Quaternary Associations of Acetylcholinesterase

II. THE POLYPROLINE ATTACHMENT DOMAIN OF THE COLLAGEN TAIL

Suzanne Bon, Françoise Coussen, and Jean Massoulie

From the Laboratoire de Neurobiologie Moléculaire et Cellulaire, Unité CNRS 1857, Ecole Normale Supérieure, 46 rue d’Ulm, 75005 Paris, France

In transfected COS cells, we analyzed the formation of heteromeric associations between rat acetylcholinesterase of type T (AChET) and various constructions derived from the NH₂-terminal region of the collagen tail of asymmetric forms, QN. Using a series of deletions and point mutations in QN, we showed that the binding of AChET to QN does not require the cysteines that normally establish intersubunit disulfide bonds with catalytic subunits and that it essentially relies on the presence of stretches of successive prolines, although adjacent residues also contribute to the interaction. We thus defined a proline-rich attachment domain or PRAD, which recruits AChET subunits to form heteromeric associations. Such molecules, consisting of one PRAD associated with a tetramer of AChET, are exported efficiently by the cells. Using the proportion of AChET subunits engaged in heteromeric tetramers, we ranked the interaction efficiency of various constructions. From these experiments we evaluated the contribution of various elements of the PRAD to the quaternary assembly of AChET subunits in the secretory pathway. The PRAD remained functional when reduced to six residues followed by a string of 10 prolines (Glu-Ser-Thr-Gly₂-Pro₁₀). We then showed that synthetic polyproline itself can associate with AChET subunits, producing well defined tetramers, when added to live transfected cells or even to cell extracts. This is the first example of an in vitro assembly of AChET tetramers from monomers and dimers. These results open the way to a chemical-physical exploration of the formation of these quaternary associations, both in the secretory pathway and in vitro.

As shown in the preceding article (1), coexpression in COS cells offers a convenient method to analyze the formation of heteromeric molecules in which tetramers of acetylcholinesterase (AChE),¹ EC 3.1.1.7) are associated with small binding proteins derived from the NH₂-terminal region of the collagen component of collagen-tailed forms (for review, see Ref. 2).

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‡ To whom correspondence should be addressed. Tel.: 33-1-44-32-3891; Fax: 33-1-44-32-3887; E-mail: jean.massoulie@biologie.ens.fr.

¹ The abbreviations used are: AChE, acetylcholinesterase; AChET, AChE catalytic subunit of type T; AChEH, AChE subunit of type H.; Q, collagenic subunit; QN, Q subunit NH₂-terminal domain; H, AChET subunit COOH-terminal domain; GPI, glyophosphatidylinositol; PI-PLC, phosphatidylinositol-phospholipase C; PRAD, proline-rich attachment domain.

When expressed alone in COS cells, AChE subunits of type T (AChET) produce mostly monomers and dimers, with smaller proportions of tetramers and higher oligomers. In the presence of the binding proteins Q₈/stop and Q₈/H₇, these subunits are recruited into tetramers that are associated with the binding domain and are efficiently secreted or attached to the cell surface by a glycolipid anchor (GPI) (1).

In this study we used a series of deletions and point mutations in the Q₈ sequence in an attempt to define the attachment domain that allows its interaction with rat AChET. We show that the cysteine residues are not required for interaction with AChET, and we narrow down the binding domain essentially to a stretch of successive prolines. Moreover, we show that polyproline itself can combine with AChET subunits, inducing their polymerization, mainly into tetramers, when added to transfected cells or to a cell extract.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Prolabo (Paris, France) or from Sigma (St. Louis, MO, U. S. A.). PI-PLC from Bacillus thuringiensis was from Immunotech (Marseille, France). Poly-L-proline with a molecular mass of 1,000–10,000 (mean 5 kDa) and >30,000 (mean 40 kDa) was purchased from Sigma.

Site-directed Mutagenesis and Transfection in COS Cells—Expression vectors encoding the binding proteins Q₈/H₇ and Q₈/stop (Q₈/stop551) were described previously (1, 3). The structure of the protein is illustrated in Fig. 1. Site-directed mutagenesis was performed with the single strand method (4). In the case of deletions, we used mutagenic 20-mer oligonucleotides consisting of 10 nucleotides complementary to each side of the deleted fragment. In the case of truncations, TGA stop codons were introduced to terminate the polypeptide chain. Transfection and culture of COS cells were performed as described in the preceding paper (1).

Analysis of AChE Forms—PI-PLC treatments, sedimentation and electrophoretic analyses, were performed as described (1).

RESULTS

Definition of the Proline-rich Attachment Domain (PRAD)

Deletions in the COOH-terminal Part of the Q₈ Domain—We examined the effect of deletions in the COOH-terminal part of the Q₈ domain on the binding of AChET subunits. For this purpose we used two strategies: introduction of stop codons at various positions in the Q₈ sequence, producing Q₈/stop proteins of various lengths; or deletions of various extents in the chimeric Q₈/H₇ protein (Fig. 1).

When coexpressed with rat AChET subunits, Q₈ molecules truncated at position 87 (Q₈/stop87) induced a significant increase in the proportion of cellular Q₈ form and more dramatically, a large increase in the released activity, where G₄₈ became predominant over G₄₂ and G₄₁ (not shown). Thus, Q₈/stop87 was able to induce the formation of heteromeric tetramers, like the Q₈/stop551 protein, containing the complete Q₈ domain, which we analyzed in the preceding paper (1). In contrast, introduction of a stop codon at position 76 totally abolished this capacity. In the case of Q₈/stop76, the pattern of

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Interaction of AChE with a Polyproline Attachment Domain

Fig. 1. Structure of the binding proteins and assembly a AChET tetramers; schematic representation of various constructions. Panel A, sequence of the chimeric QN/Hc protein. The putative signal peptide is shown in thin letters, the QN domain in bold letters, and the Hc domain in bold italics. These two domains are linked by two additional residues (GI), in thin italics, corresponding to a BamHI site introduced during the construction (3). The residues are numbered according to the original sequences of the Torpedo collagen subunit Tq, for QN (12) and of the Torpedo AChET subunit for Hc (13). Mutations are indicated by letters below the sequence. Residues that were modified en bloc are shown in boxes. Deletions are indicated by lines and arrows above the sequence. Panel B, structure of QN/Hc and QN/stop constructions. The sequence of the PRAD is indicated, and it is shown as dark boxes. The NH2- and COOH-flanking parts of the PRAD in QN are gray. The signal peptide and the Hc domain are light. Deletions are shown as thin lines. The boxes corresponding to the signal peptide and to the Hc region (GPI-addition signal) are not drawn to scale. The + and − signs indicate the capacity of each construct to assemble and bind a tetramer of AChET subunits.

AChE forms identical to that of AChET subunits alone, as illustrated in nondenaturing electrophoresis (Fig. 2). We performed similar experiments in cotransfections with Torpedo AChET subunits: the cells released essentially no activity when Torpedo AChET was expressed alone or with QN/stop76, but coexpression with QN/stop87 induced the secretion of tetramers in the culture medium (not shown).

Deletions in the QN/Hc molecule are schematically shown in Fig. 1B. Deletion of COOH-terminal segments of the QN do- main, extending up to positions 100 or 85 (QNΔ85–110/Hc), did not abolish the production of a GPI-G4 form, as shown by the sedimentation profiles of the cellular enzyme and its sensitivity to PI-PLC (not shown). However, QNΔ85–110/Hc somewhat weakened this interaction (see below). In contrast, larger deletions extending into the proline-rich sequence that follows the two vicinal cysteines of QN (QNΔ81–110/Hc and QNΔ76–110/Hc) abolished the binding of AChET.

Together these deletion experiments showed that a large part of the COOH-terminal sequence of QN may be removed without compromising the binding of AChET subunits from rat or from Torpedo. They defined a boundary of the binding domain, between positions 81 (essentially no binding) and 87 (similar to the wild type).

NH2-terminal and COOH-terminal Deletions in the QN Domain—To assess the possible role of the peptidic sequence that precedes the pair of cysteines Cys70–Cys71 in the QN domain, we removed residues 46–69 in deletion Δ1, leaving only the three residues that immediately follow the putative cleavage site of the signal peptide. In deletion Δ2, we deleted residues 90–110 in the COOH-terminal part of QN. We introduced these deletions, separately and in combination, in the QN/HC and QN/stop proteins. All of these deleted molecules were able to combine with the rat AChET subunit. For example, QN/stop551 proteins carrying one or both deletions induced the secretion of G4a rather than G1a (not shown). In the case of QN/HC, the production of GPI-anchored G4a and the release of G3a were similar for the three deleted constructs and for the complete protein, as shown by nondenaturing electrophoresis (Fig. 3) and by sedimentation analyses (not shown). Surprisingly, the Δ1 deletion actually increased the interaction with AChET, at non saturating doses of DNA encoding QN/HC or QN/stop (see below).

These observations establish that at least part of the sequence located between residues 70 and 86 plays a critical role in the interaction of QN with the AChET catalytic subunits. The most prominent feature of this short peptidic sequence is the presence of two stretches of five and three prolines, separated by two residues, Met→Phe (81). Are Disulfide Bonds Necessary for Association of QN with AChET Subunits?—The QN domain contains a pair of vicinal cysteines (Cys70–Cys71), which form disulfide bonds with cysteines located at position –4 of the COOH terminus of two AChET subunits, whereas the same cysteine residues form a disulfide bond between the other two subunits of the catalytic tetramer (11). To examine whether the formation of disulfide bonds between QN and AChET subunits was a necessary requirement for the assembly or stability of the hetero-oligomeric structure, we constructed mutants that lacked the vicinal cysteines. The double mutation C70G/C71S was introduced in the entire Q subunit and in the QN/Hc chimeric protein. In both
cases, the mutated proteins behaved exactly like those containing the pair of cysteines, producing hetero-oligomers with rat AChET subunits: collagen-tailed forms in the case of Q, GPI-anchored and released tetramers in the case of Qs/Hc (not shown).

**Mutations in the Proline-rich Region; Comparisons of the Efficiency of Interaction with AChET**: Construction of a Minimal Binding Domain—The different Qs/Hc constructions, when used at a nonsaturating dose of DNA (1 μg/dish), varied markedly in the yield of heterotetrameric cells (cellular G4, cytoplasmic G4, and secreted G4) (Fig. 4). This shows that Qs/stop85 was slightly but significantly better than Qs/stop87, which was itself less efficient than Qs/HC. The Phe 85 and Phe 86 residues therefore produced the efficiency of interaction (Fig. 4).

It was possible to obtain a more quantitative comparison, using the proportion of hetero-AChE to total activity as an index of the efficiency of interaction, if we assume that the different constructs were expressed at the same level. (Note that they all contained the same signal peptide and cleavage site.) We thus show a ladder of the different Qs/stop constructions, according to the proportion of G4 in the culture medium (Fig. 4). This shows that Qs/stop55 was slightly but significantly less efficient than Qs/stop76, which was itself less efficient than Qs/stop55. The Phe 85 and Phe 86 residues therefore contribute to the binding of AChET subunits. The effect of the Δ2(90–533) deletion also suggests that more distal residues participate in this interaction. In the case of the Qs/Hc constructs, it is possible that the interaction may depend on a sufficient distance between the binding domain and the GPI addition signal.

To assess the importance of Met 80 and Phe 81, located between the two proline stretches, in the interaction with AChET subunits, we mutated these residues into prolines. When coexpressed with rat AChET subunits, the Qs[Pro 80-Pro 81]/Hc mutant was able to produce GPI-anchored G4a, as well as released G4a, with a slightly reduced efficiency compared to the wild type Qs/Hc protein (not shown). Similarly, the Qs[Pro 80-Pro 81]/stop85, Qs[Pro 80-Pro 81]/stop87, and Qs[Pro 80-Pro 81]/stop85 mutants produced secreted G4a less efficiently than the corresponding constructs containing Met 80-Phe 81 (Fig. 4).

We also examined the importance of the proline stretches by replacing the middle residue of each group by a glycine. We found that whereas the P83G mutation had little effect on the yield of GPI-anchored G4 and released G4a, the P77G mutation reduced it markedly, and the double mutation P77G/P83G further weakened the binding. Introducing prolines at positions 80 and 81 partially compensated the effect of P77G but weakened the interaction in the case of P83G or of the double mutation P77G/P83G.

The two cysteines of the attachment domain are separated from the proline stretches by three residues, Leu 72-Leu 73-Thr 74. Mutation of these residues to glycines in complete or partially deleted Qs/Hc was found to weaken significantly the interaction with AChET. In agreement with the effects of mutations of the Cys 79-Cys 81 and Leu 72-Leu 73-Thr 74 residues, a deletion including the two cysteines (Δ46–71) was as effective as deletion Δ1(46–69) in which they were maintained, whereas removal of the following Leu 72-Leu 73 residues significantly reduced the efficiency of interaction (Fig. 4).

These observations indicate that a sufficient stretch of proline residues is essential for the association with AChET but that hydrophobic residues located before, between, and after the proline stretches (Leu 72-Leu 73-Thr 74, Met 80-Phe 81, Phe 85-Phe 86) also participate in this interaction. We constructed a mutant, Qs[Δ46–74Pro 80-Pro 81]/stop85, in which the mature protein only consisted of three residues, EST, followed by a stretch of 10 prolines, Pro 80, as in the Pro 80-Pro 81-mutant. We found that this Glu-Ser-Thr-Pro10 peptide was able to bind with AChET, decreasing the proportion of G4a and G4 and increasing markedly the level of secreted G4a, although with less efficiency than other Qs/stop constructs (Fig. 4). This suggested that the polyproline sequence may be sufficient to organize AChET tetramers in the absence of other residues.

Conversely, Qs/Hc and Qs/stop constructs in which residues 70–86 were deleted had no effect on the molecular forms of AChET, as shown by sedimentation analyses or by immunofluorescence (not shown). They did not induce the production of GPI-anchored G4 at the surface of the cells or the secretion of G4a in the medium.
Interaction of AChET Subunits with Synthetic Polyproline

Exogenous, Synthetic Polyproline Can Combine with AChET in Transfected Cells—We added various concentrations of synthetic polyproline of about 8 kDa to the culture medium after transfecting cells with AChET. We observed a dose-dependent decrease of G₂筈 and G₂筈, both in the cell extract (Fig. 5A) and in the culture medium (Fig. 5B), and a concomitant increase of G₄筈. These effects could be detected at 5 × 10⁻³ M polyproline; at higher concentrations G₄筈 became the major secreted form of AChE, as observed with Qₙ/stop constructs. In addition, a minor 16 S component appeared in the cell extract and in the medium at higher concentrations of polyproline.² The sedimentation coefficient of this form suggests that it may result from the binding of two tetramers to a sufficiently long polyproline chain.

Polyproline of higher average molecular mass (40 kDa) also interacted with AChE but induced the formation of ill defined components, sedimenting mainly between 6 and 11 S.

We wondered whether AChET and polyproline could combine spontaneously or whether the cellular biosynthetic machinery was required for this interaction. We found that α₁-dipyridyl, which inhibits hydroxylation of prolines, did not inhibit the production of G₄筈 by polyproline, even when included at 10⁻⁴ M in the culture medium for 3 days after transfection. This concentration reduced the production of total AChE activity to less than 20% of the control but did not alter the proportions of molecular forms (not shown).

Interaction of Polyproline with AChET in Cell Extracts—We also examined whether the interaction could occur in an acellular system. When a cell extract, obtained in the absence of detergent (high speed supernatant of a low salt-soluble extract), was incubated with polyproline at 37 °C for 4 h, we observed an important increase of G₄筈 at the expense of G₂筈 and G₂ (Fig. 6A). The effect of polyproline on AChE monomers and dimers in a detergent-free cell extract resembled the effect obtained with living cells, except for the absence of the 16 S component. The 13.7 S form was not observed under these conditions, probably because it disappeared during incubation at 37 °C. The efficiency of interaction was reduced markedly in the presence of Triton X-100 (Fig. 6B). In addition, the production of G₄筈, in the presence of polyproline, occurred less efficiently or more slowly at lower temperatures, 20 or 4 °C (not shown). The interaction between AChE and polyproline was therefore dose-dependent, temperature-dependent, and sensitive to the presence of detergent.

In contrast with cell extracts, we observed only a minimal effect after incubation of the secreted AChE forms with polyproline (less than 5% of G₁ and G₂ was converted into G₄筈), and this could in fact reflect the contribution of enzyme forms released by cell lysis. Experiments in which cellular extracts and media from transfected and control COS cells were combined, so that the composition of the mixtures was identical except for the cellular or secreted origin of the AChE molecules, showed that the difference in their capacity to interact with polyproline is an intrinsic property of these monomers and dimers (not shown).

**DISCUSSION**

In these experiments, we used a series of deletions and mutations in the NH₂-terminal domain of the collagen Q sub-
unit to identify the residues that are involved in the attachment of a tetramer of AChET subunits. These modifications were introduced in the QN/H subunits and Qn/stop proteins and in some cases also in the complete collagenic subunit, Q. Assembly of AChET subunits with Q is less convenient because it produces several collagen-tailed molecules (λ23, Aα, Aγ) which aggregate in low salt and cannot be analyzed in non-denaturing electrophoresis. The results were however entirely consistent with those obtained for Qn/Hc and Qn/stop proteins and have not been illustrated here.

Cysteines Are Not Required for Interaction of QN with AChET—By comparing the proportions of heteromeric tetramers we were able to rank the efficiency of interaction of different deleted and mutated constructions and thus evaluate the contribution of various elements of the QN domain. Quite unexpectedly, deletion of the peptide preceding the cysteines (Δ1) actually increased the interaction significantly. We also showed that the vicinal cysteines of QN, Cys70 and Cys71, are not required, although disulfide bonds probably stabilize the quaternary association with AChET. The fact that Cys70 and Cys71 are dispensable is consistent with our previous observation that inter-subunit disulfide bonds may be reduced without disrupting the structure of collagen-tailed AChE forms (5). We also showed that sequences following a polyproline stretch, i.e., beyond position Phe86, could be removed without compromising the binding capacity.

PRAD, a Short Conserved Peptidic Domain; Evaluation of Interaction Efficiency in the Secretory Pathway—These experiments showed that the binding domain could be reduced to a short region of 17 residues, starting with cysteines Cys70-Cys71, which we propose to name the polyproline attachment domain or PRAD. This region is remarkably well conserved from Torpedo to higher vertebrates; there are only two replacements in the rat Q subunit and none in the chicken. The presence of PRAD is sufficient for interaction with AChET subunits, and conversely its deletion abolishes this interaction completely.

The PRAD contains two conserved stretches of five and three consecutive prolines, separated by two residues, Met80-Phe81, preceded and followed by conserved residues, Leu72-Leu73. Thr74 and Phe80-Phe86. By point mutations and deletions we showed that these hydrophobic residues, as well as more distal residues, participate in the interaction with AChET subunits but are not absolutely required.

By analyzing the ratio R of secreted heterotetramers, Gα4α, to the total activity in the culture medium for nonsaturating doses of Qn/stop constructions, as described in the previous paper (1), we obtained an index of the efficiency of interaction, assuming that the binding proteins were expressed at the same level (Fig. 4). This index provides an evaluation of the influence of elements of the binding domain; for example, the M80P/F81P double mutation reduces the value of R to a similar degree in different contexts (Qn/stop87, Qn/stop85, or Qn/Gly72-Gly73, Gly73/stop85). It seems therefore possible to quantify the contributions of specific residues to quaternary assembly in the secretory pathway.

Interaction of AChE with Synthetic Polyproline—The critical feature of the binding domain PRAD appeared to be the presence of a sufficiently long series of prolines, since a simple Glu-Ser-Thr-Fru10 peptide remained functional. We showed that, in effect, AChET subunits were able to combine with synthetic polyproline. When added to cultures of COS cells expressing AChET, synthetic polyproline of about 8 kDa was able to mimic the effect of the Qα domain, even at a concentra-

3 E. Krejci, S. Thomine, C. Legay, J. Sketelj, and J. Massoulié, manuscript in preparation.

4 W. R. Randall, personal communication.

5 S. Bon, I. Cornut, J. Dufourcq, J. Grassi, and J. Massoulié, manuscript in preparation.
tracellular molecules apart from the Q collagen.

In conclusion, the present study reveals a new type of interaction that occurs intracellularly and withholds extracellular conditions. Such quaternary interactions are important for anchoring AChE and possibly other enzymes and proteins in extracellular matrices. We show that such interactions can induce molecular assembly both in the secretory pathway of living cells and in vitro.

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