol twice and dried with N2. The tips were incubated overnight in 5.6 μl of aldehyde and 0.3 ml of triethylamine. The functionalized tips were then washed twice with chloroform and dried with N2. Finally, tips were incubated in 200 μl of antibody or peptide (0.2 mg/ml in PBS) for 50 min.
to link the free NH$_2$ on the antibody or peptide to the functionalized tips. 2 μl of 1 M NaCNBH$_3$ (32 mg of NaCNBH$_3$, 50 μl of 100 mM NaOH in 450 μl of H$_2$O) were added to the solution at the very beginning of the reaction. At the end of the reaction, 5 μl of 1 M ethanolamine (in 20% NaOH) was added to the solution to passivate unreacted aldehyde groups. Functionalized tips were washed and stored in PBS buffer at 4 °C until use.

Cantilever Calibration—Cantilever spring constants were measured using two methods. For cantilevers with a nominal spring constant of 0.06 newton (N)/m (Veeco), the spring constant of the functionalized tip was estimated by measuring the spring constants with standard force calibration cantilevers as described previously (35). The spring constant of antibody- or peptide-conjugated tips was measured to be $k = 0.063 \pm 0.005$ N/m ($n = 5$) and is consistent with published results (36). Whenever feasible, we used the same conjugated tip for experiments involving internal control; for example, in mapping interactions between antibody and receptor alone, adding online inhibitor of their interactions. For cantilevers with a nominal spring constant of 0.02 N/m (Applied Nanosstructures), a modified (developed by Ben Ohler from Veeco) thermal noise method (37) was used to determine their spring constants. Briefly, the cantilever was withdrawn from the surface and was allowed to oscillate freely at its natural frequency. An AFM image (512 × 512 pixels) recording the cantilever deflection of these oscillations was obtained at a point sampling rate of ~62.5 kHz (i.e. 61 Hz lines/s). A MatLab (MathWorks, Natick, MA) code was written to obtain the power spectrum density of these oscillations and by application of the equipartition theorem, the cantilever spring constant was calculated. The measured spring constant was ~0.014 – 0.016 N/m.

AFM and Force Measurement—AFM images were collected using a NanoScope III Multimode AFM (Digital Instruments). The AFM probes were oxide-sharpened silicon nitride (Si$_3$N$_4$) tips and were operated at frequencies of 5–35 kHz with free oscillating amplitudes of ~20–35 nm. Most of the AFM imaging was carried out in tapping mode with a scanning frequency of 0.5–2 Hz that allowed simultaneous collection of the height and amplitude data. All AFM imaging of lipid bilayers with or without reconstituted Cx43 connexons was conducted at room temp (22–24 °C) in PBS buffer (pH 7.2–7.4) as described (28).

Force-volume maps were obtained to collect force curves between antibody/mimetic peptide (conjugated to the AFM tip) and connexons reconstituted in the planar lipid bilayer. One complete force curve was recorded at each position while the AFM tip was scanned across the surface of the bilayer in 64 × 64 pixels. The force curves were collected with a z-deflection rate of 1–4 nm/ms, a z-scan size of 400 nm, and a maximum cantilever deflection (relative trigger) of 20 nm.

To determine the specificity of the antibody-Cx43 or GAP26-Cx43 interaction, 100 μl of 50 μg/ml antibody or peptide (the same ligands conjugated on tips) was then added into the imaging solution, and successive force-curves were collected again after 20 min of incubation with the blocking antibody or peptide. As an additional control experiment, force curves were also recorded with a scrambled GAP26-conjugated tip in identical experimental conditions.

Data Analysis—A MatLab (MathWorks, Natick, MA) code was used to analyze the force-distance curves obtained in force-volume map as described (38). This program identifies unbinding events by searching for all significant local minima in a retraction curve. To differentiate unbinding events from noise, filters were introduced so that unbinding events with adhesion forces below a given threshold were not considered. A small fraction of force-distance curves were identified as corrupt curves, e.g. due to excessive noise or ill-defined approach and/or retraction curves, and were excluded from the analysis. Each retraction curve was analyzed by first finding the horizontal region in the approach curve. The zero force line was determined as the average value of points in the horizontal region. The intersection of this line with the retraction curve identifies the tip-surface contact point and defines the origin from which the polymer extensions and the magnitude of the adhesion/unbinding forces are measured in the retraction curve. For an unbinding event, the adhesion force corresponds to the magnitude of the force in the retraction curve measured from the zero force line. The corresponding separation distance, $d$, represents the distance traveled by the piezo from the origin of the curve (corresponding to the initial horizontal cantilever position) to the unbinding event. The polymer extension length, $L$, is simply given by subtracting the cantilever deflection, $\Delta$, from the piezo displacement, $L = d - \Delta$. The unbinding force is determined from the difference between the forces corresponding to a local minimum and the next local maximum in the direction of increasing values of the separation distance. The probability of finding either single or multiple events in a force curve (determined by the number of significant local minima) was calculated by dividing the number of curves in each category by the total number of force curves acquired. These were represented in a histogram displaying the probability for finding zero events, one event or multiple events in a force curve.

Force-extension curves were fitted with the extended FJC model (39, 40),

$$\text{Ex}(F) = N_s \left( \frac{L_p}{e^{k_s F} + 1} + \frac{L_h}{e^{k_s F} + 1} \right) \left[ \coth \left( \frac{F L_K}{k_s T} \right) - \frac{k_s T}{F L_K} \right] + \frac{N_s F}{K_s}$$

(Eq. 1)

where $\text{Ex}(F)$ is the measured extension length; $\Delta G = 3k_BT$ is the difference in Gibbs free energy between a planar and a helical PEG subunit in the presence of a force $F$ with $L_p = 0.287$ nm and $L_p = 0.358$ nm for their respective lengths (in the absence of force). The Kuhn length $L_K = 0.7$ nm, and the segment elasticity $K_s = 150$ N/m are fitted to the experimental data. $N_s$, the number of monomers for each tether (polymer contour length) is the only fitting parameter.

RESULTS AND DISCUSSION

Connexon Reconstitution in Lipid Bilayer—Hemichannels, when reconstituted in an artificial lipid bilayer, insert into the membrane with almost equal probability for both extracellular and cytoplasmic sides facing outside on a supported system.
The differing lengths and amino acid sequences for the two sides, however, enable them to be distinguished with AFM imaging (14). As shown in Fig. 1, we designed our experimental system to utilize the specific interactions either between antibody and Cx43 CT domains or between mimetic peptides and Cx43 extracellular domains in Cx43 hemichannels. Flexible polymer tethers were used to attach antibodies or mimetic peptides to the AFM tip, which spatially isolates nonspecific probe-sample interactions from the specific interactions of the tethered molecules (Fig. 1B). Anti-CT\(_{252-270}\) antibody is used to target the middle region of the Cx43 CT tail, whereas anti-CT\(_{360-382}\) is used to bind the end of the CT tail. Connexin mimetic peptide Gap26 is used to probe the Cx43 extracellular domain EL\(_{163-75}\) (Fig. 1C).

The reconstitution of connexons in the supported bilayer was confirmed by immunofluorescence labeling. Fig. 2A1 showed a planar bilayer reconstituted with hemichannels (sample) and exhibited immunofluorescence labeling (green) for Cx43, whereas no fluorescence was detected in the sample without immunofluorescence labeling (Fig. 2A2). Insignificant nonspecific immunolabeling was observed when the sample was incubated with the fluorescein isothiocyanate-conjugated secondary antibody directly (without primary anti-Cx43 antibody) (Fig. 2A3), and no fluorescence was observed in bilayers without connexons after both primary and secondary antibody labeling (Fig. 2A4). Fig. 2 (B1–B4) shows the corresponding fluorescence images of Lissamine Rhodamine B-labeled planar bilayers (red) on the coverslip. Some holes in the bilayer, where liposomes did not fuse, are also seen (arrows).

As shown in an AFM height image (Fig. 3A), the surface topography of the bilayer without any reconstituted connexons appears to be smooth and featureless with the height fluctuation of <0.3 nm (Fig. 3B). The thickness of the bilayer is \(\sim 5.0\) nm, as determined from the cross-section along the unfused bilayer holes, consistent with previous results (14). Connexons reconstituted in bilayers appear as structures protruding out from the bilayer surface in a randomly distributed pattern.
These connexons appear to fall into two groups with distinct extramembranous protrusion heights of \(0.5–1.0\) nm (red circles) and \(2.0\) nm (white circles) (Fig. 3). Fig. 3D shows cross-sections along lines in Fig. 3C: open arrows represent the first group of low protrusion heights (red circles), whereas solid arrowheads represent the second group of high protrusion heights (white circles). The height difference is due to the larger cytoplasmic CT domain protruding from the membrane. Thus, a clear distinction of the sidedness of imaged connexons is achieved that is consistent with previous biochemical and structural studies of Cx43 connexons (14, 30, 41).

Anti-CT Antibody and Mimetic Peptide Recognitions of Reconstituted Cx43—Identification of the EL- versus CT-sided hemichannels was defined by the specific interactions of either the mimetic peptide to EL domain or antibodies to CT domain for a given hemichannel in the sample. A typical force-extension plot obtained from the measurements of anti-CT\(_{252–270}\) antibody-Cx43 interaction force is shown in Fig. 4A. As the cantilever was pulled away from the surface, the probe left the tip-sample repulsive contact region (I) and entered the attractive interaction region (II) that is distinguished by a characteristic spacer stretching shape and corresponds to a specific interaction of the tethered antibody with Cx43. The non-linear parabolic increase in the interaction force prior to antibody-peptide unbinding provides the viscoelastic properties of the spacer-antibody-Cx43 connections. This interaction resembles the shape described by the extended Langevin function (red line) for characteristic polymer stretching following the Freely-Joint-Chain (FJC) model (39). In region III, the cantilever separates completely from the sample surface and returns to its equilibrium deflection. A typical force of \(\approx 100\) pN and extension of \(30–50\) nm was detected from force curves with single unbinding events.

Force curves with similar shape and unbinding force were also obtained for the anti-CT\(_{360–382}\)-Cx43 intermolecular interaction force measurements (Fig. 4B). However, there was a significant difference with respect to the anti-CT\(_{252–270}\)-Cx43 interaction: an extra extension of \(\approx 105\) nm was detected in the anti-CT\(_{360–382}\)-Cx43 interaction that is attributed to the stretching of Cx43 CT tail as discussed later. Moreover, as described later, these extensions were beyond the expected stretching of the antibody (if any) and the linkers.
The force-extension curve between the connexin mimetic peptide GAP26 and the extracellular domains of reconstituted Cx43 hemichannels showed a smaller unbinding force (Fig. 4C). This is in contrast to the large unbinding force observed for the anti-CT antibody and Cx43 interactions.

The specificity of the antibody-Cx43 interactions was confirmed when excess free anti-CT antibody was added into the imaging medium to block the antigenic sites of the Cx43 hemichannels. After addition of excess antibodies, the complicated rupture structure was abolished and no specific rupture event was observed (Fig. 4D, E). The specific rupture events were abolished by on-line addition of excess free GAP26 and when using the scrambled peptide bound to the AFM tip approaches.

The sidedness (EL versus CT) identification and the specificity of the antibody/peptide interactions to the EL and CT sides were evaluated carefully. In the reconstituted bilayer, both EL-side-up and CT-side-up Cx43 hemichannels are present (Fig. 3 in Thimm et al. (14)). In our study, using AFM tip conjugated with mimetic peptide GAP26 showed a weak force of unbinding and a short extension (~30 nm). Moreover, on-line addition of anti-CT antibodies did not affect such unbinding force or extension. On the other hand, using AFM tip conjugated with anti-CT antibodies showed strong forces of unbinding and large (~30 nm) stretching. Furthermore, these interactions were not affected by on-line addition of GAP26 but by the free anti-CT antibody. Also, in the AFM imaging and force map, we clearly observed the bimodal but random distribution of bumps with heights representing EL and CT sides up.

Our preliminary results also indicate that no apparent specific interaction is detected when the same force measurement was performed using the anti-CT antibody-conjugated AFM tip to scan the carboxyl-terminal truncated mutant of rat heart Cx43 gap junction plaques. 4

These isolated Cx43 gap junction plaques that were obtained from Dr. Mark Yeager at The Scripps Research Institute were truncated after Pro263, therefore, the antigenic binding site for anti-CT antibody was unavailable (42).

As shown in the peptide-hemichannel unbinding event histogram (Fig. 5A), ~29% of all the force curves for anti-CT252–270-Cx43 interactions were identified as single rupture events. About 60% of these single rupture events occurred with much higher forces of ~200–250 pN (Fig. 5B). Considering the relatively constant tether stretching distance in all single rupture curves, single unbinding events with higher forces would probably be attributed to ruptures of multiple parallel antibody-antigen bonds (43). In addition to the single rupture events as described above, ruptures with multiple unbinding

4 F. Liu, F. Terán Arce, S. Ramachandran, and R. Lal, unpublished data.
steps were also detected for the anti-CT252–270–Cx43 interactions. Only a few multiple rupture events, however, were observed for anti-CT360–382–Cx43 or Gap26–Cx43 interactions (Fig. 5A). The total specific rupture events (including single as well as multiple unbinding) in Gap26–Cx43 interactions were significantly reduced as well. The binding probability as well as the rupture forces in the Gap26–Cx43 map was significantly lowered, as compared with antibody–Cx43 map.

The anti-CT252–270 monoclonal antibody and the anti-CT360–382 polyclonal antibody used in this study are made against synthetic peptides corresponding to positions 252–270 (GDLSPSKDCGPYAYFNG) and 360–382 (DQRPSSRSSRPRPDDLEI) of the native sequence within the cytoplasmic CT domain of rat cardiac Cx43, respectively (44–47). As indicated in Fig. 1, the antigenic sites for both anti-CT252–270 and anti-CT360–382 antibodies are located at the CT domain. Therefore, the force curves obtained in the force maps shown in Fig. 5 (B and C) recognize the CT-side-up reconstituted Cx43 hemichannels.

The connexin mimetic peptide GAP26 used in this study corresponds to positions 63–75 of the native sequence within the extracellular loop 1 (EL1) of Cx43. The force curves
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observed (Fig. 4C) may occur between GAP26 and its complementary sequence in the extracellular loops of the Cx43 hemichannels as proposed previously (10, 48). Significantly fewer multiple unbinding events were detected in GAP26-Cx43 force measurements. Characteristic tether stretching curves and blocking competition assay as well as scrambled peptide unbinding observed in the present study suggest that the observed interactions are specific interactions between GAP26 and Cx43. Therefore, the force map adhesions in Fig. 5 observed interactions are specific interactions between GAP26 and blocking competition assay as well as scrambled peptide unbinding observed in the present study suggest that the observed interactions are specific interactions between GAP26 and Cx43. Therefore, the force map adhesions in Fig. 5 observed forces vary considerably (49–244 pN) for different systems (52). The unbinding force histogram reveals frequent occurrence of unbinding force with distinct values for different binding domains. Unbinding forces of 63 ± 11, 116 ± 22, and 222 ± 16 pN were observed for the anti-CT252–270-Cx43 interactions (Fig. 6A). The anti-CT360–382-Cx43 interactions show an average unbinding force of 125 ± 18 pN (Fig. 6B). The rupture forces for GAP26-Cx43 interactions occur at 52 ± 10 and 96 ± 14 pN. Few unbinding forces higher than 200 pN were observed for the mimetic peptide-connexon interactions (Fig. 6C).

Both the anti-CT antibodies and mimetic peptides were covalently linked to an AFM tip via the distensible aldehyde-PEG-NHS spacers to minimize the impact of nonspecific interactions. The spacers spatially isolate nonspecific probe-sample interactions from the specific interactions of the tethered molecules and allowed us to discriminate between rupture events using both the unbinding force values and rupture location. An avidin-biotin unbinding system (34) was used to estimate the PEG spacer’s average length: avidin molecules were attached to the AFM tip via the aldehyde-PEG-NHS spacer with the same method used for antibody, whereas biotinylated-bovine serum albumin was attached on the mica surface. Force curves were recorded under the same conditions as in the antibody measurements. As shown in Fig. 6 (D and E), our results are similar to the previously reported force curves for biotin-avidin unbinding events (34) and typical unbinding forces ~40 pN. The force-extension curve was fitted to the extended FJC model as described under “Experimental Procedures.” The perfect fitting in Fig. 6D indicates that the observed extension ~28 nm results mainly from the spacer stretching, even considering the ~5 nm biotin-avidin complex. Fitting parameters obtained from the extended FJC fitting as well as measured extension lengths from different interactions are listed in Table 1. The fitted contour length shows large deviations from the measured extension length in anti-CT252–270-Cx43 and anti-CT360–382-Cx43 interactions.

As shown in Fig. 6F and Table 1, an average extension of ~31 nm was observed in the GAP26-Cx43 interactions, which is very close to the PEG spacer length measured in our binitin-

![Image](image_url)

**FIGURE 6.** Probability histograms of the rupture forces of the measured anti-CT252–270-Cx43 (A), anti-CT360–382-Cx43 (B), and GAP26-Cx43 interactions (C). The histogram is fitted with a Gaussian curve, and the corresponding mean ± S.D. is indicated in the text. D, a representative force-extension curve showing specific avidin-biotin interaction in the PEG spacer extension test system: avidin molecules were attached to the AFM tip via the aldehyde-PEG-NHS spacer with the same method used for antibody; biotinylated-bovine serum albumin was attached on the mica surface; force curves were recorded under the same conditions as in the antibody measurements and fitted to the extended FJC model (red line); E, the average extension of PEG spacer stretching, ~28 nm, is estimated from the extension of the testing system. F, histograms of measured tether extensions in anti-CT252–270-Cx43, anti-CT360–382-Cx43, and GAP26-Cx43, respectively.

**TABLE 1** Parameters obtained from the extended FJC fitting

|                  | Anti-CT252–270-Cx43       | Anti-CT360–382-Cx43       | GAP26-Cx43                  |
|------------------|---------------------------|---------------------------|-----------------------------|
| **N_e**          | 94 ± 24                   | 266 ± 18                  | 125 ± 29                    |
| **L_e [nm]**     | 26.7 ± 6.8                | 75.5 ± 5.1                | 35.5 ± 8.2                  |
| **L_p [nm]**     | 56.8 ± 14.1               | 104.9 ± 20.9              | 31.1 ± 4.3                  |
| **Avidin-biotin/bovine serum albumin** | 136 ± 33                  | 38.6 ± 9.4                | 27.6 ± 8.1                  |

*Note: L_e is the measured extension of the anti-CT (or peptide)-PEG-Cx43 complex.*

Mimetic Peptide-Cx43 and Antibody-Cx43 Rupture Forces and Molecule Stretching—The Cx43-mimetic peptide and Cx43-antibody unbinding force analysis is complicated if the specific interactions arise from a number of different interacting molecules or variable strength of bonds. Previous studies have revealed a typical unbinding force of ~100 pN for single antibody-antigen pairs (19, 43, 51), although the unbinding forces vary considerably (49–244 pN) for different systems (52). The unbinding force histogram reveals frequent occurrence of unbinding force with distinct values for different binding domains. Unbinding forces of 63 ± 11, 116 ± 22, and 222 ± 16 pN were observed for the anti-CT252–270-Cx43 interactions (Fig. 6A). The anti-CT360–382-Cx43 interactions show an average unbinding force of 125 ± 18 pN (Fig. 6B). The rupture forces for GAP26-Cx43 interactions occur at 52 ± 10 and 96 ± 14 pN. Few unbinding forces higher than 200 pN were observed for the mimetic peptide-connexon interactions (Fig. 6C).
avidin testing system (Fig. 6E). This suggests that the extension in the EL experiments results only from the spacer stretching, and most likely there was very little, if any, molecular stretching of the Cx43 extracellular loop or the GAP26 peptide during the unbinding event.

A significantly larger extension of $\sim 105$ nm was observed in the anti-CT$_{360-382}$-Cx43 interactions (Fig. 6F). In this situation, the antibody binds to the CT positions 360–382, close to the end of the CT domain (Fig. 1C). The extra extension observed for this situation may reflect the total stretching of the several associated molecules, including the IgG, the PEG spacer, and the CT domains. In comparison to the mimetic peptides against the ELs, the antibody for Cx43 CT epitopes was relatively larger ($\sim 150$ kDa) and this larger size may add to some additional extension. Stretching lengths of antibodies from 5 up to 18 nm have been reported (53, 54). However, it is unlikely that the antibody three-dimensional structure can be unfolded significantly because of the external force during the unbinding events, and thereby provide the additional extension of more than 20 nm, because the intramolecular forces responsible for stabilizing antibody conformations are significantly larger than the intermolecular antibody-antigen binding force (19).

Recent NMR studies indicate that the overall structure of Cx43 CT domain is primarily random coiled with two short helical regions (17, 55). Such an elongated random coil structure might be highly flexible and easy to stretch. The additional extension length of C-tail proteins from AFM force measurement has been previously reported (56). Cx43 CT domain contains $\sim 148$ amino acid residues, and the contour length of the polypeptide chain in its fully extended conformation may extend up to 53 nm (1 amino acid $\sim 0.36$ nm) (57) and even longer if the applied external force would permit the peptide backbone to stretch (39).

Consistent with the above analysis, a shorter extension of $\sim 57$ nm was detected in the anti-CT$_{252-270}$-Cx43 interactions, where the antibody binding site is located in the middle of the flexible Cx43 CT domain (Fig. 1C). Only a small portion of the CT coil can be stretched (amino acid residues 234–252). The good fitting of measured force curve to the extended FJC model (red line in Fig. 4A) suggested that the PEG spacer stretching ($\sim 30$ nm) still contributes to the major part of the overall extension.

**Flexibility of Cx43 CT Domain: Role in Hemichannel Gating**—
Structural flexibility of the Cx43 CT domain is the basis of the particle-receptor hypothesis for Cx43 (58). Muller and coworkers (8) reported a compressing energy of 11.9 kJ/mol required for the conformational change in the CT domain of Cx26; significantly, though, the CT portion of Cx26 is much smaller compared with the CT portion of Cx43. The energy required to stretch Cx43 CT domain can be calculated by integrating the force-extension function. The area under the force-extension curve from anti-CT$_{360-382}$ interaction (Fig. 4B) corresponds to the mechanical energy required to stretch both the PEG spacer and CT domain, whereas the area under the force curve from anti-CT$_{252-270}$ interaction (Fig. 4A) corresponds approximately to the energy required to stretch only the PEG spacer; the mechanical energy for the anti-CT$_{360-382}$ interaction and for the anti-CT$_{252-270}$ interaction are $\sim 375$ kJ/mol and $\sim 130$ kJ/mol, respectively. The difference between these two values of $\sim 245$ kJ/mol is the mechanical energy required to stretch the Cx43 CT domain. This value is similar to the previously reported mechanical energy required to stretch protein molecule complex (P-selectin/PSGL-1) (53).

Electrophysiological studies have shown intracellular Ca$^{2+}$-dependent gating of Cx43 hemichannels as well as other type...
We provide a simple model for channel opening and closing that is consistent with the earlier hypothesis by Delmar and colleagues (16). In their model, the CT blocks the channel pore mouth physically by clumping as a ball (Fig. 8). Our results provide an estimate of the intrinsic distensibility of the flexible Cx43 CT domain, which is higher than the relatively inelastic EL domains. In brief, (a) higher distensibility of CT domain and (b) presence of antibody-CT domain interaction at 0 mM Ca\textsuperscript{2+} and the lack of it with 1.8 mM Ca\textsuperscript{2+}, strongly support calcium-dependent conformation change of CT domain and the ball and chain model of hemichannel gating. However, whether this estimated distensibility force translates into the required mechanical energy to move the ball from the pore mouth requires further careful investigations. The mechanical movement of the CT domain in our study would probably overestimate the amount of energy needed to remove the ball in the actual cell interior wherein several complementary physicochemical factors would modulate the overall connexin flexibility.

In summary, we examined the interactions between individual antibodies and non-crystalline, non-truncated Cx43 hemichannels reconstituted in supported lipid bilayers as well as between connexin mimetic peptides and Cx43 hemichannels using single molecule force spectroscopy. Our results provide distinct signatures of the extracellular and cytoplasmic sides of hemichannels reconstituted in bilayer. Cx-mimetic peptides recognize only the extracellular side, whereas the anti-CT antibodies recognize only the cytoplasmic side. We provided a unique system to investigate not only the intermolecular interactions (antibody-antigen) but also the intramolecular interactions (CT stretching) of the reconstituted hemichannels. Such an approach of force microscopy with a connexin mimetic peptide-functionalized probe could be used for examining the biophysical basis of gap junction formation and analyzing hemichannel structure-function in vitro as well as in cell models.

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