INTRODUCTION

Connexins are the proteins that form the gap-junctional channels that mediate cell-to-cell permeation of ions and hydrophilic molecules of M_r < 1,000 (Harris, 2001). There are 20 human connexin isoforms, varying in length from 226 to 543 amino acids, which differ in pore permeability and regulation (Harris, 2001; Hua *et al*. 2003; Willecke *et al.*, 2002). As schematically shown in Fig. 1, each connexin contains four transmembrane α helices (M1-M4), cytoplasmically-located N- and C-terminal ends, an intracellular loop (IL) and two extracellular loops (EC1 and EC2).

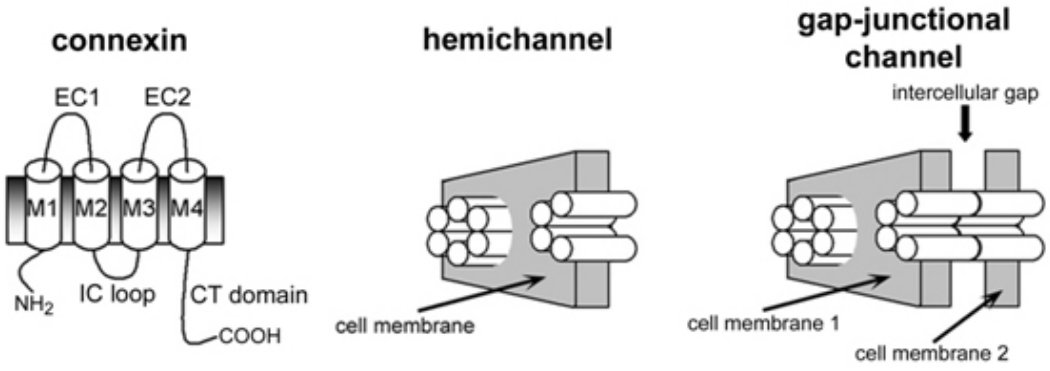

Figure 1: Connexin 43 (Cx43), hemichannels (connexin hexamers or connexons) and gap-junctional channels.

M1-M4 represent the four transmembrane α helices. The intracellular sequences include the N-terminal region, intracellular loop (IC loop) and the C-terminal domain (CT domain) that are involved in channel gating and regulation. The two extracellular loops (EC1 and EC2 loops) are essential for docking with connexons of the neighboring cell. From Bao *et al*., 2005.

Sequence analysis shows that the cytoplasmic hydrophilic regions are poorly conserved, whereas the transmembrane domains and extracellular loops are well conserved, especially the M1-EC1-M2 sequence (Hua *et al.*, 2003). Conserved Cys residues in extracellular loops form intramolecular disulfide bonds and their presence is critical for "docking" between hemichannels from adjacent cells (Foote *et al.*, 1998). Most cells that have gap junctions express more than one connexin isoform; as a result, a variety of gap-junctional channel and hemichannel arrangements can exist (Harris, 2001), as shown in Fig. 2. Hemichannels formed by one or more connexin isoforms are called homomeric and heteromeric, respectively (Harris, 2001). Gap-junctional channels formed by hemichannels of the same connexin composition are named homotypic, whereas those formed by docking of hemichannels of different composition are called heterotypic (Harris, 2001). Gap-junctional channels and hemichannels formed by different connexins display differences in permeabilities to hydrophilic solutes (Harris, 2007). Gap-junctional channels and hemichannels are poorly selective for small inorganic ions, but show selective permeability to larger molecules such as second messengers (e.g., cGMP, cAMP, ATP and IP_3) (Heyman *et al.*, 2008 and Wei *et al.*, 2004). For example, Cx26 gap-junctional channels are more permeable to cAMP than those formed by Cx32, whereas the latter are more permeable to IP3 than those formed by Cx26 (Harris, 2007). Although there is significant evidence for interaction of "large" hydrophilic substrates with the pore-lining amino-acid side chains (Weber *et al.*, 2004), the origins of the differences in selectivity between gap-junctional channels (or hemichannels) formed by different connexin isoforms is not understood (Harris, 2007).

Connexin43 (Cx43) encodes a peptide containing 382 amino acids (Yeager *et al.*, 1992; Francis *et al.*, 1999; Hua *et al.*, 2003). It is expressed in parenchymal cells of a variety of organs, such as cardiac muscle, brain, liver, kidney and myometrium, as well

as in capillary endothelial cells (Beyer *et al.*, 1987; Berthoud *et al.*, 1992; Pepper *et al.*; 1992; Sainio *et al.*, 1992; Nnamani *et al.*, 1994; Little *et al.*, 1995; Neveu *et al.*, 1995;

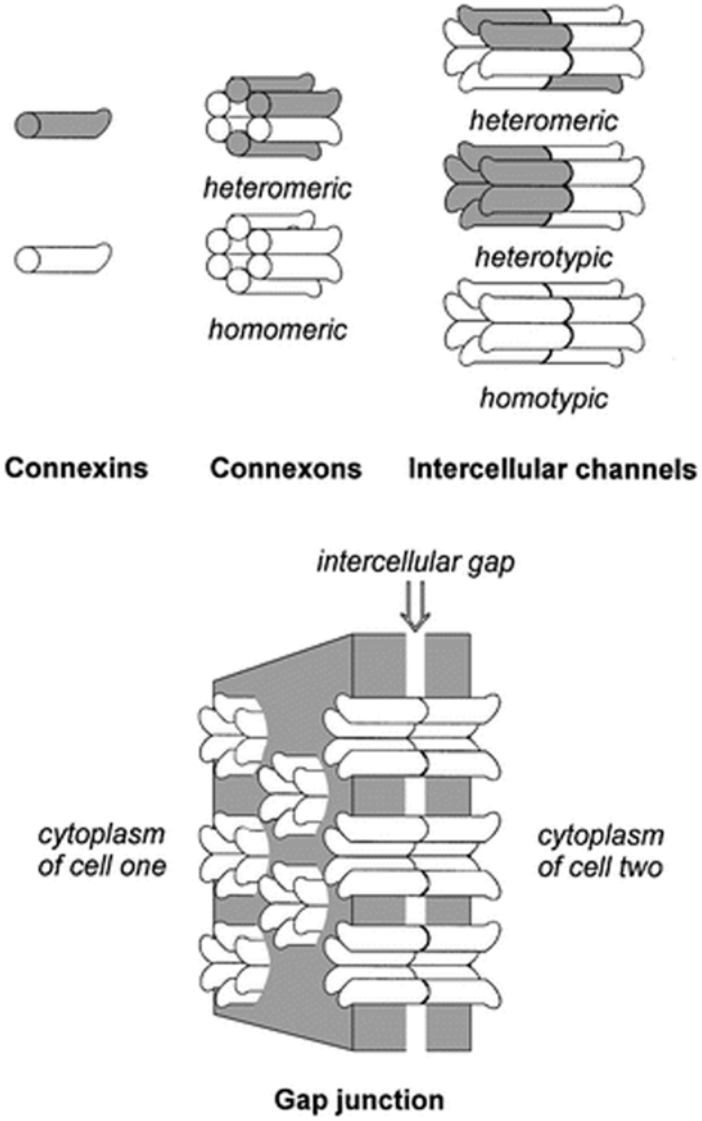

Figure 2: Nomenclature of gap-junctional channels and hemichannels formed by subunits of the same or different isoforms. Not sure about top of the figure

Venance *et al.*, 1998). Its role in the heart has been most extensively studied. Connexins are abundantly expressed in the excitation-conduction system and in the contractile myocardium, and Cx43 GJC conduct the impulse in the cardiomyocytes (Kondo et al., 2000). Cx43 is also essential for the normal development of the heart since Cx43 deletion in mice is lethal and involves cardiac malformations (Willecke *et al.*, 1999). Cx43 mutations in humans and studies in Cx43 knockout mice showed prenatal death due to cardiac malformations as well as craniofacial abnormalities, lens defects, non-syndromic deafness, hypotension and other abnormalities (Reaume *et al.*, 1995; Harris, 2001; Wei *et al.*, 2004). Interestingly, the local use of anti-sense oligonucleotides to reduce the expression of Cx43 accelerated and improved the recovery of wounds of skin or cornea (Chee et al., 2007).

Gap-junctional channels and hemichannels are regulated by a variety of factors, including intracellular divalent cations, phosphorylation, membrane voltage and pH (Harris, 2001). Regulation can also occur at the levels of connexin trafficking, folding and assembly (Segretain *et al.*, 2004). At transjunctional voltages close to 0 mV, gap-junctional channels and hemichannels are mostly open, and close at depolarizing and hyperpolarizing voltages. Intracellular acidification and increases in intracellular [Ca^{2+}] (probably through direct calmodulin-Ca^{2+} binding to connexins) close gap-junctional channels and hemichannels formed by a number of connexin isoforms, including Cx43 (Lurtz *et al.*, 2007; Zhou *et al.*, 2007; our unpublished work also). Phosphorylation blocks gap-junctional channels and hemichannels formed by several connexin isoforms, including Cx43, and extracellular divalent cations at millimolar concentrations block hemichannels formed by a number of connexin isoforms (Li *et al.*, 1996; Lampe *et al.*, 2000a; Lampe *et al.*, 2000b; Harris, 2001; Bao *et al.*, 2004a; Bao *et al.*, 2004b; Bao *et al.*, 2005; Chen *et al.*, 2005). The consequences of this regulation are that under physiological

conditions most gap-junctional channels between normal, isopotential cells are open (transjunctional voltage ~ 0 mV, low cytoplasmic [Ca^{2+}] and inaccessibility of extracellular divalent cations), whereas most hemichannels are closed (cell-membrane voltages of -60 to -90 mV, high extracellular [Ca^{2+}] and phosphorylation by PKC). During ischemic conditions, when intracellular pH decreases and intracellular [Ca^{2+}] increases, cells depolarize and phosphorylation is decreased (due to ATP depletion), gap-junctional channels between normal and ischemic cells close, whereas hemichannels open. The former seem to close because of the increases in intracellular [H^{+}] and [Ca^{2+}], whereas the latter opens because of dephosphorylation and probably S-nitrosylation (Perachia, 1990; Bevans and Harris, 1999; Bao *et al.*, 2004a; Bao *et al.*, 2004b; Bao *et al.*, 2004c; Bao *et al.*, 2005; Retamal *et al.*, 2007). Decrease in gap-junctional channel communication protects normal from damaged cells (impedes fluxes of ions and metabolites between the cells), but opening of hemichannels contributes to damage of ischemic cells (loss of metabolites, gain of Ca^{2+}). The opening of Cx43 hemichannels has been implicated in the cell injury and death in myocardial infarction, stroke and renal-tubule necrosis during ischemia (John *et al.*, 1999; Li *et al.*, 2000; Contreras *et al.*, 2002; Vergara *et al.*, 2003).

Heart disease is the most common cause of death in the USA, and many of these deaths are caused by cardiac ischemia, with arrhythmias also playing an important role. Cardiac connexins and calcium have important roles in both the damage of the heart muscle elicited by ischemia and the genesis and maintenance of arrhythmias (John *et al.*, 1999; Escobar, 2008). Block of Cx43 gap-junctional channels creates an arrythmogenic mechanism by decreasing the epicardial space constant. This generates areas of anomalous subcellular Ca^{2+} dynamics with the surrounding cells unable to maintain isopotentiality with these areas. The local abnormal Ca^{2+} release can induce extrasystoles

and alternances in the repolarization of action potentials (Pruvot *et al.*, 2004), which can eventually lead to cardiac arrest. Transplantation of embryonic cardiomyocytes in myocardial infarcts in mice protected against ventricular tachycardia, only if the transplanted cells expressed Cx43 (Roell *et al.*, 2007).

Cryoelectron microscopy and image analysis of two-dimensional crystals of a recombinant Cx43 with truncation of the C-terminal domain produced a three-dimensional density map at 7.5 Å in the membrane plane and 21 Å in the vertical direction contradiction with Fig. 3 (Unger *et al.*, 1999). It showed that the hemichannels are hexamers and that the transmembrane pore of each hemichannel is formed by 24 closely packed α helices. A 7.5 Å resolution crystal structure obtained by cryoelectron microscopy has established that connexins have four transmembrane helices (Unger *et al.*, 1999 and Fig. 3). The molecular organization of gap-junctional channels formed by Cx43 truncated at residue 263 shows that the diameter of the hydrophilic cavity in gap-junctional channels narrowed from ~40 Å (cytoplasmic side of pore) to ~15 Å (extracellular side of pore) excluding the amino acid side chains. The narrowest constriction of pore is ~ 5 Å in diameter if the amino acid side chains are considered (Fig. 3 and Unger *et al.*, 1999).

Since the original structure of Cx43 gap-junctional channel was determined by electron crystallography, image analysis improved the resolution to 5.7Å in plane and 19.8Å vertical (Fleishman *et. al.*, 2004). This resolution, in combination with the lack of structural information on the intracellular regions, was insufficient for helix assignment, and the four helices have been named A, B, C and D (Fig. 3).

In summary, two transmembrane helices per subunit line the pore: helices B and C (Unger *et al.*, 1999 and Fleishman *et al.*, 2004). The narrowest region of the

hemichannel pore appears to be located near the extracellular surface of the membrane (Unger *et al.*, 1999 and Fleishman *et al.*, 2004).

A number of attempts have been made to assign the helices based on phylogenetic analysis, mutagenesis and engineering of chimeras as well as the use of the substituted cysteine accessibility method (SCAM), but these efforts have not produced a reliable hemichannel model based on experimental measurements. Details on these competing models and their problems are presented later in specific chapters of this dissertation.

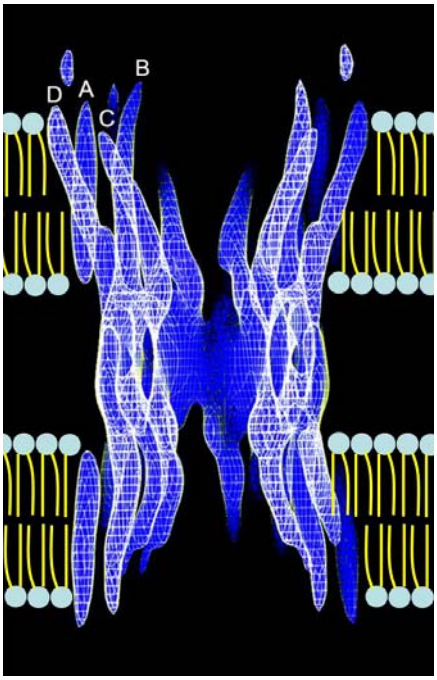

Figure 3: Model of gap-junctional channels based on the electron density map from Unger *et al.*, 1999.

The recombinant Cx43 gap-junctional channel has 48 transmembrane helices embedded in the lipid bilayers of adjacent cells. This structure shows four transmembrane helices per Cx43 subunit (arbitrarily named A, B, C and D), two of which line the pore: helices B and C. The density has been cropped to show the water-filled cavity. Modified from Unger *et al.*, 1999.

The main goal of my studies was to generate a reliable hemichannel model that can be used as a basis for analyzing diseases caused by connexin mutations and predict permeability properties of different connexin isoforms, as well as to have a model framework that can be tested experimentally.

Based on site-directed mutagenesis studies, the extracellular loops have been suggested to be β sheets (Foote *et al.*, 1998), but the structure may be complex, perhaps a combination of β sheets with α helices that extend from the membrane-pore helices (Kronengold *et al.*, 2003; Manthey *et al.*, 2001). Structural studies using atomic-force microscopy imaging confirmed the hexameric structure of the hemichannels and revealed a Ca^{2+}-dependent conformational change (Thimm *et al.*, 2005; Muller *et al.*, 2002).

In this Dissertation, I address primarily the assignment of the Cx43 hemichannel transmembrane helices, with emphasis on the identification of the pore-lining helices. Secondarily, I show how parts of the Cx43 hemichannel move during gating by phosphorylation. In order to accomplish these goals, I contributed to the development of certain methodologies, including the purification and reconstitution of functional hemichannels, the characterization of a cysteine-less version of Cx43, the generation of hemichannels of controlled subunit composition (e.g., 3 phosphorylated and 3 dephosphorylated subunits *per* hemichannels), improvements of SCAM, and the development of luminescence resonance energy transfer (LRET) methodology for the assessment of subunit composition and measurements of distances between specific selected connexin residues. In Chapter 1, I tested the notion that phylogenetic sequence analysis can be used to identify connexin pore-lining helices using poly-alanine helix scanning mutagenesis; in Chapter 2, I tested a modified SCAM approach to attempt the experimental identification of pore-lining helices; in Chapter 3, I present the development and characterization of a method to generate hemichannels of controlled subunit

composition and the bases for the use of LRET in hemichannels; in Chapter 4, I present LRET data that serve as bases for helix assignment and a new model for Cx43 folding in hemichannels; and in Chapter 5, I present data on the molecular mechanism of Cx43 hemichannel gating by PKC-mediated phosphorylation.

My long-term goal is to understand the molecular bases for the permeability properties of hemichannel pores, which will serve as an important step towards our understanding of the function of gap-junctional channels and hemichannels. In my dissertation, I focus on the first step of identifying the helices and residues that line the transmembrane region of the pore. My specific aims are to:

1) Identify the residues that line the Cx43 hemichannel pore based on the residue's side-chain environment. To determine which side chains face the water-filled pore the reactivity of introduced single Cys residues to hydrophilic thiol reagents was measured. Accessibility of the thiol reagents was evaluated by a combination of permeability studies in *Xenopus laevis* oocytes and purified hemichannels using the SCAM.

2) Develop and test a method based on LRET for structural studies of hemichannels. In order to identify the pore-lining helices (Aim 3), it was necessary to develop a purified hemichannel preparation where the subunit composition can be controlled and determined, and energy transfer studies can be performed.

3) Assign the Cx43 hemichannel pore-lining helices of the cryoelectron-microscopy structure from inter-helical distances. Analysis of the low-resolution Cx43 structure available shows that the distances between selected homologous residues (same residues) in diametrically-opposed subunits of the transmembrane helices are sufficiently different to allow helix assignment. Distances between homologous residues in diametrically-opposed subunits of transmembrane helices were determined using LRET

in purified and reconstituted hemichannels formed by Cx43 mutants containing a single-Cys residue at selected positions. LRET is a technique that allows for calculation of distances between selected residues labeled with donor and acceptor luminescence probes with sub-Angstrom resolution. These measurements should allow for assignment of the Cx43 helices in the hemichannel.