Structural bases for the chemical regulation of Connexin43 channels

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Abstract

Connexins proteins associate with a variety of catalytic and non-catalytic molecules. Also, different domains of connexin can bind to each other, providing a mechanism for channel regulation. Here, we review some of these associations, placing particular emphasis on the intramolecular interactions that regulate Connexin43 (Cx43). We also describe some novel methods that allow for the characterization of protein–protein interactions such as those observed in the cardiac gap junction protein Connexin43. Overall, intra- and inter-molecular interactions may regulate gap junctions to filter the passage of molecular messages between cells at the appropriate time and between the appropriate cells. As a potential area for future investigations, we also speculate as to whether some of the inter-molecular interactions involving connexins lead to modifications in the function of the associated protein, rather than on the function of connexin itself.

1. Regulation of cardiac connexins

Given the diversity of connexin isoatypes, and their heterology in terms of regulatory functions, we will focus the present article on one particular isoatype, Connexin43 (Cx43). This is the most abundant connexin in the heart as a gap junction. We place particular emphasis on the concept that interactions within the connexin molecules (i.e., intramolecular interactions) may be essential for Cx43 regulation. These interactions may regulate the permeability and selectivity of the channel, as well as the ability of the connexin molecules to interact with other partners. Given the growing interest in the study of protein–protein interactions as a mechanism of gap junction regulation, we also discuss some novel methods that have been recently applied to the identification and characterization of the inter- and intra-molecular interactions involving gap junction proteins. Molecular promiscuity may be a key mechanism by which gap junctions filter the passage of information between cells according to specific cues provided by changes in the intracellular space.
well as other tissues such as brain. Moreover, there is a wide variety of regulatory mechanisms that can alter the conductivity of gap junctions [6]. A thorough review of all those different mechanisms is beyond the scope of this article. Here, we will review some of the molecular interactions that occur both inside a Cx43 molecule (intra-molecular) as well as between connexins and other associated molecules (inter-molecular) and that can modify channel function.

1.1. Structural bases for Cx43 regulation. The “ball-and-chain” hypothesis

There is general consensus that the carboxyl terminal (CT) region of Cx43 acts as the regulatory domain. Initial studies showed that truncation of the CT domain at amino acid 257 prevented acidification-induced uncoupling [7]. That work was followed by a study indicating that uncoupling could be restored if the CT domain was co-expressed as a separate fragment [8]. Based on these and other data, we proposed that pH regulation of Cx43 is consistent with a “ball-and-chain” model of gating. This model, originally proposed for voltage gating of the sodium channel [9] and later demonstrated for the N-type inactivation of Shaker [10] and other potassium channels, states that the channel is blocked by the interaction between an intracellular particle (a “ball”)—which is tethered to a flexible element (a “chain”)—and a receptor affiliated with the pore. We therefore proposed that pH gating of Cx43 functions as a particle–receptor interaction, where the CT domain acts as a gating particle (see Fig. 1). At normal pH, the gating particle would be away from the pore and the channel would be open. Upon acidification, the particle would bind to a separate region of the protein (a “receptor”) affiliated with the pore. The particle–receptor interaction would lead to channel closure. Later on, studies from this and other laboratories expanded this model by showing that it applies to the regulation of Cx43 by insulin and insulin-like growth factor [11] as well as by src [12]. Further studies have shown that some (but not all) other connexins follow a similar model of pH gating. In fact, the CT domain of Cx40 can also rescue the pH sensitivity of a truncated Cx40 channel [13]. These heterodomain interactions may be more efficient than homodomain interactions at closing the channel upon acidification [13,14]. A ball and chain-like mechanism seems to be responsible for the presence of the residual state in both Cx43 and Cx40 [15,16].

Indeed, studies from our laboratory looking at single channel transitions between the closed, open and residual states show that truncation of the CT domain eliminate the residual state, whereas this state is restored after the CT is coexpressed as a separate protein. These observations suggest that the ball and chain model applies to the regulation of Cx43 and of Cx40 not only by chemical factors, but by transjunctional voltage as well [15,16].

The results outlined above strongly support the hypothesis that the carboxyl terminal domain acts as a gating particle that binds to a “receptor” affiliated with the pore to regulate Cx43 channels. These data point to the existence of an intra-molecular interaction regulating connexins. Moreover, the intra-molecular interactions may be concurrent with changes in the association of connexins with other molecules. Novel methods for characterization of protein–protein interactions have become critical in understanding the regulation of gap junctions. Because of their relevance, some of these methods are reviewed below.

2. Identification and characterization of connexin-interacting proteins

A number of connexin-interacting proteins have been identified. A thorough characterization of the kinetic properties of those associations is a fundamental aspect of their analysis. Here we review one additional method for the screening of molecular partners and then we focus on a series of novel spectroscopic methods for characterizing the kinetics of interaction, the stoichiometry of association and potentially, the specific amino acids that are structurally involved in the association.

2.1. Antibody array: a screening method to search for potential molecular partners

Antibodies are often used to detect the presence of a protein within a complex. A limitation of conventional methods (such as co-immunoprecipitation) to study protein–protein interactions is that the identity of the potential partner needs to be known. Moreover, co-immunoprecipitation is a slow and tedious process that cannot be used for high-throughput screening of large numbers of potential binding partners. The recent development of antibody arrays has increased the speed at which previously unknown partners can be identified. The arrays consist of up to 400 different antibodies that are immobilized on a nitrocellulose membrane. A cell lysate is incubated with the array and then washed. Proteins in the cell lysate whose antibodies are in

Fig. 1. Diagram illustrating the concept of a ball and chain model for chemical regulation of connexins. An intracellular flexible domain (the carboxyl terminal domain) acts as a gating particle. Under normal conditions, the gate is away from the pore. Under the appropriate stimulus, the gate would swing toward the mouth of the channel, bind to a “receptor” affiliated with the pore, and close (or modify) the channel.
2.2. Kinetics, stoichiometry and amino acid identity in protein–protein interactions

2.2.1. Enzyme-Linked Sorbent Assay (ELSA)

A novel way of determining the concentration dependence of association between two proteins is by an assay that we refer to as Enzyme-Linked Sorbent Assay (ELSA). This colorimetric method (similar to the conventional “ELISA”) takes advantage of the property of many proteins to non-specifically bind to plastic dishes. The ligand of interest (e.g., the carboxyl terminal of Cx43; see Ref. [19]) is immobilized to the surface of Immulon plates (Thermo-Electron) and a potential ligate (i.e., a molecule capable of interacting with the ligand) is biotinylated. The ligate is presented to the ligand and the non-bound molecules are washed. The bound ligate is then detected using horseradish peroxidase-conjugated streptavidin and enzymatic conversion of ABTS (2,2’-azino-bis[3-sulfonic acid]) (Rockland Immunochemicals). Emission of color light can then be measured on a plate reader (Molecular Devices VMAX). This system was used to confirm the pH dependent binding of the carboxyl terminal of Cx43 to a peptide corresponding to the cytoplasmic loop [19]. Alternatively, the potential ligate is produced as a recombinant protein fused to the maltose binding protein (MBP) using the pMAL system (New England Biolabs). MBP acts both as a handle for purification and a target for analysis. After allowing the ligate (i.e., the MBP-fused protein) to bind to the ligand (e.g., the Cx43CT), the excess is washed away and the bound ligate tagged with HRP-conjugated anti-MBP. Detection is then based on colorimetry as described above. This ELSA assay is amenable to automation with the use of automated plate washers and plate readers thus making it potentially useful as a high throughput system for the study of protein–protein interactions.

2.2.2. Surface plasmon resonance

Surface plasmon resonance (SPR) is a spectroscopic method to determine binding amplitude and kinetics in real time. The ligand of interest (in our case the carboxyl terminal domain of Cx43) is covalently bound to a carboxymethyl dextran matrix. The matrix is attached to a thin film of gold (see diagram in Fig. 3). A beam of incident laser light is focused on the metal film, and a sensor collects the light reflected off the metal film. Various angles of incidence of light are scanned. There is a particular angle of incidence at which the photons in the light resonate with the electrons in the gold. This resonance creates a thin electromagnetic film that dissipates within a short distance from its source. The device detects the angle of incidence suited for resonance as a decrease in the amplitude of the reflected light. More importantly, the angle of incidence at which resonance occurs is highly sensitive to changes in the molecular content occurring in the immediate vicinity of the matrix [20]. Thus, binding of a ligate (added to the flow chamber) to the matrix-bound ligand is immediately sensed as a shift in the angle of incidence required for resonance. As a result, a plot of angle of incidence (in arc seconds) as a function of time directly represents the amplitude and kinetics of ligate–ligand binding in real time. This method represents a powerful approach to the study of protein–protein interactions (see, e.g., Ref. [21]). An alternative method, which we have used in the past, [19] is called Mirror Resonance Spectroscopy. We have previously validated this technique using antibodies specific (or not) to Cx43CT [22].
2.2.3. Mirror resonance spectroscopy

Mirror resonance spectroscopy takes advantage of the change in direction suffered by a light wave when moving between two media of different refractive indices. For waves traveling from high to low refractive index materials, there is a particular incident angle at which the wave is completely reflected (total internal reflection). However, the electromagnetic field penetrates into the low refractive index material. This “evanescence field” dies away exponentially with distance. If a region of high refractive index material \((n_2)\) is bounded on both sides (“cladded”) by low index material \((n_1)\), the light will be constrained within the high index material through successive total internal reflections. This “guiding” will occur only at a discrete angle \(\theta\), which is in turn extremely sensitive to the physical parameters of the system. The principle of this biosensor is that protein binding at or near the surface of the cladding material changes the value of \(\theta\). Under this principle, a ligand (Cx43CT in our case) is immobilized to the surface of a cuvette. A potential binding partner is added. If the two molecules do bind, this is reported as a change in the value of \(\theta\) (in arc-sec). This method allows for real time measurement of protein–protein interactions and a direct measurement of binding kinetics. From concentration-dependence curves, the dissociation constants can be calculated. Results obtained using this technique can be found in previous references [19,22].

2.2.4. Sedimentation equilibrium

Sedimentation equilibrium is a method for the direct measurement of molecular weight of a protein or a protein complex in its native state in solution. One of the most powerful advantages of sedimentation analysis is that molecules that are non-covalently bound are detected as a complex. The method allows for identification of the complex as well as characterization of the stoichiometry, the strength of the interaction between subunits, and the thermodynamic nonideality of the solution. Sedimentation equilibrium is capable of analyzing protein–protein interactions over a wide range of solute concentrations without perturbing the chemical equilibrium.

Sedimentation equilibrium analysis is performed using an analytical ultracentrifuge (ex. Beckman Optima XL-A) fitted with a high-intensity xenon flash lamp optical system to provide light. Typically, \(\sim 100 \, \mu\text{L}\) of purified protein \(A_{280} = 0.5\) and 125 \(\mu\text{L}\) of buffer are placed in a two sector cell. Readings are obtained by a photomultiplier tube, which detects light absorbance through the sample sector relative to the reading from the reference sector. The ultracentrifuge is run at a low rotor speed (ex. 18,000 rpm) so as not to pellet the protein at the bottom of the cell, but to allow for a gradient of increasing concentrations of the protein as the distance from the center of rotation increases. After an initial period of time, sedimentation and diffusion reach an equilibrium and no apparent movement of solute occurs throughout the cell. The equilibrium distribution depends on the buoyant weight of the molecules regardless of the shape of the protein. Typically, multiple scans are taken for each sample at 22 and 24 h to ensure equilibrium has been reached. Data is represented as radial distance as a function of protein concentration. Algorithms for curve-fitting are performed using the Beckman XL-A/ XL-I software package within Microcal, ORIGIN v4. Values for buffer density and protein partial specific volume are calculated using a Mettler DE40 density meter and the program Sednterp v1.03, respectively. Since the method does not require the denaturation of the proteins, molecular complexes are not disrupted and are detected because of the added molecular weights of the individual components. A number of studies have applied this technique to the detection of molecular complexes (see, e.g., Ref. [23]).

2.2.5. Translational diffusion analysis

An alternative approach to determine the molecular weight of a protein or a protein complex is by translational diffusion analysis using nuclear magnetic resonance (NMR). This technique characterizes the diffusion coefficient \((D_0)\) of a given molecule within a pulsed magnetic field gradient, based on the size of the molecular complex. A basic premise in this method is that smaller molecules will move further than larger molecules along the gradient. The faster the protein diffuses, the greater the defocusing and weaker the measured signal. The rationale is similar to that of proteins moving along an electric gradient in conventional SDS-PAGE. The key difference (for the purpose discussed in this chapter) is that proteins do not need to be denatured and so complexes remain intact, as in the native solution. Hence, if two molecules are non-covalently bound the diffusion rate of the molecule that is being monitored will be much slower than the one expected for the single unit. It is fundamental for the appropriate application of this method that the resonance peak that is followed corresponds to a single molecule of the complex, so that its diffusion properties can be monitored when in the presence of a potential associated molecule. This will allow for a direct comparison between control (only one protein) and experimental (protein–protein) spectra. Translational diffusion coefficients are measured by a bipolar pulse pair longitudinal-eddy-current delay experiment [24]. We have applied NMR translational diffusion analysis to the interaction between the carboxyl terminal domain of Cx43 and a peptide corresponding to the cytoplasmic loop [19]. Further details on those results are discussed in a separate section of this chapter.

2.2.6. \(^{15}\text{N}-\text{Heteronuclear Single Quantum Coherence (HSQC)}\)

An NMR application that is very effective at detecting protein–protein interactions is Heteronuclear Single Quantum Coherence (HSQC) of \(^{15}\text{N}\)-labelled proteins. The \(^{15}\text{N}\)-HSQC spectrum of a protein provides a fingerprint for each
amino acid except proline. (There are excellent textbooks that provide a general introduction to the concepts of NMR; see, e.g., Ref. [25]) Specifically, the spectra display the chemical shift of the amide protons as a function of the amide nitrogens. If the chemical environment around a residue is disturbed (because of the presence of a partner molecule), the fingerprint resonance signal for that particular residue would decrease in intensity and/or would be shifted in the $^{15}$N-HSQC spectra. We have applied this technique to demonstrate that Cx43CT is able to bind to two well-known binding partners of Cx43: the second PDZ domain of ZO-1 and the SH3 domain of c-Src (unpublished).

Application of the techniques detailed above have allowed us to further our understanding of the intra- and inter-molecular interactions that regulate Cx43. Below, we describe some of the results that have led us to better understand the structures involved in Cx43 regulation.

3. Intra-molecular interactions in the chemical regulation of Cx43

We used resonance mirror spectroscopy as well as translational diffusion analysis and ELSA to test whether Cx43CT binds in a pH-dependent manner to other intracellular regions of Cx43 (see Ref. [19] for a detailed description of these results). All three methods converged in demonstrating that Cx43CT binds in vitro, in a pH dependent manner, to a peptide corresponding to the second half of the cytoplasmic loop (Cx43L2). Fig. 4 shows the concentration-dependence of the binding as determined under resonance mirror spectroscopy. The consistency of these results is demonstrated by confirming the binding using the ELSA method (Fig. 5). Recombinant Cx43CT was used as a ligand. Clearly, the amplitude of the binding responses obtained at low pH was significantly higher than those recorded at a higher pH. Table 1 shows the results obtained from translational diffusion. Three specific non-overlapping resonances (A, B and C in Table 1) of Cx43L2 were followed both in the absence and in the presence of Cx43CT. The diffusion coefficient obtained from a Cx43CT resonance is shown for comparison (D). No pH dependent differences in mobility were detected for either Cx43CT or Cx43L2 alone. However, the Cx43L2 diffusion coefficient decreased in the presence of Cx43CT at low pH (0.59, 0.56, $0.51 \times 10^{-6}$ cm$^2$/s for resonances A, B and C, respectively) when compared to the data obtained at pH 7.4 (1.62, 1.45, 1.52 $\times 10^{-6}$ cm$^2$/s). Altogether, the results demonstrate selective, pH-dependent and concentration-dependent binding between the carboxyl terminal domain and the second half of the cytoplasmic loop of Cx43.

Our studies on CT–L2 interaction so far have been conducted in vitro, using recombinant protein fragments and synthetic peptides. Whether these interactions occur within the environment of a functional channel remains to be determined. Yet, preliminary data from our laboratory show that a peptide from the L2 region, delivered via the patch pipette, can modify channel function. This observa-

![Fig. 4. Binding of a peptide corresponding to amino acids 119–144 of Cx43 to the carboxyl terminal domain of Cx43. Concentration-dependence curves obtained by mirror resonance spectroscopy. The data show that the amplitude of the binding increased at low pH.](image)

![Fig. 5. Binding of peptide 119–144 of Cx43 (peptide “Cx43L2”) to the carboxyl terminal domain of Cx43. Data obtained by an Enzyme-Linked Sorbent Assay. Together with data obtained by translational diffusion analysis, these results show that there is a pH dependent association of the carboxyl terminal domain with a region of the cytoplasmic loop of Cx43.](image)
tion supports the notion that the L2 region binds to a separate region of the connexin molecule and by this interaction can modify the activity of the gap junction pore. We propose that the L2 region is the receptor that binds to the gating particle during channel regulation.

4. Structure of the Cx43 pore and pH gating

The data described above strongly suggest that pH gating results from an interaction between the carboxyl terminal domain, acting as a gating particle, and the second half of the cytoplasmic loop, acting as a receptor for such particle (see Fig. 1). The question remains as to whether this interaction interrupts cell–cell communication by inducing a transformation of the pore structure, or by placing a “plug” on an otherwise unaltered channel pore. A wooden model of Cx43 introduced by Unwin and Zampighi [26] and later applied the regulation of a gap junction channel by calcium [27] suggested that the channel opens and closes by a coordinated tilting and sliding motion of the protein subunits along their lines of contact. This observation, however, was not confirmed by the X-ray diffraction analysis of Makowski [28]. This author proposed instead a model by which channel regulation would be mediated by intracellular, flexible domains present at the mouth of the channel that would interfere with the channel pore. Similarly, based on their X-ray diffraction analysis, Tibbitts et al. [29] concluded that “...it appears unlikely that gating involves twisting and tilting of the transmembrane part of the connexin molecules as previously inferred from... electron microscopy data” [26,27]. Instead, it was the authors’ contention that “Some portions of the connexon near the cytoplasmic membrane surface and facing the transmembrane channel may be more mobile, in particular, the part forming the gating structure identified at the mouth of the channel” [28]. These results would tend to support the notion of chemical gating as mediated by channel block, such as it may occur in a ball-and-chain type of interaction. The high resolution structure of Unger et al. [30] presents only one configuration. Whether an alternative structure is present, corresponding to a channel in a different conductive state, remains to be determined. Our results do suggest that a starting point for better understanding the structural bases for the chemical regulation of gap junctions may be the characterization of the two structures that seem to be involved: the cytoplasmic loop and the carboxyl terminal domain.

4.1. The structure of the cytoplasmic loop

Our results led us to characterize the secondary structure of the Cx43L2 peptide (amino acids 119–144 in the Cx43 sequence) by means of NMR. A key finding was the fact that acidification of the solvent had a dramatic effect on the structural organization of this region. At pH of 7.5 the peptide was mostly a random coil, whereas at pH of 5.8, structural order was observed [19]. The solution to the structure is presented in Fig. 6. The amino acid sequence of the Cx43L2 region is presented on the top. The sequence of the alpha-helical regions is indicated in red. The position of the imidazole rings in H126 and H142 are noted in the structure. These amino acids are protonated upon acidification, likely enhancing the ability of the molecule to acquire its alpha-helical structure.

The structure shown was obtained from an isolated peptide in solution. Whether this is the structure of the same fragment when integrated in the whole protein remains

| Table 1 |
| Translation diffusion coefficient ($D_s$) |
| Cx43L2 | Cx43 | Cx43L2/Cx43 |
| pH 5.8 | pH 8.0 | pH 5.8 | pH 8.0 | pH 5.8 | pH 8.0 |
| A | 1.45<sup>a</sup> | 1.24 | 0.37 | 0.40 | A | 0.59 | 1.62 |
| B | 1.37 | 1.40 | B | 0.56 | 1.45 |
| C | 1.48 | 1.35 | C | 0.51 | 1.52 |

<sup>a</sup> ($D_s$) $\times 10^{-6}$ cm$^2$/s.

Fig. 6. Secondary structure of a peptide corresponding to amino acids 119–144 of Cx43, as solved by nuclear magnetic resonance. We propose that this structure acts as a receptor for the gating particle during chemical regulation of Cx43 channels.

![Diagram illustrating the principle of surface plasmon resonance.](Fig. 3)
to be determined. The solved structure of the gap junction channel mostly reveals the transmembrane domains [30]. Yet, the images do show an alpha-helical cytoplasmic region that projects as a continuation of the “C” transmembrane domain. Some have speculated that the “C” helix in the structure of Unger et al. [30] corresponds to the region of the primary sequence identified as the third transmembrane domain [31]. If that is indeed the case, then the cytoplasmic extension of the C helix may correspond to the “L2” region as characterized by NMR.

4.2. The carboxyl terminal domain

We have applied NMR to the study of the carboxyl terminal domain [19,32]. Resonance assignments were recently published and deposited in the protein data bank [32]. Recombinant Cx43CT was produced using a bacterial system, purified on a glutathione column and then concentrated by filtration. The structural analysis suggests two regions of high order, likely alpha-helical, flanked by areas of random coil. It is interesting to note that changing the pH of the solvent from 8.0 to 5.8 did not cause a substantial modification on the predicted structure. Moreover, unpublished data from our laboratories suggest that the CT domain dimerizes, leading to the interesting possibility that the hexameric connexon may in fact be a “trimer of dimers.” This would be consistent with other transmembrane protein complexes that organize as “dimers of dimers” [33–35]. Our early indications suggest that one of the areas of dimerization may include the PDZ binding domain, used to bind to the second PDZ domain of ZO-1.

5. Inter-molecular interactions

Since the early reports on the primary sequence of Cx43, it has been thought that the carboxyl terminal region might be involved in the regulation of the channel, given the number of putative consensus sites for phosphorylation. Moreover, truncation of the CT domain prevented the closure of the channel upon intracellular acidification [7]. More recently it has been shown that Cx43 binds (directly or indirectly) to a number of intracellular proteins including ZO-1, [3] src, [36] caveolin, [37] β-catenin, [38] p120-catenin [39] and tubulin [2]. At least in the case of tubulin and ZO1, the binding domain of Cx43 is thought to be contained within the carboxyl terminal region. Techniques such as NMR, SPR and ELSA will be fundamental in characterizing the nature of these interactions and the structural modifications that may come about as a result.

6. Conclusions and potential future directions

The image of a gap junction as an inert, passive pore for the passage of electrical charge is slowly being replaced for that of a highly regulatable filter that controls the traffic of molecular information between cells. The properties of the filter are likely to be modified according to complex intra- and inter-molecular interactions that occur in response to changes in the microenvironment. As such, a gap junction is better described as a multi-molecular complex of constantly changing composition. All of these interactions, so far, focus on the connexin molecule as a substrate, a recipient of regulatory messages, catalytic or not. Still to be determined is whether by associating to a molecule, the function ascribed to such a molecule changes as well. Though no catalytic activity has ever been assigned to connexins, it is hard to imagine a functional structure that only receives and responds, without also giving and commanding. The field of gap junction regulation may one day be extended to understand not only how gap junctions are regulated by other molecules, but how other molecules change their function consequent to their interaction with connexins.

References


