In this study we have used cultured muscle cells to investigate the role of disulfide bond formation in the sequence of molecular events leading to nicotinic acetylcholine receptor (AChR) assembly and surface expression. We have observed that disulfide bond formation in newly synthesized AChR α-subunits occurs 5–20 min after translation and that this modification can be blocked by dithiothreitol (DTT), a membrane-permeant thiol-reducing agent. DTT treatment was found to arrest AChR α-subunit conformational maturation, assembly, and appearance on the cell surface, showing that these events are dependent on prior formation of disulfide bonds. Subunits prevented from maturation by the reducing agent do not irreversibly misfold or aggregate, since upon removal of DTT, AChR α-subunits undergo formation of disulfide bonds and resume folding, oligomerization, and surface expression. We have previously found that nascent α-subunits undergo transient complexes with the molecular chaperone calnexin immediately after subunit synthesis (Gelman, M. S., Chang, W., Thomas, D. Y., Bergeron, J. J. M., and Prives, J. M. (1995) J. Biol. Chem. 270, 15085–15092) and have now observed that both the formation and the subsequent dissociation of these complexes are unaffected by DTT treatment. Thus, α-subunits appear to dissociate from calnexin independently of their undergoing disulfide bond formation and achieving conformational maturation. This finding together with the absence of irreversible misfolding of DTT-arrested α-subunits suggests that calnexin may act to prevent misfolding by aiding in the initial folding events and is not an essential participant in the late stages of α-subunit maturation.

The extracellular domains of many transmembrane proteins contain intrachain disulfide bonds, which are thought to contribute to the formation and stabilization of their mature conformation (for reviews see Refs. 1–3). In some cases these bonds are established co-translationally, as soon as the participating cysteine residues emerge into the lumen of the endoplasmic reticulum (ER), yet in other cases the formation of disulfide bonds does not occur until an appreciable interval after translation (4–7), or even after assembly of monomers (6, 7). The oxidizing microenvironment necessary for this modification is provided by the lumen of the ER. This organelles also contains the enzymes that facilitate formation of disulfide bonds and assist in protein folding, including “foldases,” such as protein disulfide isomerase and peptidylprolyl isomerase, and molecular chaperones such as calnexin and BiP (8–11). In addition, the ER is the site of assembly of most oligomeric membrane and secretory proteins, which exit to the Golgi complex and are transported to the cell surface only after the completion of assembly (12).

The nicotinic acetylcholine receptor (AChR) is a hetero-oligomeric complex of four different transmembrane glycoproteins assembled in the stoichiometry α2β2 (for reviews see Refs. 13–16). The assembly of subunits into pentamers in a precise stoichiometry and order of subunits does not occur immediately upon synthesis of the subunits, but only after a lag period of ~30 min (17, 18). During this interval AChR subunits undergo post-translational modifications leading to conformational maturation and acquisition of the capacity to assemble (18–20). Conformational maturation has been studied in the α-subunit, which besides its prominence from a stoichiometric standpoint, has binding properties distinct from those of the other subunits. The extracellular domain of each α-subunit contains a region essential for acetylcholine binding, as well as a binding site for the elapid venom neurotoxin α-bungarotoxin (α-Bgt) and a characteristic sequence termed the main immunogenic region, the epitope for most antibodies made against native AChR (21). All of these sites are initially absent in newly translated α-subunit polypeptides in intact cells (22, 23), nor are they present in α-subunit polypeptides expressed in a cell-free system (24). In intact cells the toxin binding site and main immunogenic region epitope are acquired by the α-subunit monomers in the interval between biosynthesis and subunit assembly, while the agonist binding sites are not acquired until the assembly of α- with γ- or δ-subunits (23). On this basis the binding of mAb 35, a monoclonal antibody that selectively recognizes the main immunogenic region (21), as well as acquisition of the ability to bind α-Bgt (25, 26) can be used to monitor the course of α-subunit folding toward conformational maturation.

The reducing agent dithiothreitol (DTT) has played a major role in the molecular characterization of AChR. Before sequence information became available, the presence of disulfide bonds in AChR was first inferred from studies demonstrating that DTT treatment altered functional properties of AChR on the surface of intact electrogenic cells from electric eel (27). This effect was demonstrated to be due to the in situ reduction of a disulfide bond near the acetylcholine binding site (28). After AChR α-subunit was cloned and sequenced (29) the precise location of this disulfide bond was determined by the

The abbreviations used are: ER, endoplasmic reticulum; AChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; DTT, dithiothreitol; mAb, monoclonal antibody.

* This research was supported in part by National Institutes of Health Grant NS25945. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by a training grant in pharmacological sciences (GM07518) from the National Institutes of Health.

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covalent binding of a radioactive affinity alkylation agent to a pair of cysteine residues (Cys\textsubscript{102}, Cys\textsubscript{139}) after DTT treatment (30). This disulfide bond is unique to \(\alpha\)-subunit; an additional disulfide bond between Cys\textsubscript{228} and Cys\textsubscript{342}, forming a 15-residue loop in the N-terminal extracellular domain, is conserved among all four AChR subunits (30).

Recently, interest in DTT has been renewed by the demonstration that it can permeate across cellular membranes and prevent formation of disulfide bonds on nascent proteins in the ER of living cells (31, 32). This approach has been applied to study the contribution of the ER redox environment to the posttranslational processing and intracellular transport of individual disulfide-containing secretory and membrane proteins. Under these conditions DTT blocked disulfide bond formation in newly synthesized proteins without adverse effects on most cellular functions, including ATP synthesis and transport of proteins through the secretory pathway (32, 33). We have now used this approach to investigate the relationship between disulfide bond formation and AChR subunit folding and assembly in cultured myotubes.

**MATERIALS AND METHODS**

**Reagents—**\(\text{[^{35}S]}\)methionine/\(\text{[^{35}S]}\)cysteine protein labeling mix (specific activity 1050–1200 Ci/mmol) and \(\text{[^{35}S]}\alpha\)-bungarotoxin (specific activity 13–15 mCi/\mu g) were purchased from DuPont NEN. Polyacrylamide gel electrophoresis reagents were from Bio-Rad. All other reagents were from standard vendors.

**Cell Culture—**Muscle primary cultures were prepared from breast muscle of 12-day chick embryos and plated on collagen-coated culture dishes (initial density = 6 × 10\textsuperscript{5} cells/100-mm culture dish) as described previously (18, 34). Cultures were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum and 2% chick embryo extract at 37°C in an atmosphere of 92% air, 8% CO\textsubscript{2}. Under these conditions the myoblasts fused to form multinucleated myotubes on the second day after plating, initiated rapid synthesis of AChR on the third day, and were used 3–4 days postplating.

**Antibodies—**Anti-chick AChR \(\alpha\)-subunit antibody and anti-chick AChR \(\beta\)-subunit antibody were raised in rabbits against the individual subunits purified on SDS-polyacrylamide gel electrophoresis from denatured chick leg muscle and were shown to be non-cross-reactive (18). Anti-\(\alpha\)-bungarotoxin antibody was raised in rabbits and affinity-purified on \(\alpha\)-bungarotoxin-Sepharose (18). The monoclonal antibody mAb 35, which recognizes the main immunogenic region of AChR \(\alpha\)-subunit (21) was isolated from the supernatant of hybridoma TIB 175 (American Type Culture Collection). Anti-calnexin antibody was raised against a synthetic peptide corresponding to a highly conserved stretch near the C terminus (amino acids 487–505) of calnexin (35, 36).

**AChR Surface Labeling—**For surface labeling of AChR on intact muscle cells, cultures were washed once with DMEM and incubated with \(\text{[^{35}S]}\alpha\)-Bgt (10 \textsuperscript{8} \mu M) in DMEM containing bovine serum albumin (1 mg/ml) for 1 h at 37°C (18, 37). At the end of this period, cultures were washed five times with 3-ml volumes of Dulbecco’s phosphate-buffered saline to remove unbound toxin. Surface labeling was quantitated by \(\gamma\)-counting of the cell extracts.

**Metabolic Labeling and Immunoprecipitation—**For metabolic labeling, cultures were methionine/cysteine-depleted by incubation with methionine- and cysteine-free DMEM (Life Technologies, Inc.) for 1 h and then labeled at 37°C with a mixture of \(\text{[^{35}S]}\)methionine and \(\text{[^{35}S]}\)cysteine for the specified time. In the pulse-chase experiments, chase was performed by washing cells once with DMEM, followed by incubation in DMEM supplemented with 5 m\textsubscript{M} \(\lambda\)-methionine. Where specified, freshly prepared 5 m\textsubscript{M} DTT was added to the chase medium or the labeling medium. Cells were harvested as follows. Cultures were washed twice with ice-cold phosphate-buffered saline, scraped, and extracted for 30 min at 4°C in STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA) containing 1 mg/ml apronitin, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonfonyl fluoride and supplemented with 1% Triton X-100. Clarification of the extracts was achieved by centrifugation for 15 min in the microcentrifuge at 4°C. The clarified supernatants were incubated at 4°C with the specified antiserum for 3 h. Then protein A-Sepharose beads were added, and incubation at 4°C was continued for a further 1 h. Where indicated, sequential immunoprecipitations were carried out essentially as described before (20, 38). Briefly, cells were extracted in HBS buffer (50 mM HEPES, pH 7.5, 200 m\textsubscript{M} NaCl, 1 mM CaCl\textsubscript{2}, 10% glycerol, 1% aprotinin, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonfonyl fluoride) supplemented with 2% sodium cholate. After immunoprecipitation of cell extracts with anti-calnexin or anti-\(\alpha\)-subunit antibody, proteins were eluted from the protein A-Sepharose beads by denaturing conditions (38 M sodium cholate). After elution, the eluates were loaded on SDS-polyacrylamide gels and subjected to electrophoresis. For the analysis of disulfide bonds in the AChR, SDS-polyacrylamide gels were subjected to electroblotting on nitrocellulose filters. The blots were incubated with anti-AChR \(\alpha\)-subunit antibody, then washed, and incubated with secondary antibody conjugated to alkaline phosphatase. The spots were revealed by substrate treatment.

**RESULTS**

**DTT Treatment Blocks AChR Surface Appearance—**To determine the effect of DTT on the cell surface expression of newly made AChR, cultured myotubes were pulse-labeled with \(\text{[^{35}S]}\)methionine/\(\text{[^{35}S]}\)cysteine for 15 min and then incubated for the specified intervals in chase medium in the absence or presence of DTT (5 m\textsubscript{M}). To monitor the appearance of labeled receptors on the external surface of myotubes, the pulse-labeled cells were incubated with the AChR ligand \(\alpha\)-Bgt and subsequently immunoprecipitated with anti-\(\alpha\)-Bgt antibody. As can be seen in Fig. 1, in the absence of DTT, labeled AChR \(\alpha\)-subunit was detected at the cell surface after chase intervals of 3 h and longer following its synthesis (lanes 2 and 3). No labeled AChR was detected at the cell surface after chase intervals of 2 h or less (not shown), consistent with a duration of between 2 and 3 h between AChR biosynthesis and its surface appearance (40, 41). When DTT was present in the chase medium (lanes 4 and 5), significant amounts of labeled receptors could not be immunoprecipitated from the cell sur-
face, showing that DTT treatment blocks the appearance of newly made AChR at the plasma membrane. The faint bands in lanes 4 and 5 that comigrate with α-subunit probably correspond to the small population of pulse-labeled subunits that underwent oxidation and assembly during the 15-min pulse period before application of DTT. It should be noted that DTT was removed immediately prior to labeling of DTT-treated cells with α-Bgt to prevent reduction of the disulfide bonds in α-subunit and its consequent inactivation. The block of AChR surface appearance by DTT was reversible: upon removal of the reducing agent from the chase medium and incubation of myotubes in DTT-free medium for an additional 3 or 4 h, labeled AChR was detected at the cell surface (lanes 6 and 7). To test whether the block in AChR surface expression upon exposure to DTT occurred before or after AChR assembly, DTT was added to the culture medium 90 min after the end of the pulse, at a time after completion of AChR assembly but before the receptor had reached the cell surface. As seen in lanes 8 and 9, application of DTT after assembly does not block surface appearance of AChR (or impair the binding of α-Bgt to these receptors). Therefore, in order to block AChR surface expression DTT must be present before the assembly of AChR takes place.

DTT Treatment Arrests AChR Assembly—To test directly the effect of DTT treatment on AChR subunit assembly, myotubes were pulse-labeled and then chased in the absence or presence of DTT, and AChR assembly was measured by immunoprecipitation of α-subunit with a non-cross-reactive antibody directed against the δ-subunit. Figure 2 (left panel) shows the time course of AChR assembly under control conditions, as monitored by the accumulation of α-subunit immunoprecipitated with anti-δ-subunit antibody with increasing chase times. The δ-subunit itself is not visible in [35S]methionine-labeled preparations due to its diffuse migration as a heterogenous band, high susceptibility to proteolysis, and nonspecific background in this region of the gel, as noted previously by ourselves (18, 20) and others (43). However, the δ-subunit is phosphorylated and is clearly visible in immunoprecipitates from cultures labeled with [32P]Pi, (18). As can be seen, appreciable AChR assembly was not detected immediately after the pulse but was clearly evident by 40 min and continued to increase with the further chase intervals. In contrast, when DTT was present in the chase medium (right panel) no labeled α-subunit was immunoprecipitated with anti-δ-subunit antibody at any time point. The ability of anti-δ-subunit antibody to recognize δ-subunit was not impaired by this DTT treatment, as determined by immunoprecipitation of this subunit from cultures labeled with [32P]Pi, in the presence of DTT (data not shown). Therefore, we conclude that the absence of α-subunit in anti-δ-subunit immunoprecipitates reflects a block in AChR assembly in the ER in the presence of the reducing agent. The effect of DTT treatment on the rate of AChR assembly is shown in Fig. 3. As can be seen, upon removal of DTT from the culture medium, the assembly of the labeled AChR subunits resumed at the same rate as in untreated cultures (Fig. 3). This finding indicates that DTT treatment does not induce the subunits to misfold permanently, but rather arrests them in a partially folded conformation that remains conducive to assembly upon withdrawal of the reducing agent. When DTT treatment was followed by brief exposure to the cell-permeant alkylating agent N-ethylmaleimide (20 mM), the assembly of the AChR subunits was not restored even after prolonged incubation in the absence of both drugs (not shown), likely due to the alkylation of the -SH groups and indicating that the recovery of assembly requires the formation of disulfide bonds.

DTT Treatment Prevents AChR α-subunit Folding—To determine if the arrest of AChR assembly by DTT treatment is due to impaired subunit folding, pulse-labeled cultures were chased in the absence or presence of 5 mM DTT as described above, and cellular extracts were then immunoprecipitated with mAb 35, an antibody specific for conformationally mature AChR α-subunit (21). As shown in Fig. 4, α-subunits were not recognized by mAb 35 to an appreciable extent immediately after the pulse (lane 1), but became immunoprecipitable with this antibody by 60 min of chase in untreated cells (lane 2). In contrast, when DTT was present during the chase α-subunit was not recognized by mAb 35 even after 60 min of chase (lane 4), although equivalent amounts of labeled α-subunit were immunoprecipitable by anti-chick α-subunit antibody from untreated and DTT-treated cell extracts (not shown). Replicate cultures were chased in the presence of DTT for 30 min and then rinsed and shifted to medium lacking DTT. Upon removal of the reducing agent, the labeled α-subunits acquired the mAb 35 epitope at a rate and to an extent comparable with that observed under control conditions. These results show that, as in the cases of AChR subunit assembly and surface expression, α-subunit folding is dependent on disulfide bond formation and thus susceptible to DTT. Furthermore, in all three cases the DTT arrest is reversed when the oxidizing environment in the ER is restored.

Temporal Relationship between α-subunit Translation, Disulfide Bond Formation, and Conformational Maturation—In order to determine the time course of the formation of disulfide...
bonds on the nascent \( \alpha \)-subunits, we used nonreducing SDS-polyacrylamide gel electrophoresis in an attempt to distinguish the oxidized and reduced forms of \( \alpha \)-subunit. Cells were pulse-labeled for 5 min to isolate a relatively synchronous population of newly made subunits, chased for the specified intervals, and then extracted and immunoprecipitated with anti-\( \alpha \)-subunit antibody. The immunoprecipitates were fractionated in SDS-polyacrylamide gels under nonreducing conditions. To detect the difference in migration between the nascent and disulfide-bonded forms of \( \alpha \)-subunit, samples harvested immediately after pulse were run next to each sample harvested after the specified chase interval (Fig. 5, lanes 1–10). To compare the migration pattern of the fully reduced and oxidized forms of \( \alpha \)-subunit, replicate cultures were chased in the presence or absence of 5 mM DTT for 1 h, an interval sufficient for the completion of \( \alpha \)-subunit folding in the untreated cultures. As can be seen in Fig. 5, lanes 11 and 12, a small but clearly detectable difference in the mobility of reduced versus oxidized \( \alpha \)-subunit is evident under these conditions.

In untreated cultures the shift in migration of \( \alpha \)-subunit indicative of disulfide bond formation was only marginally detectable after 5 min of chase (Fig. 5, lanes 1 and 2), and it was more clearly seen after 10 min (lanes 3 and 4), and appeared to be maximal at 15 min of chase and longer (lanes 5–10). These results indicate that disulfide bonds on \( \alpha \)-subunits are established before the acquisition of mAb 35 epitope. Since DTT treatment that prevents formation of these disulfide bonds results in the block of folding and assembly it is likely that the formation of intrachain disulfides is essential for conformational maturation of the individual subunits.

Relationship between Disulfide Bond Formation and the Interaction of AChR \( \alpha \)-Subunit with Calnexin—We have recently found that nascent AChR \( \alpha \)-subunit binds to the molecular chaperone calnexin immediately after \( \alpha \)-subunit synthesis (20). The time course of \( \alpha \)-subunit-calnexin dissociation was observed to coincide with the conformational maturation of \( \alpha \)-subunit, consistent with a role for calnexin in mediating \( \alpha \)-subunit folding. Since DTT has now been shown to block the folding of \( \alpha \)-subunit (Fig. 4), it is of interest to determine the fate of \( \alpha \)-subunit-calnexin complexes in the presence of DTT. In the experiment shown in Fig. 6 cells were pulse-labeled with \( [35S] \)methionine/\( [35S] \)cysteine for 15 min and chased in the absence or presence of 5 mM DTT. To determine the amount of radiolabeled \( \alpha \)-subunit associated with calnexin at each time point, cells were extracted in HBS, 2% sodium cholate buffer, which preserves interactions of calnexin with substrate proteins, and immunoprecipitated with anti-calnexin antibody. The immunoprecipitated calnexin-substrate complexes were eluted from the pellet and reprecipitated with anti-\( \alpha \)-subunit antibody. The rate of \( \alpha \)-subunit dissociation from calnexin in DTT-treated cells, with a half-time of 18 min, is indistinguishable from that observed in the absence of DTT (20). Thus, no difference is seen in the amounts of \( \alpha \) subunit bound to calnexin after 15 min of chase in DTT-treated and untreated cultures (Fig. 6, lanes 7 and 8). Therefore, \( \alpha \)-subunit-calnexin dissociation does not appear to be altered by the presence of DTT in the chase medium.

Finally, we tested whether or not the initial binding of calnexin to \( \alpha \)-subunit is affected by DTT when the reducing agent is present in the medium during the pulse period. A measure-
Disulfide bond formation in AChR biogenesis in the absence and presence of DTT. a, AChR α-subunit is translated on ER-bound polysomes and is cotranslationally inserted into the ER membrane. b, immediately or soon after synthesis α-subunit binds to the ER-resident chaperone calnexin. c–f, disulfide bonds are established 5–20 min after α-subunit translation. Disulfide-bonded α-subunit dissociates from calnexin and continues to fold, achieving conformational maturation by 60–75 min after translation. Correctly folded subunits assemble into pentameric AChR and exit the ER. g–j, in the presence of DTT, formation of disulfide bonds on α-subunit is blocked, but calnexin dissociation is not impaired. Subunit folding and assembly are suspended for as long as DTT is present in the medium. Aggregation or permanent misfolding of subunits do not occur during this interval. Upon removal of DTT, disulfide bond formation is restored, and the subunits undergo conformational maturation and oligomerization.

**DISCUSSION**

In the present study we have measured the time course of disulfide bond formation in AChR α-subunits and have shown that this posttranslational modification is crucial for further folding, assembly, and surface expression of AChR. As established previously under similar experimental conditions, AChR α-subunit conformational maturation is first detectable at 30 min after translation (interval measured from the beginning of the pulse) and increases linearly, achieving a maximum by 60–75 min after translation (20). Assembly into pentameric complexes takes place shortly after maturation, beginning 30–45 min after translation and attaining completion by 90 min (18, 20). The assembled AChR reaches the cell surface 2.5–3 h after subunit synthesis (Refs. 40 and 41; our present results). We have now determined that disulfide bond formation on α-subunit is completed by 20 min after subunit translation, preceding subunit maturation as measured with the conformation-specific antibody. Our observation that the AChR α-subunit needs to undergo additional folding after this disulfide bond formation to acquire the mAb 35 epitope indicates that the newly oxidized subunit constitutes a discrete folding intermediate that is a precursor of the mature, assembly-competent α-subunit.

Our results show that DTT treatment blocked the appearance of newly made AChR at the plasma membrane, but only when the reducing agent was present in the culture medium within the first few minutes after pulse labeling. Under these conditions DTT blocked disulfide bond formation on α-subunit and arrested subsequent subunit folding and assembly. The addition of DTT after the disulfide bonds have formed (at 30 min after the pulse; data not shown) no longer had any effect on the extent and timing of assembly. Similarly, adding DTT after the completion of assembly (90 min) did not affect the transport of pulse-labeled AChR to the cell surface. Although the block of disulfide bond formation on nascent secretory and membrane proteins in the ER is a general consequence of DTT treatment, the time course data strongly suggest that DTT exerts its effects on AChR directly, by preventing the oxidation of AChR subunits. Together these results indicate that formation of disulfide bonds on AChR α-subunit is a fundamental step required for subunit maturation and assembly into pentameric AChR.

The contribution of disulfide bonds to AChR biogenesis has been addressed in recent studies using recombinant subunits expressed in Xenopus oocytes (42) or in transfected fibroblasts (43). In these experiments mutant α-subunits lacking the conserved disulfide bond between Cys128 and Cys142 failed to form α-Bgt binding sites, which like the mAb 35 epitope, are dependent on the acquisition of the correctly folded conformation (25, 26). However, both studies reported association of these mutant α subunits with normal δ subunits, suggesting that formation of this disulfide bond is not an absolute prerequisite for AChR assembly. In contrast, under the present experimental conditions DTT treatment abolished detectable assembly of α- and δ-subunits. How can these apparent differences be reconciled? First, elimination of disulfide bond formation either by DTT or by mutagenesis of the α-subunit may result in a significant decrease in the affinity between subunits. Consequently, in DTT-treated cultured muscle cells the assembly of endogenously expressed AChR subunits is diminished to undetectable levels, while in transfected cells association between mutant α- and δ-subunits, although markedly less efficient, could remain detectable due to overexpression of the recombinant subunits. In addition, since DTT is anticipated to prevent formation of the conserved disulfide bond in all four AChR subunits, it is possible that the impaired folding of other subunits as well as the α-subunit contributes to the block in assembly. As α-subunits directly assemble with both γ- and δ-subunits (23), it would be of interest to determine if α-γ binding is also vulnerable to DTT treatment.

We found that the described effects of DTT on AChR biogenesis are fully reversible. Removal of the reducing agent leads to the resumption of disulfide bond formation on the arrested subunits, with subsequent folding and assembly into pentameric AChR, followed by transport to the cell surface. Moreover, upon reversal of the DTT effect, the time course of these events is indistinguishable from that of subunit folding, assembly, and cell surface appearance in untreated cultures. These results indicate that DTT treatment at least for the durations used in this experiment does not cause irreversible misfolding or ag...
It is possible that the prevention of irreversible misfolding of proteins reduced by DTT is mediated by one or more ER-resident molecular chaperones, proteins that are thought to prevent misfolding and facilitate correct folding of nascent polypeptides. The ER chaperone calnexin forms complexes with newly made AChR subunit, and these complexes subsequently dissociate with a t1/2 of approximately 20 min, concomitantly with α-subunit folding (20). In the present study we have observed that formation of α-subunit-calnexin complexes was not impaired by DTT. Moreover, the kinetics of calnexin dissociation were apparently unchanged in the presence of DTT, although the folding and assembly of the α-subunit remained arrested for as long as DTT was present, indicating that calnexin dissociation from α-subunit is independent of the completion of the α-subunit folding. This finding supports the possibility that calnexin performs its function mainly during the transatlional and early posttranslational stages of AChR subunit folding, when nascent subunits may be most susceptible to misfolding and aggregation. In a similar manner, calnexin has been reported to mediate early folding of influenza virus hemagglutinin (HA), dissociating prior to conformational maturation of HA monomer (44). In an earlier study a mechanism has been proposed in which the ER-resident molecular chaperone BiP forms transient complexes with nascent HA during the folding process and prevents its misfolding by blocking formation of inappropriate disulfide bonds (7).

Recent studies describe divergent effects of DTT on calnexin interaction with substrate proteins (36, 45, 46). In two instances, the major secretory glycoprotein in Madin-Darby canine kidney cells (36) and thyroglobulin in thyroid epithelial cells (45), DTT has been reported to lock substrates onto calnexin by blocking the dissociation of the complexes. In contrast, in the case of HA, the addition of DTT to a cell-free translation system has been reported to cause detachment of calnexin from unfolded HA, apparently due to reduction of the disulfide bond(s) in calnexin itself (46). Since this susceptibility of calnexin to DTT is not restricted to cell-free preparations (46), it is possible that the ER in different cell types varies with respect to the ability of DTT to alter its redox potential. In addition, since the structural requirements for complexing with calnexin apparently differ among its various substrate proteins (45, 47, 48), calnexin-substrate interactions may also vary with respect to their sensitivity to disulfide bond reduction.

Our present findings summarized schematically in Fig. 7 indicate that formation of disulfide bonds is an essential aspect of the quality control mechanism that ascertains that only correctly folded and assembled AChR pentamers exit the ER and are transported to the cell surface. The ability to interrupt AChR folding and assembly by preventing disulfide bond formation in intact cells offers a means for isolation of folding intermediates as well as the potential for identification of additional ER-resident proteins that participate in the quality control process.

Acknowledgments—We are grateful to Dr. J ohn J. M. Bergeron for helpful discussions and a generous gift of anti-calnexin antibody. We thank Sandeep Mody for preparation of muscle cell cultures.

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J. Biol. Chem. 1996, 271:10709-10714.
doi: 10.1074/jbc.271.18.10709

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