Acetylcholinesterase tetramers are inserted in the basal lamina of neuromuscular junctions or anchored in cell membranes through the interaction of four C-terminal t peptides with proline-rich attachment domains (PRADs) of cholinesterase-associated collagen Q (ColQ) or of the transmembrane protein PRiMA (proline-rich membrane anchor). ColQ and PRiMA differ in the length of their proline-rich motifs (10 and 15 residues, respectively). ColQ has two cysteines upstream of the PRAD, which are disulfide-linked to two AChE$_T$ subunits (“heavy” dimer), and the other two subunits are disulfide-linked together (“light” dimer). In contrast, PRiMA has four cysteines upstream of the PRAD. We examined whether these cysteines could be linked to AChE$_T$ subunits in complexes formed with PRiMA in transfected COS cells and in the mammalian brain. For comparison, we studied complexes formed with N-terminal fragments of ColQ, N-terminal fragments of PRiMA, and chimeras in which the upstream regions containing the cysteines were exchanged. We also compared the effect of mutations in the t peptides on their association with the two PRADs. We report that the two PRADs differ in their interaction with AChE$_T$ subunits; in complexes formed with the PRAD of PRiMA, we observed light dimers, but very few heavy dimers, even though such dimers were formed with the PQ chimera in which the N-terminal region of PRiMA was associated with the PRAD of ColQ. Complexes with PQ or with PRiMA contained heavy components, which migrated abnormally in SDS-PAGE but probably resulted from disulfide bonding of four AChE$_T$ subunits with the four upstream cysteines of the associated protein.

Mammalian cholinergic tissues mostly express the T (“tailed”) variant of acetylcholinesterase (AChE$_T$)$_2$, in which the C-terminal 40-residue t peptide allows the formation of AChE$_T$ tetramers, associated with the collagen ColQ and the transmembrane protein PRiMA (1, 2). Heteromeric complexes containing ColQ are attached to the basal lamina at neuromuscular junctions, whereas complexes containing PRiMA are anchored in cell membranes and represent the major AChE species in the brain (3).

We have previously shown that the t peptide forms an amphiphilic α-helix, with a sector containing seven aromatic residues that are strictly conserved in all vertebrate cholinesterases (AChE, as well as butyryl cholinesterase (BChE)) (4). Although AChE$_T$ and other AChE variants can form dimers through an association zone located in the catalytic domain (the “four-helix bundle,” formed by two helices from each subunit) and an intercatenary disulfide bond through a C-terminal cysteine (5), only AChE$_T$ subunits form tetramers and associate with anchoring proteins through an assembly of four C-terminal t peptides, also called tryptophan amphiphilic tetramerization domains. Aromatic residues play a major role in the association of four t peptides with proline-rich motifs that exist in the N-terminal regions of ColQ and PRiMA (3, 6). These motifs are sufficient for the association with t peptides, and have been called proline-rich attachment domains (or PRADs). It should be noted that these quaternary associations do not require intercatenary disulfide bonds, although such bonds can be formed between the structural proteins and cysteines located near the C terminus of the t peptides.

The assembly of AChE$_T$ with ColQ has been studied in detail using site-directed mutagenesis and biochemical analyses of oligomers produced in transfected cells (6, 7), as well as crystallographic studies of a complex formed between synthetic t and PRAD peptides (8). In this complex, 4 α-helical t peptides form a coiled coil around the PRAD of ColQ, organized as a polyproline II helix. The aromatic residues of the t peptides are oriented toward the inside of this compact cylinder, and in particular, three evenly spaced tryptophans of each t peptide are closely stacked with the proline rings. The four t peptides are parallel and the PRAD is oriented in the opposite direction, in agreement with the fact that cysteines located at position -4 from the C terminus of each t peptide can form disulfide bonds either between pairs of t peptides or with two adjacent cysteines located upstream of the prolines in ColQ (2, 4).

Although ColQ and PRiMA appear to form similar quaternary complexes with AChE$_T$ subunits, they differ markedly in the number of prolines in their PRADs (8 in ColQ, 14 in PRiMA). A recent analysis of deletions in PRiMA showed that the recruitment of AChE$_T$ subunits into tetramers was severely reduced by the removal of the last three prolines and the residues that follow, even though the resulting mutant still contained a longer proline-rich motif than ColQ (9). In addition, the numbers and dispositions of the cysteines are very different in ColQ (2 adjacent cysteines, located 4 and 5 residues...
upstream of the first proline) and in PRIIMA (4 cysteines located 2, 4, 8, and 15 residues upstream of the first proline, plus a cysteine located 13 residues downstream of the last proline). The organization of the complexes may thus be somewhat different.

PRIIMA was first discovered as a 20-kDa hydrophobic protein associated with membrane-bound AChE tetramers purified from bovine brain (10, 11). This protein could be labeled with the hydrophobic reagent [125I]TID, and after denaturation under nonreducing conditions, it was found to be disulfide-linked with a dimer of AChET subunits. This arrangement under nonreducing conditions, it was found to be disulfide-linked with a dimer of AChET subunits. This arrangement appeared similar to that observed with ColQ, in which half of the catalytic subunits are linked to the binding protein, forming a “heavy” dimer, and the other half are associated together, forming a “light” dimer (4, 12).

However, the cysteines of PRIIMA that could form intercatenary disulfide bonds with the C-terminal cysteine of the t peptides were not identified. We now report an analysis of the AChET-PRIIMA complexes. To compare the PRADs of PRIIMA and ColQ, we also studied the soluble, secreted complexes formed by co-expressing AChET subunits with N-terminal fragments of PRIIMA and ColQ and with chimeras in which we exchanged the regions preceding the PRADs and containing the cysteines that could form intercatenary disulfide bonds. We mutated the four cysteines of PRIIMA individually and in combination, and we analyzed the presence of intercatenary disulfide bonds in nonreducing SDS-gel electrophoresis. We had previously shown that the deletion of the entire N-terminal region of PRIIMA does not abolish its quaternary association with four AChET subunits (9), and we confirmed that the cysteines are not essential. Contrary to our expectations, we found very few heavy dimers in the complexes formed with the PRAD of PRIIMA. In some complexes, all four AChET subunits appeared to be disulfide-linked to PRIIMA, whereas in others, they were associated only in pairs (light dimers). In addition, a mutation of the last aromatic residue in the t peptide (Y31G) differently affected the formation of complexes with the PRADs of ColQ and PRIIMA. Therefore, the quaternary organizations of AChET tetramers associated with ColQ and PRIIMA appear to be somewhat different.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs and Site-directed Mutagenesis—All constructs were expressed in the pEF-BOS vector (13, 14). Mutagenesis was performed according to the method of Kunkel et al. (15). Rat AChET subunits and full-length mouse PRIIMA (PRIIMA I) were tagged with an HA epitope (YPYDVPDYA) inserted before the stop codon at the C terminus of the t peptide of rat AChET, and of PRIIMA I. We also added the HA epitope between the putative signal sequence and the N terminus of these proteins. In this case, the signal peptide is predicted by the SignalP site to be cleaved immediately upstream of the HA epitope, and this was verified by the fact that the anti-HA antibody recognized the mature proteins. Tryptophan 17 and tyrosines 20 and 31 (numbering of the t peptide) were mutated to glycines. All or some of the cysteines in the N-terminal domain of PRIIMA were mutated to serines. Constructs encoding N-terminal domains of ColQ and PRIIMA, containing the PRADs, were obtained by inserting stop codons at the indicated positions (e.g. Q45R, P46W) (Fig. 1). In the QN-HCS construct, which was described previously (16), the N-terminal region of ColQ, containing the PRAD, is fused to a GPI addition signal derived from the Torpedo AChE, subunit. This construct produces GPI-anchored AChE tetramers, which can be compared with PRIIMA-anchored tetramers. The regions preceding the PRADs were exchanged in the chimeric constructs QP and PQ. The sequences of the mature proteins (without signal peptides) are shown in Fig. 1.

Transfection of COS Cells—COS cells were transfected by the DEAE-dextran method as described previously (17), using 2 μg of DNA encoding rat AChET and various amounts of pEF-BOS vector encoding the binding proteins per 60-mm dish. In all series of transfections, which differed in the level of binding proteins, we completed to the same amount of total vector DNA with pEF-BOS encoding a noninteracting protein (ΔPRAD), a ColQ N-terminal fragment from which the PRAD was deleted (6). After transfection, COS cells were incubated for 3–4 days at 37 °C in a medium containing 10% NuSerum (Inotech, Dottikon, Switzerland) that had been pretreated with 10−5 M soman to inactivate serum cholinesterases.

Extracts from Transfected COS Cells and from Rat and Mouse Brain—Three days after transfection, the cells were collected and homogenized in extraction buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2) containing 2.5 mM benzamid, a protease inhibitor, at 20 °C for 15 min. The supernatant was collected after centrifugation at 13,000 rpm for 10 min at 4 °C. The culture media were also collected for analysis of secreted AChE.

Two g of brain dissected from 2-month-old rats or from adult mice were frozen in liquid N2, cut in small pieces, and homogenized in a Potter glass-Teflon homogenizer with 20 ml of ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 2.5 mM benzamidine). The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant constituted the low salt-soluble fraction. The pellet was subjected to another cycle of extraction in 1.9 ml of ice-cold buffer A containing 1% Triton X-100, yielding the detergent-soluble fraction.

Determination of AChE Activity—AChE activity was determined by the colorimetric method of Ellman et al. (18) using acetylthiocholine as substrate. Enzyme samples (usually 10 μl) were added to 0.2 ml of Ellman assay medium, and the reaction was monitored at 414 nm with a Labsystems Multiskan RC automatic plate reader (Helsinki, Finland). Optical density was recorded at 20-s intervals over a period of 10 min. Under those conditions, 1 M OD corresponds to the hydrolysis of 22.5 pmol of acetylthiocholine.

Sedimentation Analysis in Sucrose Gradients, Gel Filtration Chromatography, and Nondenaturing Electrophoresis—For sedimentation analyses, 100–300-μl samples were mixed with Escherichia coli β-galactosidase and alkaline phosphatase as internal sedimentation markers, deposited onto 5–20% sucrose gradients (50 mM Tris-HCl, pH 7.5, 20 mM MgCl2, in the presence of 1% Brij-97 or 0.2% Triton X-100), and centrifuged in a Beckman SW41 rotor at 36,000 rpm for 17.5 h at 6 °C. Fractions of −100 μl were collected and assayed for AChE, β-galactosid-
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ase, and alkaline phosphatase activities as described previously (9).

Gel filtration was performed in a Biogel A 15-m column (95 cm long, 1.5 cm in diameter) to determine the Stokes radii of complexes. The flow rate was about 10 ml/h, and fractions were collected every 10 min. The void volume was determined with blue dextran and the total volume with potassium ferricyanide. The standards used were thyroglobulin (8.6 nm, 19.4 S, 669 kDa), β-galactosidase (6.9 nm, 16 S, 464 kDa), apoferritin (6.3 nm, 17.6 S, 443 kDa), and alkaline phosphatase (3.3 nm, 6.1 S, 87 kDa). Electrophoresis in horizontal nondenaturing polyacrylamide gels was performed as described previously (19), and AChE activity was revealed by the histochemical method of Karnovsky and Roots (20).

Western Blots—For analysis of proteins by Western blotting, samples were submitted to electrophoresis in 10% polyacrylamide gels under reducing or nonreducing conditions. We used colored proteins as mass standards (PAGE regular prestained protein ladder SM0671 from Fermentas; HiMark prestained HMW protein standard LC5699 from Invitrogen). After electrophoresis, proteins from the gel were electroblotted with the Bio-Rad mini-Protean II Trans-Blot system onto polyvinylidene difluoride membranes (Roche Applied Science) for 1.5 h. After transfer, the membrane was saturated with 5% milk powder in a buffer containing Tween-20 (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.6) for 2 h. The membrane was then incubated overnight at 16 °C with the appropriate antibodies: goat polyclonal anti-AChE antibody E-19, raised against the N-terminal region of mouse AChE (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and rabbit polyclonal anti-HA antibody N-terminal region of the protein. We checked the specificity of this antibody in Western blots of brain extracts from normal and AChE−/− mice (21); this was necessary because other commercial anti-AChE antibodies detected non-AChE proteins, notably in extracts from brains of knock-out mice lacking the AChE gene (supplemental Fig. 1S).

To analyze complexes formed in transfected cells, we inserted an HA epitope (YPYDVPDYA) at the C terminus of AChET. By immunoreaction with anti-HA antibodies, we verified that the HA epitope was present and accessible in the mature AChET subunits. The presence of the added epitope had no effect on the formation of complexes with binding proteins.

We studied the formation of heteromeric complexes in which AChE tetramers are associated with proteins derived from PRiMA (Fig. 1B) and from ColQ (Fig. 1C). We used wild type or mutated mouse PRiMA I as well as N-terminal fragments of mouse PRiMA in which the cytoplasmic and transmembrane domains were deleted by inserting stop codons at different positions. As shown previously, fragments P s46 and P s38 produced soluble, secreted complexes (9). Note that a downstream cysteine, Cys48, and a putative N-glycosylation site at Asn44 were deleted in Ps46 and P s38. For detection in Western blots, PRiMA I and its fragments were epitope-tagged with HA at their C termini. These epitopes did not interfere with the association with AChET subunits.

The formation of membrane-bound complexes containing full-length PRiMA was compared with the formation of GPI-anchored complexes containing the previously described QN-Hc construct in which the N-terminal region of Torpedo ColQ was fused to the C-terminal GPI addition signal of the Torpedo AChET variant (Fig. 1C). The formation of soluble complexes with N-terminal PRiMA fragments (P s46, P s38) was compared with the formation of similar complexes with N-terminal fragments of Torpedo ColQ (Q s71, Q s45). In addition, we analyzed chimeras PQ and QP, in which the regions preceding the proline-rich motives were exchanged, as shown in Fig. 1D (note that we obtained identical results for QP chimeras containing or not the 3 residues (LLT) that precede the prolines in ColQ).

ColQ contains two cysteines upstream of the PRAD, Cys28 and Cys29, and PRiMA contains four cysteines (Cys6, Cys13, Cys17, and Cys19). The upstream cysteines in P s46 and in PQ were mutated to serines, separately and in various combinations, as indicated for each experiment. To assess the possible importance of the distance between the cysteines and the PRAD, we constructed a Q s45 mutant in which Cys28 and Cys29 were replaced by serines, and 2 cysteines were introduced at positions 14 and 15, similar to upstream cysteines of PRiMA.

Differential Effects of a t Peptide Mutation (Y31G) on the Formation of Tetramers with the PRADs of ColQ and PRiMA—In previous studies, we had found that mutations of the central aromatic residues (W17P, Y20P) in the AChET subunits from Torpedo marmorata essentially abolished the association of AChET subunits with an N-terminal fragment of ColQ (Qs3), whereas mutation of the distal tyrosine (Y31P) had little or no effect (7). We now compared the effects of mutating this tyrosine on the formation of complexes with constructs containing the PRADs of ColQ and of PRiMA. In agreement with our pre-
vious results, tetrers associated with the binding protein were produced and secreted when wild type or Y31G AChE$_T$ subunits were co-expressed with constructs containing the PRAD of ColQ (QN and PQ) and could be visualized in non-denaturating electrophoresis (Fig. 2). Such complexes were also produced with wild type AChE$_T$ and constructs containing the PRAD of PriMA (Ps46 and PQ) but not with mutated Y31G AChE$_T$ subunits (Fig. 3). A comparison between the PQ and QP chimeras suggests that the Y31G mutation does not interfere with the N-terminal region preceding the proline motifs but that its effect depends on the length of the polyproline motifs and/or on the residues located immediately downstream.

**Influence of the Cysteines Preceding the PRADs on the Recrui-tment of AChE$_T$ Subunits—**Nondenaturing gel electrophoresis showed that the various binding proteins (QN, Q$_{71}$, P$_{46}$, PQ, and QP) induced the formation of AChE$_T$ tetramers. All of these complexes were soluble and secreted, appearing less abundant in the cell extracts than in the culture media. We analyzed the effect of mutating the cysteines in the N-terminal region of PriMA and found that mutants of P$_{46}$ and PQ containing the 4 cysteines (Cys$_6$, Cys$_{13}$, Cys$_{17}$, Cys$_{19}$), only 2 cysteines, a single cysteine, or no cysteine, all induced the formation of heteromeric AChE$_T$ tetramers. This shows that cysteines are not necessary for the formation of the complexes, in agreement with the fact that the entire N-terminal region of PriMA, which contains the cysteines, could be deleted (9).
10 S increased gradually with the amount of binding protein to reach a saturating level of about 80%, and this was not significantly modified by mutations of the cysteines (see supplemental Fig. 3S).

Comparison of the Recruitment of AChET Subunits by Proteins Containing the PRADs of ColQ and PRiMA—We also compared the recruitment of AChET subunits by Ps46, Ps38, Qs71, Qs45, and the chimeras PQ and QP in the cells and the media (see supplemental Fig. 4S). The total AChE activities were similar in all cases in both the cells and the media, but the proportions of tetramers increased with the amount of vector encoding the co-transfected binding protein and appeared to saturate at similar concentrations. However, the maximal proportion of tetramers was considerably lower for QP than for the other constructs, both in the cells and in the medium. PQ and Ps46 appeared essentially equivalent, producing a proportion of tetramers that was slightly higher than with Qs71 and Qs45 in the cells, but lower in the media, perhaps indicating a difference in the secretability of the complexes. In this series, the PRAD of ColQ associated more efficiently with tetramers than the PRAD of PRiMA, and each PRAD functioned better when associated with its own N-terminal region.

Equivalent Amounts of Light and Heavy Dimers Are Formed with Binding Proteins Containing the PRAD of ColQ, but Not the PRAD of PRiMA, in Transfected COS Cells—It is well established that in complexes of four AChE subunits with ColQ or of its N-terminal fragment QN, two catalytic subunits are disulfide-linked with the structural protein through their C-terminal cysteines, and the other two are disulfide-linked together. Thus, in nonreducing SDS-PAGE, the complex gives rise to one dimer of AChE subunits (the light dimer) and one dimer associated with the binding protein (the heavy dimer).

We analyzed intercatenary disulfide bonds in complexes formed by co-expressing AChE subunits and PRiMA in COS cells by nonreducing electrophoresis of cell extracts and Western blotting. For comparison, we studied AChE expressed with a nonbinding protein (ΔPRAD) and with the QN-HC construct, which, like PRiMA, produces membrane-bound AChE tetramers. Fig. 4 shows Western blots in which AChE was detected with a specific commercial anti-AChE antibody, E-19. Although AChE alone produced monomers and only a small amount of dimers, the association with QN-HC or PRiMA induced the formation of oligomers. In the case of QN-HC, we observed light and heavy dimers as reported previously. In the case of PRiMA, we observed mostly light dimers and heavy components (HC), which migrated with a very high apparent mass, above the 500-kDa mass standard. A faint band was identified as consisting of heavy dimers because it contained PRiMA (shown in Fig. 7), but its intensity was much weaker than that of light dimers.

We also analyzed the complexes formed with N-terminal fragments of PRiMA as well as QN and the chimeras QP and PQ. Because the resulting complexes were not membrane-bound but secreted, we analyzed both the cell extracts and the
culture media. Nonreducing SDS-PAGE and immunodetection of HA-labeled AChE\textsubscript{T} clearly showed the presence of heavy dimers in complexes formed with Q\textsubscript{N} and PQ but not with P\textsubscript{s46} (Fig. 5) or with QP (Fig. 6A); this is due to the fact that the two types of dimers are not readily resolved because of the small size of the binding proteins, as small fraction of HA-labeled P\textsubscript{s46} or QP could be detected at this position (not shown). In addition, HC resembling those formed with PRiMA were formed and secreted with PQ and at a lower level with P\textsubscript{s46} (in the latter case, secreted HC were barely detectable, as shown in Fig. 6A) but not with Q\textsubscript{N} or QP. Thus, the formation of heavy components depends on the N-terminal region of PRiMA.

The apparent mass of AChE\textsubscript{T} monomers and light dimers was higher in the medium than in cell extracts but became indistinguishable after deglycosylation, showing that the difference is due to their N-linked glycans, which are elongated during maturation in the secretory pathway (Fig. 5). However, the secreted heavy dimers formed with Q\textsubscript{N} and PQ still appeared about 30 kDa heavier than the cellular species after deglycosylation. This suggests that an additional component, perhaps a disulfide-linked protein, might be coupled with the secreted AChE\textsubscript{T}–PQ complex. This difference did not occur with Q\textsubscript{s71} (not shown), indicating that it depends on the C-terminal peptide of Q\textsubscript{N} and is not relevant to the association with AChE\textsubscript{T} subunits.

Mutation of Cysteines in Q\textsubscript{N}, P\textsubscript{Q}, PQ, and QP—Mutations of the cysteines preceding the PRADs, which could be involved in intercatenary disulfide bonds, showed that PQ mutants containing any pair of cysteines produced heavy dimers (Fig. 6A). A mutant of Q\textsubscript{N}, in which Cys\textsubscript{28} and Cys\textsubscript{29} were replaced by cysteines at positions 14 and 15, i.e. at a distance from the prolines similar to the upstream cysteines of PRiMA, also formed heavy dimers (Fig. 6B). The formation of intercatenary disulfide bonds by cysteines located at various positions suggests that the N-terminal regions of PRiMA and ColQ containing the cysteines are very flexible. Each of the 4 cysteines of the PQ chimera could form disulfide bonds with AChE\textsubscript{T} subunits, but PQ mutants containing a single cysteine were not disulfide-linked with one AChE\textsubscript{T} subunit, as we did not observe any “heavy monomers” that would migrate between monomers and light dimers (Fig. 6A); this indicates that the disulfide bonds of AChE\textsubscript{T} dimers can be replaced by disulfide bonds with PQ only when this does not leave an unpaired AChE\textsubscript{T} subunit.

Heavy components were produced and secreted with PQ and at a lower level with P\textsubscript{s46} when these proteins contained all 4 cysteines of the N-terminal region of PRiMA but not when some of the cysteines were mutated (Fig. 6B). Similarly, they were not produced with full-length PRiMA when the four cysteines were mutated (not shown). This is consistent with the idea that they contain four AChE\textsubscript{T} subunits, disulfide-linked to the 4 cysteines preceding the PRAD of PRiMA.

The Components of High Apparent Mass Are Derived from the 9 S AChE\textsubscript{T}–PRiMA Complex and Contain Both AChE\textsubscript{T} and PRiMA—The apparent mass of HC observed in nonreducing SDS-PAGE is too high for a complex of four AChE\textsubscript{T} subunits associated with a small protein, PRiMA, PQ, or P\textsubscript{s46}. To deter-

![Figure 4. Analysis of intercatenary disulfide bonds in membrane-bound complexes of AChE\textsubscript{T} subunits with PRiMA and Q\textsubscript{N}–H\textsubscript{C}. AChE\textsubscript{T} subunits (2 \(\mu\)g of DNA/60-mm dish) were expressed in COS cells without binding protein and with PRiMA or Q\textsubscript{N}–H\textsubscript{C} (3 \(\mu\)g of DNA) forming membrane-anchored complexes. Disulfide-linkages between AChE\textsubscript{T} subunits and between AChE\textsubscript{T} subunits and binding proteins were analyzed in cell extracts by nonreducing SDS-PAGE and Western blotting with the E-19 anti-AChE antibody. In the absence of binding protein (i.e. with \(\Delta\)PRAD), we observed monomers and dimers of AChE\textsubscript{T}, in the presence of Q\textsubscript{N}–H\textsubscript{C}, light and heavy dimers (composed of two AChE\textsubscript{T} subunits linked with Q\textsubscript{N}–H\textsubscript{C}), and in the presence of PRiMA, light dimers and HCs, with an apparent mass superior to that of the 500-kDa standard. A faint band about 10-kDa heavier than the light dimers appears to contain PRiMA (see Fig. 7) and therefore represents a small proportion of heavy dimers.](image)

![Figure 5. Cellular and secreted complexes of AChE\textsubscript{T} subunits with P\textsubscript{s46}, Q\textsubscript{N}, and PQ showing the effect of deglycosylation. As described in the legend for Fig. 4, COS cells were transfected with 2 \(\mu\)g of DNA encoding AChE\textsubscript{T} and 3 \(\mu\)g of DNA encoding binding proteins. AChE\textsubscript{T} subunits were labeled with a C-terminal HA epitope, and the resulting AChE\textsubscript{T} species were analyzed by nonreducing SDS-PAGE and immunodetected with an anti-HA antibody, both in the cells and in the culture medium, with and without deglycosylation with N-glycanase. Q\textsubscript{N} produced light and heavy dimers; P\textsubscript{s46} produced light dimers and components of high apparent mass (HC); PQ produced all of these species. All AChE\textsubscript{T} monomers and oligomers were N-glycosylated, as their apparent masses decreased after deglycosylation. The masses of secreted monomers and light dimers were higher than those of corresponding cellular species but became identical after deglycosylation, as indicated by the thin dotted lines. The situation appears more complex for heavy dimers as discussed under “Results.”](image)
mine their origin, cell extracts containing the AChE₇–PRiMA complex were fractionated in sucrose gradients, and individual fractions were analyzed by SDS-PAGE. We analyzed in parallel complexes formed with HA-labeled AChET subunits and with HA-labeled PRiMA. Under reducing conditions, we observed only the expected monomeric HA-labeled AChET and PRiMA subunits migrating around 70 and 20 kDa, respectively (not shown). Western blots performed under nonreducing conditions showed that the distribution of the HC coincided with that of the 9 S PRiMA-linked AChET complex, indicating that they were derived from this molecular species and that they contained both AChET and PRiMA (Fig. 7, left panels). An analysis of complexes containing HA-labeled AChET and PRiMA in the same gel indicated that the two proteins were present in the HCs (Fig. 7, right panel). We detected HA-labeled PRiMA in a band about 10 kDa heavier than the light AChET dimers, which probably corresponds to heavy dimers.

Abnormal Migration of the Heavy Components in Nonreducing SDS-PAGE—The presence of components with an apparent mass exceeding 500 kDa in the complexes formed between AChET subunits and PRiMA or PQ was paradoxical, because these complexes sediment around 9 or 10.5 S, in agreement with the idea that they consist of an AChE tetramer (4 × 70 kDa) associated with the structural PRAD-containing protein (20 kDa). Their total mass would therefore be about 300 kDa. A much higher mass might be reconciled with the observed sedimentation coefficient only if these complexes incorporate very elongated components and possessed a very high Stokes radius. We therefore determined the Stokes radii of the AChE₇–PRiMA and AChET–PRQ complexes by gel filtration chromatography (Fig. 8) and evaluated the mass of the AChE₇–PQ complex by comparison with standard proteins, assuming that the mass is approximately proportional to the product of its sedimentation coefficient and its Stokes radius (S × Rₛ) for proteins of similar density (this would not hold for AChE₇–PRiMA complexes, which are associated with detergent micelles). The Stokes radius of the AChE₇–PQ complex, was about 6.7 nm, which together with a sedimentation coefficient of 10.5 S indicates a mass of about 300 kDa, as expected for a tetramer of AChET subunits associated with the structural protein.

It thus appeared that the heavy components must be composed of four AChET subunits, disulfide-linked to the 4 cysteines of PRiMA or of PQ. This, however, does not explain the existence of at least two bands in the gels, and it is therefore possible that other proteins might be associated with the complexes.

In any case, the apparent mass of HCs deduced from nonreducing SDS-PAGE is much too high, indicating an abnormal electrophoretic migration. It appeared possible that this could reflect an aggregation of the protein and the formation of disulfide bonds upon denaturation. However, when the samples were pretreated with 5 or 10 mM N-ethylmaleimide, iodoacetamide, or iodoacetic acid for 90 min at 20 °C to block any free cysteines before denaturation and electrophoresis, we observed the same heavy components (not shown). Another explanation could be that the assembly of four disulfide-linked AChET subunits with small associated proteins was not totally denatured and bound less SDS than a fully unfolded protein, resulting in a slower migration rate. We therefore performed denaturation and electrophoresis in the presence of 6 M urea, which was expected to induce a complete denaturation. This again had no effect on the migration of the heavy components. It has been found that acidic proteins such as α-synuclein bind less SDS than classical proteins and migrate with a very high apparent mass in SDS-PAGE (22); in our case, the migration of individual subunits in reducing SDS-PAGE was in fact normal.

Analysis of PRiMA-anchored AChE in the Mammalian Brain—The preceding experiments were performed with various mutated and/or epitope-tagged PRiMA and AChET subunits expressed in transfected COS cells. To see whether the
of oligomers. We verified that after reduction, the totality of AcE immunoreactivity migrated as monomers, as in the case of transfected COS cells (not shown).

**DISCUSSION**

**Conserved Differences between the PRADs of ColQ and PRiMA: Role of the Tyr31 Residue of the t Peptide**—The present results indicate that the quaternary association of a tetramer of AChET subunits with PRiMA differs from that with ColQ. The difference between ColQ and PRiMA mainly appears to be due to the length of their proline-rich motifs, to residues immediately following the PRAD, and to the numbers of upstream cysteines. As shown in supplemental Fig. 2S, the overall length of the proline-rich motifs seems to be conserved among species for each protein (10 for ColQ, 15 for PRiMA), whereas the intercalated residues vary, suggesting that they do not play a critical role in agreement with previous mutagenesis studies on ColQ and on PRiMA (6). In contrast, two residues located downstream of the prolines (FF for ColQ, RL for PRiMA) are conserved, and deletion experiments have shown that they participate in the interaction (6). In fact, deletion of the residues following the prolines in PRiMA markedly reduced the recruitment of AChET subunits (9).

Crystallographic studies revealed that the complex formed by t peptides and a ColQ-PRAD peptide is a very tight coiled coil of four α-helical t peptides, forming a left-handed spiral staircase around the more extended polyproline II helical PRAD (8); the overall length of the 40-residues α-helices corresponds approximately to that of the 10-residues PRAD. It is possible that, in the case of the longer PRAD of PRiMA, the t peptide α-helices are more staggered, forming a longer cylinder. In any case, we found that, although the distal Tyr31 residue is dispensable for the interaction with the PRAD of ColQ, it plays an important role in the association with the longer PRAD of PRiMA.

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PRiMA, and each PRAD functions better with its own N-terminal region.

Disulfide Bonds between AChEₜ Subunits and PRiMA—Previous results had suggested that, in the same manner as ColQ, PRiMA is disulfide-linked to a pair of AChEₜ subunits in the complex (heavy dimer), the other two being disulfide-linked to each other (light dimer) (10, 11). We did observe such heavy dimers in nonreducing polyacrylamide gels, revealed either by radioactive metabolic labeling (not shown) or by Western blotting of complexes formed with constructs containing the PRAD of ColQ, such as Q₍ or PQ, but very little with PRiMA or with constructs containing the PRAD of PRiMA, such as P₄₆ or QP. Even though the regions preceding the PRAD contain cysteines that might form intercatenary disulfide bonds, they are not responsible for this difference, as shown by comparison of the chimeras PQ and QP in which they were exchanged. In the case of PQ, we observed the formation of heavy dimers with mutants containing all 4 cysteines or any pair of cysteines from the upstream region of PRiMA, indicating that this region is quite flexible. When a single cysteine was present, it was not disulfide-linked to an AChEₜ subunit, probably because it would be thermodynamically unfavorable to leave an unpaired AChEₜ subunit.

The apparent mass of secreted monomers and dimers, as indicated by SDS-gel electrophoresis, was significantly higher than that of the corresponding cellular components. This difference was abolished after deglycosylation, in the case of monomers and light dimers, showing that it reflects the maturation of glycans in the secretory pathway. However, the difference between secreted and cellular heavy dimers formed with Q₄ or PQ was much higher and was maintained after deglycosylation, suggesting that an additional component, possibly a protein, may be added to the secreted complexes, forming a “super heavy” dimer. This association appears specific for the C-terminal region shared by Q₄ and PQ and therefore does not concern the association of AChEₜ subunits with PRiMA.

FIGURE 8. Determination of the Stokes radius of the AChEₜ-PQ complex by gel filtration chromatography. The main frame of this figure shows the elution of protein standards and of the AChEₜ-PQ complex using a gel filtration column (Bio gel A15m). The void volume (Vᵥ) and the total volume (Vₜ) correspond to the elution of blue dextran and of potassium ferricyanide, respectively. As shown in the inset, the linear relationship between the Stokes radius (Rₛ) and the square root of the logarithm of the elution parameter (Kₑ = (Vᵥ – Vₛ)/(Vₜ – Vₛ)) showed that for the complex, Rₛ = 6.7 nm. We obtained the same Rₛ value when analyzing gradient fractions corresponding to the AChEₜ-PQ complex (10.5 S). We also verified that fractions from the gel filtration column (Rₛ = 6.7 nm) sedimented at 10.5 S as expected for the AChEₜ-PQ complex and produced the same pattern of bands in SDS-PAGE as shown for AChEₜ-PQ in Fig. 5, including the components of high apparent mass (HC). The AChEₜ-PRiMA complex was analyzed in the same way in the presence of 0.1% Triton X-100; the deduced Stokes radius was ~7 nm (not shown).

FIGURE 9. Analysis of AChE from rat, mouse brain, and PRiMA⁻/⁻ mouse brain. A, sedimentation profiles. Low salt-soluble (LSS) and detergent-soluble (DS) brain extracts were analyzed by sedimentation in sucrose gradients in the presence of 1% Brij-97. All sedimentation profiles are drawn to the same scale, relative to the total AChE activities in the extracts. In wild type rat and mouse brain, the predominant AChE species corresponds to detergent-soluble AChEₜ-PRiMA complexes sedimenting at 9 S. This species does not exist in PRiMA⁻/⁻ mouse, revealing the presence of tetramers sedimenting at 12 S, which may exist in wild type brains but in such a low proportion that they would be difficult to detect. B, analysis by nonreducing SDS-PAGE and immunodetection with the anti-AChE antibody E-19. For wild type rat and mouse brains, we observed light dimers, possibly a small proportion of heavy dimers and HCs, probably composed of four AChEₜ subunits disulfide-linked to PRiMA. This pattern is identical to that observed for AChEₜ-PRiMA complexes formed in transfected COS cells. The intensity is very low in the case of PRiMA⁻/⁻ mouse brain, but even after prolonged exposure, we could not detect any heavy components (not shown).
Abnormal Migration of an Assembly of Four AChET Subunits with PRiMA in Nonreducing SDS-PAGE—We also found that components of very high apparent mass (HC) contain both AChET subunits and PRiMA or PQ; these components were observed both in the cell extracts and in the medium, indicating that their assembly precedes secretion. Similar heavy components had been observed previously in Western blots of purified brain AChE under nonreducing conditions and were considered to represent various oligomers or aggregates of AChET and PRiMA subunits (11, 23–25). Sedimentation analyses indicate that these heavy components derive from the 9–10.5 S AChET20-kDa protein labeled with the [125I]TITD hydrophobic reagent species in mammalian brain. The present results differ from the PRiMA or AChET-PQ complexes. Although the apparent mass of these components in nonreducing SDS-PAGE was higher than that of a 500-kDa standard, this clearly was the result of an abnormal migration, since the Stokes radius of the AChET-PQ complex was found to be 6.7 nm; combined with its sedimentation coefficient (10.5 S), this indicates a mass of ~300 kDa (26), i.e. the value expected for an assembly of four AChET subunits disulfide-linked to the structural protein. However, the presence of at least two distinct heavy components suggests that they may incorporate other small proteins.

We obtained a similar Stokes radius for the AChET-PRiMA complex. The determination of its mass is less straightforward because the binding of detergent micelles affects its hydrodynamic parameters (sedimentation coefficient, Stokes radius, density), but it cannot be as high as suggested for the heavy components in nonreducing SDS-PAGE. The abnormal migration of an AChE tetramer attached to PRiMA or PQ, under denaturing but nonreducing conditions, was not due to an unusual behavior of individual components, as they migrated according to their mass after reduction. This migration does not appear to be due to the formation of aggregates during denaturation or to incomplete denaturation, because it was not modified by blocking free cysteines or by 6 M urea. It may rather depend on the organization of the complex, even in the presence of 6 M urea.

The hypothesis that these components contained four disulfide-linked AChET subunits is supported by the fact that they were produced when AChET subunits were co-expressed with PRiMA itself and also with fragments of PRiMA (P46) and with the PQ construct, which contains the N-terminal region of PRiMA, but only when the four cysteines were present. Another cysteine, Cys46, located downstream of the PRAD, is not involved in this association, as it is not present in P46 or in PQ.

AChET-PRiMA Complexes from Rat and Mouse Brain—We obtained identical results in transfected COS cells and in brain extracts from rat or mouse; the AChET-PRiMA complex was of course absent from the brain of mice in which the PRIMA gene was knocked out. This shows that the conclusions drawn from our studies of complexes formed in transfected COS cells are generally valid for the physiological PRiMA-anchored AChE species in mammalian brain. The present results differ from the original description of PRiMA, which was discovered as a 20-kDa protein labeled with the [125I]TITD hydrophobic reagent in purified preparations of membrane-bound AChE tetramers isolated from bovine brain (10, 11, 25). Under nonreducing conditions, this protein appeared to be disulfide-linked with two AChET subunits forming heavy dimers, which were 20 kDa heavier than the light dimers and equally abundant. The published Western blots also showed the presence of apparently heavy bands, which were not interpreted but resemble the HCs described here.

The present study showed that nonreducing electrophoresis of AChET-PRiMA complexes, either produced in transfected COS cells or extracted from rat or mouse brain, produced a faint PRiMA-containing band that might correspond to heavy dimers, but it contained a small fraction of total AChET subunits. In AChET-PRiMA complexes from rat and mouse brain, the AChET subunits were distributed nearly equally between components of high apparent mass (HC) and dimers. It therefore appears that intercatenary disulfide bonds in AChET-PRiMA complexes are organized in three possible manners: between all four AChET subunits and PRiMA; only between pairs of AChET subunits; and very modestly in light and heavy dimers, as in the case of AChET-ColQ complexes. The major difference between our experimental procedures and those used in previous studies is that we analyzed the brain samples immediately after extraction without prior fractionation or purification. It is possible that disulfide bonds were remodeled during the purification procedure so that heavy dimers were produced secondarily.

In conclusion, we have found that the organization of AChET tetramers associated with the PRiMA membrane anchor is probably different from that of ColQ-associated tetramers. In the AChET-PRiMA complex, formed either in transfected COS cells or in the mammalian brain, four AChET subunits may be disulfide-linked to PRiMA. The presence of a doublet of HCs in nonreducing SDS-PAGE suggests that the complex may incorporate additional small proteins. Such hypothetical partners interacting with AChE or with the extracellular domain of PRiMA may play a role in the functional localization of membrane-bound AChE at cholinergic synapses and therefore deserve further study.

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