

## Perspective

# Ion Channel Assembly: Creating Structures that Function

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With advances in recombinant DNA, structural, and electrophysiological techniques, much progress has been made in understanding the structure and, in particular, the function of ion channels. Less progress has been made in resolving the cell biological events that guide the assembly and trafficking of these proteins and that have a major impact on both structure and function. The assembly of an ion channel refers to the processes that transform newly synthesized, unfolded channel subunits into functional ion channels. The precise mechanisms by which any protein folds and assembles are unknown and the question of how proteins fold remains a major challenge in biology, attracting widespread attention (e.g., Brooks et al., 1998; Dobson and Ellis, 1998). Because single ion channels control the flow of  $\sim 10^7$  ions/s, the malfunction or improper targeting of even a few channels can be disastrous for a cell. To avoid this, ion channel assembly and targeting must occur with almost perfect fidelity. A good example of the consequences of channel misassembly is the  $\Delta F508$  mutation of the cystic fibrosis transmembrane regulator (CFTR), the cause of most cases of cystic fibrosis. The disease results from misfolding of the protein, which prevents CFTR delivery to the cell surface (Cheng et al., 1990).

The assembly of ion channels shares many features with other proteins produced in the secretory pathway. Certain viral membrane proteins, such as influenza hemagglutinin (HA), have been particularly well studied (Hammond and Helenius, 1995). It has been established that specialized mechanisms exist to assist in the folding and assembly of proteins produced in the secretory pathway. The same mechanisms appear to apply to ion channels. To start, mRNAs are selectively targeted to the endoplasmic reticulum (ER) membrane where assembly begins. The initial assembly events are cotranslational. These events include: (a) membrane insertion of subunits, (b) a set of different processing events such as attachment of the core N-linked glycan and signal sequence cleavage, and (c) initial rapid folding. Because these events are cotranslational, they proceed from the  $\text{NH}_2$  terminus to the  $\text{COOH}$  terminus and establish a vectorial order to the assembly. The rapid cotranslational events are followed by slower folding reactions where different domains can interact and other types

of processing occur, such as disulfide bond formation and proline isomerization. For the homotrimeric viral glycoproteins, such as HA and vesicular stomatitis virus glycoprotein, the subunits undergo a series of slow folding reactions and disulfide rearrangements (Braakman et al., 1992; de Silva et al., 1993). The slow posttranslational folding and processing precede and are a prerequisite for subunit oligomerization. Posttranslational folding, processing, and ultimately oligomerization of virtually all secretory pathway proteins occur in the ER, which provides "quality control" by identifying and degrading any misassembled proteins (Hurtley and Helenius, 1989; Helenius et al., 1992; Kopito, 1997).

While the assembly of ion channels in many respects is similar to that of other proteins produced in the secretory pathway, important differences are beginning to emerge (see Green and Millar, 1995). One reason for these differences is that ion channels are larger and more complex than most proteins. Almost all ion channels are heteromeric and appear to require a set subunit composition, stoichiometry, and the correct positioning of each subunit within the oligomer for proper function. Another complication with respect to assembly is that subunits are typically polytypic, with anywhere from 2 (e.g., inward-rectifying  $\text{K}^+$  channels) to 24 (e.g., voltage-gated  $\text{Na}^+$  channels) membrane spanning domains. As a consequence of their complex structure, ion channel assembly is slower and less efficient than that of many other membrane proteins. For nicotinic acetylcholine receptors (AChRs; Merlie and Lindstrom, 1983), voltage-gated  $\text{Na}^+$  channels (Schmidt and Catterall, 1986), and the cystic fibrosis transmembrane regulator (Ward and Kopito, 1994), assembly occurs in 2–3 h and only 20–30% of synthesized subunits are assembled.

Another feature of ion channels that distinguishes them from many other secretory pathway proteins is that their production, degradation, and subcellular location are under tight regulatory control. In addition to the subunits that form the functional ion channel unit, there are a host of "auxiliary" subunits (Gurnett and Campbell, 1996; Sheng and Wyszynski, 1997; Colledge and Froehner, 1998; Trimmer, 1998b) that help to regulate the expression, targeting, and stability of ion channels. While some of these subunits assemble with

ion channels in the ER (Nagaya and Papazian, 1997), others assemble in the Golgi stacks (Schmidt and Catterall, 1986) and at the plasma membrane (Froehner et al., 1990; Phillips et al., 1991). Thus, many ion channels differ from other secretory pathway proteins in that they continue to oligomerize with auxiliary subunits after release from the ER. The addition of these subunits at sites closer to or at the plasma membrane is likely to be important for the regulation of ion channel function.

#### *Difficulties in Assaying Assembly*

A variety of techniques are used to assay ion channel assembly (for recent reviews see Sheng and Deutsch, 1998; Trimmer, 1998a; Xu and Li, 1998), but few directly assay assembly. The most common methods measure the expression of the fully assembled ion channel either by a functional assay such as electrophysiological or flux measurements, or by tagging the channel by means such as metabolic labeling. Subunit regions involved in assembly are inferred by alterations using recombinant DNA methods and measuring how these changes affect expression. Alternatively, subunits and/or subunit fragments are expressed in isolation. By expressing less than the full complement of subunits and finding the combination that results in partially assembled complexes with properties expected of an intermediate structure, potential assembly intermediates can be identified. The yeast two-hybrid or protein overlay binding methods can screen for new proteins and subunit regions that associate with a particular channel subunit. All of these techniques are powerful means to identify potential assembly intermediates, subunit regions, and new components in assembly. However, because they do not directly assay assembly, other methods must be used to validate the findings.

Ion channel assembly is a dynamic process. To establish the precursor–product sequence of events that occurs during assembly, one must isolate and identify intermediates and follow them as they form and disappear during assembly. The only way to achieve this objective is through kinetic measurements. Small soluble proteins can be studied at high concentrations in a test tube, which allows precise biophysical measurements of protein folding and unfolding. Unfortunately, large biological ion channels do not assemble in a test tube. Most ion channels cannot even be assembled using *in vitro* translation methods (for an exception, see Rosenberg and East, 1992). Full assembly of an ion channel has only been studied using cultured cells. Even though cultured cells appear to be the system of choice, they too are problematic. For example, with the transient expression of heterologous AChR subunits, <1% of the subunits assemble into AChRs because of cell-to-cell variations in the ratio of the subunit

cDNAs taken up by the cells (Eertmoed et al., 1998). This inefficiency in the assembly process prevents direct measurements of many aspects of the assembly process. Nonetheless, cell cultures combine two critical features: (a) high enough levels of expression to assay assembly, and (b) the expression of a set of chaperone and processing enzymes required for assembly. Presently, the only kinetic assay used to study assembly in cultured cells is pulse–chase metabolic labeling of subunits (Millar et al., 1996; Trimmer, 1998a). By first pulse-labeling subunits, and then following the labeled subunits, subunit folding and oligomerization can be assayed directly (e.g., Merlie and Lindstrom, 1983; Schmidt and Catterall, 1986; Green and Claudio, 1993).

Another problem inherent in assays of assembly is the need to solubilize cells to isolate assembly intermediates formed intracellularly. Detergents used to solubilize membranes may cause the dissociation of subunits in partially assembled complexes even though the fully assembled ion channel is stable in the detergent. The associations between chaperone proteins and channel subunits also can be dissociated by detergent (Ou et al., 1993). Certain nonionic detergents such as CHAPS and digitonin tend to be better than other detergents at preserving associations. Detergent-induced dissociation also can be prevented by phospholipid-detergent mixtures (Helenius and Simons, 1975).

#### *Models of AChR Assembly*

The muscle-type AChR continues to be the best characterized ion channel in terms of its assembly, though much is also known about K<sup>+</sup> channel and CFTR assembly. AChRs are composed of four distinct, yet homologous subunits,  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  that assemble into AChR  $\alpha_2\beta\gamma\delta$  pentamers. The consensus membrane topology of the AChR subunits is shown in Fig. 1. At the amino terminal end, each subunit has a large extracellular domain that comprises approximately half the subunit's mass. All ligand binding sites, N-linked glycosylation sites, and disulfide bonds lie within this domain. There are four transmembrane regions and a large intracellular domain between the third and fourth transmembrane regions. At the carboxy terminus, there is a short stretch of amino acids on the subunit's extracellular side just after the fourth transmembrane region. This membrane topology, as well as other structural features, are shared by a family of neurotransmitter-gated ion channels that also includes neuronal AChRs, serotonin-3, glycine, and GABA<sub>A</sub> receptors, and it is likely that many aspects of AChR assembly are common to the whole family.

As shown schematically in Fig. 2, there are currently two models that describe the assembly of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits into the native AChR. In both models, assembly occurs along a pathway where a defined subset of

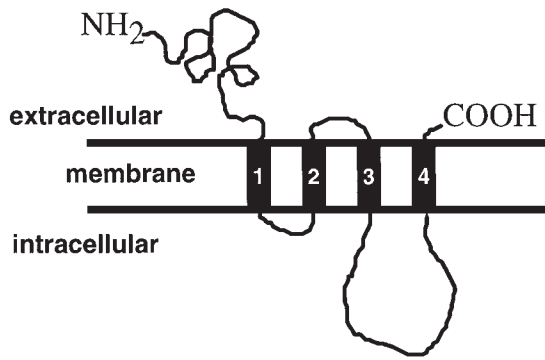


FIGURE 1. Consensus membrane topology of the AChR subunits. M1–M4 represent putative transmembrane regions.

the four subunits assemble into intermediates that then assemble into the  $\alpha_2\beta\gamma\delta$  pentamer. In the first model (Fig. 2 A), the “heterodimer” model (Blount and Merlie, 1991; Gu et al., 1991b; Saedi et al., 1991; Kreienkamp et al., 1995), the assembly is similar to that of HA in that most subunit folding is completed before oligomerization can occur. Posttranslationally, the subunits undergo a series of slow folding reactions before oligomerization, the best characterized being the formation of the  $\alpha$ -bungarotoxin (Bgt) binding site and the mAb 35 epitope on the  $\alpha$  subunit. Afterwards, the “mature”  $\alpha$  subunit associates with  $\gamma$  or  $\delta$  subunits to assemble  $\alpha\gamma$  or  $\alpha\delta$  heterodimers, and the heterodimers assemble with  $\beta$  subunits into  $\alpha_2\beta\gamma\delta$  pentamers. In this model, the two ACh binding sites, distinguishable by a difference in affinity for ligands such as d-tubocurarine (dTC), form on the  $\alpha\delta$  and  $\alpha\gamma$  heterodimers. The evidence for  $\alpha\gamma$  and  $\alpha\delta$  intermediates comes from studies where  $\alpha$  and either  $\gamma$  or  $\delta$  subunits were expressed in the absence of the other two subunits. Using steady state protocols, it was shown that heterodimeric complexes bind Bgt and that binding is blocked appropriately by agonists and antagonists.

In the second model (Fig. 2 B), the “sequential” model (Green and Claudio, 1993; Green and Wanamaker, 1997, 1998),  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits rapidly assemble into trimers. The slow posttranslational folding of the  $\alpha$  subunit occurs only after trimers are assembled. Soon after the Bgt binding site forms, the  $\delta$  subunit joins the complex to make  $\alpha\beta\gamma\delta$  tetramers. The first ACh binding appears on tetramers, after which the second  $\alpha$  subunit is added to make  $\alpha_2\beta\gamma\delta$  pentamers, and the second Bgt and ACh sites form on the pentamer. The evidence for this model is based on pulse–chase protocols in which assembly intermediates were identified by coimmunoprecipitation using subunit-specific antibodies, by immunoprecipitation with conformation-dependent antibodies, or by precipitation with affinity resin. Once they are formed, most  $\alpha\beta\gamma$  trimers could be

“chased” into  $\alpha\beta\gamma\delta$  tetramers, then into  $\alpha_2\beta\gamma\delta$  pentamers, and finally onto the cell surface as  $\alpha_2\beta\gamma\delta$  pentamers that demonstrated a precursor–product relation between each intermediate and the surface pentamers (Green and Wanamaker, 1998).

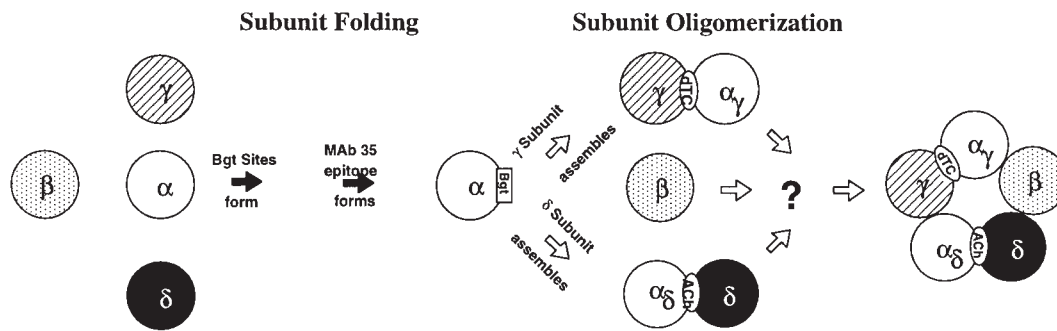
Although there are fundamental differences in the two AChR assembly models, there are no disagreements about the data on which either model is based. Similar data was obtained by all groups when cells expressing less than the full complement of subunits were studied (see also Green and Claudio, 1993). Contradictions with the heterodimer model only arose when cells expressing all four AChR subunits were studied. With all four subunits present, two features of the methods were critical in overcoming difficulties involved in isolating assembly intermediates. First, AChR complexes were solubilized in a detergent other than Triton-X 100 to prevent the dissociation of most AChR assembly intermediates (Green and Claudio, 1993). Instead, subunit complexes were solubilized with a mixture of Lubrol PX and phosphatidylcholine. Second, the *Torpedo* AChR assembly is temperature sensitive (Claudio et al., 1987). When the temperature is lowered to 20°C, the rate of assembly is slowed by more than an order of magnitude, and the slow kinetics greatly aided in the isolation of assembly intermediates. Although the *Torpedo* AChR subunits were used to obtain most of the data in support of the sequential model, many features of the sequential model were verified with the mouse  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\delta$  subunits at 37°C (Green and Claudio, 1993).

#### *Ion Channel Subunit Associations and Folding*

One difference between the two models is that in the sequential model subunits rapidly associate into trimers before most of the posttranslational folding occurs. The associations are so fast that they could be cotranslational (Green and Claudio, 1993).  $K^+$  channel subunits similarly associate rapidly, perhaps cotranslationally (Deal et al., 1994; Shi et al., 1996). If subunit associations occur cotranslationally, it is likely that the associating regions are at the  $NH_2$ -terminal end of the subunits. This is consistent with studies that have shown that regions near the  $NH_2$  terminus of AChR subunits (Gu et al., 1991a; Yu and Hall, 1991; Sumikawa, 1992) and  $K^+$  channel subunits (Li et al., 1992; Shen et al., 1993) mediate subunit associations. One reason why subunits might associate so rapidly is to protect critical domains from exposure to either the membrane or the aqueous environment, which should help to prevent misfolding of these domains.

Another difference between the two models is that, in the sequential model, subunits continue to fold during subunit assembly and even after all of the subunits have assembled together into pentamers. There is considerable evidence that subunit folding reactions occur

## A Heterodimer Model



## B Sequential Model

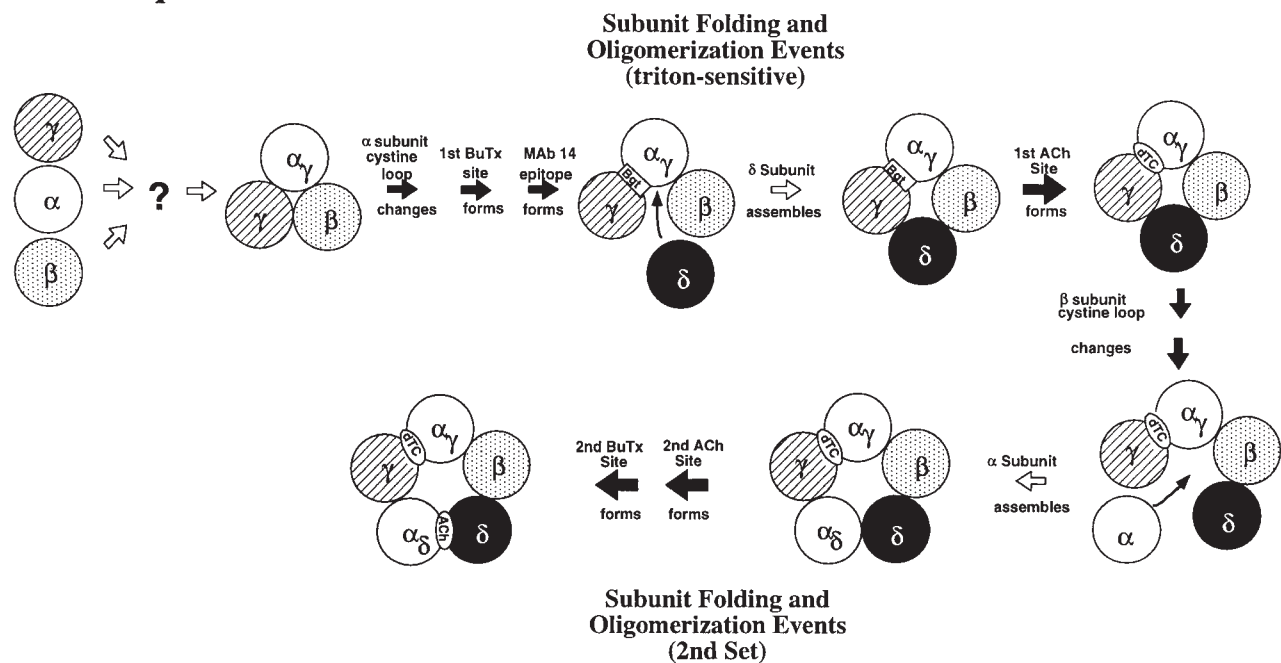


FIGURE 2. The two models of AChR assembly. Subunit folding reactions are denoted by the filled arrows and oligomerization events by the open arrows. (A) Formation of the Bgt binding sites and the mAb 35 epitope precedes all subunit associations. The two different ACh binding sites, the high affinity dTC site (dTC) and the low affinity dTC site (ACh), appear on  $\alpha\gamma$  or  $\alpha\delta$  heterodimers, respectively. (B) A conformational change in the  $\alpha$  subunit cysteine loop region and the formation of the first Bgt binding site and mAb 14 epitope occur on  $\alpha\beta\gamma$  trimers. A conformational change in the  $\beta$  subunit cysteine loop region and formation of the first ACh binding site, the high affinity dTC site, occur on  $\alpha\beta\gamma\delta$  tetramers. Finally, the second Bgt and ACh binding sites appear on  $\alpha_2\beta\gamma\delta$  pentamers. Note that we are not proposing that subunits in the trimers and tetramers reposition to allow the insertion of unassembled  $\delta$  and  $\alpha$  subunits. This is only shown to illustrate the possible links between the subunit additions and the conformational changes on the trimers and tetramers.

after each oligomerization step (Fig. 2 B). Furthermore, if a specific disulfide bond on  $\alpha$  subunits does not form, assembly is completely blocked after assembly of  $\alpha\beta\gamma$  trimers. If the homologous  $\beta$  subunit disulfide bond does not form, assembly is blocked at a later step, after assembly of  $\alpha\beta\gamma\delta$  tetramers (Green and Wanamaker, 1997). Thus, the processing and folding events between oligomerization steps are required for assembly. The picture that has emerged from this work

is that subunit associations and folding during assembly are continuous and interdependent processes. The assembly of the  $K^+$  channel appears to be similar in that subunit folding occurs during and after the assembly of the  $\alpha\beta$  subunit tetramer (Schulteis et al., 1998). This interdependence between subunit associations and folding is shown schematically in Fig. 2 B by a major rearrangement of the trimer and tetramer subunits to allow for the insertion of unassembled  $\delta$  and  $\alpha$  subunits.

Note that we know little about the structure of these assembly intermediates, and we are not proposing that such a drastic structural change is actually occurring.

The available evidence indicates that AChRs (Smith et al., 1987) and K<sup>+</sup> channels (Nagaya and Papazian, 1997) are assembled in the ER. However, it is uncertain whether the late folding reactions occur in the ER since it has been recently shown that subunit folding of AChR and K channel subunits continues after formation of the AChR pentamer and K<sup>+</sup> channel tetramer. For both AChRs (Green and Wanamaker, 1998) and K<sup>+</sup> channels (Schulteis et al., 1998), the late folding appears to be required for normal channel function. Thus, late folding events may serve to regulate whether channels are in their functional state after release from the ER at sites closer to where the channel will be targeted.

### Conclusions and Perspective

Several features of ion channel assembly appear to be different from that of other secretory pathway proteins. Subunit associations can occur rapidly after synthesis, and posttranslational folding and processing of subunits can occur throughout assembly, even after the final oligomeric complex is formed. That ion channel subunits continue to fold after associating with other subunits should be considered when designing and interpreting experiments about channel structure and assembly. This is particularly relevant for experiments in which less than the full complement of subunits are studied or in which a subunit fragment is used to substitute for the full-length subunit. For example, the crystal structure of the NH<sub>2</sub>-terminal K<sup>+</sup> channel region, T1, thought to mediate associations between subunits, was obtained by studying the T1 fragment (Kreusch et al.,

1998). T1 formed a tetramer 20 Å in length with a central aqueous pore that was suggested to be the structure of the channel's cytoplasmic vestibule. Another interpretation, however, is that this is a structure that forms rapidly to initiate K<sup>+</sup> channel assembly, and that the subunits may continue to fold during assembly, ultimately forming a different structure.

Yet to be determined is the identity of the factors required for the assembly of ion channels in addition to the subunits themselves. Presently, ion channels can assemble only in the environment of a cell. Some nicotinic receptors are properly assembled only in cells of neuronal origin containing additional unidentified factors (Cooper and Millar, 1997, 1998; Rangwala et al., 1997). Chaperone proteins are likely to be some of the unidentified cellular factors required for assembly. The ER chaperone proteins BiP (Blount and Merlie, 1991; Paulson et al., 1991; Forsayeth et al., 1992) and calnexin (Gelman et al., 1995; Keller et al., 1996, 1998) associate with unassembled AChR subunits and may directly aid the assembly process. Interestingly, the proline isomerase, FKBP12, is a subunit in the functional ryanodine receptor complex (Brillantes et al., 1994). Furthermore, NSF and α and β SNAPS associate with AMPA receptors in the dendrites of hippocampal pyramidal cells (Osten et al., 1998) and appear to function as "chaperones" that are required for channel function or recycling (Nishimune et al., 1998). These findings raise important questions for future investigations about ion channel assembly. At what point does channel assembly end? Does it continue at the plasma membrane? Finally, can the folding and oligomerization events that occur during assembly be distinguished from conformational changes and protein associations that occur at the plasma membrane?

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