The C-terminal 40-residue t peptide of acetylcholinesterase (AChE) forms an amphiphilic α helix with a cluster of seven aromatic residues. It allows oligomerization and induces a partial degradation of AChE subunits through the endoplasmic reticulum-associated degradation pathway. We show that the t peptide induces the misfolding of a fraction of AChE subunits, even when mutations disorganized the cluster of aromatic residues or when these residues were replaced by leucines, indicating that this effect is due to hydrophobic residues. Mutations in the aromatic-rich region affected the cellular fate of AChE in a similar manner, with or without mutations that prevented dimerization. Degradation was decreased and secretion was increased when aromatic residues were replaced by leucines, and the opposite occurred when the amphiphilic α helix was disorganized. The last two residues (Asp-Leu) somewhat resembled an endoplasmic reticulum retention signal and caused a partial retention but only in mutants possessing aromatic residues in their t peptide. Our results suggested that several “signals” in the catalytic domain and in the t peptide act cooperatively for AChE quality control.

The major function of acetylcholinesterase (AChE) is to regulate the cholinergic transmission by cleaving the neurotransmitter acetylcholine. In mammals, alternative splicing of a single gene gives rise to different variants (types R, H, and T), which possess the same catalytic activity but different tissue-specific distributions (1–3). The catalytic subunits of type T, which possess the same catalytic activity but different tissue-specific distributions (1–3). The catalytic subunits of type T possess the same catalytic activity but different tissue-specific distributions (1–3). Type T, AChE subunit of type T; DEPQ, 7-[(2R,3R,4S,5R)-3-((1R,2S,3R)-1-methyl-2,3-dihydroxypropoxy)-1-methylquinolinium methyl sulfate; ER, endoplasmic reticulum; PRAD, proline-rich attachment domain; t peptide, C-terminus of AChE; WAT, tryptophan amphiphilic tetramerization domain (8). Its homo- and hetero-oligomerization properties depend on two major features, which are conserved in all vertebrates: 1) a series of seven aromatic residues (Trp10, Phe14, Trp17, Tyr20, Trp24, Phe28, and Tyr31) and 2) a cysteine near its C terminus (Cys37) (9). Spectroscopic and crystallographic studies showed that the t peptide is organized as an amphiphilic α-helix in which all of the aromatic residues are grouped on one side (10). Cys37 may form disulfide bridges between two t peptides in homodimers and homotetramers or between two t peptides and the two PRAD cysteines in the heteromeric PRAD-linked tetramers (10–16).

The formation of AChE subunits depends on contact between subunits at the level of two α-helices from each catalytic domain (αT,αR and αT,αT), forming the “four-helix bundle” (17), and on the formation of a disulfide bridge between the C-terminal cysteines of two t peptides (18), but it does not require the aromatic residues of the t peptide. It has been shown that rat AChE subunits, which carry a mutation in the αT,αR helix (F535A), 9 amino acids upstream of the t peptide), are deficient in dimerization despite the presence of Cys37 (18). In contrast, the formation of homomeric or PRAD-linked tetramers depends on the cluster of aromatic residues. It is facilitated by intercatalytic disulfide bonds but also occurs in their absence (9).

The presence of a t peptide induces an intracellular degradation of AChE subunits, which is strongly increased with the F535A mutation (AChE*), unless they assemble with a PRAD-containing N-terminal fragment of ColQ (Qα), forming PRAD-linked complexes. This degradation process follows the ER-associated degradation pathway. It can be blocked by proteasome inhibition and is reduced by inhibiting ER-mannosidase I, indicating that sugar chains are involved in its mechanism (9).

Furthermore, the t peptide seems to play a role in delaying protein secretion. Indeed, we recently showed that deletion of the last two residues increased the secretion of Torpedo AChE, suggesting that its C terminus behaves as a weak retention signal, perhaps facilitating the assembly of oligomers. This may be due to the similarity of the C-terminal tetrapeptide (CAEL) with the classical endoplasmic reticulum retention signal, KDEL (9, 20).

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In this study, we designed mutations to assess the importance of the cluster of aromatic residues in the α-helical structure and of the aromatic versus hydrophobic nature of these residues in rat AChE<sub>E</sub> subunits. We investigated the synthesis, secretion, degradation, and interaction with a PRAD for wild type and F535A mutants with and without the C-terminal Cys<sup>37</sup>. We showed that distinct features of the t peptide determine these different processes.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Transfection**—CDNAs encoding the AChE<sub>E</sub> subunit of rat AChE and an N-terminal fragment of CoIQ (Q<sub>N</sub>) were inserted in the expression vector (21). The primary sequence of the Q<sub>N</sub> protein is 5'-ESTFLDKAFSLQALLPMEMHKKVSNKCCLETITPPPPPPMFPFFFTETTNLQVEQDLLNLNLPEKTEPS-3' (6) (the signal peptide is not shown; the PRAD and the two cysteines are underlined in boldface).

Site-directed mutagenesis was realized with the method of Kunkel (22). The C2 mutant was derived by deletion of most of the C-terminal peptide from the H variant of AChE, leaving a cysteine at position 6 downstream of Cys<sup>37</sup>. We showed that distinct features of the t peptide determine these different processes.

**Results**

**Mutations in the t Peptide**—We introduced mutations in the t peptides of rat AChE<sub>E</sub> and of the dimerization-defective mutant carrying the F535A mutation in the α<sub>10</sub> helix (AChE<sup>*</sup>), as illustrated in Fig. 1A. We modified the cluster of aromatic residues of the t peptide in various ways. The segment containing these residues was deleted in the Del<sub>10–31</sub> and Stp10 mutants, the seven aromatic residues were replaced by leucines in the 7L mutant, and two phenylalanine residues (Phe<sup>14</sup> and Phe<sup>28</sup>) were replaced by tryptophans in the 5W mutant. In the “scrambled” (Scr) mutant, the positions of the seven aromatic residues were exchanged with those of other residues so that they could be dispersed around the helix (Fig. 1B). In the Scr mutant, the composition of the t peptide and the positions of acidic and basic residues were not modified to maintain potential salt bridges, which may stabilize the helical structure of its oligomeric associations. The 10–31 region is predicted to form an α-helix in the case of the 7L, 5W, and Scr mutants as in the wild type t peptide (see “Experimental Procedures”).

In this paper, WT refers to the wild type aromatic region of the t peptide combined or not with the F535A mutation in the catalytic domain (AChE<sub>E</sub> or AChE<sup>*</sup>) and with mutations in the C-terminal tetrapeptide, which contained a cysteine or a serine at position 37 (indicated by indices C or S, for example: 5WC, 5W<sub>C</sub>). In the stop39 mutant, we removed the last two residues (Asp-Leu) so that the C terminus would be Ser-Ser. We also replaced the last four residues by the canonical ER retention signal KDEL. For comparison, we studied mutants lacking the t peptide consisting of the catalytic domain only (C1) or of the catalytic domain followed by a short peptide containing a cysteine (C2). We found that C1 formed exclusively monomers, whereas C2 was largely dimerized.

All of the mutants produced active AChE. We compared the activities of these mutants by titrating their active sites with an irreversible inhibitor, DEPQ. We found that the catalytic turnover per site was identical for cellular and secreted enzymes and was not affected by the presence of the F535A mutation, by the presence or absence of the t peptides, or by the different mutations that were introduced in the t peptide (data not shown).

**Dimerization Requires Cys<sup>37</sup>, Whereas Tetramerization with or without Q<sub>N</sub> Mainly Depends on the Aromatic Cluster**—Fig. 2A shows the electrophoretic patterns obtained in non-denaturing electrophoresis for the different mutants of rat AChE<sub>E</sub> subunits expressed in transfected COS cells with or without Q<sub>N</sub>. As previously described (6, 30), cellular extracts from cells expressing wild type AChE<sub>E</sub> subunits contained monomers (T<sub>1</sub>), dimers (T<sub>2</sub>), and a small proportion of tetramers (T<sub>4</sub>), whereas the culture medium contained comparable proportions of monomers, dimers, and tetramers. When the cells co-expressed AChE<sub>E</sub> subunits with the Q<sub>N</sub> protein, both cell extract and medium contained Q<sub>N</sub>-linked tetramers (T<sub>4</sub><sub>Q<sub>N</sub></sub>).

The molecular forms of AChE produced with the 5WC mutant were very similar to those obtained with the wild type, although the formation of homomeric tetramers (T<sub>4</sub>) was slightly reduced. When this mutant was co-expressed with Q<sub>N</sub>, the formation of T<sub>4</sub><sub>Q<sub>N</sub></sub> complexes was reduced. The Scr<sub>T</sub> mutant produced monomers and dimers but no tetramers, either homomeric or associated with Q<sub>N</sub>. This mutant was secreted at a very low level, either without or with Q<sub>N</sub>. Cells expressing the 7L<sub>E</sub> mutant also produced monomers and dimers but secreted mostly dimers. It did not form any tetramer, but a slowly migrating component (perhaps hexamers) appeared in the medium (Fig. 2, A and B). The Scr<sub>T</sub> and 7L<sub>T</sub> mutants illustrate the necessity of an aromatic cluster for tetramerization.

Without Cys<sup>37</sup>, dimerization was abolished in all of the cases. The WT<sub>Q<sub>N</sub></sub> subunits (which only differed from the wild type by the C37S mutation) mainly produced monomers (T<sub>1</sub>) together with a slowly migrating component (Fig. 2B). The C1 and C2 cells produced exclusively monomers (T<sub>1</sub>).
with lower levels of tetramers (T₄) and T₄-QN complexes than the wild type. The ScrS and 7LS mutants only produced monomers. It is noteworthy that the 5WS mutant did not form homomeric tetramers or QN-linked tetramers, in contrast to the 5WC mutant. This shows that intercatenary disulfide bonds contribute to the stability of both homomeric and QN-associated tetramers.

Tetramerization was also abolished in the case of the dimerization-deficient AChE*-T subunits containing the Scr and 7L mutations with or without Cys37. Transfected cells co-expressing AChE*-5WC with QN secreted T₄-QN complexes but at a lower level than with the wild type, and these complexes were not detectable in cellular extracts. The AChE*-5WS mutant, similar to AChE-5WS, did not form such heteromeric complexes.

In Fig. 2, the relative intensities of AChE bands in the medium reflect the relative levels of activity of the different mutants, as documented below. For example, the Del and 7L mutants were more actively secreted than the WT, 5W, and particularly Scr mutants.

The Amphiphilicity of Monomers and Dimers Depends on a Cluster of Hydrophobic Residues in the t Peptide—Fig. 2 shows the influence of a charged detergent, DOC, on the migration of AChE molecular forms in the presence of the non-ionic detergent Triton X-100. The migrations were normalized to those of the Del₄ and Del₅ mutants, which do not possess the cluster of hydrophobic residues and constitute non-amphiphilic standards. The migration of amphiphilic molecules such as the wild type monomers and dimers was slower in the presence of Triton X-100 alone compared with Triton X-100 plus DOC. Only those produced by the ScrC mutant retained a fast migration under these conditions. Fig. 2C shows the ratio of migration in the presence and absence of DOC as an index of amphiphilicity. We obtained similar values for cellular and secreted molecular species. Dimers were systematically less accelerated by DOC than monomers, as expected if their aromatic clusters partially had occluded each other, and tetramers were non-amphiphilic (data not shown). The wild type and 5W mutants presented similar values, the 7L mutants were significantly less sensitive to the detergents, and the Scr mutants were essentially not affected. Thus, the amphiphilic character depends on the clustering of hydrophobic residues. It is interesting that leucines provide a weaker amphiphilic character than tryptophans, which most authors classify as less hydrophobic.

Influence of the Aromatic-rich Segment on Cellular and Secreted AChE Activities—Fig. 3 shows the levels of AChE activity obtained in cellular extracts and in the medium for the different mutants expressed in COS cells normalized to those obtained for the wild type AChE-T subunit.

**Fig. 1. Mutations analyzed in this study.** A, mutations in the C-terminal t peptide of rat AChE in the presence or absence of mutation F535A (respectively noted AChE* and AChE). This mutation, located in the C-terminal α₁₀ helix of the catalytic domain, compromises dimerization through the four-helix bundle. In mutant C1, the entire t peptide was deleted, and in mutant C2, it was replaced by the first seven residues from the C-terminal peptide of the AChE₉ splice variant containing a cysteine (see “Experimental Procedures”). In this paper, WT refers to the wild type aromatic-rich region. B and C, wheel diagrams of the 9–32 segment of the wild type and Scr mutant. The preceding mutations were combined with mutations in the C-terminal tetrapeptide (underlined), replacement of Cys³⁷ by a serine as noted by an index (e.g. WT₉, or WT₁₀), deletion of the last two residues with the C37S mutation (e.g. WT₉₁₀), and replacement of the last four residues by the ER retention signal KDEL (e.g. WT₉₋₉₁₀).
When Cys37 was present, the cellular enzymatic activity was increased to 130 and 160% for ScrC and 5WC, respectively, whereas the corresponding secreted activity was reduced to 30 and 50%. On the contrary, the cellular activity was reduced to 40% or less for 7LC, whereas the corresponding secreted activity was increased to 150%. Secretion was increased even higher...
for the DelC mutant (280%) and reached the same level as that for the truncated mutants, which lacked the t peptide, either without a C-terminal cysteine, (C1: 220%) or with cysteine near the end of the catalytic domain, allowing dimerization (C2: 270%). The cellular activities of these mutants were either slightly increased (110% for DelC) or decreased (70% for C2). In the absence of cysteine Cys37, both cellular and secreted activities were lower, suggesting an increased degradation, but the effects of the mutations were qualitatively similar.

Thus, secretion was strongly increased when the aromatic sector was removed (Del, C1, or C2) and to a lesser extent when aromatic residues were replaced by leucines (7L). In contrast, secretion was reduced and we observed an increase of the cellular activity when two phenylalanines were replaced by tryptophans (5W). Also, this effect was stronger when the clustering of aromatic residues was suppressed by changing their positions (Scr).

The cellular activity obtained with the dimerization-deficient mutant AChE* carrying a wild type t peptide with or without Cys37 was <40% wild type, and secretion was almost abolished compared with that of the C2* mutant, indicating a strong degradation as previously reported (18). Despite the fact that the Scr mutation did not increase the cellular activity, possibly because of intracellular degradation, the effects of the different mutations in the t peptide were remarkably similar to those observed without the F535A mutation. It is particularly noteworthy that the dimerization-deficient mutants AChE* 7L,C and AChE* 7L,5W were significantly secreted, in contrast to AChE* WT and AChE* WT5.

Cells expressing the wild type AChE produced and secreted somewhat more activity than cells expressing the C37S mutant; however, Fig. 4 shows that the ratio of secreted to cellular activities were the same, suggesting that the cysteine exerts no retention effect, in agreement with the fact that monomers possessing the CSDL C terminus were actively secreted along with disulfide-linked dimers. In the case of the dimerization-deficient mutants (AChE*), the ratio of secreted to cellular activities was clearly lower with a cysteine for WT, Scr, and 5W.

Fig. 3. Effect of mutations in the aromatic-rich region on the levels of cellular and secreted AChE activity. The mutants contained a wild type or a dimerization-deficient catalytic domain noted, respectively, as AChE (plain bars) and AChE* (hatched bars) and possessed Cys37 or not (C37S) as indicated. The cellular and secreted activities were measured after 3 days of transfection. Because of variation in transfection efficiencies, the values obtained in each experiment were normalized to those obtained for the wild type (AChE-WT), which was always used as a control taken at 100%. The values indicated are the means ± S.E. of at least three totally independent transfection experiments. Note that secretion was increased by the deletion of the aromatic-rich region (C1, C2, stop10, or Del) and, to a lesser degree, by the 7L mutation. Because the synthesis of AChE protein was the same for all of the mutants as shown in Fig. 6A and all of the mutants possessed the same catalytic activity (data not shown), the differences observed reflect the production of the active enzyme.

Fig. 4. Ratio of secreted to cellular activities: the combined effects of mutation F535A, of mutations in the aromatic-rich domain, and of the C-terminal Cys37. Because of their wide range, the values were plotted on a logarithmic scale. Dark and light gray, with and without Cys37; plain and hatched bars, AChE and AChE* as in Fig. 3. C represents truncated species, essentially reduced to the catalytic domains C1 and C2.
C-terminal mutations (without cysteine C37)

|       | cell | medium | medium/cell |
|-------|------|--------|-------------|
|        | wt   | stop39 | SSDL        |
|        |      |        | KDEL        |
|        | 7L   | stop39 | SSDL        |
|        |      |        | KDEL        |
|        | Scr  | stop39 | SSDL        |
|        |      |        | KDEL        |
|        | 5W   | stop39 | SSDL        |
|        |      |        | KDEL        |
|        | Del  | stop39 | SSDL        |
|        |      |        | KDEL        |

**Fig. 5.** Effect of the C-terminal tetrapeptide motif on cellular activity and secretion. The cellular and secreted activities are shown as in Fig. 3 for mutants of the aromatic-rich region containing the C-terminal sequences Ser-Ser (stop39), SSDL, or KDEL. A, cellular activity. B, secreted activity. C, ratio of secreted to cellular activities, arbitrarily taken as 1 for the 7L and Del mutants, terminating with Ser-Ser or SSDL. The values were plotted on a logarithmic scale as in Fig. 4.

mutants, but it was equal for 7Lc and 7Ls as well as for Delc and Dels.

**Effect of the C-terminal Extremity of the t Peptide on Secretion**—We compared mutants in which the cysteine was replaced by a serine (SSDL) with mutants lacking the last two residues (stop39, ending with Ser-Ser) and with mutants in which the last four residues were replaced by the classical ER retention signal, KDEL. These C-terminal endings were combined with the different mutations of the aromatic-rich region as described in Fig. 1A.

The KDEL tetrapeptide increased the cellular activity but only moderately for 5W with or without mutation F535A (Fig. 5A). It reduced the secreted activity in all of the mutants but only marginally for 7L (Fig. 5B). The stop39 mutation increased the secretion of WT and Scr but had no clear effect on 7L, 5W, or Del. This mutation did not affect the cellular activity of WT, 7L, or Del and decreased it very weakly for 5W and quite significantly for Scr. The ratio of secreted to cellular activities provided a more convenient index of these effects (Fig. 5C). This ratio was decreased by KDEL in all cases but less markedly for 7L. The stop39 mutation increased it for WT, Scr, and 5W but not for Del and 7L.

**Synthesis of AChE Protein and Production of AChE Activity**—Metabolic labeling experiments showed that all of the mutant proteins were synthesized at the same rate, as expected, because the vectors and the coding sequences were identical with the exception of a few codons in the region encoding the short non-catalytic C-terminal t peptide. It reduced the secreted activity in all of the mutants but only marginally for 7L (Fig. 5B). The stop39 mutation increased the secretion of WT and Scr but had no clear effect on 7L, 5W, or Del. This mutation did not affect the cellular activity of WT, 7L, or Del and decreased it very weakly for 5W and quite significantly for Scr. The ratio of secreted to cellular activities provided a more convenient index of these effects (Fig. 5C). This ratio was decreased by KDEL in all cases but less markedly for 7L. The stop39 mutation increased it for WT, Scr, and 5W but not for Del and 7L.

We also studied the recovery of AChE activity after its inhibition by soman. Within two hours after washing the inhibitor, the cell activity increased linearly, recovering ~25% original cell content per hour in the case of WT5. Fig. 6B shows that the presence of a t peptide decreased the rate of production of newly synthesized active AChE compared with mutants that contained no t peptide (C1, C1*) or a t peptide from which the aromatic region was deleted (Del). In contrast, the F535A mutation in the dimerization zone of the catalytic domain had no influence. Since all AChE mutant proteins were synthesized at the same rate and all mutant enzymes possessed the same catalytic turnover, the fact that the presence of a complete t peptide reduced the production of AChE activity implies that some of the synthesized AChE polypeptides remain inactive.

Because AChE-WT5 subunits assemble as T4-QN tetramers in which the t peptides are occluded, we examined the effect of co-expression with QN. We found that this did not change the rate of recovery of AChE activity (data not shown).

**Secretion of AChE Protein and Activity**—We also monitored the secretion of a newly synthesized enzyme after treatment of transfected COS cells with soman (Fig. 6D). Although the cellular activity increased linearly after the removal of the inhibitor, secretion resumed after a lag period of ~3 h, corresponding to the time required for the transport of secreted proteins from the ER to the medium. Secretion then resumed and essentially recovered its original level after ~5 h. The highest secretion was observed for the Del mutant followed by 7L and then 5W and wild type with Scr being the least secreted.
in agreement with the relative amounts of activities in the medium (Fig. 3).

Degradation of AChE Protein and Activity—We analyzed the degradation of AChE in transfected cells, which had approximately reached a steady state 4 days after transfection. After metabolic labeling for 30 min., we followed the evolution of AChE protein during a chase period of several hours both in the medium (Fig. 7A). All of the mutants progressively disappeared from the cells. The WT and Scr mutants did not appear in the medium, and the 5W mutant was barely detectable. In contrast, the 7L and Del mutants were clearly secreted, in agreement with the histograms of Fig. 3.

To analyze the intracellular degradation of AChE activity as well as protein, synthesis was blocked by cycloheximide and secretion was suppressed by brefeldin A. These experiments were performed with mutants lacking Cys37 with or without the F535A mutation. Fig. 7B and C, shows that AChE activity was degraded faster for all of the mutants that contained the F535A mutation (AChE*) compared with corresponding mutants without this mutation (AChE). Fig. 7D shows that the decrease of radioactively labeled protein (AChE*) was similar to that of enzymatic activity under the same conditions. In all of the cases, the Del mutant appeared more stable than the wild type and the 7L mutant was intermediate. The Scr and 5W mutants were similar to the wild type or were less stable. We also obtained similar results for mutants possessing a C-terminal KDEL tetrapeptide without brefeldin A (data not shown).

**DISCUSSION**

Previous studies established that the t peptide of AChE represents an autonomous interaction domain that allows the formation of homo-oligomers as well as hetero-oligomers with the anchoring proteins ColQ and PRiMA, which both possess a PRAD. The t peptide contains 40 amino acids and forms an amphiphilic α-helix with a cluster of seven aromatic residues. Besides its role in oligomerization, the t peptide can target AChE subunits for degradation through the ER-associated degradation pathway rather than secretion, particularly when the catalytic domain contains the F535A mutation in its dimerization zone.

In this study, we investigated the features of the t peptide that determine the cellular fate of rat AChE toward degradation or secretion. For this purpose, we modified the aromatic-rich region of the t peptide of AChE subunits in various ways, disorganizing the aromatic cluster (Scr), replacing phenylalanines by the more bulky and less hydrophobic tryptophans (5W), replacing aromatic by the aliphatic hydrophobic leucines (7L), or deleting the entire aromatic-rich region 10–31 (Del). We also modified the last four residues, which contain the Cys37 and two C-terminal residues resembling those of the ER retention signal, KDEL.

**Amphiphilic Properties and Oligomerization—**Mutations in the aromatic-rich region of Torpedo AChE previously showed that the clustering of aromatic residues explains the amphiphilic properties of AChET monomers and dimers (10). In this study, amphiphilicity was conserved when the two phenylalanines were replaced by tryptophans (5W), even though these residues are less hydrophobic, or when the seven aromatic residues of the t peptide were replaced by leucines (7L). In these mutants, dimers appeared less amphiphilic than monomers, indicating a partial occlusion of the hydrophobic clusters. Amphiphilicity was suppressed when the aromatic-rich region was deleted (Del and Stop10 mutants) but also when the cluster of aromatic residues was disrupted by displacement of these aromatic residues around the predicted helix without changing the amino acid composition (Scr).

In agreement with a previous analysis of oligomeric associations of Torpedo AChE subunits, we found that aromatic residues, clustered on one side of an amphiphilic α-helix, are necessary for the assembly of homomeric or PRAD-associated tetramers, whereas the presence of a cysteine (Cys37) is sufficient for the formation of stable dimers (9). Accordingly, the 5W mutants retained the capacity to form homomeric or PRAD-linked tetramers but not the Del, Scr, or 7L mutants.

We particularly focused our attention on the synthesis, degradation, and secretion of the various mutants. Very clearly,
the effects of mutations in the aromatic-rich region were qualitatively the same with or without the C-terminal cysteine (Cys37 or C37S) and with or without the F535A mutation, which perturbs dimerization (AChE or AChE*). Thus, the observed effects were not related to dimerization.

Synthesis of Active AChE—Titration experiments with an organophosphate reagent of the active site (DEPQ) showed that mutations in the t peptide do not affect the catalytic turnover of the enzyme, as expected, because the catalytic domain and the t peptide are functionally independent. In addition, the F535A mutation, which affects dimerization of the catalytic domain, had no influence on its catalytic turnover. Therefore, the catalytic activities produced by the different mutants were directly proportional to the numbers of active AChE subunits.

We found that mutations or deletions in the t peptide with or without the F535A mutation do not affect the rate of synthesis of AChE proteins in COS cells, in agreement with the fact that the mutations were restricted to a very small part of the protein in the non-catalytic C-terminal region.

However, the production of AChE activity and thus active AChE subunits was sensitive to the presence of a t peptide. After irreversible inhibition of the preexisting enzyme, the synthesis of newly synthesized active AChE was not affected by the F535A mutation but was increased by deletion of the t peptide or of its aromatic-rich region (Del). Co-expression with QN did not increase the rate of production of active wild type enzyme despite the formation of the T₄-Q₅ complexes in which the t peptides are engaged in a tight interaction with the PRAD in T₄-Q₅ complexes (11) in such a way that their aromatic-rich regions are occluded. The lack of effect of QN on the production of active AChE suggests that complete folding precedes the assembly of the complex, although this association has also been shown to occur in the ER (9).

Degradation—Previous studies showed that degradation was increased by the F535A mutation and by the presence of aromatic residues in the t peptide (19). Although degradtion was faster for the dimerization deficient mutant (AChE*) than for the wild type catalytic domain (AChE), the influence of mutations in the aromatic-rich region was similar in both cases. We obtained similar degradation half-lives by studying the decrease of cellular AChE activity or of labeled AChE protein when secretion was blocked either by a KDEL retention signal or by brefeldin A (Fig. 7, C and D). This finding suggested that inactive and active AChE molecules were degraded...
in the same manner and that brefeldin A did not perturb the degradation process. In particular, the 7L mutant, which produced the highest proportion of misfolded polypeptides, was degraded less rapidly than the wild type and the Scr or 5W mutants but more rapidly than the Del mutant. Thus, degradation is triggered by the presence of hydrophobic residues but much more efficiently by aromatic residues than by leucines. It does not depend on the organization of an amphiphilic α-helix as shown by the rapid degradation of the Scr mutant. It is interesting to emphasize the cooperativity between the F535A mutation in the catalytic domain and the functionally distinct t peptide for inducing degradation.

Effect of the C-terminal Tetrapeptide—We found that the C-terminal residues of the t peptide with or without the cysteine (CSDL or SSDL) do not influence the acquisition of AChE activity. Cys
 does not prevent secretion of monomers. The ratio of secreted to cellular activity was only reduced when the presence of this cysteine was combined with the 5W mutation or with the F535A mutation in the catalytic domain and a t peptide containing aromatic residues (WT, Scr, or 5W). This finding indicates a cooperativity among various “signals” in different parts of the protein.

The role of the C-terminal tetrapeptide of AChE as a retention signal is not clear, and retention by an appended KDEL motif was reversed by dimerization through Cys
 (20). We showed that the removal of the last two residues (Glu-Leu) of Torpedo AChET subunits decreased the cellular activity and concomitantly increased the secreted activity ~4-fold (9). To examine a possible intracellular retention of monomeric rat AChE subunits, we compared the influence of the C-terminal tetrapeptide SSDL with that of the canonical ER retention signal, KDEL, and we studied the effect of deleting the last two residues (Asp-Leu). The KDEL tetrapeptide did exert a retention effect, because it nearly blocked secretion and largely increased the cellular activity. It did not change the half-life of cellular AChE compared with that observed in brefeldin A-treated cells. However, retention was much less marked in the 7L mutant, which remained nearly secreted as with the SSDL terminus, indicating that the action of a KDEL “signal” may be strongly modulated by upstream elements. It is possible that the C-terminal leucine is partially occluded because of interactions with the other leucines. Removal of the Asp-Leu residues from SSDL clearly increased the secreted activity of the Scr mutant, but had no effect on secretion of the 7L and Del mutants. In addition, we observed no corresponding increase of their cellular activity, except for Scr. Therefore, it is not possible to consider that an SSDL tetrapeptide systemically acts as a retention signal but these observations confirm that it may exert an effect that is modulated by mutations in the aromatic-rich region.

Effect of the Aromatic-rich Region on Secretion—We studied the rate of recovery of AChE activity and the rate of secretion after irreversible inhibition of the cellular AChE. Secretion resumed after a lag period of ~3 h and recovered its initial rate after 5 h.

For the Del and 7L mutants, the rate of secretion into the medium is nearly equal to the rate of synthesis of AChE activity in the cells observed after the removal of the inhibitor, indicating that most of the newly synthesized enzyme is secreted. In contrast, the ratio of secretion to synthesis is ~40–50% for WT and 5W and ~25% for Scr, indicating that the presence of aromatic residues reduces the efficiency of secretion, especially when the helical cluster is disorganized. However, the strongly hydrophobic leucine residues do not divert the active 7L molecules from secretion. Therefore, secretion is not limited by the presence of an amphiphilic helix but rather by exposed aromatic residues.

Thus, it is possible to identify the features of the t peptide that affect the folding, degradation, and secretion of AChE subunits. Interference with a correct folding of the catalytic domain depends primarily on hydrophobicity, not on the aromatic nature of residues, or on their organization in a helical cluster. Degradation is stronger with aromatic residues than leucines and again is not sensitive to their α-helical organization. The influence of aromatic residues reinforces the strong effect due to the mutation F535A in the dimerization zone of the catalytic domain, although this is apparently not due to the capacity of catalytic domains for dimerization. Secretion efficiency was less reduced by leucines than by aromatic residues, and this effect was much stronger when the α-helical clustering of these residues was disorganized. The C-terminal Asp-Leu residue exerts a moderate retention effect, which is much weaker than that of a KDEL signal and only occurs in the presence of aromatic residues.

The characteristics of the t peptide that control folding are quite different from those that control degradation or secretion. AChE and its t peptide thus offer an extremely interesting model for analyzing the mechanisms of cellular trafficking of a secreted protein, with or without oligomerization.

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