Synthesis, assembly and structure of gap junction intercellular channels

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Gap junction membrane channels assemble as dodecameric complexes, in which a hexameric hemichannel (connexon) in one plasma membrane docks end to end with a connexon in the membrane of a closely apposed cell. Steps in the synthesis, assembly and turnover of gap junction channels appear to follow the general secretory pathway for membrane proteins. In addition to homo-oligomeric connexons, different connexin polypeptide subunits can also assemble as hetero-oligomers. The ability to form homotypic and heterotypic channels that consist of two identical or two different connexons, respectively, adds even greater versatility to the functional modulation of gap junction channels. Electron cryocrystallography of recombinant gap junction channels has recently provided direct evidence for α-helical folding of at least two of the transmembrane domains within each connexin subunit. The potential to correlate the structure and biochemistry of gap junction channels with recently identified human diseases involving connexin mutations makes this a particularly exciting area of research.

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Abbreviations

CMTX disease	X-linked Charcot–Marie–Tooth disease
Cx	connexin
ER	endoplasmic reticulum

Introduction

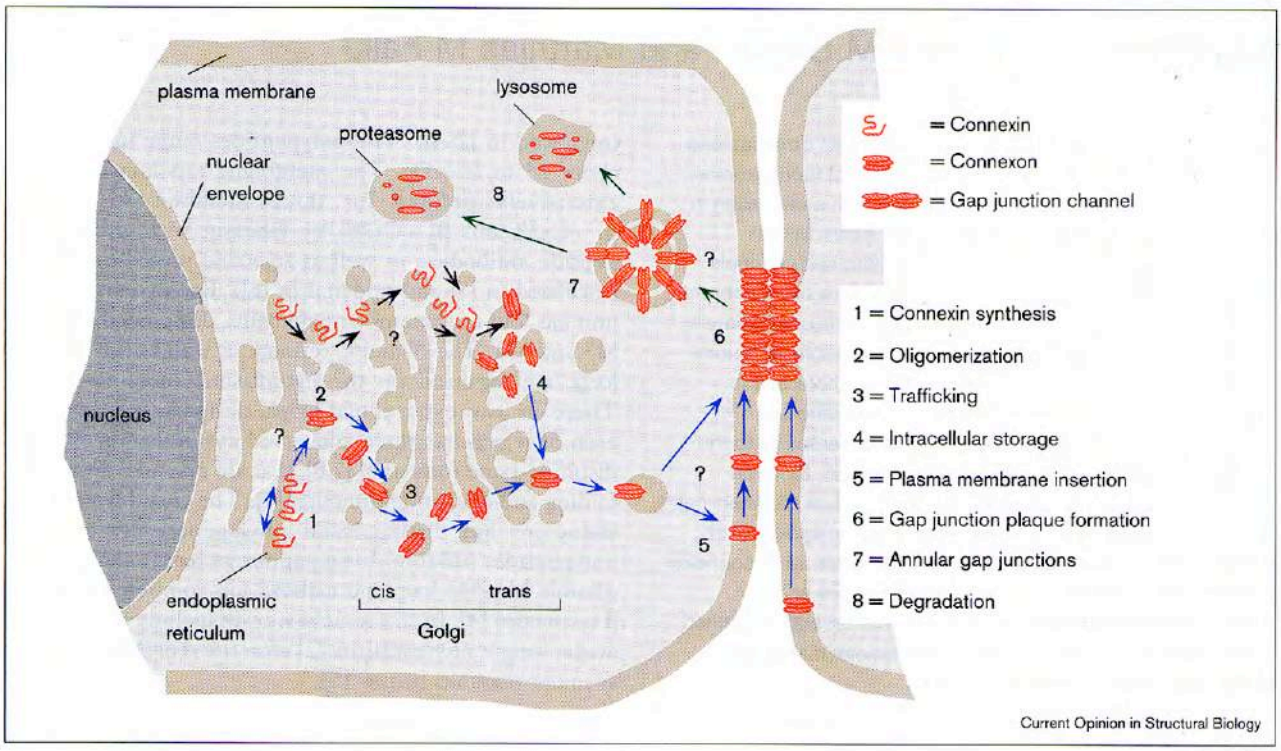
Gap junctions are formed by the aggregation of hundreds of protein oligomers into so-called plaques in the plasma membrane that are generally about one to two microns in diameter. Six gap junction protein subunits (termed connexins, Cx) assemble into a hemichannel (termed a connexon) that spans the lipid bilayer. The end to end interaction or 'docking' of two connexons from adjacent cells generates an intercellular aqueous channel that allows the exchange of nutrients, metabolites, ions and small molecules up to ~1000 Da [1]. Gap junction connexins constitute a multigene family with over a dozen members (reviewed in [2–4]). The polypeptides range in size from about 25 to 50 kDa. The membrane topology has been extensively defined for three members (α_1 = Cx43, β_1 = Cx32 and β_2 = Cx26) by labeling with site-specific peptide antibodies, as well as selective protease cleavage (reviewed in [5] and updated in [6]). Each connexin subunit has four transmembrane domains, designated M1, M2, M3 and M4. The N and C termini, as well as the M2–M3 loop, are localized on the cytoplasmic membrane face. There are two extracellular loops, designated E1 and E2, each of which has three conserved cysteines, involved in disulfide-bond formation [7,8], that are essential for normal folding and/or channel function [9]. The α and β classes of connexins are distinguished phylogenetically and the polypeptides differ in that α connexins have a longer cytoplasmic M2–M3 loop and carboxy tail compared with the β connexins [4]. In this review, we will include the Cx molecular weight nomenclature [2] after the α or β designation, as in our previous review [5].

Several reviews describe our current knowledge regarding the structure and function of gap junctions [5,6,10–13]. Recent analysis of two-dimensional crystals has provided structural information at 7 Å resolution [14•]. Despite this progress towards understanding the gap junction structure, the interpretation of intermediate resolution density maps relies extensively on additional biochemical results that establish structure/function relationships within the molecule. Here, we summarize recent progress in areas encompassing the synthesis, assembly and structure of gap junction channels.

Synthesis of connexins and maturation into gap junction channels

With few exceptions, all plasma membrane proteins studied to date are synthesized at the endoplasmic reticulum (ER), co-translationally integrated into the ER membrane and transported by successive vesicle budding and fusion events from the ER, through the Golgi stacks to the plasma membrane. This general intracellular transport route, referred to as the secretory pathway (reviewed in [15]), has also been shown to apply to connexins (Figure 1). The delineation of the steps in the secretory pathway for connexins has relied on an *in vitro* system that utilizes translation-competent cell lysates, supplemented with ER-derived membrane vesicles (microsomes). Such experiments have demonstrated that connexins are co-translationally integrated into the ER membrane in a signal-recognition particle-dependent reaction [16–18]. During the process of integration into the ER membrane, connexins adopt their final transmembrane topology [19]. Western immunoblot analysis

Figure 1



Schematic representation of the synthesis, assembly and degradation of gap junction membrane channels, based on the current literature. Steps 1 to 8 show (1) connexin synthesis at the endoplasmic reticulum membrane, (2) oligomerization into gap junction connexons (hemichannels), (3) trafficking along the secretory pathway, (4) intracellular storage within the Golgi apparatus, (5) insertion of the gap junction connexons into the plasma membrane, (6) formation of the gap junction plaque comprising many individual gap junction channels, (7) proposed internalization of the plaque via formation of annular gap junctions and (8) degradation of gap junction channels via lysosomal and/or proteasomal pathways. Some areas of current discussion have been highlighted with a question mark. Evidence has been obtained for oligomerization within the membranes of either the endoplasmic reticulum or the Golgi apparatus. It is unclear how and where gap junction connexons are inserted into the plasma membrane and how the gap junction plaque is formed. Annular gap junctions may play a role in gap junction plaque degradation by lysosomal and/or proteasomal pathways. Blue arrows indicate synthesis and assembly in the endoplasmic reticulum membrane and the transport of assembled connexons through the Golgi stacks to the plasma membrane. Black arrows indicate the transport of unassembled connexins to the *trans* side of the Golgi apparatus. Green arrows indicate degradation pathways of gap junction channels.

of cell fractions and immunoprecipitation of radiolabeled proteins demonstrated that connexin polypeptides can be detected in the ER, Golgi and plasma membranes. This localization pattern has been replicated in a variety of tissues, such as rat liver [16,20], dog pancreas [16] and mouse preimplantation embryos [21], as well as tissue culture cell lines that express endogenous [22,23] or recombinant connexins [24,25•]. Furthermore, no gap junction channel assembly and/or plaque formation has been observed in cells treated with drugs that interfere with the secretory pathway [21,23,26].

Since gap junction channels are oligomeric protein complexes, the connexin subunits have to assemble before they can function. For almost all known oligomeric membrane proteins, including voltage- and ligand-gated ion-channel subunits [27], assembly in the ER is a necessary prerequisite for their further transport through the secretory pathway (reviewed in [28]). Using tissue culture cells that express α_1[Cx43], Musil and Goodenough [26] presented evidence that connexins may not assemble following their synthesis in ER membranes, but instead assemble later, after their exit from the ER, in the late Golgi membranes. In a cell-free translation system, however, Falk *et al.* [29•] observed the functional assembly of connexons composed of α_1[Cx43] or β_1[Cx32] in isolated ER membrane vesicles (microsomes). This result is consistent with studies on the heterologous expression of β_1[Cx32] in stably transfected baby hamster kidney (BHK) cells. In this system, ER membranes contain not only connexons, but also complete gap junction plaques — the viability of the cells is unaffected [24]. Nevertheless, it cannot be ruled out that the elevated concentration of connexin polypeptides may be responsible for the observed assembly in the ER. Hence, additional experimentation will be required in order to determine the actual site of connexin oligomerization *in vivo*.

How connexons are transported and localized in the plasma membrane is not well understood, although an interaction of α_1[Cx43] with cytoskeletal proteins ZO-1 and α-spectrin was recently described [30]. A linkage between α_1[Cx43] polypeptides and α-spectrin, via ZO-1, implies the involvement of the cytoskeleton in the trafficking and plasma membrane localization of connexins. After the delivery of assembled connexons to the plasma membrane [31•], the hemichannels in the membranes of apposed cells are believed to pair via interactions of the extracellular loop domains of the connexin subunits, forming the complete dodecameric intercellular channel. This process is enabled or at least facilitated by calcium-dependent cell adhesion molecules such as E-cadherins [32–36]. The turnover of gap junctions is thought to occur via internalization of complete dodecameric channels in a complex process that involves lysosomal as well as proteasomal pathways [20,35,37–40].

Regulated assembly of gap junction connexons

The assembly of connexin into connexons is a complicated process. Homo-oligomeric connexons and homotypic channels composed of only one connexin subtype probably exist *in vivo* since some cell types express only one connexin polypeptide. Support for the presence of homo-oligomeric connexons has been provided by immunofluorescence microscopy [41] and mass measurements using scanning transmission electron microscopy [42]. In addition, gap junction structures assembled from a single type of connexin that are expressed in either transfected tissue culture cells [24,43] or insect cells infected with baculovirus [44,45] are morphologically indistinguishable from *bona fide in vivo* gap junctions. More than a dozen different connexins have been cloned from rodents, however, and it is common for many cells to express more than one connexin, which can be localized to the same gap junction plaque [41,46]. For instance, Risek *et al.* [41] used double-label confocal immunofluorescence microscopy to provide compelling evidence that the two connexins α_1[Cx43] and β_2[Cx26] can be localized to the same gap junction plaque when expressed simultaneously in developing rat epidermal cells. Choosing developing epidermis was an important prerequisite for allowing such an analysis since the gap junction plaques assembled in these cells were large enough to allow a clear distinction between plaque areas composed predominantly of α_1[Cx43], β_2[Cx26] and both connexin isotypes.

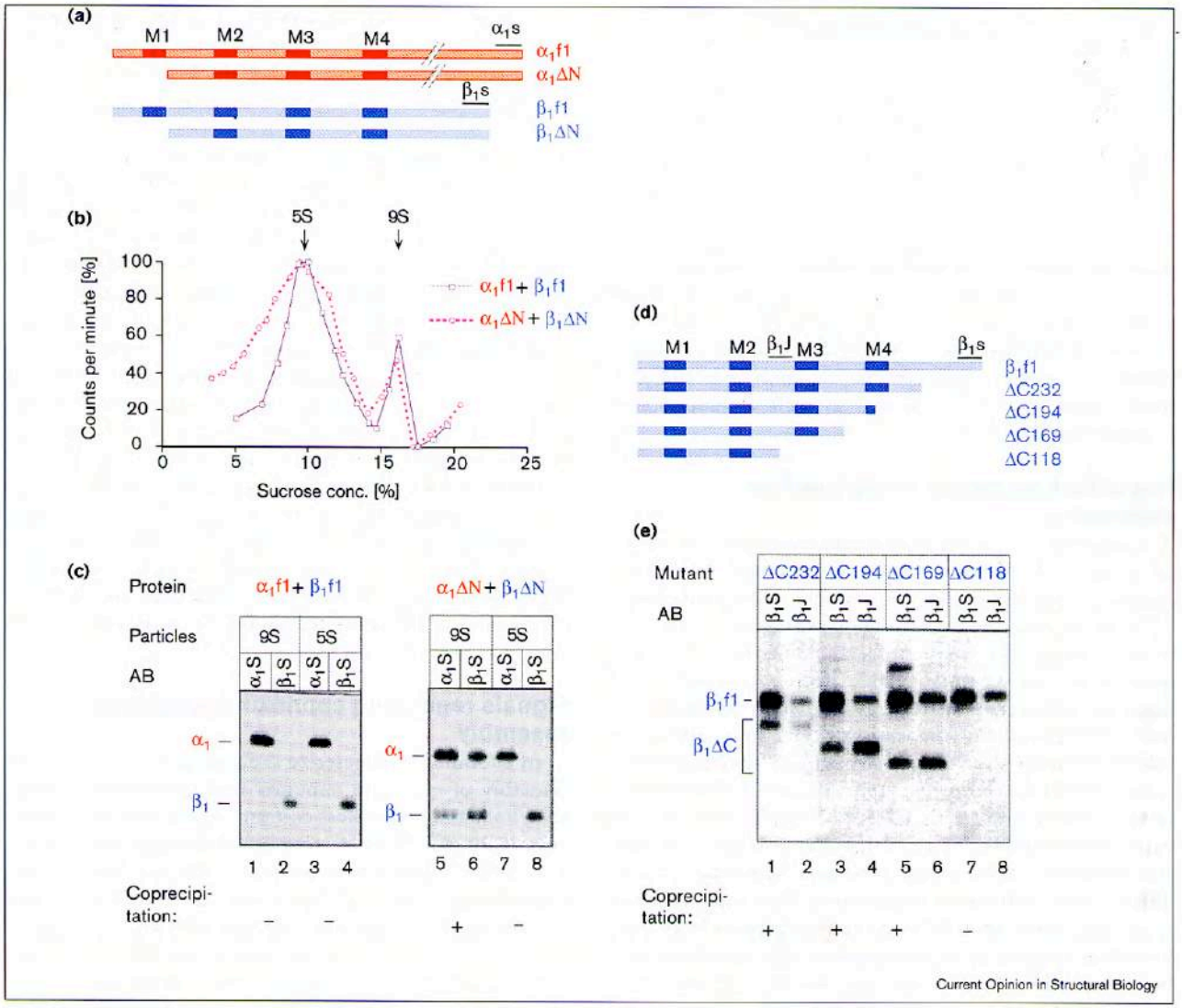
Hetero-oligomerization of different connexin polypeptides was demonstrated by Western immunoblot analysis of connexons that were isolated from lens fiber cells, which contain both α_8[Cx50] and α_3[Cx46] [47•], and liver gap junctions, which contain β_1[Cx32] and β_2[Cx26] [48]. Further evidence for the existence of hetero-oligomeric connexons has been obtained from the analysis of *in vitro* connexin subunit assembly in heterologous and cell-free expression systems [29•,49–51]. By analogy, numerous subunits of vertebrate ligand- and voltage-gated ion channels have been cloned. Within a given channel type (e.g. K^+ channel), different subunits may oligomerize into many different channel isotypes that are believed to be specifically adapted to precisely regulate the function of the cells in which they are expressed (reviewed in [27]).

A unique feature for diversity among gap junction channels is the formation of heterotypic gap junction channels that involve the docking of two homomeric connexons, each formed by a different connexin subtype. *In vitro* evidence for heterotypic interactions has been suggested by the formation of functional gap junction channels when two types of cells that express different connexin polypeptides are co-cultured [43], as well as from mass measurements of liver gap junctions that contain both β_1[Cx32] and β_2[Cx26] [42]. The formation of heterotypic channels appears to not only depend on the extracellular loops but may also be influenced by the C terminus and the M2–M3 cytoplasmic loop [52,53]. The possibility also exists that two hetero-oligomeric connexons could form a gap junction channel, although this has not been explicitly demonstrated. The subunit composition, stoichiometry and organization within heteromeric connexons are likely to play a critical role in determining the properties of gap junction channels.

Signals regulating connexin polypeptide assembly

In an attempt to characterize the signals that regulate the assembly of connexin subunits into gap junction channels, Falk and co-workers co-translated different connexin isotypes, and N and C-terminal truncated connexins in a cell-free translation system. Hydrodynamic and immunoprecipitation analyses were performed in order to investigate the interaction and assembly of Cx subunits [19,29•] (Figure 2). When α_1[Cx43] and β_1[Cx32] were co-translated, no co-precipitation was observed, indicating that the assembled connexons were homo-oligomers, being composed of either α_1[Cx43] or β_1[Cx32] subunits (Figure 2a–c). On the other hand, co-translation of truncated α_1[Cx43] and β_1[Cx32] molecules that had been cleaved immediately after M1 (concomitant with their membrane integration) [19] allowed co-precipitation of the two connexins by monoclonal antibodies directed against either α_1[Cx43] or β_1[Cx32] (Figure 2a–c). These results suggest that different connexin isotypes do not assemble randomly with each other, but interact selectively, only allowing the assembly of homo-oligomers and certain types of hetero-oligomers. The observed loss of selectivity also suggests that the N-terminal domain of the connexin polypeptide (N terminus, M1 and/or the E1 domain) plays a role in the selective assembly of certain connexin isotypes into distinct hetero-oligomers. Similarly, 'assembly signals' that are critical for mediating subunit interactions have been identified both in the N-terminal domains and in the first transmembrane segments of various ion-channel proteins. Specific structural

Figure 2



The characterization of selectivity and assembly signals within the the connexin polypeptide sequence. Different connexin isotypes were translated in cell-free translation systems in the presence of ER-derived membrane vesicles (microsomes). The assembly and interaction of the polypeptides was analyzed by hydrodynamic and immunoprecipitation experiments. **(a)** Schematic representation of the full length and N-terminal truncated α_1[Cx43] ($\alpha_1$fl and $\alpha_1\Delta$N) and β_1[Cx32] ($\beta_1$fl and $\beta_1\Delta$N) polypeptides used in this analysis. The transmembrane regions are marked M1 to M4. **(b)** α_1[Cx43] and β_1[Cx32] were co-translated either as full length or N-terminal truncated polypeptides. Assembly into connexons (9S particles) was observed in both instances. Unassembled connexins have a sedimentation coefficient of 5S. **(c)** The composition of the assembled connexons was analyzed by immunoprecipitation techniques using α_1[Cx43] (α_1S) and β_1[Cx32] (β_1S)-specific monoclonal antibodies. No co-precipitation of the other connexin isotype was observed when full-length α_1[Cx43] and β_1[Cx32] were co-translated, indicating the assembly of only homo-oligomeric connexons. Co-precipitation of the other connexin isotype was observed, however, when N-terminal truncated α_1[Cx43] and β_1[Cx32] were co-translated, indicating hetero-oligomeric connexon assembly under these conditions. **(d)** Schematic representation of the C-terminal truncated β_1[Cx32] polypeptides generated and used in this analysis. **(e)** Full-length β_1[Cx32] was co-translated with the individual C-terminal truncated β_1[Cx32] polypeptides, and the interaction and assembly was investigated using β_1[Cx32]-specific monoclonal antibodies. Note that the β_1S antibodies only recognize the full-length β_1[Cx32] polypeptides, whereas the β_1J antibodies also recognize truncated polypeptides ΔC232 to ΔC169. Co-precipitation of the truncated β_1[Cx32] polypeptides ΔC232 to ΔC169, but not ΔC118, was observed, indicating that a C-terminal portion including the third transmembrane-spanning domain (M3) may be required for successful connexin subunit recognition and assembly. Adapted with permission from [29•].

motifs, which are formed by stretches of amino acids or individual residues, provide the basis for high-affinity interactions between individual subunits and thereby discriminate between different subunit isotypes (see [27] for a review). Accordingly, the deletion or disruption of potentially similar signals in the connexins would reduce the selectivity during oligomerization. Since oligomers still form when the N-terminal domain has been cleaved,

this region may not be essential for the general assembly of oligomers. Similarly, tetrameric K^+ channels can still form in the absence of their C-terminal membrane-spanning domains [54].

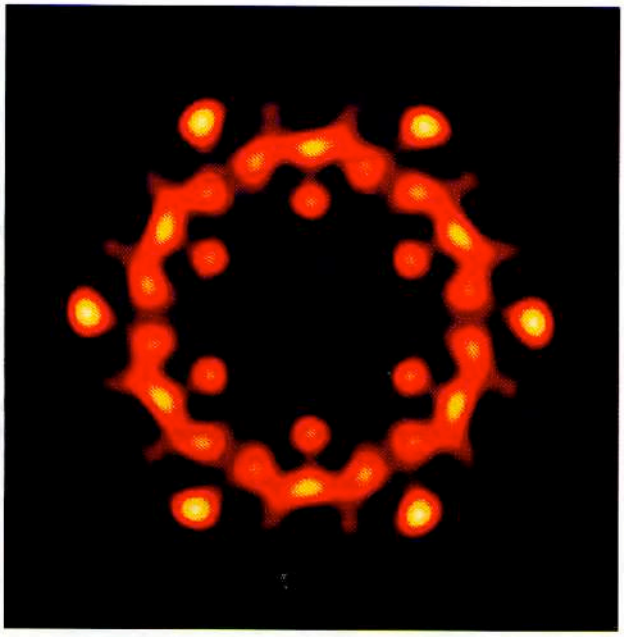
In order to characterize which portion(s) of the connexin polypeptide is required for subunit recognition and assembly of connexons, a set of β_1[Cx32] proteins that were progressively truncated from the C terminus were co-translated with full-length connexins. Potential protein–protein interactions were detected by immunoprecipitation [55] (Figure 2d,e). Truncated β_1[Cx32] proteins extending from the N terminus past transmembrane domain M3 co-precipitated and assembled with the full-length β_1[Cx32] protein (Figure 2e, lanes 1–6), although no assembly was observed when the truncated proteins extended only from the N terminus past transmembrane domain M2 (Figure 2e, lanes 7 and 8). This behavior was not restricted to homomeric interactions. For instance, the two conditions for the assembly of α and β hetero-oligomers were the N-terminal cleavage of at least one of the polypeptides and the extension of both polypeptides beyond transmembrane domain M3 [29•,55].

Taken together, these results suggest that distinct signals located within the connexin polypeptide sequence regulate the assembly of connexin subunits into gap junction channels. Parts of the C-terminal portion of the subunit may be important for the formation of intersubunit contacts and may serve as an 'assembly' signal, while a 'selectivity' signal, regulating the potential assembly of hetero-oligomeric connexons, may be located in the N-terminal portion.

Transmembrane α helices in gap junction channels

Electron cryocrystallography of recombinant gap junction channels has recently provided direct evidence of α-helical folding for at least two of the transmembrane domains in each connexin subunit [14•]. A projection density map at 7 Å resolution, based on the analysis of frozen hydrated two-dimensional crystals, shows that recombinant connexons formed from a C-terminal truncation mutant assemble as a hexameric cluster of subunits, with a diameter of ~65 Å (Figure 3). At a radius of 17 Å, the channel is lined by circular densities with the characteristic appearance of transmembrane α helices that are oriented perpendicular to the membrane plane [56–64]. A similar appearance for densities at 33 Å radius suggests the presence of α helices at the interface with the membrane lipids. The two rings of α helices are separated by a continuous band of density at a radius of 25 Å. In the hexamer, each connexin polypeptide must have defined packing interactions with its two neighbors. Hence, it seems clear that individual connexin polypeptides interact with one or two transmembrane-spanning domains of the adjacent connexin polypeptides within the connexon. It therefore seems likely that signals regulating connexin assembly are located within the transmembrane domains and/or immediately adjacent sequences.

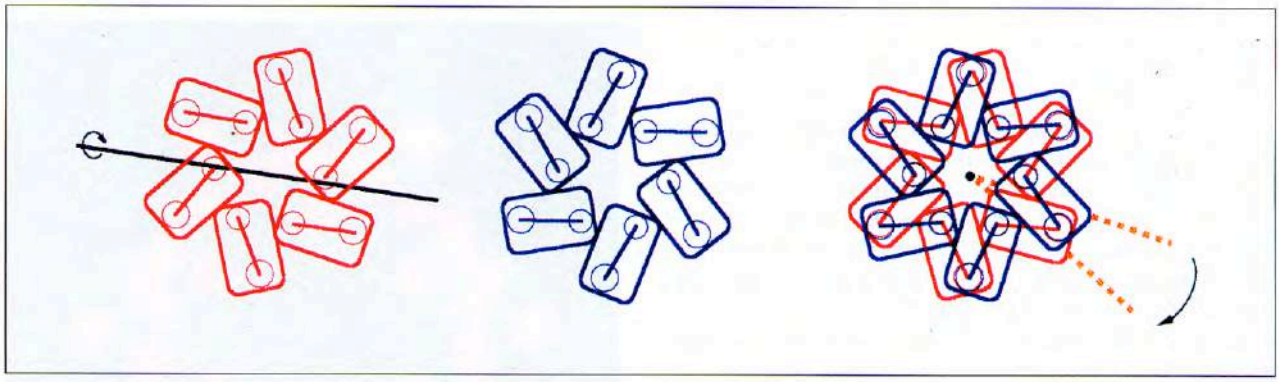
Figure 3



Projection density map at 7 Å resolution of a recombinant cardiac gap junction channel, as determined by electron cryocrystallography. This end-on view shows that each connexon is formed from a hexameric cluster of subunits with three major features: a ring of circular densities centered at a radius of 17 Å, interpreted as the α helices that line the channel; a ring of densities centered at a radius of 33 Å, interpreted as the α helices that are most exposed to the lipid; and a continuous band of density at a radius of 25 Å separating the two groups of helices, which may arise from the superposition of projections of additional transmembrane α helices and polypeptides density arising from the extracellular and intracellular loops within each connexin subunit. The structural details revealed by such studies are essential for understanding the general molecular design of gap junction channels. The rank order of mass density is white > yellow > red > violet > blue. Reproduced with permission from [14•].

Residues that are accessible to the aqueous pore were recently examined using cysteine-scanning mutagenesis [65]. In this study, connexons formed from α_3[Cx46] and a chimaeric connexin (formed from α_1[Cx43] with E1 inserted from β_1[Cx32]) were expressed in *Xenopus* oocytes. The thiol reagent maleimido-butyryl-biocytin was used to probe cysteine replacement mutants in M1, the N-terminal segment and M3. Channel activity was blocked for two positions in M1, suggesting that at least part of this transmembrane domain is accessible to the pore. Since M3 contains a hydrophilic strip of residues, it was predicted to form the lining of the aqueous pore [66]; however, only minimal labeling of the transmembrane residues in M3 was detected. It is possible that the mutated residues in M3 reside in a narrow portion of the pore, which may sterically prevent labeling by a cysteine reagent with a molecular weight of 537 Da.

Figure 4



A schematic model for the packing of α helices, connexins and connexons in the gap junction intercellular channel [14•]. Each connexin subunit is represented by a rectangle and the transmembrane α helices are depicted as circles. The twofold symmetry axes located in the extracellular gap generate the views of the apposed connexons in cell 1 (red) and cell 2 (blue) that form the intercellular channel. The superposition is in accordance with the observed projection density map and predicts that the connexons within the channel will be rotationally staggered, as shown by the dashed lines and the arc. The superposition of the helices and this rotation dictate that the α helices within one connexin will be superimposed with helices within two connexin subunits in the apposed connexon (i.e. one red subunit makes contact with two blue subunits and *vice versa*). Reproduced with permission from [14•].

Docking of connexons involves rotational stagger

A notable feature of the projection density map (Figure 3) is the 30° displacement between the rings of α helices at 17 and 33 Å radius. This places constraints on structural models for the docking of the two connexons when forming the intercellular channel. As a consequence of the 30° displacement, the two connexons forming the channel are rotationally staggered with respect to each other (as shown by the arcs in Figure 4), if a roughly square shape is assumed for the connexin subunit. The amount of rotational stagger will dictate whether six or 12 peaks are resolved in the outer ring of α helices at 33 Å radius. For instance, models with less than 30° of rotational stagger between connexons are not consistent with the map in Figure 3 since there would be 12 rather than six peaks in the outer ring of α helices. In addition, the 30° displacement between the rings of helices is not consistent with models in which the α helices are co-linear through the center of the channel. In contrast, the model shown in Figure 4 is in agreement with the projection density map. Note that the superposition of resolved α helices between the apposed connexins predicts that each subunit of one connexon will interact with two connexin subunits of the apposing connexon of the dodecameric intercellular channel. Such an arrangement may confer stability in the docking of the connexons.

Electron microscopy and image analysis of negatively stained gap junction plaques that had been split in the extracellular gap by urea treatment showed that the extracellular surface of each connexon contains six protrusions [67]. In order to form a tight seal with the apposed connexon, computer modeling of the map at ~18 Å resolution predicted that these protrusions interdigitate in such a way that requires a 30° stagger [68], as predicted from the projection map at 7 Å resolution [14•].

Recent analysis by Foote *et al.* [69] has provided insight into the folding of the two extracellular loops. These loops display the most similarity in sequence between connexins but still influence the allowed docking between different connexons across the extracellular gap [43,52]. A notable feature of the extracellular loops is that they each contain three strictly conserved cysteine residues that are essential for normal folding and/or channel function. Site-directed mutagenesis was used to shift four of the six conserved cysteines in the extracellular loops of β_1[Cx32], both individually, in all possible pairwise combinations and even some quadruple combinations. The results support a model in which all the disulfides are intramolecular, with bridges between the first cysteine in E1 and the third cysteine in E2, and the third cysteine in E1 with the first cysteine in E2. In addition, the pattern of periodicity for the cysteine positions that produced functional channels was consistent with a model in which the extracellular loops fold as antiparallel β sheets.

Perspectives

Several human diseases have now been related to connexin mutations. The X-linked form of Charcot–Marie–Tooth (CMTX) disease, an inherited demyelinating neuropathy, is caused by mutations of β_1[Cx32] [70,71]. Mutations of β_2[Cx26] are associated with the most common form of nonsyndromic neurosensory autosomal recessive deafness [72]. Some patients with developmental anomalies of the cardiovascular system may have mutations in the C-terminal domain of α_1[Cx43], the principal gap junction protein in the heart [73]. Obviously, the successful synthesis, trafficking, folding and assembly of gap junction channels is a prerequisite for normal gap junction channel function. The

potential to correlate the structure and biochemistry of gap junction channels with human disease makes this a particularly exciting area of research.

Acknowledgements

In accordance with the policies of Current Biology Publications, we regret that some papers published prior to 1996 could not be highlighted as being of special interest. This work was supported by grants from the National Institutes of Health (MMF and MY) and a Clinical Scientist Award in Translational Research from the Burroughs Wellcome Fund (MY). MMF and VMU are recipients of postdoctoral fellowships from the Deutsche Forschungsgemeinschaft and the American Heart Association, respectively. MY is an Established Investigator of the American Heart Association and Bristol–Myers Squibb.

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- •• of outstanding interest

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