CHAPTER 2: IDENTIFICATION OF THE PORE-LINING RESIDUES OF CX43 HEMICHANNELS BASED ON THEIR ACCESSIBILITY TO HYDROPHILIC THIOL REAGENTS

INTRODUCTION

The 3D electron density map calculated from cryoelectron microscopy data revealed that the hemichannels have 24 rod-like densities of length and diameter compatible with the expected transmembrane α-helices. Unfortunately, the helices could not be assigned because of the low resolution and lack of structural detail of the extra- and intracellular regions (Kovacs *et al*., 2007). Although attempts to obtain higher resolution structures from 2D and 3D crystals have been going on for years in our and other laboratories, they have not been successful yet. This is a common problem for the study of membrane proteins due in part to their amphipathic nature, which makes it difficult to obtain well-ordered crystals (Rosenbaum *et al*., 2007). Recently, there has been some progress on the structure of purified Cx26 hemichannels using cryoelectron microscopy, but the resolution is still well below that required to assign individual helices (Muller *et al.*, 2002; Yu *et al.*, 2007; Oshima *et al.*, 2007). In the absence of detailed structural information, I resorted to biochemical methods that have been successfully used in the past to identify residues with side chains exposed to a hydrophilic environment. In this chapter, I present data using a modified version of SCAM. In the traditional use of SCAM to study ion channels, single native residues at selected positions are substituted with Cys, to take advantage of the highly-specific chemistry for reaction of reduced sulfhydryls (He *et al.*, 1996). Ideally single-Cys are introduced in a functional Cys-less protein, and the effects of small thiol reagents of different properties (charge, hydrophilicity) are determined electrophysiologically, assessing the changes in whole-

cell or better single-channel properties (conductance, ionic selectivity). SCAM in all its variations has been very useful to identify residues accessible from aqueous transmembrane transport pathways (see Karlin, 2001). However, when applied to connexins, it has not yielded clear results. Gap-junctional channels are two hemichannels in series, which complicates the interpretation of the results (Skerrett *et al*., 2002), and the mutant connexins used retained the native Cys residues, which can affect the results (Zhou *et al*., 1997; Frillingos *et al*., 1998; Pfahnl and Dahl, 1998; Skerrett *et al*, 2002; Kronengold *et al*., 2003). In addition, gap-junctional channels and hemichannels have a large pore whose diameter may not be decreased enough by thiol reagents, resulting in small effects on the currents carried by small inorganic ions (Zhou *et al*., 1997; Pfahnl and Dahl, 1998; Hille, 2001; Skerrett *et al*., 2002; Kronengold *et al*., 2003).

It is for the reasons above that SCAM with some simple modifications was used. First, the focus was on hemichannels, which are simpler to study than gap-junctional channels. Second, a functional Cys-less version of Cx43 was used. This forms functional hemichannels blocked by extracellular [Ca^{2+}] and phosphorylation of Ser368, just as wild-type Cx43 hemichannels. This avoids potential problems related to reaction to native Cys residues. Third, the relatively large thiol reagent maleimidobutylryl biocytin (MBB) was employed. This is a hydrophilic reagent (M_r 537) that has been employed before to study membrane proteins, including connexins (Zhou *et al*., 1997; Skerrett *et al*., 2002; Bao *et al*., 2004c). Since Cx43 hemichannels allow passage of hydrophilic solutes of molecular weight of up to 1,000 Da, MBB can get into the open pore, react with the introduced Cys residue, and as a result reduce the effective pore diameter and solute permeability. Forth, relatively large hydrophilic solutes were used as permeability probes. Reaction of thiol reagents with Cys introduced in helices that line the pore is expected to reduce the effective pore diameter, but small ions can still permeate through

the smaller-size pores. As a consequence, the changes in currents elicited by the Cys modification are small, and changes as small as ~5% have been considered positive (i.e., indicative that the thiol reagent reacted with a pore-lining residues). Using larger hydrophilic compounds as permeability probes, we obtained large effects, approaching or reaching abolishment of permeability. Therefore, the effects are easily measured and more reliable. Fifth, the combined use of thiol reagents of different sizes and properties to confirm that the introduced Cys are indeed facing the pore. The sole use of MBB as a thiol reagent is insufficient because its hydrophobic moiety can penetrate into the lipid bilayer and label residues that do not line the pore (see Skerret *et al*., 2002). Pre-treatment with a small hydrophilic thiol reagent such as iodoacetic acid (IAA) will prevent MBB labeling only if the reaction occurred with a water-accessible residue. The idea here is that if MBB reacts with a Cys in a hydrophobic environment, instead of in the pore, IAA will not have access to it and will fail to prevent the reaction of MBB with that Cys.

In this chapter, I present SCAM data on transmembrane helices 1 through 3 (M1-M3) of Cx43 hemichannels.

MATERIALS AND METHODS

Generation of single-Cys mutants for expression in *Xenopus laevis* oocytes

To generate the single cysteine mutants of M1, the Cx43 construct used to generate the poly-Ala mutants described in Chapter 1 was employed. This construct (Cx43NRS) has unique restriction sites flanking each transmembrane segment. First, the 6 Cys in the extracellular loops were replaced with Ala, using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), using the following mutagenic

primers: 5'-GCCTTTCGCGCTAACACTCAACAACCTGGCGCCGAAAACGTCGCC TATGACAAGTCC-3' (to remove the 3 Cys of the first extracellular loop); 5'-GTCTAC ACCGCCAAGAGAGATCCC*GC*CCCTCACCAGGTAGACGCCTTCCTCTCACG-3' (to remove the 3 Cys of the second extracellular loop). For replacement of the C-terminal Cys with Ala, the *PvuII*/*XcmI* fragment was replaced with the equivalent Cys-less fragment of pOcyt-CL Cx43D2, a Cys-less Cx43 variant without the unique restriction sites flanking the transmembrane segments (Bao *et al*., 2004c). Expression of this new Cys-less Cx43 mutant yielded functional hemichannels, undistinguishable from those formed by wild-type Cx43 or the original Cys-less Cx43 (see **RESULTS**). To generate single-Cys M1 mutants, the DNA fragment containing the M1 sequence was excised with *SacI*/*BamHI* and replaced with sets of oligonucleotide adaptors ready for ligation to the cut sites (*SacI*/*BamHI*). Each set of adaptors contained a single Cys replacing one of the native residues of M1. The 21 sets of adaptors were: 5'-GTGCGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTCAGAATCCTGCTCCTGG GGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3' (G21C sense); 5'-GATC CTTCA TCACCCCAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGAA TATGAAGAGCACTGACAGCCACACCTTCCCGCACGC-3' (G21C antisense); 5'–GGGATGCAAGGTGTGGCTGTCAGTGCTCTTCATATTCAGAATCCTGCTCCT GGGGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3' (G22C sense); 5'-GA TCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTG AATATGAAGAGCACTGACAGCCACACCTTGCATCCCGC-3' (G22C antisense); 5'-GGGAGGGTGCGTGTGGCTGTCAGTGCTCTTCATATTCAGAATCCTGCTCCT GGGGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3' (K23C sense); 5'-GA TCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGA ATATGAAGAGCACTGACAGCCACACGCACCCTCCCGC-3' (K23C antisense);

5’-GGGAGGGAAGTGCTGGCTGTCAGTGCTCTTCATATTCAGAATCCTGCTCCT GGGGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (V24C sense); 5’-GA TCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGA ATATGAAGAGCACTGACAGCCAGCACTTCCCTCCCGC-3’ (V24C antisense); 5’- GGGAGGGAAGGTGTGCCTGTCAGTGCTCTTCATATTCAGAATCCTGCTCC TGGGGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (W25C sense); 5’-GA TCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGA ATATGAAGAGCACTGACAGGCACACCTTCCCTCCCGC-3’ (W25C antisense); 5’- GGGAGGGAAGGTGTGGTGCTCAGTGCTCTTCATATTCAGAATCCTGCTCCTGG GGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (L26C sense); 5’-GATCCTT CATCACCCCAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGAATAT GAAGAGCACTGAGCACCACACCTTCCCTCCCGC-3’ (L26C antisense); 5’-GGGA GGGAAGGTGTGGCTGTGCGTGCTCTTCATATTCAGAATCCTGCTCCTGGGGAC AGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (S27C sense); 5’-GATCCTTCATC ACCCCAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGAATATGAAG AGCACGCACAGCCACACCTTCCCTCCCGC-3’ (S27C antisense); 5’-GGGAGGGA AGGTGTGGCTGTCATGCCTCTTCATATTCAGAATCCTGCTCCTGGGGACAGCT GTTGAGTCAGCTTGGGGTGATGAAG-3’ (V28C sense); 5’-GATCCTTCATCACCC CAAGCTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGAATATGAAGAGGC ATGACAGCCACACCTTCCCTCCCGC-3’ (V28C antisense); 5’-GGGAGGGAAGGT GTGGCTGTCAGTGTGCTTCATATTCaGAATCCTGCTCCTGGGGACAGCTGTTG AGTCAGCTTGGGGTGATGAAG-3’ (L29C sense); 5’-GATCCTTCATCACCCCAAG CTGACTCAACAGCTGTCCCCAGGAGCAGGATTCTGAATATGAAGCACACTGA CAGCCACACCTTCCCTCCCGC-3’ (L29C antisense); 5’-GGGAGGGAAGGTGTGG CTGTCAGTGCTCTGCATATTCaGAATCCTGCTCCTGGGGACAGCTGTTGAGTC

AGCTTGGGGTGATGAAG-3’ (F30C sense); 5’-GATCCTTCATCACCCCAAGCTGA CTCAACAGCTGTCCCCAGGAGCAGGATTCTGAATATGCAGAGCACTGACAGC CACACCTTCCCTCCCGC-3’ (F30C antisense); 5’-GGGAGGGAAGGTGTGGCTGT CAGTGCTCTTCTGCTTCAGAATCCTGCTCCTGGGGACAGCTGTTGAGTCAGCT TGGGGTGATGAAG-3’ (I31C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCA ACAGCTGTCCCCAGGAGCAGGATTCTGAAGCAGAAGAGCACTGACAGCCAC ACCTTCCCTCCCGC-3’ (I31C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGT GCTCTTCATATGCAGAATCCTGCTCCTGGGGACAGCTGTTGAGTCAGCTTGGG GTGATGAAG-3’ (F32C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAG CTGTCCCCAGGAGCAGGATTCTGCATATGAAGAGCACTGACAGCCACACCTT CCCTCCCGC-3’ (F32C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTC TTCATATTCTGCATCCTGCTCCTGGGGACAGCTGTTGAGTCAGCTTGGGGTGA TGAAG-3’ (R33C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAGCTGT CCCCAGGAGCAGGATGCAGAATATGAAGAGCACTGACAGCCACACCTTCCCT CCCGC-3’ (R33C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCA TATTCAGATGCCTGCTCCTGGGGACAGCTGTTGAGTCAGCTTGGGGTGATGA AG-3’ (I34C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCC AGGAGCAGGCATCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCG C-3’ (I34C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTC AGAATCTGCCTCCTGGGGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (L35C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCCAGG AGGCAGATTCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCGC-3’ (L35C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTCAG AATCCTGTGCCTGGGGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (L36C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCCAGG

CACAGGATTCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCGC-3’ (L36C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTCAG AATCCTGCTCTGCGGGACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (L37C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAGCTGTCCCGCAG AGCAGGATTCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCGC-3’ (L37C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTCAG AATCCTGCTCCTGTGCACAGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (G38C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAGCTGTGCACAGG AGCAGGATTCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCGC-3’ (G38C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTCAG AATCCTGCTCCTGGGGTGCGCTGTTGAGTCAGCTTGGGGTGATGAAG-3’ (T39C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACAGCGCACCCCAGG AGCAGGATTCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCGC-3’ (T39C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTCAG AATCCTGCTCCTGGGGACATGCGTTGAGTCAGCTTGGGGTGATGAAG-3’ (A40C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCAACGCATGTCCCCAGG AGCAGGATTCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCGC-3’ (A40C antisense); 5’-GGGAGGGAAGGTGTGGCTGTCAGTGCTCTTCATATTCAG AATCCTGCTCCTGGGGACAGCTTGCGAGTCAGCTTGGGGTGATGAAG-3’ (V41C sense); 5’-GATCCTTCATCACCCCAAGCTGACTCGCAAGCTGTCCCCAGG AGCAGGATTCTGAATATGAAGAGCACTGACAGCCACACCTTCCCTCCCGC-3’ (V41C antisense).

Single-Cys M2 mutants in which all 20 native residues were substituted with Ala, were obtained by site-directed mutagenesis using the template plasmid described before, in this section. The single-Cys M3 mutants were engineered using oligonucleotide

adaptors, as described above for the M1 mutants. The *NcoI*/*SpeI* fragment containing the M3 sequence was replaced with the following 15 adaptor sets: 5'-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTGCATCATCAGCATCCTCTTCAAGTCTGTCTTCGAGGTGGCCTTCCTA-3' (Y155C sense); 5'-CTAGTAGGGAGGCCACCTCGAAGACAGACTTGAAGAGGATGCTGATGATGCAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3' (Y155C antisense); 5'-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACTGCATCAGCATCCTCTTCAAGTCTGTCTTCGAGGTGGCCTTCCTA-3' (I156C sense); 5'-CTAGTAGGAAGGCCACCTCGAAGACAGACTTGAAGAGGATGCTGATGCAGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3' (I156C antisense); 5'-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACATCTGCAGCATCCTCTTCAAGTCTGTCTTCGAGGTGGCCTTCCTA-3' (I157C sense); 5'-CTAGTAGGAAGGCCACCTCGAAGACAGACTTGAAGAGGATGCTGCAGATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3' (I157C antisense); 5'-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACATCATCTGCATCCTCTTCAAGTCTGTCTTCGAGGTGGCCTTCCTA-3' (S158C sense); 5'-CTAGTAGGAAGGCCACCTCGAAGACAGACTTGAAGAGGATGCAGATGATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3' (S158C antisense); 5'-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACATCATCAGCTGCCTCTTCAAGTCTGTCTTCGAGGTGGCCTTCCTA-3' (I159C sense); 5'-CTAGTAGGAAGGCCACCTCGAAGACAGACTTGAA GAGGCAGCTGATGATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3' (I159C antisense); 5'-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACATCATCAGCATCTGCTTCAAGTCTGTCTTCGAGGTGGCCTTCCTA-3' (L160C sense); 5'-CTAGTAGGAAGGCCACCTCGAAGACAGACTTGAAGCAGATGCTGATGATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3'

(L160C antisense); 5’-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAAC CTACATCATCAGCATCCTCTGCAAGTCTGTCTTCGAGGTGGCCTTCCTA-3’ (F161C sense); 5’-CTAGTAGGAAGGCCACCTCGAAGACAGACTTGCAGAGGATG CTGATGATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3’ (F161C antisense); 5’-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACA TCATCAGCATCCTCTTCTGCTCTGTCTTCGAGGTGGCCTTCCTA-3’ (K162C sense); 5’-CTAGTAGGAAGGCCACCTCGAAGACAGAGCAGAAGAGGATGCTGA TGATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3’ (K162C antisense); 5’-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACA TCATCAGCATCCTCTTCAAGTGCGTCTTCGAGGTGGCCTTCCTA-3’ (S163C sense); 5’-CTAGTAGGAAGGCCACCTCGAAGACGCACTTGAAGAGGATGCTGAT GATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3’ (S163C antisense); 5’-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACA TCATCAGCATCCTCTTCAAGTCTTGCTTCGAGGTGGCCTTCCTA-3’ (V164C sense); 5’-CTAGTAGGAAGGCCACCTCGAAGCAAGACTTGAAGAGGATGCTGA TGATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3’ (V164C antisense); 5’-CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACA TCATCAGCATCCTCTTCAAGTCTGTCTGCGAGGTGGCCTTCCTA-3’ (F165C sense); 5’-CTAGTAGGAAGGCCACCTCGCAGACAGACTTGAAGAGGATGCTGAT GATGTAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3’ (F165C anti-sense); 5’- CATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACATC ATCAGCATCCTCTTCAAGTCTGTCTTCTGCGTGGCCTTCCTA-3’ (E166C sense); 5’-CTAGTAGGAAGGCCACGCAGAAGACAGACTTGAAGAGGATGCTGATGATG TAGGTTCTCAGCAAGCCGCCCCTCATTTTCACCTTGC-3’ (E166C antisense); 5’-C ATGGCAAGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACATCATCAGCAT

CCTCTTCAAGTCTGTCTTCGAGTGCGCCTTCCTA-3’ (V167C sense); 5’-CTAGTA GG AAGGCGCACTCGAAGACAGACTTGAAGAGGATGCTGATGATGTAGGTTC TCAGCAAGCCGCCCCTCATTTTCACCTTGC-3’ (V167C antisense); 5’-CATGGCA AGGTGAAAATGAGGGGCGGCTTGCTGAGAACCTACATCATCAGCATCCTCTT CAAGTCTGTCTTCGAGGTGTGCTTCCTA-3’ (A168C sense); 5’-CTAGTAGGAAG CACACCTCGAAGACAGACTTGAAGAGGATGCTGATGATGTAGGTTCTCAGCA AGCCGCCCCTCATTTTCACCTTGC-3’ (A168C antisense); 5’-CATGGCAAGGTGA AAATGAGGGGCGGCTTGCTGAGAACCTACATCATCAGCATCCTCTTCAAGTC TGTCTTCGAGGTGGCCTGCCTA-3’ (F169C sense); 5’-CTAGTAGGCAGGCCACC TCGAAGACAGACTTGAAGAGGATGCTGATGATGTAGGTTCTCAGCAAGCCGC CCCTCATTTTCACCTTGC-3’ (F169C antisense).

DNA sequencing of all constructs was performed at the Protein Chemistry Core Laboratory of the University of Texas Medical Branch.

Oocyte preparation, cRNA injection and measurements of 5(6)-carboxyfluorescein uptake

See **MATERIALS AND METHODS** in Chapter 1.

RESULTS

Activity of hemichannels formed by single-Cys Cx43 mutants

Cx43 mutants in which one transmembrane residue of a Cys-less, functional, Cx43 was replaced with a Cys, were engineered to obtain single-Cys M1, M2 and M3 mutants. The mutants were expressed in *Xenopus* oocytes injected with the corresponding cRNA, and the functionality of the mutants was assessed by measuring

carboxyfluorescein uptake in low-[Ca^{2+}] medium. Extracellular [Ca^{2+}] at millimolar concentrations blocks of hemichannels, which open in the low-[Ca^{2+}] medium, allowing the uptake of carboxyfluorescein and other "large" hydrophilic solutes (Bao *et al*., 2005, and Figs. 2.1, 2.2 and 2.3). Figs. 2.1-2.3 show that all of single-Cys mutants were functional (i.e., there was a significant increase in carboxyfluorescein uptake in low-[Ca^{2+}] medium). These data are consistent with the results in the poly-Ala mutant Cx43 hemichannels presented in Chapter 1, supporting the notion that Cx43 has a high tolerance for transmembrane helix mutations, and that the protein does not have essential amino acids in its transmembrane sequences. In the following sections, I show SCAM data using the functional mutants.

Identification of pore-lining residues

For the attempts to use the modified SCAM described under **METHODS**, I focused on 3 of the 4 transmembrane α helices, M1, M2 and M3. These helices have been implicated as pore-forming helices (Zhou *et al*., 1997; Pfahnl and Dahl, 1998; Hille, 2001; Skerrett *et al*., 2002; Kronengold *et al*., 2003), whereas the data on M4 do not support it as a pore-lining helix (Zhou *et al*., 1997; Pfahnl and Dahl, 1998; Hille, 2001; Skerrett *et al*., 2002; Kronengold *et al*., 2003). The latter conclusion is also supported by the results presented under Chapter 1 that show that the Cx43 M4 poly-Ala mutant was the only one capable of forming heterotypic gap-junctional channels with wild-type Cx43. Additional data supporting a non-pore role of M4 will be presented in Chapter 4. In order to identify the pore-lining residues in three of the transmembrane helices, the effect of MBB on carboxyfluorescein uptake *via* the single-Cys hemichannels was tested. The modifications that introduced to the "basic" SCAM have been delineated in the **INTRODUCTION** of this chapter. As shown in Figs. 2.1 and 2.2, no positive results

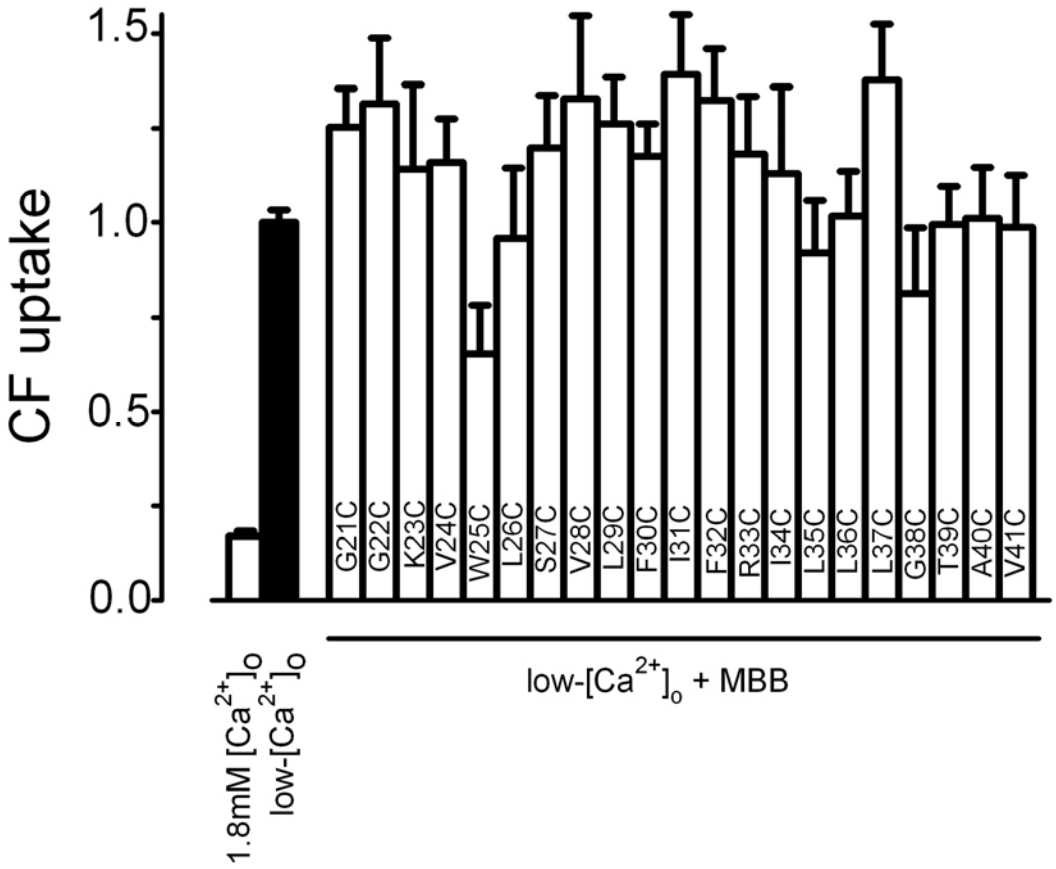

Figure 2.1: Effects of MBB on carboxyfluorescein uptake *via* hemichannels formed by M1 single-Cys Cx43 mutants.

The carboxyfluorescein uptake elicited by lowering extracellular Ca^{2+} was measured in single frog oocytes in the absence or presence of 100 μM MBB (+ MBB). Data were normalized to the uptake in low-[Ca^{2+}] solution. Data are means ± SEM of >30 oocytes *per* condition. For simplicity, we show the control and low-[Ca^{2+}] data as means ± SEM of all groups. * $P < 0.05$ compared to the control value, in the presence of 1.8 mM Ca^{2+}. The results do not show significant effects of MBB.

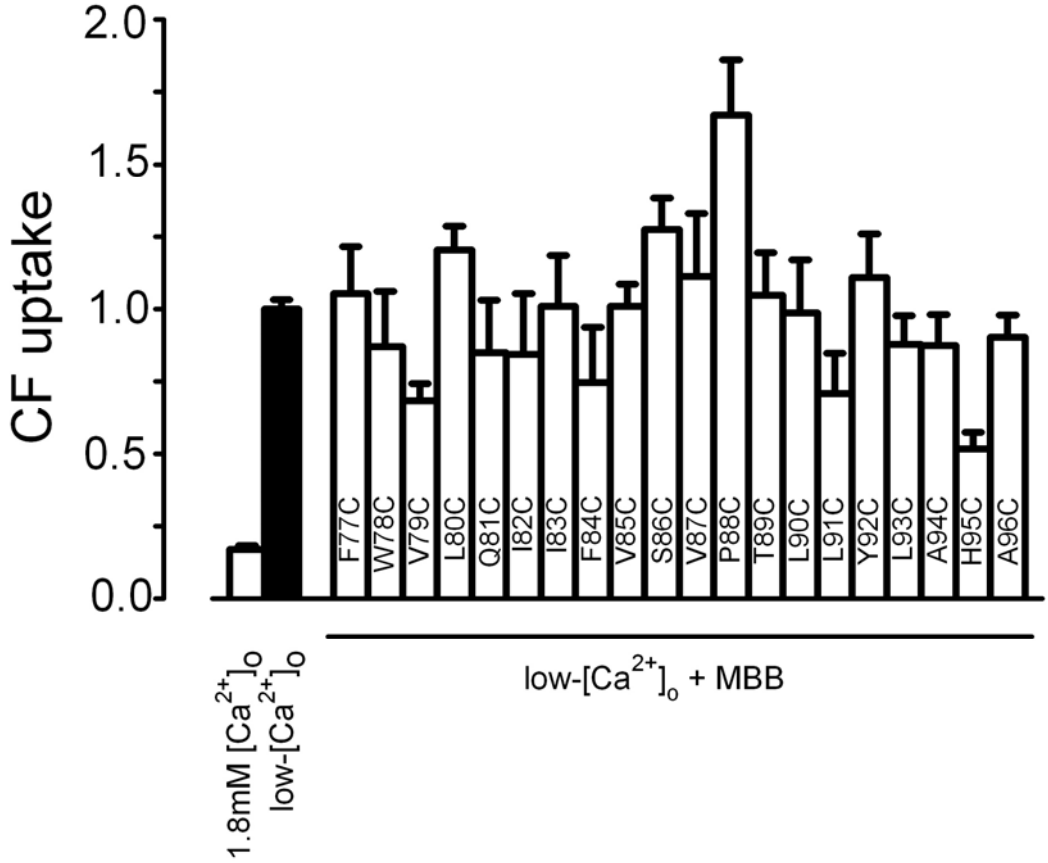

Figure 2.2: Effects of MBB on carboxyfluorescein uptake *via* hemichannels formed by M2 single-Cys Cx43 mutants.

For details see legend to Fig. 2.1. Data are means ± SEM of >30 oocytes per condition. The results do not show significant effects of MBB.

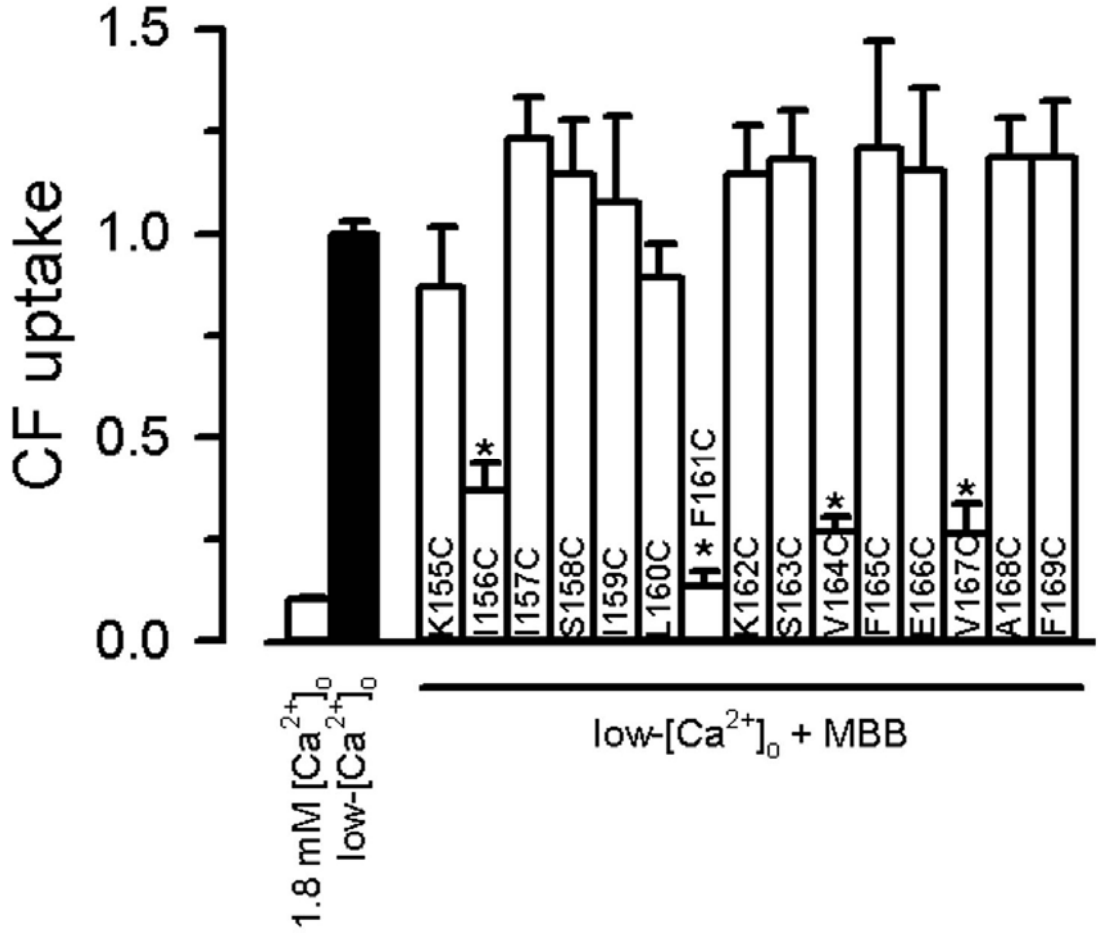

Figure 2.3: Effects of MBB on carboxyfluorescein uptake *via* hemichannels formed by M3 single-Cys Cx43 mutants.

For details see legend to Fig. 2.1. Data are means ± SEM of >30 oocytes *per* condition. The asterisks depict a $P < 0.05$ compared to the corresponding result in the absence of MBB. MBB produced significant block of carboxyfluorescein uptake in several single-Cys mutants.

were obtained with the M1 and M2 mutants, positive meaning a block by MBB of carboxyfluorescein uptake elicited in low-[Ca^{2+}] medium. These disappointing results were not entirely unexpected because of the problems encountered in the past with the use of SCAM to study gap-junctional channels and hemichannels, which were reviewed in the **INTRODUCTION** to this chapter. However, the results obtained using the M3 single-Cys mutants were more encouraging, implying that some of the modifications of the SCAM that were instrumented had a positive impact. The results illustrated in Fig. 2.3 show that, of the 15 M3 mutants studied, in four MBB produced a significant decrease of the carboxyfluorescein uptake elicited by low-[Ca^{2+}] medium. The mutants in which MBB blocked carboxyfluorescein uptake were I156C, F161C, V164C and V167C, suggesting that residues of M3 at these positions are pore-lining.

MBB is a cell-membrane impermeable amphipatic compound with a hydrophobic moiety, and it is therefore possible for MBB to react with a Cys that do not line the pore. To rule out this possibility, it was determined whether previous reaction with IAA protects from the MBB effect. IAA is a small (M_r 186) hydrophilic thiol reagent that is not expected to partition significantly into the lipid bilayer. Fig. 2.4 shows that pre-treatment with IAA does prevent the inhibition of carboxyfluorescein uptake by MBB in the Cx43 mutants F161C, V164C and V167C. IAA was not effective in the I156C mutant. The possibility that IAA by itself blocks the carboxyfluorescein uptake elicited by low-[Ca^{2+}] medium in hemichannels formed by the I156C mutant was ruled out experimentally. The difference in carboxyfluorescein uptake *via* I156C hemichannels in response to lowering medium [Ca^{2+}] between oocytes exposed and not exposed to IAA was 3 ± 16% (n = 6, not significant). In any case, the results on the hemichannels formed by single-Cys mutants of Cx43 strongly suggest that residues at positions 161, 164 and 167 are pore- lining.

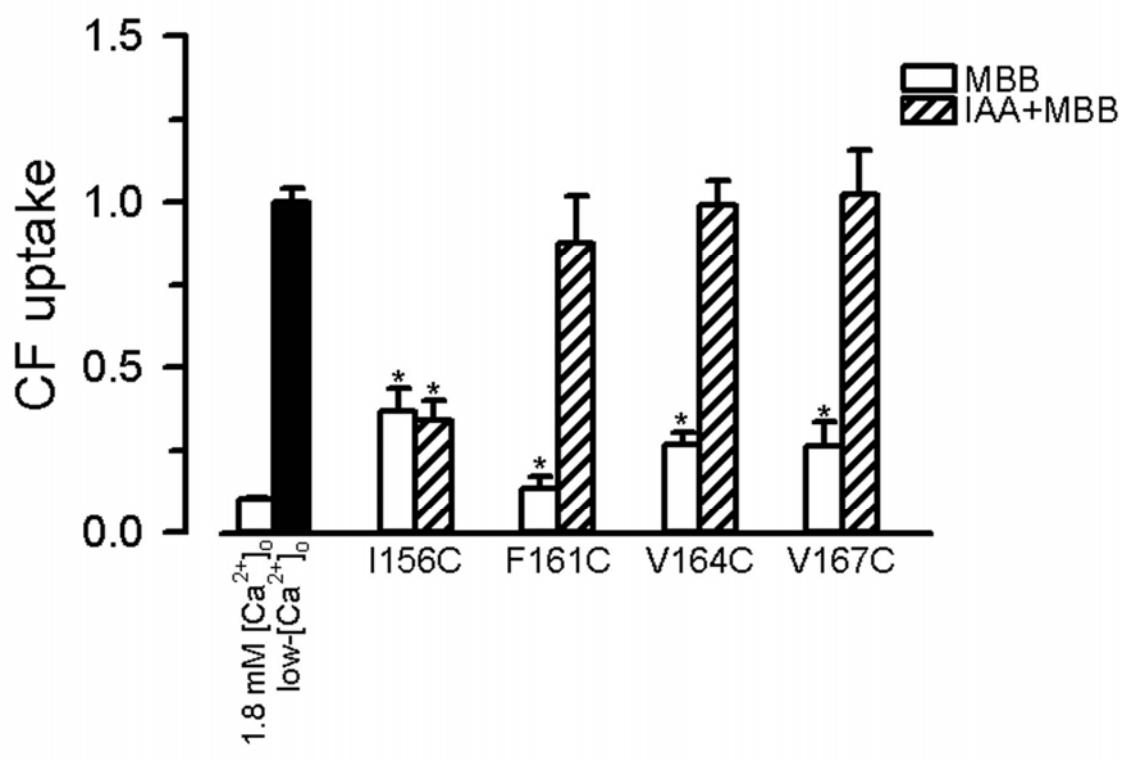

Figure 2.4: Protection of the inhibition by MBB of carboxyfluorescein uptake by prior reaction with IAA.

For details see legend to Fig. 2.1. Data are means ± SEM of >20 oocytes *per* condition. The asterisks depict a $P < 0.05$ compared to the corresponding result in the absence of MBB. IAA prevented the block of carboxyfluorescein uptake by MBB in three of the four single-Cys mutants responsive to MBB.

DISCUSSION

The original plan was to identify the 2 pore-lining helices of the transmembrane pore hemichannel using a modified SCAM approach (see **INTRODUCTION**). However,

as detailed under RESULTS, this approach was only partially successful. Residues in M3 were identified as pore lining by showing a significant decrease in the uptake of carboxyfluorescein by the cell-membrane impermeable thiol reagent MBB. An important point about our functional assay using "large" hydrophilic molecules as probes is that the magnitude of the block by MBB is much larger (65-90%) that those reported for current measurements that use "small" inorganic ions as probes (frequently <30%, e.g., see Kronengold *et al*., 2003, and Skerret *et al*., 2002).

As mentioned under **RESULTS**, an alternative possibility for the block by MBB is the reaction with the Cys from the bilayer, instead of from the pore (Skerret *et al*., 2002). This seems unlikely because the small hydrophilic thiol reagent IAA, which is short and has no hydrophobic moiety, and thus cannot label Cys residues from the lipid phase, prevents the block by MBB in 3 of the 4 "positive" residues. At the pH of the experiments, the concentration of iodoacetate is more than four orders of magnitude higher than that of iodoacetic acid, and the anionic form is likely the form that carboxymethylates the sulfhydryls. Since IAA can only label from the aqueous phase, F161, V164 and V167 must line the pore.

As mentioned under **RESULTS**, the absence of reversal of the carboxyfluorescein uptake block by MBB in the 4th MBB "positive" residue (I156C mutant) is not due to equal magnitude of block by MBB and IAA. Two possibilities can explain the result: 1) The Cys introduced does not line the pore and MBB blocked carboxyfluorescein uptake by reaction with this residue by penetration of its hydrophobic tail away from the pore, and therefore IAA cannot react with the Cys. 2) The Cys introduced is a pore-lining residue and both MBB and IAA are bound to the Cys (there are six Cys residues in the hemichannel ring), resulting in carboxyfluorescein uptake block (e.g., two MBBs or one MBB plus two IAAs could block carboxyfluorescein

uptake). Our LRET data shown in Chapter 4 suggest that the residue at position 156 is a pore-lining residue.

It is interesting to note that all "positive" M3 positions are located on the same face of the helix (Fig. 2.5). Although the positions of residues 161, 164 and 167 are entirely compatible with the hemichannel model presented of Fleishman *et al*. (2004), it is not possible to determine which of the 2 pore-forming helices is M3 because residues at positions equivalent to F161, V164 and V167 of M3 can belong to helices B or C (M3 and M1 in the Fleishman *et al*., (2004) model (Fig. 2.6). This is exemplified in Fig. 2.7, where the SCAM "positive" residues were considered to belong to helices C (blue) or B (red). In both cases the residues are clearly accessible from the pore, and therefore the SCAM results cannot distinguish between them. In summary, the "positive" residues are pore-lining independently on whether M3 corresponds to helix C, as proposed in the model of Fleishman *et al*. (2004) (Fig. 2.7, shown in blue), or helix B, as we will propose based on LRET distance measurements presented in Chapter 4. (Fig. 2.7, shown in red).

Because of the partial failure of the SCAM experiments and the impossibility to separate clearly the individual pore-lining helices, we decided to perform distance measurements between selected residues using LRET, as a way to identify individual helices. This required the use of purified and reconstituted hemichannels, the generation of hemichannels with well-established number of donor and acceptors) and acceptor, and the implementation of LRET for the studies of these hemichannels. Bao *et al*. (2004b) in our laboratory have already worked the basic development of the techniques to obtain highly-purified, reconstituted and functional Cx43 hemichannels. In Chapter 3, I will present some improvements in the purification methodology and additional characterization of the preparation as well as the methodology for the generation and characterization of hemichannels of controlled subunit composition. In Chapter 3 I will

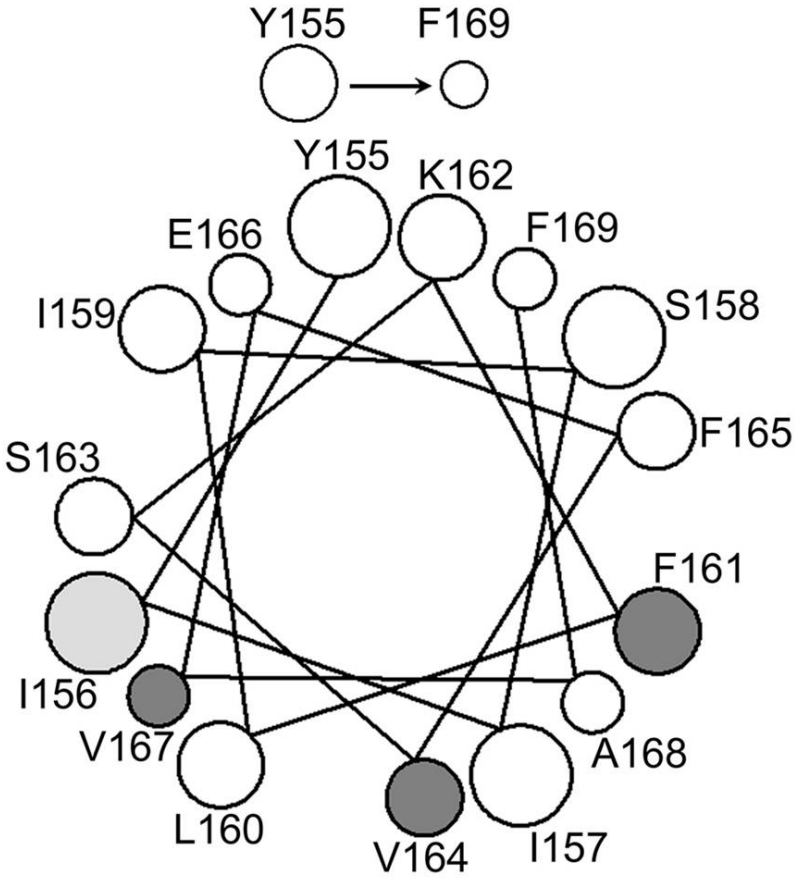

Figure 2.5: M3 helix-wheel summarizing the SCAM experiments. The residue positions sensitive to MBB are in gray (both lighter and darker gray). The positions where IAA prevented the effect of MBB are shown in dark gray. Note that all the MBB-sensitive lie on one helix face.

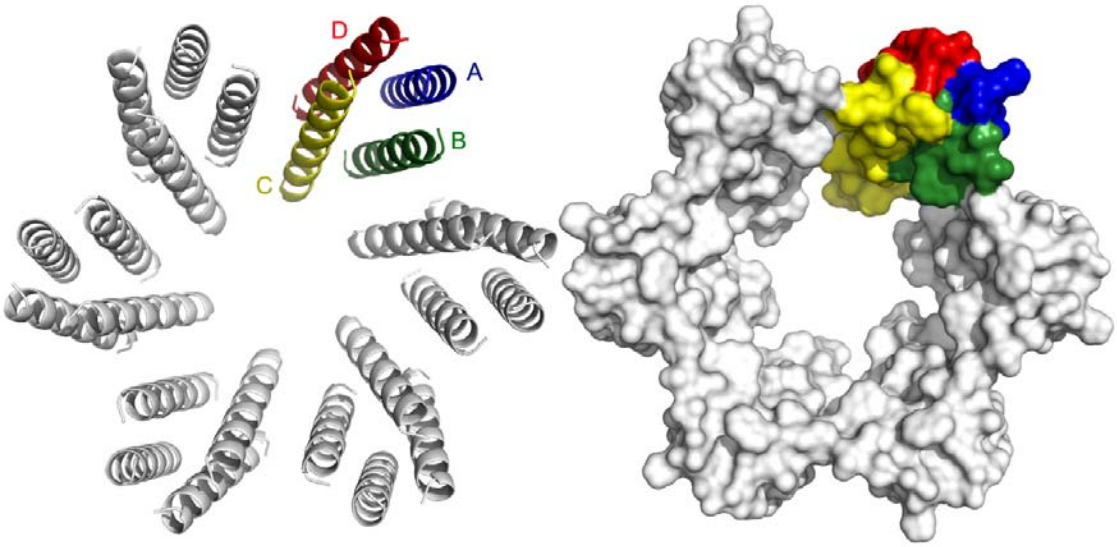

Figure 2.6: Structural model of the Cx32 GJH pore.

Schematic representation of the transmembrane α helix GJH model from Fleishman *et al*. (2004), viewed from the cytoplasmic side. The cartoon on the left panel shows the proposed model for Cx32 helix packing. Although coordinates from Cx43 gap-junctional channels were employed, the model of Cx32 was presented by Fleishman *et al*. because experimental SCAM data (not reliable, see Chapter 3), were used to support the model. The helices from one monomer are labeled with different colors (green, blue, yellow and red represents to helix 1, 2, 3, and 4, respectively). The coordinates were provided by Dr. Mark Yeager from The Scripps Institute in La Jolla, CA. This figure is a copy of Fig. 1.1, placed here to facilitate reading.

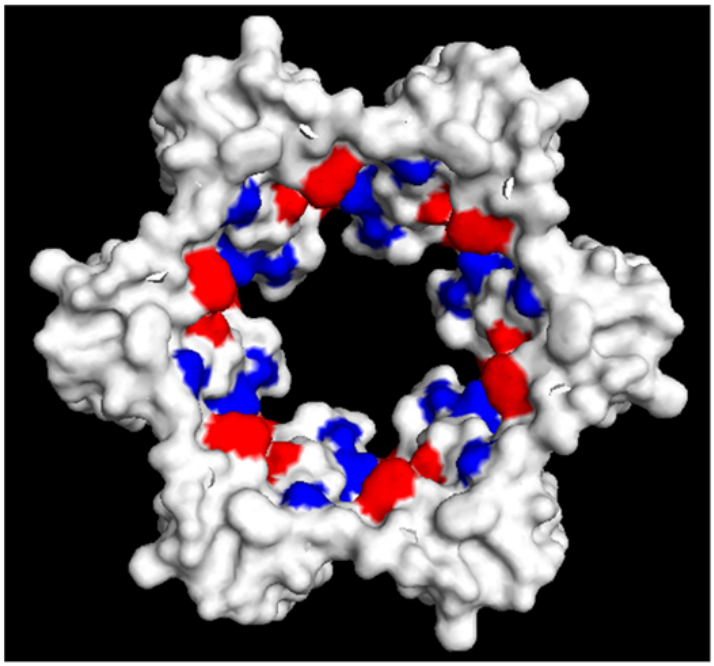

Figure 2.7: Location of MBB "positive" residues in either of the pore-lining helices (helices B and C) of the model of Fleishman *et al.* (2004).

Space-filling representation of the GJH viewed from the cytoplasmic side (based on the coordinates provided by Dr. M. Yeager, see Fleishman *et al.*, 2004). The MBB sensitive positions identified from the experiments in Fig. 2.3 are shown in blue and red for the assignment of M3 as helix C (Fleishman *et al.*, 2004) or helix B (our model, Chapter 4), respectively.

also present the use of LRET in hemichannels. In Chapter 4, I will show LRET data supporting a new model of connexin folding into the hemichannels, with the first helix assignment based on experimental measurements. In Chapter 5 I will present data on the molecular mechanism of regulation of Cx43 hemichannels by PKC-mediated phosphorylation, which include movement of a pore-lining helix determined by LRET.