The acetylcholine receptor (AChR) at the motor synapse is a pentamer of homologous subunits with the composition α₂βγδ. Owing to the circular arrangement of subunits that forms a central ion channel, each subunit interface contains contributions from opposite faces of each subunit, designated + and −. Common to all subunits of the AChR and members of its superfamily is a disulfide loop formed between cysteines 128 and 142 of the major extracellular domain. To gain insight into the structural contribution of the disulfide loop and its possible location, we mutated the invariant proline at position 136 to glycine (P136G) and examined subunit assembly. When introduced into any AChR subunit, P136G disrupted assembly by affecting the − face of the subunit, suggesting equivalent positioning of the loop in each subunit and localization to the − face. Also, the contribution of the loop in the overall assembly process differed for each subunit. In the β and γ subunits, P136G prevented assembly of higher order heteroligomers, whereas in the α and δ subunits, P136G prevented transport of assembled pentamers to the cell surface. The results demonstrate asymmetry in the contribution of the disulfide loop to formation of subunit interfaces, and that the loop in each subunit contributes at different stages of assembly.

Receptors for acetylcholine, glycine, γ-aminobutyric acid, and 5-hydroxytryptamine-3 bind a nerve-released transmitter to elicit excitatory or inhibitory responses in postsynaptic cells. The acetylcholine receptor (AChR) at the motor synapse has served as a model for understanding the structure and function of the superfamily of neurotransmitter-operated channels. It is a pentamer of homologous subunits with the composition α₂βγδ (1). Studies from several laboratories have shown that the subunits are positioned equivalently within the pentamer such that each subunit interface contains contributions from opposite faces of the subunit (2), designated + and − in Fig. 1A.

The most conserved structural domain in subunits of the AChR superfamily is a disulfide loop formed between cysteines 128 and 142 of the major extracellular domain of the subunit (Ref. 3 and Fig. 1B). This conserved motif has attracted considerable attention, yet its structural contribution and functional significance remain unknown. The presence of an N-linked glycosylation site at position 141 indicates that Asn141, the adjacent Cys142, and the disulfide-linked Cys128 are at the surface of the subunit. Also, the loop lies between stretches of residues found to interact with cholinergic ligands, including residues 111–117 and 161–180, which contribute to the − face of the subunit (2, 4, 5). Studies of exposure of the loop to antipeptide antibodies showed that the loop is not accessible to antibodies in intact cells or membrane fragments but that it becomes accessible following treatment with low concentrations of Triton X-100 (6). Mutation of cysteines α128 or α142 to serine or deletion of the loop abolishes a-bungarotoxin binding but does not prevent these mutant subunits from associating with the δ subunit (7, 8); because the + face of the α subunit contributes to the α-δ interface, these findings suggest that the loop is not located on the + face of the subunit. Thus the available data indicate that the loop is flanked by residues that contribute to the − face of the subunit, that the disulfide-linked portion of the loop is located at the surface of the subunit, but that part of the loop is buried, perhaps at the subunit interface.

To gain insight into the structural and functional significance of the disulfide loop and its possible location within the subunit, we mutated the invariant proline at position 136 to glycine (P136G) and examined the ability of mutant and wild type subunits to form stable associations. The results show that when incorporated into each of the four subunits, P136G disrupts subunit assembly through a specific effect on the − face of the subunit. Furthermore, the nature of the effect of P136G was subunit specific. In the β and γ subunits P136G blocked formation of higher order heteroligomers, whereas in the α and δ subunits P136G allowed formation of pentamers but prevented their expression on the cell surface. The overall results demonstrate asymmetry in the contribution of the disulfide loop to formation of subunit interfaces and suggest that the loop of each subunit contributes to forming the subunit interface at different stages of assembly.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The 293 human embryonic kidney cell line (293 HEK) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in the presence of 5% CO₂ at 37 °C.

**Mutagenesis and Expression**—Mouse AChR subunit cDNAs were subcloned into the cytomegalovirus-based expression vector pRBG4 (9). Mutations were constructed by bridging naturally occurring or mutagenically installed restriction sites with synthetic double stranded oligonucleotides (4). For constructs without convenient restriction sites, mutations were introduced using the overlap polymerase chain reaction method. All constructs were confirmed by restriction mapping and dyeoxy sequencing. AChRs were expressed in 293 HEK cells by cotransfecting wild type and/or mutant subunit cDNAs in various combinations using calcium phosphate precipitation. Cells at about 75% confluence were transfected with a 2:1:1:1 ratio of α, β, γ, and δ subunits for expression of pentamers, or 1:1 for expression of αγ or αδ dimers, where 1 corresponds to 6.8 μg of DNA/10-cm plate of cells. Cells were
incubated in the calcium phosphate-DNA medium for 5–10 h, changed back to normal growth medium, and incubated for another 48 h prior to expression experiments.

**Ligand Binding Measurements**—Two days after transfection, cells were permeabilized in low ionic strength buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM EDTA, 0.1% bovine serum albumin, and 1% saponin, pH 7.5), harvested by gentle agitation in phosphate-buffered saline containing 5 mM EDTA, briefly centrifuged, resuspended in high potassium Ringer’s solution (140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 1.7 mM MgSO<sub>4</sub>, and 25 mM HEPES, pH 7.4), and divided into aliquots for ligand binding measurements. When binding to surface receptors was measured, cells were harvested without permeabilization with saponin. Specified concentrations of ligands were added to cells 30 min prior to adding <sup>125</sup>I-labeled α-BTX. To measure the initial rate of toxin binding, <sup>125</sup>I-labeled α-BTX was allowed to bind for 30 min to occupy at most one-half of the receptors. The total number of binding sites was determined by incubating with α-BTX for 120 min. Unbound α-BTX was removed by washing twice with potassium Ringer’s solution containing 300 μM d-tubocurarine, followed by centrifugation. Radioactivity bound to the cell pellet was measured with a γ counter. Nonspecific binding was determined in the presence of saturating concentrations of dimethyl-d-tubocurarine (DMT).

Rates of α-BTX binding in the presence and absence of competing ligand were calculated from binding measured at 30 and 120 min (10). These rates are related to ligand occupancy by

\[ h_{\text{obs}} = h_{\text{max}}(1 - Y) \]

where \( h_{\text{obs}} \) is the rate of toxin binding in the presence of a specified concentration of competing ligand, \( h_{\text{max}} \) is the rate in the absence of competing ligand, and \( Y \) is the occupancy function for the competing ligand, in these studies given by the Hill equation. Data were fitted to the Hill equation by least squares nonlinear regression using Sigma Plot (Jandel Scientific, Inc.).

**Metabolic Labeling, Immunoprecipitation, and SDS-Polyacrylamide Gel Electrophoresis**—Prior to labeling with <sup>35</sup>S-methionine, cells were washed twice with methionine-free Dulbecco’s modified Eagle’s medium and incubated for 30 min. Cells were then covered with 3 ml of methionine-free Dulbecco’s modified Eagle’s medium supplemented with 200 μCi of <sup>35</sup>S-methionine and incubated at 37 °C in an atmosphere containing 5% CO<sub>2</sub> for 4 h. Labeling was stopped by addition of Dulbecco’s modified Eagle’s medium containing 5 mM methionine and 10% fetal bovine serum, and cells were incubated for an additional 12 h. Cells were washed with ice-cold 5 mM EDTA/phosphate-buffered saline, harvested, and then pelleted by centrifugation. Pellets were resuspended in 900 μl of lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02% NaN<sub>3</sub>, and 0.8% Triton X-100) plus a mixture of protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 35 μg/ml phenylmethylsulfonyl fluoride). After incubation for 30 min on ice, insoluble material was removed by centrifugation. Monoclonal antibodies mAb 35 or mAb 148 and protein A-Sepharose or protein G-Sepharose were added to the Triton X-100 extract. After mixing for 2 hours, the antibody-protein AG-Sepharose complex was pelleted and washed three times with lysis buffer. The immunoprecipitates were placed in a denaturing buffer and heated to 100 °C for 3 min, loaded on an SDS-polyacrylamide gel (7.5% resolving gel and 4% stacking gel), and electrophoresed. The gel was fixed, treated for 30 min with Amplify, dried, and exposed to Kodak XRP film for 48 h at −80 °C using an intensifying screen.

**Sucrose Density Gradients**—Three days after transfection, 293 HEK cells expressing various combinations of AChR subunits were permeabilized in low ionic strength buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM EDTA, 0.1% bovine serum albumin, and 1% saponin, pH 7.5), harvested, and resuspended in high potassium Ringer’s solution. Following labeling with <sup>125</sup>I-α-BTX, cells were washed free of unbound radioactivity and solubilized in 1 ml Triton X-100 buffer (0.6% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.5). Extracts were layered on sucrose gradients (3–30%) and centrifuged for 22 h at 40,000 rpm, and fractions were collected and counted with a γ counter. Radioactivity in each fraction was normalized to that of the fraction containing the maximum counts in each gradient. The S value of each peak was calculated from the midpoint of the count distribution. The S value of each peak was then normalized to that of the fraction containing the maximum counts in each gradient. The S value of each peak was calculated from the midpoint of the count distribution.

**RESULTS**

**Asymmetry in the Effect of P136G on Association of α and γ Subunits**—We focused on the invariant proline at position 136 within the disulfide loop and chose to mutate it to glycine in each subunit (Fig. 1). We mutated Pro<sup>136</sup> rather than Cys<sup>136</sup> or Cys<sup>142</sup> because mutation of Pro<sup>136</sup> does not interfere with α-BTX binding (7), thus allowing subunit association to be monitored by <sup>125</sup>I-α-BTX. Mutation to glycine is the most conservative natural substitution for proline, as both proline and glycine can promote formation of β turns (17). To look for effects of P136G on subunit association, we took advantage of previous work showing that coexpression of α and γ subunits leads to formation of α monomers, αγ dimers, and αγγ tetramers. Coexpression of α and δ subunits, on the other hand, leads to formation of α monomers and αδ dimers but no higher order complexes (7, 11). Formation of dimers reflects the ability of one face of each α and non-α subunit to stably associate, whereas formation of tetramers reflects the specificity of the γ subunit to contribute both faces for association with the α subunit; tetramers form by association of pairs of αγ dimers in which the face of the α subunit of one dimer interacts with the + face of the γ subunit of the other. Formation of tetramers is thought to reflect the unique position of the γ subunit in the native AChR as the single subunit between the two α subunits (Fig. 1A). We therefore chose to examine complexes formed by α and γ subunits to monitor both faces of the subunits for changes in subunit association.

Coexpressed combinations of mutant and wild type α and γ subunits in 293 HEK cells, labeled saponin-permeabilized cells with <sup>125</sup>I-α-BTX, and centrifuged cell extracts on sucrose density gradients. Coexpression of wild type α and γ subunits leads to formation of α monomers, αγ dimers, and αγγ tetra-
type and mutant profiles of complexes formed by coexpressing the following pairs of wild type and mutant α and γ subunits in 293 HEK cells: ● α-γ-∇, αP136G-γ; □, α-γP136G; □, αP136G-γP136G; ○, αβγ8·8. Cells were permeabilized with saponin, labeled with [125I]α-BTX, and extracted with Triton X-100, and the cell extracts were layered on a 5–30% linear sucrose gradient and centrifuged as described under “Experimental Procedures.” Radioactivity in each fraction is normalized to the peak value in each gradient. The average of three gradients is presented for each combination of subunits; vertical lines, S.E. Arrows, positions of peaks corresponding to α-BTX (Tx, 1.3S), α monomer (M, 3.3S), αγ dimer (D, 5.4S), αγγ tetramer (T, 8.3S), and αβγ8·8 pentamer (P, 9.5S). Note that αP136G prevents formation of tetramers but not dimers, whereas γP136G prevents formation of tetramers and dimers but not monomers. B, SDS-polyacrylamide gel electrophoresis analysis of pairs of wild type and mutant α and γ subunits. The indicated subunits were coexpressed, metabolically labeled with [35S]methionine, extracted, and immunoprecipitated with mAb 35. The immunoprecipitates were electrophoresed as described under “Experimental Procedures.” αm and γm indicate the P136G mutation. Note that αP136G associates with the γ subunit, whereas γP136G prevents association with the α subunit. C, binding of DMT to cells cotransfected with pairs of the indicated wild type or mutant subunits. DMT binding was determined by competition against the initial rate of [125I]α-BTX binding. Fitting to the Hill equation yielded the following parameters: αγ, Kd = 0.28 ± 0.03 μM; n = 1.03 ± 0.10; αγγ, Kd = 0.27 ± 0.02 μM; n = 1.05 ± 0.06. Note that αP136G does not affect DMT binding; D, schematic diagram of the effect of the P136G mutation on association of α and γ subunits. The P136G mutation acts on the γ-face of the subunit to prevent association, as indicated by shading.
mation of αβδ2 pentamers. We transfected cells with α, β, and δ subunits, permeabilized them with saponin, labeled with 125I α-BTX, and centrifuged cell extracts on sucrose density gradients (Fig. 3A). To promote formation of αβδ2 pentamers, cells were incubated at 31°C for 24 h prior to labeling, as described previously (4, 16). Coexpression of wild type α, β, and δ subunits produced both dimer (D, 5.4S) and pentamer (P, 9.5S) peaks, whereas the presence of βP136G resulted in only a dimer peak (D, 5.4S). Results from a single experiment are shown with maximum cpm values of: αβ, 2658; and αβP136G-δ, 1876. B, association of α and δ subunits examined by SDS-polyacrylamide gel electrophoresis. Cells were cotransfected with the indicated combinations of subunits, labeled with 35S-methionine, extracted, and immunoprecipitated with mAb 148. The immunoprecipitates were electrophoresed on a polyacrylamide gel. αδ and βδ indicate the P136G mutation. Note that when present in either the α or δ subunit, P136G fails to prevent association of α and δ subunits. C, schematic diagram showing how the βP136G prevents formation of αβδ2 pentamers but not αβ and αδ dimers.

The absence of pentamers in the presence of βP136G may result from failure to form either the δ-β or the β-α interface (Fig. 1A). To determine whether the β-α interface forms in the presence of βP136G, we coexpressed mutant or wild type α and β subunits, metabolically labeled cells with [35S]methionine, immunoprecipitated cell extracts with the δ subunit-specific antibody mAb 148 (12), and electrophoresed the immunoprecipitates on SDS-polyacrylamide gels. Fig. 3B shows that both wild type and mutant β subunits associate with the α subunit, indicating that βP136G-α dimers form and are likely to be present in the dimer peak of Fig. 3A.

To determine the composition of oligomers within the dimer peak, we noted that α-BTX binds to both αδ and αβ complexes, but that small competitive ligands inhibit α-BTX binding to αδ but not to αβ complexes (19). We coexpressed α, βP136G, and δ subunits, permeabilized the cells with saponin, and measured the amount of 125I-α-BTX binding protectable by DMT. Of the total α-BTX bound, 51% was protectable by DMT, indicating about one half of α-δ dimers, with the remaining nonprotectable binding corresponding to βP136G-α dimers. Thus the results presented in Fig. 3 show that when coexpressed with α and δ subunits, βP136G blocks formation of the δ-β interface to prevent formation of αβδ2 pentamers, as depicted in Fig. 3C. As observed for the α and γ subunits, βP136G affects the – face of the β subunit to prevent subunit association.

The experiments illustrated in Fig. 3B also produced the unexpected result that α and β subunits associate even with P136G in both subunits. The basis for this persistent association of α and β is examined further in Fig. 5.

Effect of P136G on Association of α and β Subunits—Having localized the effect of P136G in subunit assembly to the – face of the α, β, and γ subunits, we next studied the effect of P136G on association of α and δ subunits. Only the α-δ interface could be examined in these experiments because lysine residues (K147 and K150) in the extracellular domain of the δ subunit prevent formation of αδ-αδ tetramers (11). We coexpressed wild type α and δP136G, permeabilized cells with saponin, and measured the number of 125I-α-BTX binding sites protectable by DMT. Relative to the wild type δ subunit, δP136G decreased the number of DMT-protectable α-BTX binding sites by about 40%, in contrast to the greater than 90% decrease produced by γP136G (Fig. 4A). Coexpression of αP136G with δ, on the other hand, increased the number of DMT-protectable α-BTX binding sites. Thus unlike the αγ, αδ, and βδ interfaces, the αδ interface forms with P136G in either one or both subunits.

Because the – face of the δ subunit contributes to the ligand binding site formed at the αδ interface, we examined the integrity of the ligand binding interface of the α-δP136G oligomer by measuring the concentration dependence of DMT binding. Oligomers formed by α and δP136G exhibit a broad DMT binding curve, indicating heterogeneity of binding sites, whereas oligomers formed by αP136G and wild type δ exhibit a single affinity for DMT, which is identical to that of the wild type αδ dimer (Fig. 4B). Thus the effect of δP136G is again specific to the – face of the subunit. Although δP136G does not prevent
Moreover, a tertiary wild type subunits and measured the number of cell surface. To determine whether this subunit dependence is observed with our observation that P136G in these subunits prevents them from associating with δ and α subunits, respectively. Formation of pentamers in the presence of mutant α or δ subunits correlates with our observation that P136 in these subunits still allows them to associate with β and α subunits, respectively. These results further support our conclusion that in the β and γ subunits, the correct structure of the disulfide loop is important in stages of assembly in which the β-δ and α-γ interfaces form; thus at these interfaces the loop appears essential for the initial association of subunits. In the α and δ subunits, however, the structure of the loop is not important for the initial association of subunits to form the β-α and α-δ interfaces, but it is essential for the assembled pentamers to be transported to the cell surface.

**DISCUSSION**

The experiments described herein demonstrate asymmetry in the contribution of the conserved disulfide loop of each AChR subunit to assembly of mature AChR pentamers. By examining formation of each subunit interface, we found that mutation of the invariant proline at position 136 of the α, β, γ, and δ subunits affects subunit assembly through a structural disturbance of the α face of the subunit. We also found that association of subunits to form the β-δ, α-γ, and γ-α interfaces relies on an intact loop only in the subunit that contributes the α face. Thus at these three interfaces, the correct structure of the disulfide loop is required for the initial association of subunits to form a stable heteroligomer. Association of subunits to form the two remaining interfaces, β-α and α-δ, does not require an intact loop in either subunit of the pair. At these interfaces, αP136G or δP136G allows assembly of pentamers but blocks their transport to the cell surface; thus in the α and δ subunits the loop appears to be required in later steps of assembly, such as protein folding, which are necessary for transport of the assembled pentamer to the cell surface. The overall results demonstrate subunit-specific contributions of the disulfide loop to subunit oligomerization, as well as contributions to maturation of either the subunit or the oligomer.

The effect of P136G on AChR assembly suggests that a portion of the disulfide loop is located at the subunit interface, where it contributes to the α face of the subunit. Although the perturbation caused by P136G could be either global or local, the following observations favor a local perturbation. First, alanine does not change the overall conformation of the disulfide-linked backbone (18). Third, we found that a series of mutants in the α subunit did not effect binding of the competitive ligand DMT, which senses the α face of the α subunit, indicating that the effect of the mutation does not propagate to the α face of the subunit. Fourth, we found that deletion of the entire disulfide loop from the α subunit (αΔ) does not prevent association with the β subunit, nor does it prevent binding of mAb 35, an antibody sensitive to the conformation of the α subunit (12). These observations with αΔ indicate that the disulfide loop is not essential for maintenance of the overall tertiary structure of the α subunit, suggesting that the loop is superficial rather than buried within the subunit. Blount and Merlie (7) also found that deletion of the loop from the α subunit did not prevent association with the β subunit. Thus the effect of P136G is expected to be local, suggesting that the disulfide loop is located near the α face of the subunit.

Our second major finding is that although the disulfide loop of each subunit is essential for assembly of the mature penta-
mer, the contribution of the loop in forming subunit interfaces depends on the interface in question. Such selectivity of interface formation is not likely to be encoded by the amino acid sequence of each loop, given the high degree of homology of the loops across subunits (see Fig. 1A), suggesting the existence of additional structures that confer selectivity. Our observation that α and β subunits associate even when the loop is deleted from the α subunit shows that structures distinct from the loop allow stable association of these two subunits. Structures underlying this additional type of association may contribute to the specificity of subunit association and thus to the sequential nature of subunit assembly (14). In the context of a sequential pathway of subunit assembly, our findings suggest that the disulfide loop is essential for two types of interactions. The first is oligomerization of subunits where in the γ and β subunits the loop is essential for establishing stable contacts with the α and δ subunits, respectively. The second is maturation, or perhaps subunit folding, where in the α and δ subunits the loop is not involved in forming stable contacts with the β and α subunits, respectively, but is required for formation of a pentamer with the correct structure for transport to the cell surface.

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FIG. 6. Expression of individual P136G mutant subunits with complementary wild type subunits. A, subunits containing the P136G mutation were coexpressed with complementary wild type subunits, and specific binding of 125I-α-BTX to intact cells was determined. Note that mutation in any single subunit markedly reduces surface expression. B, sucrose gradient profiles for saponin-permeabilized cells expressing the following mutant subunits with complementary wild type subunits, labeled with 125I-α-BTX: αP136G (○); βP136G (●); γP136G (▲); and δP136G (▼). Maximum radioactivity in each peak were (cpm): wild type, 2445; αP136G, 1929; βP136G, 1905; γP136G, 1779; and δP136G, 2021. Note that primarily dimers form when P136G is present in β and γ subunits, whereas primarily pentamers form when P136G is present in the α and δ subunits.