The Role of the Cystine Loop in Acetylcholine Receptor Assembly*

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Nicotinic acetylcholine receptors (AChRs) are composed of α, β, γ, and δ subunits, assembled into αβγδ pentamers. A highly conserved feature of ionotropic neurotransmitter receptors, such as AChRs, is a 15-amino acid cystine “loop.” We find that an intact cystine loop is necessary for complete AChR assembly. By preventing formation of the loop with 5 mM dithiothreitol, AChR subunits assemble into αβγ trimers, but the subsequent steps in assembly are blocked. When α subunit loop cysteines are mutated to serines, assembly is blocked at this step, i.e. after assembly of αβγδ tetramers and before the addition of the second α subunit. After formation of the cystine loop, the α subunit undergoes a conformational change, which buries the loop. This conformational change is concurrent with the step in assembly blocked by removal of the disulfide bond of the cystine loop, i.e. after assembly of αβγ trimers and before the addition of the δ subunit. The data indicate that the α subunit conformational change involving the cystine loop is key to a series of folding events that allow the addition of unassembled subunits.

Based on their findings, the “heterodimer” model was proposed, where the α subunit must first fold or “mature,” as assayed by the formation of the α-bungarotoxin (BuTx) binding site and antigenic epitopes, before assembling with other subunits. The mature α subunit assembles with γ or δ subunits in parallel to form αγ and αδ heterodimers, and the heterodimers associate together and with β subunits to form αβγδ pentamers. We have developed techniques that have allowed isolation of assembly intermediates in cells stably expressing all four AChR subunits (7, 8) and have obtained results at odds with the heterodimer model. Instead of heterodimers, two partially assembled complexes, αβγ trimers and αβγδ tetramers, were isolated. αβγ trimers, which assemble extremely rapidly, were then assembled first into αβγδ tetramers and then into αβγδ pentamers. Our data demonstrated that assembly occurs sequentially, each step being the addition of an uncomplexed subunit. We also demonstrated that the α subunit maturation steps, which were thought to precede its assembly, occurred after assembly into αβγ trimers but prior to the addition of the δ subunit. These folding events require a specific combination of subunits and correlate in time with the δ subunit addition. The data led us to suggest that the α subunit maturation steps are folding events forming the δ subunit recognition site, i.e. the site where the δ subunit associates with the αβγ trimer.

Our goal has been to identify posttranslational processing sites and regions on the AChR subunits involved in AChR subunit folding and assembly. A good candidate is the highly conserved region defined by a pair of cysteine residues separated by a stretch of 13 amino acids, which is found on the neurotransmitter-binding, extracellular domain of the subunits (Fig. 1, A and B). The two cysteines form a disulfide bridge on all four Torpedo AChR subunits (9, 10). Analogous cystine “loops” appear to form on other neurotransmitter-gated ion channel subunits, which include all muscle and neuronal AChR subunits and all GABA_α, γ, and 5HT_3 receptor subunits. Other residues in the loop are identically conserved across species from Caenorhabditis elegans to mammals and are even conserved on some of the glutamate receptor subunits (11). Site-directed mutations of the cysteines in the α subunit prevented BuTx binding site formation and reduced AChR expression (12), which suggested that the cystine loop is critical for subunit conformational stability or assembly. A recent study where the conserved proline in the cystine loop (see Fig. 1, A and B) was mutated also suggested that the cystine loop may play a role in subunit assembly (13). However, mutated α or β subunits lacking the cystine loop disulfide bond assembled with other wild type subunits (14, 15) and produced functional receptors (15). Based on these data, it was suggested that formation of the cystine loop is not required for subunit assembly but instead plays an important role in the rate that subunits are degraded and in the efficiency of transport of AChRs to the cell surface. Contrary to this viewpoint, we demonstrate in this paper that an intact cystine loop is essential for proper AChR subunit assembly. Elimination of the cystine loop separately on the α and β subunits blocks different steps during

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EXPERIMENTAL PROCEDURES

Cystine Loop Mutations, Cell Lines, and AChR Subunit Expression—The cystine loop mutations, α128C→A128, α128G→A128, and β128G→A128 (15), which were the subject indicates which cystine residues were replaced by serine residues, were a generous gift from Dr. Sumikawa. The cystine loop mutation, α128C→A128, was created by inserting the α128C→A128 fragment back into α128G→A128 and α128G→A128 subunits, the C. elegans nAChR subunit δ-3 (33), the calf brain GABA-A receptor α1 and β1 subunits (34, 35), the rat brain glycine receptor α1 subunit (36), the mouse 5HT3 receptor subunit (37), and the rat brain glutamate receptor subunit Glu R1 (11). Furthermore, the cycline loop is expressed in the extracellular region. The putative secondary structure of AChR subunits is displayed, showing the four membrane-spanning regions, M1–M4 (for a recent review, see Ref. 38).

assembly. For the α subunit, the cystine loop undergoes a conformational change, which appears to be an event required for assembly to continue.

RESULTS

Reduction of Disulfide Bonds Alters Subunit Assembly—When added extracellularly to cultured cells, DTT reaches the endoplasmic reticulum and prevents the formation of protein disulfide bonds without altering most other cellular functions (18). Cells stably expressing the four Torpedo AChR subunits were subjected to an [35S]methionine pulse-chase protocol in the absence or presence of 5 mM DTT (Fig. 2, A and B). Labeled subunits were immunoprecipitated with either a γ subunit-specific monoclonal antibody (mAb 168) or a novel, conformation-dependent mAb 14, to assay for subunit assembly and formation of the mAb 14 epitope. During a 30-min pulse of [35S]methionine, αβγ trimeric forms were shown as the coprecipitation of predominantly α and β subunits with the γ subunits (Fig. 2A). During the chase in the absence of DTT, progressively more δ subunits are added to the trimers, followed by the addition of the second α subunit as shown by the doubling in the amount of α subunit relative to the other coprecipitated subunits. These two subunit additions are better resolved by the mAb 14 immunoprecipitations (Fig. 2, A and C). Since the mAb 14 epitope forms on αβγ trimeric forms just prior to the addition of the δ subunit (8), these immunoprecipitations show the complete time course of both the addition of the δ and second α subunits to the αβγ trimeric.

In the presence of DTT, the subunits clearly retain the ability to assemble into αβγ trimers and with approximately the same efficiency as occurs in the absence of DTT (Fig. 2B). This result is at odds with a recent study (19), which suggested that 5 mM DTT completely blocks AChR subunit assembly. There
are several differences between this and the other study that explain the conflicting results. Probably the most important difference in terms of the ability to observe $\alpha\beta\gamma$ trimers was that their only assay for measuring the assembly of $\alpha$ subunits with other subunits was an immunoprecipitation with $\alpha$ subunit-specific antibodies. Obviously, $\alpha\beta\gamma$ trimers could never be observed with this assay. Another difference was the protocol used to solubilize the AChR subunit complexes. We have shown previously that solubilization in 1% Triton X-100 causes the assembly intermediates to dissociate and that the subunit associations are stable when solubilized with a combination of Lubrol PX and phosphatidylcholine (8).

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**FIG. 2.** Reduction of disulfide bonds blocks AChR subunit assembly. A and $B$, AChR subunit assembly in the absence or presence of 5 mM DTT. A mouse L cell line, stably expressing all four Torpedo AChR subunits (the $\alpha\beta\gamma\delta$ cell line; Ref. 7), was pulse-labeled with $[^{35}\text{S}]$methionine for 30 min at 37 °C and chased at 20 °C in the absence or presence of 5 mM DTT for the indicated times. Labeled subunits were immunoprecipitated with either the $\gamma$ subunit-specific mAb 168 or the $\alpha$ subunit, conformation-dependent mAb 14 (24). The band labeled as $\alpha'$ has previously been shown to be different from the $\delta$ subunit (8) (see also Fig. 5B), although it migrates just above the $\delta$ subunit. Further evidence that this band is not the $\delta$ subunit is displayed in lane 1 of Fig. 2A. Cells stably expressing only the Torpedo $\alpha$, $\beta$, and $\gamma$ subunits were subjected to the same pulse-chase protocol as the $\alpha\beta\gamma\delta$ cells and immunoprecipitated with mAb 168. The $\alpha'$ band coprecipitates with the other subunits in the absence of any $\delta$ subunit expression. $C$, time course of mAb 14 epitope formation. Displayed is the quantification of the mAb 14-precipitated $\alpha$, $\beta$, $\gamma$, and $\delta$ subunit bands analyzed by SDS-polyacrylamide gel electrophoresis and quantified from resultant fluorographs by scanning densitometry. Also displayed for comparison are the scanned values of the $\delta$ subunits coprecipitating with the precipitated $\gamma$ subunits. The percentage assembled values are the scanned values divided by the value for the $\beta$ subunit at the 48-h chase time (100%) and are shown to emphasize the time course of the $\alpha$ subunit doubling. $D$, the rate of $\gamma$ subunit degradation in the absence or presence of DTT. Displayed are the scanned values for the $\gamma$ subunit bands chased in the absence or presence of DTT and precipitated by the $\gamma$ subunit-specific mAb (Fig. 2, A and $B$). Also displayed are values for the $\beta$ subunits that coprecipitate with the $\gamma$ subunits in the absence of DTT (Fig. 2A). The data are plotted on a semilog scale. In the absence of DTT, the decay of the $\gamma$ subunit signal is biphasic. The slowly decaying component corresponds to assembled $\gamma$ subunits as shown by the similar rate of decay of the assembled $\beta$ subunits. To estimate the rate of decay of unassembled $\gamma$ subunits, the 48-h value was subtracted from the other values and plotted as the $\gamma$ adjusted symbols. $E$, the presence of DTT blocks BuTx site formation. $^{125}\text{I}$-BuTx binding to the cell lysate of $\alpha\beta\gamma\delta$ cells was measured for cells grown in the absence or presence of 5 mM DTT for different lengths of time. The 0 time point is the time at which the cultures were shifted from 37 to 20 °C to start subunit assembly. A single 10-cm culture was used for each time point.
assembly after the association of the α subunit with DTT. We conclude that the addition of DTT blocks subunit assembly of 128/142AChR subunits, which contain an additional cysteine formed between adjacent cysteines. These findings suggest that the loss of AChR binding was due to the formation of the mAb 35 epitope during subunit assembly in the 128/142 subunit, and the efficiency of these interactions was reduced 2–3-fold relative to wild-type subunit assembly (see Fig. 2B, lane 2, and Fig. 6C, lane 1). Formation of the mAb 35 epitope during subunit assembly in the 128/142 subunit results in the loss of AChR binding, subunit assembly, or cystine loop disulfide. The efficiency of these interactions was reduced 2–3-fold relative to wild-type subunit assembly (see Fig. 2A, lane 2, and Fig. 6C, lane 1). Formation of the mAb 35 epitope during subunit assembly in the 128/142 subunit was significantly lower than in wild-type subunit bands (Fig. 4A). The data were plotted on a semilog scale. The half-life was estimated to be 10.9 h for the wild-type subunits based on a least squares fit of an exponential function to the data, which is within the range of values found for wild-type subunits under the same conditions (8).

**Assembly of Cysteine Loop Disulfide Bond on the α Subunit Blocks Assembly**—The addition of DTT in the above experiments prevents disulfide bond formation for all AChR subunits as well as for other proteins that might affect AChR subunit assembly. To test whether the cysteine loop on AChR subunits is involved in the DTT block of assembly, we obtained mutations of the Torpedo AChR α and β subunits in which cysteines forming the cysteine loop were replaced by serines (15). Of the four AChR subunits, only α subunits contain an additional cysteine formed between adjacent cysteines (cysteines 192 and 193) located at the ACh binding site (20) (Fig. 1). Deletion mutations eliminating that cysteine cause the loss of ACh binding but have no effect on BuTx binding, subunit assembly, or cell surface expression (12, 14). An α subunit construct was created, α128/142g, where both cysteine loop cysteines were replaced by serines (15). Of the four AChR subunits, only α subunits contain an additional cysteine formed between adjacent cysteines (cysteines 192 and 193) located at the ACh binding site (20). Formation of the α128/142gα subunit was significantly lower than in wild-type subunit bands (Fig. 4A). The data were plotted on a semilog scale. The half-life was estimated to be 10.9 h for the wild-type subunits based on a least squares fit of an exponential function to the data, which is within the range of values found for wild-type subunits under the same conditions (8).
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The band were pulse-labeled with [35S]methionine and chased for the indicated times. The half-life was estimated to be 15.4 h for abgd complexes. This indicates that intracellular aβ128γδ subunit-specific mAb 148 (lanes 2–6), or mAb 14 (lane 7) coprecipitates with the other subunits. The band (~43 kDa) that migrates between the alpha and beta subunit bands is believed to be actin. B, the rate of beta28 subunit degradation. Displayed are the scanned values for the beta28 subunit bands and the alpha subunits that coprecipitated with the beta28 subunits in Fig. 5A. The data are plotted on a semilog scale. The half-life was estimated to be 15.4 h for beta28 subunits based on a least squares fit of an exponential function to the data, which is within the range of values found for the wild type subunits under the same conditions. C, sedimentation of aβ128γδ subunit complexes. aβ128γδ and aβ28γδ cells were bound with 125I-BuTx as in Fig. 5B and size-fractionated on a 5–20% linear sucrose gradient. Shown are 125I counts from the 125I-BuTx-bound intracellular complexes in fractions 4–14, which were immunoprecipitated with the beta subunit-specific mAb 148. aβ28γδ cell surface complexes were first bound with cold BuTx to block 125I-BuTx binding to the surface AChRs. Also displayed on the figure are the standards, alkaline phosphatase (5.4 S), catalase (11 S), and surface aβ28γδ complexes (9 S; dashed line). The aβ128γδ complexes peak at 8 S as estimated from a least squares linear regression fit to the S values of the standards. The shape of the intracellular aβ128γδ profile can be duplicated by the sum of the intracellular aβ128γδ profile reduced by 60% plus the 9 S peak cell-surface aβ28γδ complexes. This indicates that intracellular aβ128γδ complexes are similar in size and composition to intracellular aβ28γδ complexes with the exception of the aβ28γδ complexes in the 9 S peak. The broad profile observed for the intracellular AChR complexes relative to the cell surface aβ28γδ 9 S peak has been seen in other studies both for the Torpedo subunits at reduced temperature (4, 8, 40) and the mouse subunits at 37 °C (3, 5, 6, 8, 10). D, the effects of the a28/142 and beta28 subunits on subunit assembly are consistent with the model shown. The a28/142 subunit and DTT block assembly after the formation of trimers but before the formation of the BuTx binding site and mAb 14 epitope. The beta28 subunit blocks assembly after the addition of the beta subunit but before the addition of the second alpha subunit.
wild type subunit assembly (see Fig. 2A, lane 2, and Fig. 6C, lane 1). A similar reduction in assembly efficiency was observed for subunit complexes containing the β128 subunit (Fig. 5A). The decrease in assembly efficiency could be caused by misfolding of some of the mutated subunits, which has been observed for other proteins where cysteines have been mutated (21). To examine whether α128/142 subunits are misfolded, we tested whether the α128/142 subunits are recognized by mAb 35. mAb 35 is a conformation-dependent antibody specific for the α subunit (2). mAb 35 differs from mAb 14 in that its epitope forms on unassembled α subunits, and it forms well before the mAb 14 epitope (8). As shown in Fig. 4B, the results obtained with the mAb 35 precipitation are similar to the results with a subunit-specific polyclonal antibodies (Fig. 4A, lanes 2–6). mAb 35 recognizes a large percentage of unassembled α128/142 subunits as well as the α128/142βγ complexes, which indicates that the unassembled α128/142 subunits are not grossly misfolded.

Elimination of the Disulfide Bond on the β Subunit Blocks Assembly at a Later Step—The finding that the BuTx binding site forms on complexes that contain the β128 subunit (Fig. 3B) suggests that subunit assembly with the β128 subunit progresses to a later step than assembly with the α128/142 subunit. The assembly of the β128 subunit with the wild type α, γ, and δ subunits was characterized using an [35S]methionine pulse-chase protocol with the αβ128γδ cells as shown in Fig. 5A, lanes 1–7. In contrast to subunit assembly with the α subunit mutation or after DTT treatment, assembly continued after the formation of αβγ trimers. Immuno precipitation of the labeled subunits with β subunit-specific antibodies (lanes 5–6) coprecipitated the δ subunit as well as the α and γ subunits. The mAb 14 epitope forms on the assembled complexes as shown by precipitation of the [35S]methionine-labeled subunits by mAb 14 (Fig. 5A, lane 7), and as with the β subunit-specific antibodies, all four subunits are precipitated. The BuTx binding site also forms on these complexes as shown by the 125I-BuTx-bound complexes precipitated by β subunit-specific antibodies (Fig. 3B).

The assembly of αβ128γδ complexes differs from the assembly of the four wild type subunits in that the second α subunit is not added to the αβ128γδ complexes. The pulse-chase experiments demonstrate that the amount of α subunit coprecipitated with β128 subunits (Fig. 5B) is constant throughout the pulse-chase protocol, in contrast to the doubling observed with wild type subunit assembly. To further investigate the nature of the αβ128γδ subunit complexes formed, their sedimentation on a linear sucrose gradient was determined (Fig. 5C). The αβ128γδ complexes, bound with 125I-BuTx and immunoprecipitated with β subunit-specific antibodies as in Fig. 3B, migrated in a peak at a value of 8.3 S. The size of the αβ128γδ complexes is consistent with tetramers (8), which sediment at ~8 S and are smaller than the cell-surface αβγδ complexes, which sediment at 9 S.

The effects of the two subunit mutations and DTT on subunit assembly are summarized in Table I and are consistent with the model displayed in Fig. 5D. Based on the results of both the pulse-chase experiment and the sedimentation of the mature αβ128γδ complexes, the end product of subunit assembly in the αβ128γδ cells is an αβ128γδ tetramer. Thus, the β128 subunit, like the α128/142 subunit, causes a block in subunit assembly. However, the β128 subunit blocks assembly at a later step, after the formation of αβγδ tetramers and before the addition of the second α subunit. The failure of αβ128γδ complexes to fully assemble into pentamers provides an explanation for why αβ128γδ complexes are not transported to the cell surface (Fig. 5A). Since αβ128γδ complexes never fully assemble, most likely they are retained and degraded in the endoplasmic reticulum (22).

The α Subunit Cystine Loop Changes Conformation during Assembly—To address when the α subunit cystine loop forms during assembly, we made use of a mAb (mAb 259) that selectively recognizes the α subunit only when the cystine loop is intact (23). This specificity of the mAb is demonstrated in Fig. 6A, where the mAb failed to recognize either the reduced α subunit or the mutated α subunit with the cystine loop elimi-
nated. In Fig. 6, B and C, the αβγδ cells were pulse-labeled with [35S]methionine and chased to test at what point in assembly the α subunit is recognized by the cystine loop mAb. Immediately following the half-hour [35S]methionine pulse, the cystine loop mAb precipitated about the same amount of labeled α subunit as our α subunit-specific polyclonal antibodies (Fig. 6B, compare lanes 1 and 3). The α subunit cystine loop, thus, must form shortly after the subunit is synthesized. Since the events blocked by DTT and by the α subunit mutation occur several hours after the synthesis of the α subunit, the block occurs after the formation of the cystine loop.

The ability of the cystine loop mAb to precipitate α subunits diminished with time. The loss of the epitope occurs during subunit assembly as shown by the difference in the subunits precipitated by the cystine loop mAb compared with the subunits precipitated with the α subunit-specific polyclonal antibodies (Fig. 6, B, lanes 3–7, and C, lanes 1–5). During the chase, the amount of α subunit precipitated by the cystine loop mAb is progressively reduced. By the last chase time, the cystine loop mAb precipitated very little α subunit (Fig. 6B, lane 7) and also was unable to precipitate any significant amount of cell surface AChRs (Fig. 7A). The mAb epitope, although inaccessible to the mAb, is still present on the α subunit as demonstrated by the cystine loop mAb precipitating as much α subunit as the α subunit-specific antibodies after the subunits were denatured with SDS (Fig. 6, B and C, compare lanes 8 and 5). The loss of the epitope thus results from a conformational change that buries the epitope.

The Conformational Change Occurs before Formation of the BuTx Binding Site and the Addition of the δ Subunit—The only AChR subunit complexes that appear to be recognized by the cystine loop mAb are αβγδ trimers. This is most clearly observed at the times when the δ subunit is maximally assembled with the other subunits, i.e. 24–48 h after assembly begins (Fig. 2, A and C). At these times, only δ and γ subunits coprecipitate with the α subunits recognized by the cystine loop mAb (Fig. 6B, lanes 6 and 7), although complexes containing δ as well as the other three subunits are present when all α subunits are precipitated (Fig. 6C, lanes 4 and 5) or when the subunits are precipitated by the γ-specific mAb or mAb 14 (Fig. 2A). At earlier times in the pulse-chase experiments of Fig. 6, B and C, the α′ band, which is specifically recognized by the cystine loop mAb and α subunit-specific antibodies, may be obscuring the presence of any δ subunit. The DTT block of assembly was used to address whether δ subunits are present at the earlier times during assembly. As shown in Fig. 2B, the addition of 5 mM DTT blocks assembly so that only αβγδ trimers assemble. In Fig. 6D, subunit complexes were precipitated with the α-specific antibodies after cells were treated with 5 mM DTT. There are no significant differences between the results in Fig. 6B (lanes 4–7) and those of Fig. 6D. From this we conclude that the complexes recognized by the cystine loop mAb are identical to the αβγδ trimers precipitated by the α-specific antibodies in Fig. 6D.

To further characterize the subunit complexes recognized by the cystine loop mAb, we tested whether the BuTx binding site and the mAb 14 epitope form on these complexes. The cystine

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**TABLE I**

Characteristics of assembly intermediates isolated in different cell lines and under different conditions

| Subunit Complexes | αβγδ Trimers | αβγδ Tetramers | αβγδ Pentamers | Cell Surface BuTx Binding | Total Cell BuTx Binding | mAb 14 Epitope |
|-------------------|-------------|----------------|----------------|-------------------------|------------------------|----------------|
| αβγδ Cells        | Yes         | Yes            | Yes            | Yes                     | Yes                    | Yes            |
| αβγδ Cells with DTT | Yes        | No             | No             | Yes                     | Yes                    | Yes            |
| α121α213βγδ Cells | Yes         | No             | No             | Yes                     | Yes                    | Yes            |
| αβγδ Cells        | Yes         | Yes            | Yes            | Yes                     | Yes                    | Yes            |
| Cystine loop-specific mAb complexes | Yes | No | No | No | No | No |

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**FIG. 7.** The BuTx binding site does not form on subunit complexes recognized by the cystine loop mAb. A and B, the mAb that specifically recognizes the α subunit cystine loop does not precipitate [125I]BuTx-bound complexes containing the γ subunit and mAb 14 epitope. Both cell surface (A) and total cell (B) [125I]BuTx binding were performed on the αβγδ cells. The cystine loop mAb (mAb 259) precipitated less than the cell surface counts measured in the presence of carbamylcholine (αβγδ cells + carb), while mAb 35 precipitated 60% of the total number of cell surface counts (αβγδ cells). The cystine loop mAb precipitated a significant number of total cell [125I]BuTx binding sites, about 12% of the sites precipitated by mAb 14 and the γ subunit-specific mAb, mAb 168. However, the number of sites precipitated by the cystine loop mAb was not reduced if the precipitation was performed after the precipitation by mAb 14 or the γ subunit-specific mAb. The data indicate that the intracellular [125I]BuTx binding sites precipitated by the cystine loop mAb do not contain the γ subunit or the mAb 14 epitope. These BuTx sites appear to be a small population of the unassembled α subunits. Each bar is the mean and S.D. determined from measurements on 4–6 wells of a 6-well plate (A) or 4–6 6-cm plates (B).
FIG. 8. Cystine loop “switch” model. Based on our results, we propose the cystine loop “switch” model, where the formation of the subunit recognition sites for the δ subunit and second α subunit depend on the conformation of the α and β subunit cystine loops as diagrammed. The subunit recognition sites are created by a series of folding events. The folding events that create the δ subunit recognition site include the formation of the BuTx site and the mAb 14 epitope and the change in conformation of the α subunit cystine loop. The change in conformation of the α subunit cystine loop is essential and occurs early in this chain of events. The cystine loop is modeled as a switch. In the cystine loop “up position,” δ subunit recognition site formation and the rest of assembly are blocked. In the cystine loop “down position,” subunit recognition site formation and assembly continue. A similar role for the β subunit cystine loop is proposed for the formation of the second α subunit recognition site.

plexes recognized by the cystine loop mAb appear to be αβγ δ trimers. The data further indicate that the cystine loop conformational change occurs prior to the formation of the BuTx binding site and the mAb 14 epitope. The point during subunit assembly when the cystine loop conformational change takes place is evident in the pulse-chase experiment of Fig. 6B. The loss of the cystine loop epitope during assembly, shown by decreasing amounts of α, β, and γ subunits that are precipitated by the cystine loop mAb, begins at 3-h time point and is almost completed by the 24-h time point (Fig. 6B, lanes 4–6). This is the time period during which the BuTx site and mAb 14 epitope form and are quickly followed by the addition of δ subunits to the trimers (Fig. 2, A and C; see also Ref. 8). The similarity in the kinetics of the cystine loop conformational change and these assembly events together with the block of these events by α126V12 DTT demonstrate a strong correlation between the α subunit cystine loop conformational change and the assembly events. As summarized in Table I, there is a close identity between the subunit complexes precipitated by the cystine loop mAb and those assembled in the presence of DTT and with the α subunit mutation. Altogether the data indicate that after the α subunit cystine loop forms, it undergoes a conformational change, which is essential for the BuTx site and mAb 14 epitope to form and for the δ subunit to associate with the αβγ δ trimer.

DISCUSSION

The strong conservation of the cystine loop among the different ionotropic neurotransmitter receptors and throughout evolution suggests that this structure plays a vital role with respect to the receptors. In this paper, we demonstrate that the cystine loop is essential for different events that take place during subunit assembly. Events occurring during the assembly of the AChR can be broadly classified as either subunit folding or oligomerization. Previously, we observed that AChR subunits continued to fold after associations with other subunits (8). The data suggested a link between subunit folding and subsequent subunit associations. We proposed that the folding events, BuTx binding site, and mAb 14 epitope formation, are part of the process to create a recognition site for the incoming δ subunit. In support of this hypothesis, we find that eliminating the α subunit cystine loop blocks these folding events and also blocks the subsequent addition of the δ subunit to the αβγ δ trimer. As shown in the model in Fig. 8, we suggest that a subunit recognition site for the δ subunit is created concurrently with the α subunit cystine loop conformational change. Because elimination of the β subunit cystine loop blocks the addition of the second α subunit to the αβγ δ tetramer, we further propose that the recognition site for the second α subunit is created in parallel with a change in the conformation of the β subunit cystine loop.

Formation of the different subunit recognition sites is likely to involve large rearrangements of the assembly intermediates. Such a rearrangement occurs on the α subunit. Three folding events, 1) the cystine loop conformational change, 2) the formation of the BuTx binding site, and 3) the formation of the mAb 14 epitope, occur at about the same time and precede the addition of δ subunits to αβγ δ trimers. The three events occur on three separate regions that span the length of the N-terminal domain of the α subunit. The mAb 14 epitope overlaps or is very near to the main immunogenic region (24) at amino acids 67–76 (25, 26), while the BuTx binding site is at the other end of this domain in the region of amino acids 185–196 (27, 28). Regions of γ subunits are also involved in these events, since the presence of this subunit is necessary for the mAb 14 epitope to form and greatly enhances formation of the BuTx binding site (8). Since the α subunit cystine loop conformational change is part of a large rearrangement of the α subunit, it is possible that the cystine loop itself does not directly associate with the δ subunit during the assembly of the αβγ δ tetramer. Instead, other regions, involved in subsequent folding events and distant from the cystine loop, could associate with the δ subunit.

A feature of our model (Fig. 8) is that AChR subunit assembly is controlled by the ordered formation of subunit recognition sites. These events are rate-limiting, as shown by the kinetics of the δ and second α subunit additions, and as such provide checkpoints during assembly where the fidelity of the assembly process can be tested. Each subunit recognition site would form only if assembly had proceeded properly up to that point. Any misfolded or misassembled intermediates would be prevented from participating in later stages of assembly, and these improperly assembled subunits would be selectively retained and targeted for degradation by the endoplasmic reticulum “quality control” mechanisms (29). This paradigm, where the formation of subunit recognition sites is rate-limiting and contributes to quality control mechanisms during oligomer assembly, is likely to be found in the assembly of other ionotropic neurotransmitter receptors and ion channels (1) and may also apply in the assembly of other complex oligomeric proteins. Key
to the formation of subunit recognition sites appears to be the cystine loop conformational change. As shown in Fig. 8, we envision the role of the cystine loop as a switch in subunit assembly. In the “up position,” the cystine loop blocks subunit recognition site formation and assembly. In the “down position,” subunit recognition site formation and assembly continue. In such a role, the cystine loop conformational change is an essential part of the assembly process, which allows the subunits themselves to guide proper subunit folding and maintains the correctly ordered pathway by which subunits oligomerize.

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