Conserved Aromatic Residues of the C-Terminus of Human Butyrylcholinesterase Mediate the Association of Tetramers†

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ABSTRACT: Human butyrylcholinesterase (BChE) in serum is composed predominantly of tetramers. The tetramerization domain of each subunit is contained within 40 C-terminal residues. To identify key residues within this domain participating in tetramer stabilization, the interaction between C-terminal 46 residue peptides was quantitated in the yeast two-hybrid system. The wild-type peptide interacted strongly with another wild-type peptide in the yeast two-hybrid system. The C571A mutant peptides interacted to a similar degree as the wild-type. However, the mutant in which seven conserved aromatic residues (Trp 543, Phe 547, Trp 550, Tyr 553, Trp 557, Phe 561, and Tyr 564) and C571 were altered to alanines showed only 12% of the interaction seen with the wild-type peptide. The seven mutations (aromatics-off) were incorporated into the complete BChE molecule, with or without the C571A mutation, and expressed in 293T and CHO-K1 cells. Expression of wild-type BChE in these cell lines yielded 10% tetramers. The aromatics-off mutant formed dimers and monomers but no tetramers. The aromatics-off/C571A mutant yielded only monomers. Addition of poly-L-proline to culture medium, or coexpression with the N-terminus of COLQ including the proline-rich attachment domain (Q_NPRAD), increased the amount of tetrameric wild-type BChE from 10 to 70%, but had no effect on the G534stop (lacking 41 C-terminal residues) and the aromatics-off mutants. Recombinant BChE produced by coexpression with Q_NPRAD was purified by column chromatography. The purified tetramers contained the FLAG-tagged Q_NPRAD peptide. These observations suggest that the stabilization of BChE tetramers is mediated through the interaction of the seven conserved aromatic residues and that poly-L-proline and PRAD act through these aromatic residues to induce tetramerization.

Butyrylcholinesterase (BChE)[1] (EC 3.1.1.8, human serum cholinesterase) occurs in human serum primarily as a soluble homotetrameric glycoprotein (*1*). The physiological function of this enzyme has not yet been established, but it is known to hydrolyze drugs such as aspirin, succinylcholine, heroin, and cocaine (*2*). In addition, BChE has a demonstrated protective effect against organophosphate poisons when administered as a prophylactic treatment in mice (*3*), rats (*3*, *4*), and nonhuman primates (*5*, *6*). This protective effect is conferred by the covalent binding of organophosphates to BChE in a one-to-one ratio, precluding binding and inactivation of acetylcholinesterase at nerve synapses of the central and peripheral nervous systems. Recently, a mutant BChE, the G117H mutant, has been produced which can hydrolyze organophosphates (*7*, *8*). A recombinant BChE would be most stable in the bloodstream in the tetrameric form (*9*) and, therefore, would be most effective as a therapeutic agent if it were a tetramer. However, recombinant BChE is a mixture of less stable monomers and dimers and is composed of only about 10−30% tetramers (*10*). To enhance the level of recombinant BChE tetramer, it is necessary to obtain a better understanding of the elements involved in producing and stabilizing the tetrameric form.

Previous experiments have isolated the tetramerization domain of BChE and AChE to the C-terminus of the molecule, within the final 40 residues of each subunit (*10*−*13*). There is currently a lack of structural information for cholinesterases in this region since this domain is absent from all cholinesterase crystals studied to date. The *Torpedo californica* acetylcholinesterase structure is that of phospholipase C-cleaved homodimeric H-subunits (*14*). H-subunits form glycolipid-anchored dimers and do not contain the C-terminal domain required for tetramerization. The mouse acetylcholinesterase structure is that of truncated H-subunits produced by recombinant expression in human embryonic kidney cells (*15*). The eel AChE structure is that of the tetramer, but the tetramerization domain is missing (*16*).

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[1] Abbreviations: BChE, butyrylcholinesterase; PRAD, proline-rich attachment domain; Q_NPRAD, N-terminus of the collagen tail including the 17 residue PRAD peptide; rQ45, 45 residues from the N-terminus of the collagen tail including PRAD; rQ117, 117 residues from the N-terminus of the collagen tail including PRAD; ONPG, *o*-nitrophenyl β-D-galactopyranoside; 3-AT, 3-amino-1,2,4-triazole; IGP, imidazole glycerol phosphate; SD, synthetic dextrose; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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            529                                                 574
Human BChE  VLEMTGNIDEAEWEWKAGFHRWNNYMMDWKNQFNDYTSKKESCVGL
Human AChE  LLSATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHY-SKQDRCSDL
Rabbit BChE VLEMTGNIDEAEQEWKAGFHRWNNYMMAWKNHFNDYTSKKERCAGF
Rabbit AChE LLSATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHY-SKQDRCSDL
Mouse BChE  VLEMTGDIDETEQEWKAGFHRWSNYMMDWQNQFNDYTSSKESCTAL
Mouse AChE  LLSATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHY-SKQERCSDL
Cat BChE    VLEMTGNIDEAEREWRAGFYRWNNYMMDWKNQFNDYTSKKESCAGL
Cat AChE    LLSATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHY-SKQDRCSDL
Horse BChE  VLELTGNIDEAEREWKAGFHRWNNYMMDWKNQFNDYTSKKESCSDF
Rat AChE    LLSATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHY-SKQERCSDL
Bovine AChE LLNATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHY-SKQDRCSDL
Chicken AChE LLNATGPPEDAEREWRLEFHRWSSYMGRWRTQFEHY-SRQQPCATL
Snake AChE  LLNATDNIEEAERQWKLEFHLWSAYMMHWKSQFDHY-NKQDRCSEL
Eel AChE    LLNVTENIDDAERQWKAEFHRWSSYMMHWKNQFDHY-SKQERCTNL
Torpedo AChE LLNATETIDEAERQWKTEFHRWSSYMMHWKNQFDHY-SRHESCAEL
conserved aromatic        W   F  W  Y   W   F  Y
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FIGURE 1: Conserved aromatic residues of cholinesterases. The C-termini of tetrameric AChE and BChE were aligned to the C-terminus of human BChE beginning at residue 529 and ending at residue 574 (human BChE numbering). A peptide containing residues 529 through 574 of human BChE was used in the yeast two-hybrid system. Sequences are from http://meleze.ensam.inra.fr/cholinesterase/.

Masson and Cléry have suggested that aromatic−aromatic interactions stabilize BChE tetramers (*17*). They based their conclusion on the observation that BChE tetramers did not dissociate at high pressure.

In the present study, we probed the importance to BChE tetramerization of seven selected conserved C-terminal aromatic residues: Trp 543, Phe 547, Trp 550, Tyr 553, Trp 557, Phe 561, and Tyr 564. These seven residues are conserved in tetrameric AChE and BChE from multiple species including human, rabbit, mouse, cat, horse, rat, cow, chicken, snake *Bungarus fasciatus*, electric eel, and *Torpedo californica*, illustrated in Figure 1. We report that wild-type BChE C-terminal peptides interacted with one another independently from the remainder of the molecule in the yeast two-hybrid system. Substitution of key aromatic residues in this region with alanine decreased interaction by 8.6-fold. Tetramers of BChE could not be formed when these seven aromatic residues were replaced with alanines. Bon et al. (*12*) demonstrated that addition of poly-L-proline increased the amount of AChE tetramers secreted by COS cells in culture. When we added poly-L-proline to the media of 293T cells transfected with wild-type BChE, the level of BChE tetramers increased from 10 to 70%. Cotransfection of the rat Q_NPRAD peptide (*18*) with wild-type BChE in CHO-K1 cells resulted in a comparable increase in BChE tetramers. The increase in tetramers in both cases was dependent on the presence of the conserved aromatic residues of the BChE C-terminus as the addition of poly-L-proline or cotransfection with the Q_NPRAD peptide did not influence the level of tetramer for the aromatics-off mutant. We conclude that the conserved aromatic residues of the C-terminus are responsible for the stabilization of BChE tetramers and mediate the interactions to increase tetramers of BChE in the presence of poly-L-proline and PRAD.

MATERIALS AND METHODS

Two-Hybrid System Plasmids and Yeast Strain. Yeast two-hybrid system plasmids pBTM116 (*19*, *20*), encoding the bacterial LexA DNA binding domain (residues 1−211) and the TRP1 gene, and pACTII (*21*), encoding the yeast Gal4 transcriptional activation domain (residues 768−881) and the LEU2 gene, as well as *S. cerevisiae* yeast strain L40 [Mat**a** his3-Δ200 trp1−901 leu2−3,112 ade2 LYS2::(4lexAop- -HIS3) URA3::(8lexAop--lacZ)] (*19*) were generously provided by Dr. Cheng Kao. DNAs encoding 46 residues of the C-terminal of human BChE (529−574) or 48 residues (527−574), each with flanking *Eco*RI/*Pst*I and *Eco*RI/*Xho*I restriction sites for in-frame insertion into pBTM116 and pACTII, respectively, were made by PCR using Pfu polymerase (Stratagene, La Jolla, CA). Amplified DNA was purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA), cleaved at appropriate restriction sites, and ligated into the proper vectors. Large-scale purifications of plasmid DNA were made using the Qiagen plasmid purification kit. The C571A mutant contained alanine in place of cysteine 571. The aromatics-off/C571A mutant had alanine in place of seven conserved aromatic residues at the C-terminus of BChE at Trp 543, Phe 547, Trp 550, Tyr 553, Trp 557, Phe 561, and Tyr 564 along with the C571A mutation. All mutants were sequenced to confirm that the mutation was present.

The 2 hybrid proteins tested for interaction in this system were (1) the DNA binding domain of bacterial LexA joined to the 46 residue C-terminal BChE peptide and (2) the transcriptional activation domain of Gal4 fused to the 48 residue C-terminal BChE peptide. L40 yeast cells dependent on tryptophan and leucine for growth were cotransfected with two-hybrid system plasmids and selected for harboring these plasmids by growth on synthetic medium lacking tryptophan and leucine. L40 yeast had two chromosomal integrated reporter constructs, the yeast HIS3 gene and the bacterial lacZ gene, both under regulation of the LexA operator. When the BChE C-terminal peptides interacted, they tethered the two attached transcriptional domains to the LexA operator sequence to drive the expression of the reporter proteins under the regulation of the LexA operator; i.e., both HIS3 and lacZ were expressed. Since the L40 yeast had a deletion in its endogenous HIS3 gene, it was histidine auxotrophic unless protein interaction occurred in the two-hybrid system to induce HIS3 expression. Therefore, the double transformants were also screened for interaction of hybrids on medium lacking Leu, Trp, and His (SD-Leu-Trp-His) and

finally on SD-Leu-Trp-His with 25 mM 3-AT (3-amino-1,2,4-triazole). 3-AT inhibits the product of the HIS3 gene, IGP dehydratase (imidazole glycerol phosphate dehydratase). At 25 mM 3-AT, IGP dehydratase is only partially inhibited in yeast containing hybrids that interact strongly. Therefore, 25 mM 3-AT allows qualitative discrimination between hybrids that interact weakly or strongly. The other reporter of protein interaction in this system, β-galactosidase, could be assayed qualitatively in X-gal filter assays and quantitatively in ONPG assays.

Transformation of Yeast Cells and Selection of Double Transformants. Yeast cells were transformed by a method based on that of Schiestl and Geitz (*22*). Five milliliters of sterile yeast extract peptone dextrose (10 g/L yeast extract peptone dextrose, 20 g/L peptone, and 20 g/L dextrose medium) was inoculated with a colony of L40 yeast and incubated in a shaker incubator at 30 °C for 12−16 h. This starter culture was diluted 1:25 into fresh yeast extract peptone dextrose and grown to OD $\approx$ 0.5 at 600 nm. The culture was centrifuged at 500*g* (1500 rpm in a Beckman GPR centrifuge equipped with a GH 3.7 rotor) for 5 min. The supernatant was poured off, and the cells were washed with 5 mL of sterile freshly prepared LiAc/TE mixture (0.1 M lithium acetate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The cells were resuspended in 1 mL of sterile LiAc/TE and incubated for 1 h at 30 °C with shaking to yield competent cells. For each transformation, the following were combined in a sterile microfuge tube: 100 μL of yeast competent cells, $\sim$1 μg of plasmid DNA, and 50−100 μg of carrier DNA (sheared salmon sperm DNA, freshly boiled). The tubes were incubated at 30 °C for 30 min before adding 600 μL of poly(ethylene glycol) mix [40% poly(ethylene glycol) 3350, 1 M Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM lithium acetate] to each tube. The suspensions were incubated at 30 °C for 30 min. This was followed by a 15 min heat shock at 42 °C. The cells were pelleted for 1 min in a microfuge, resuspended in 200 μL of TE, and plated onto the appropriate selection medium −SD (synthetic dextrose) lacking Leu, Trp, or His or a combination of these amino acids and 25 mM 3-amino-1,2,4-triazole (3-AT) (Sigma CAT# A8056), as required. The plates were incubated at 30 °C for 3−5 days until 1−2 mm colonies appeared. As a negative control, plasmids without BCHE inserts (called "empty") were also transfected into L40 yeast.

X-gal Filter Lift Assays. Qualitative assay of β-galactosidase activity within yeast cells was performed according to the method of Staudinger et al. (*23*). Yeast double transformants from selective plates were transferred to circular 0.45 μm nitrocellulose filters (Millipore HA filter CAT# HATF 082 25) by pressing a clean filter to each dish. Cells on each filter were permeabilized by floating the filter in liquid N_2 for 10−20 s. The filters were placed, colony side up, on Whatman No. 3 filter paper presoaked in Z-buffer I (60 mM $Na_2HPO_4 \cdot H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM $Mg_2SO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol) with 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) and incubated at 30 °C in a covered Petri dish for up to 12 h to allow for color development.

Yeast Lysate Preparation and ONPG Assays. A method based on that of Rose and Botstein (*24*) was used to prepare yeast lysates and assay β-galactosidase activity by ONPG hydrolysis. One hundred milliliters of cultured cells was generated from single colonies selected for the presence of both pBTM116 and pACTII plasmids. For each mutant, wild-type, and control cell line, at least three colonies were grown to a concentration of $\sim 1 \times 10^7$ cells/mL ($OD_{600\ nm} \approx 0.5$) in selective medium at 30 °C. Cells were chilled on ice and harvested by centrifugation at 900*g* (2000 rpm in a Beckman GPR centrifuge with a GH 3.7 rotor) for 5 min. Cells were then transferred to 1.5 mL Eppendorf microfuge tubes and resuspended in 250 μL of breaking buffer (100 mM Tris-HCl, pH 8, 1 mM dithiothreitol, and 20% glycerol). Then 12.5 μL of phenylmethylsulfonyl fluoride stock solution (40 mM in 2-propanol) was added to each tube and mixed well. Autoclaved and acid-washed glass beads (0.425−0.600 mm, Sigma CAT# G-8772) were added to this mixture to a level just below the meniscus of the liquid. Tubes were vortexed 6 times at top speed in 15 s bursts and chilled on ice between bursts. An additional 250 μL of breaking buffer was added to each tube and mixed in well. Lysates were withdrawn after plunging the tip of a 1000 μL Pipetman to the bottom of each tube. Clarified lysates, prepared by centrifuging the extracts, were tested for activity, but the majority of the activity was found in the particulate fraction. Therefore, unclarified lysates were used for ONPG assays.

To perform the ONPG hydrolysis assay, 100 μL of unclarified lysate was added to 0.9 mL of Z-buffer II (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $Mg_2SO_4 \cdot 7H_2O$, 40 mM β-mercaptoethanol) and preincubated at 28 °C for 5 min. The reaction was initiated by the addition of 0.2 mL of ONPG stock solution (4 mg/mL *o*-nitrophenyl β-D-galactopyranoside in Z-buffer II), and the mixture was incubated at 28 °C until it acquired a pale yellow color. The reaction was terminated by the addition of 0.5 mL of 1 M Na_2CO_3, and the time for each reaction was noted precisely. The samples were immediately chilled on ice, and particulate matter was subsequently pelleted by spinning the reaction tube at top speed in a microfuge for 2 min. The optical density of the yellow supernatant solution was measured at 420 nm and used to calculate the change in absorbance per minute. The protein concentration of each sample was measured using the dye-binding assay of Bradford (*25*) to normalize β-galactosidase activity to the total amount of protein. Bovine serum albumin diluted in breaking buffer to 0−1 mg/mL served as standard. Two microliters of unclarified lysates from each sample was incubated with 5 mL of Bradford reagent (0.10 mg/mL Coomassie brilliant blue G-250, 4.25% ethanol, and 8.5% phosphoric acid) at room temperature for 10 min. The absorbance of this solution at 595 nm was recorded and used to determine protein concentration against the albumin standard curve. Average β-galactosidase specific activity was expressed in nanomoles of *o*-nitrophenol released per minute per milligram of protein.

Construction of BChE with Aromatics-off and Aromatics-off/C571A. The aromatics-off and aromatics-off with C571A mutations were incorporated into pGS-BCHE by PCR site-directed mutagenesis as described elsewhere (*7*). The pGS vector has rat glutamine synthetase for selection with 50 μM methionine sulfoximine. The aromatics-off mutant of BChE had alanine in place of seven conserved aromatic amino acids at the C-terminus of BChE: W543A, F547A, W550A, Y553A, W557A, F561A, Y564A. The aromatics-off/C571A BCHE contained the above mutations as well as alanine in

place of cysteine 571. DNA sequencing revealed that the aromatics-off BChE contained only the desired mutations but that the aromatics-off/C571A BCHE contained an unwanted mutation altering Lys 568 to Met.

Transient Transfection of 293T Cells and Assay for Butyrylcholinesterase Activity. 293T/17 embryonic kidney cells (ATCC CRL 11268) were maintained in DMEM with 5% fetal bovine serum. This cell line, derived from 293 cells (*26*), was used because 70−100% of cells are transfectable. 293T cells contain the simian virus 40 large tumor antigen. Calcium phosphate-mediated transfection (*27*) introduced the desired BCHE mutants [aromatics-off, aromatics-off/C571A, C571A, and G543stop (*10*)] into 293T cells.

At the time of transfection, the cells were ~50% confluent in 10 cm dishes. The DNA/$CaCl_2$/HEPES mixture was left on cells for 5 days. Medium was collected from cells on the fifth day after transfection and assayed for butyrylcholinesterase activity with 1 mM butyrylthiocholine iodide in 100 mM potassium phosphate buffer, pH 7.0 (*28*). A unit of activity hydrolyzes 1 μmol of butytyrylthiocholine per minute.

Activity-Stained Gels. Nondenaturing 4−30% gradient polyacrylamide gels were prepared with an SE 600 apparatus (Hoefer Scientific), and samples were applied and electrophoresed at 120 V for 36 h at 4 °C. It was important to run gels at low temperature to maintain the activity of dimers and monomers. The gels were stained for butyrylcholinesterase activity with 2 mM butyrylthiocholine iodide by the method of Karnovsky and Roots (*29*). The gels were dried onto Whatman filter paper, and band intensity was quantitated by densitometry.

Densitometry. Gel scans were obtained by photographing gels using a Kodak CCD (charge caption device) camera (BioImage 110S System, Millipore). Scans were transferred to a Sun SPARC station 1+ computer and analyzed using BioImage Whole Band software. The IOD (integrated optical density) of each BChE species was expressed as a percentage of the total IOD in each lane.

Western Blots. Recombinant wild-type (5 ng) and C571A (10 ng) BChE expressed in CHO-K1 cells (*10*) were electrophoresed on a native 4−30% polyacrylamide gel as above. Protein bands were transferred from the gel to a PVDF membrane (Millipore) using the BIO-RAD Trans-Blot 49BR apparatus at 480 mA for 1 h and 4 °C in transfer buffer (25 mM Trizma, 192 mM glycine). Blots were prehybridized in blocking buffer [TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) with 0.2% (v/v) Tween 20] containing 5% (w/v) instant nonfat dry milk for 2 h at 37 °C. Blots were then hybridized with polyclonal anti-BChE in rabbit [1:1000 dilution in blocking buffer with 5% (w/v) dry milk], overnight at 4 °C with rocking. The antibody was custom-made for us against denatured human BChE by HRP Inc., now renamed Covance Research Products Inc. (Denver, PA). Blots were washed sequentially with TBS alone, blocking buffer, TBS with 3% (v/v) Tween 20, TBS alone, blocking buffer, and blocking buffer with 5% (w/v) milk. Blots were then hybridized with horseradish peroxidase-conjugated goat anti-rabbit antibody [1:2000 dilution in blocking buffer with 5% (w/v) milk] at 4 °C for 1 h. The blots were washed again as described above except the last wash contained no Tween 20. Blots were developed using the LumiGLO Chemiluminescent Substrate Kit from Kirkegaard and Perry Laboratories (Gaithersburg, MD) and exposed to film for up to 2 min.

Poly-L-proline Experiment. Poly-L-proline with a molecular mass of 1000−10 000 Da (mean 8 kDa) was purchased from Sigma (CAT# P-2254). Poly-L-proline, dissolved in phosphate-buffered saline, was added to the culture media of 293T cells to a final concentration of 10^{-7} or 10^{-5} M immediately prior to calcium phosphate-mediated transient transfection with pGS-BCHE plasmids. As described above, samples were applied to native polyacrylamide gels which were activity-stained and analyzed by densitometry.

Coexpression of the Rat Collagen Tail Q_NPRAD with Wild-Type and Mutant BChE. cDNA for the 5′ end of the rat collagen tail (partial COLQ) read-through was obtained from Dr. Eric Krejci (*18*). DNA encoding the amino-terminal 45 (rQ45) or 117 (rQ117) residues of the rat collagen tail was amplified by PCR using primers that placed the FLAG epitope DYKDDDDK at the C-terminus. The amplified fragments, which included the proline-rich attachment domain (PRAD) and the FLAG epitope, were inserted into the *Hin*dIII/*Not*I sites of the neomycin selection vector pRc/RSV (Invitrogen). The PRAD domain has two cysteines as well as five and three consecutive prolines in the sequence CCLLMPPPPPLFPPPFF (*12*, *18*). The rQ45 plasmid included 22 codons for the signal peptide, 45 for the N-terminus of COLQ, and 8 for the FLAG peptide. The rQ117 plasmid included 22 codons for the signal peptide, followed by 117 codons for the read-through, and 8 for the FLAG peptide. The 117 codons have the amino acid sequence: QPTFINSVLPISAALPGLDQKKRGNHKACCLLMPPPPPLFPPPFFRGSRSPVSTLDSVSEKSPDSATSAPFPARHGHLRTPLKRGSSDVTRGQRPGVGIVSVVPIHSHVCSLARSGL, where the final 73 residues are encoded by intron 1. Stable CHO-K1 cell lines expressing wild-type, aromatics-off, aromatics-off/C571A, and C571A BChE were transfected with either the rQ45 or the rQ117 plasmid and selected with 0.8 mg/mL G418 (Geneticin) and 50 μM methionine sulfoximine.

RESULTS

Interaction of BChE Peptides in the Yeast Two-Hybrid System. Since previous experiments revealed that up to 40 amino acids of the C-terminus of BChE are necessary for the formation of tetramers (*10*), the in vivo interaction of these C-terminal peptides in a eukaryotic system was tested using the yeast two-hybrid assay. The yeast system was selected over a mammalian two-hybrid system because of the ease of transformation and rapid growth of yeast. The peptides studied in this system were composed of 46 and 48 amino acids encompassing residues 529−574 and 527−574, respectively, of the human BChE C-terminus.

The wild-type BChE C-terminal peptides showed interaction in the yeast two-hybrid system in both qualitative (Table 1) and quantitative assays (Figure 2). Wild-type BChE double transfectants capable of propagating on SD-Leu-Trp also grew on SD-Leu-Trp-His as well as SD-Leu-Trp-His with 25 mM 3-AT (Table 1), indicating that the wild-type BChE hybrids interacted strongly. As expected, the negative controls [untransfected cells, cells transfected with a single plasmid, and cells transfected with both plasmids containing no BChE inserts (empty)] were unable to thrive in medium

Table 1: Qualitative Assays for Interaction of C-Terminal BChE Peptides in the Yeast Two-Hybrid System[a]

L40 yeast transfection	DNA binding domain hybrid	activation domain hybrid	growth on histidine-negative and 3-AT plates[b]	colony color on X-gal filter assay[c]
untransfected	−	−	−	white
pBTM116 alone	LexA (1−211)-wt BChE (529−574)	−	−	white
pACTII alone	−	Gal4 (768−881)-wt BChE (527−574)	−	white
empty plasmids	LexA (1−211)	Gal4 (768−881)	−	light blue
wild-type	LexA (1−211)-wt BChE (529−574)	Gal4 (768−881)-wt BChE (527−574)	+	blue
C571A	LexA (1−211)-C571A BChE (529−574)	Gal4 (768−881)-C571A BChE (527−574)	+	blue
aromatics-off/C571A	LexA (1−211)-AO[d]/C571A BChE (529−574)	Gal4 (768−881)-AO[d]/C571A BChE (527−574)	−	light blue

[a] Qualitative estimation of β-galactosidase activity in multiple (>100) colonies of intact yeast cells was conducted by permeabilizing yeast cells and incubating them with X-gal. L40 yeast transfected with pBTM116 and pACTII plasmids were selected on medium lacking the appropriate amino acids before being tested in this assay. [b] Growth on synthetic dextrose medium lacking leucine, tryptophan, and histidine and containing 25 mM 3-amino-1,2,4-triazole (3-AT). [c] Color seen after 12 h incubation at 37 °C of permeabilized transfected/untransfected L40 *S. cerevisiae* on filters containing 1 mg/mL X-gal dissolved in Z-buffer. [d] AO, aromatics-off, contains alanine in place of seven conserved aromatic residues, Trp 543, Phe 547, Trp 550, Tyr 553, Trp 557, Phe 561, and Tyr 564, of BChE.

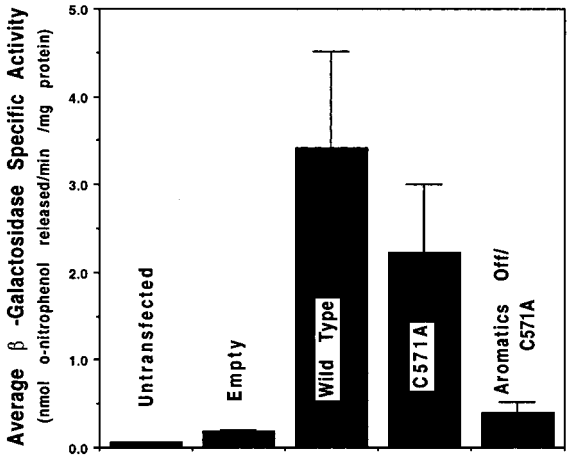

FIGURE 2: Interaction of C-terminal BChE mutants in the yeast two-hybrid system: quantitative assay of β-galactosidase activity. Average specific activity is the average activity of at least three colonies for each mutant with each colony lysate tested in triplicate. Standard deviations for each measurement are indicated by error bars.

lacking His. The qualitative screen for β-galactosidase activity revealed that the wild-type BChE C-terminal peptides could interact in the absence of the remainder of the BChE protein to produce a bright blue color on nitrocellulose filters. In contrast, the untransfected cells, cells transfected with a single plasmid, or cells transfected with plasmids containing no BChE inserts (empty) showed no significant interaction in the two-hybrid system, as revealed by their lack of blue color in this assay. To quantitate the degree of this interaction, β-galactosidase activities were assayed by hydrolysis of the chromogenic substrate ONPG using cell lysates from each yeast double transformant. In these assays, the wild-type BChE peptide chimeras released 3.4 ± 1.1 nmol of *o*-nitrophenol min^{-1} (mg of protein)$^{-1}$ and showed 18-fold greater interaction than the empty control, which could release only 0.19 ± 0.02 nmol of *o*-nitrophenol min^{-1} (mg of protein)$^{-1}$ (Figure 2).

The C571A mutant, altering the cysteine involved in interchain disulfide bonding (*30*) to alanine, was studied in this system to evaluate the importance of the altered residue to BChE C-terminal peptide interaction. All evidence indicated strong interactions between these peptides, similar to that of the wild-type peptides. Yeast transfected with the C571A mutant grew well in the absence of Leu, Trp, and His and also in the presence of 25 mM 3-AT (Table 1). Double transfectant yeast colonies of the C571A mutant produced a strong β-galactosidase signal as revealed by their bright blue color in X-gal colony filter lift assays. This β-galactosidase specific activity was quantitated to be 2.2 ± 0.8 nmol of *o*-nitrophenol min^{-1} (mg of protein)$^{-1}$ (Figure 2). Therefore, this mutant showed a similar degree of interaction to the wild-type peptide in this in vivo system. The interchain disulfide bond was not required for interaction of the 40 residue C-terminal domain.

When the conserved aromatic residues and Cys 571 (Figure 1) were replaced with alanines (aromatics-off/C571A mutant), the interaction of the BChE peptides in the yeast two-hybrid system was significantly decreased. Yeast transfected with the aromatics-off/C571A constructs were able to grow on SD-Leu-Trp but grew poorly on SD-Leu-Trp-His. Their failure to thrive on medium lacking His indicated a decreased interaction between the aromatics-off peptides as compared to the wild-type peptides and low levels of IGP dehydratase reporter expression. When IGP dehydratase was inhibited with 25 mM 3-AT, no growth of these yeast was observed (Table 1). In X-gal filter assays, the aromatics-off/C571A transfectants showed only a light blue color in comparison to the wild-type colonies, indicating reduced β-galactosidase expression. This reduced β-galactosidase activity was confirmed by the ONPG assays revealing that the aromatics-off/C571A mutant showed only 12% [0.4 ± 0.1 nmol of *o*-nitrophenol min^{-1} (mg of protein)$^{-1}$] of the β-galactosidase activity, of wild-type (Figure 2). These assays indicated that the aromatic residues were crucial for interac-

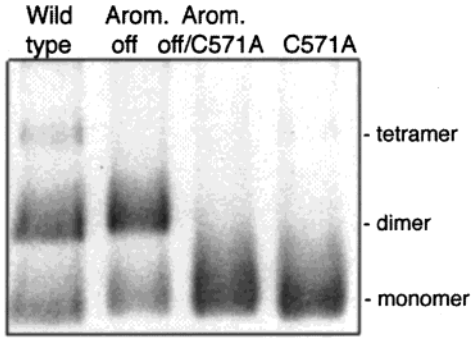

FIGURE 3: Oligomeric composition of wild-type and mutant BChE. Wild-type and mutant BChE secreted by 293T cells were tested for activity with butyrylthiocholine iodide, and 10 milliunits of each sample was loaded onto a 4−30% native polyacrylamide gel. After electrophoreses at 120 V at 4 °C for 36 h, gels were stained for activity with butyrylthiocholine iodide. Locations of tetramers, dimers, and monomers are indicated.

tion between the C-terminal peptides encompassing the tetramerization domain of BChE.

Aromatics-off Mutations in BChE. To evaluate the importance of the seven aromatic residues in the C-terminus to the oligomeric structure of BChE, we mutated these aromatics to Ala in the BCHE cDNA and expressed them transiently in 293T cells. As a control, the wild-type BChE was expressed in 293T cells as well. As seen on activity-stained gels, the wild-type BChE secreted into culture medium was composed of three forms: tetramer, dimer, and monomer (Figure 3). These appeared in a ratio of approximately 10−30%, 40−50%, and 30−40%, respectively, with the tetramer being least abundant. The aromatics-off mutant contained only dimer and monomer, and the corresponding ratio of these forms was 60−70% and 30−40%, respectively. The absent tetramers of the aromatics-off mutant could be accounted for by the increased proportion of dimers, with no accompanying change in the proportion of monomers. Tetramers of aromatics-off BChE were not detected in Western blots (data not shown), indicating that this mutant produced neither active nor inactive tetramers. As a result of these mutations, BChE was incapable of assembling into tetramers.

Cys 571 Mutation. Expression of the C571A mutant in CHO-K1 cells had previously revealed a decrease in the proportion of active dimers (from 55% to 2−7%) but no significant difference in the proportion of active tetramers (5−30%) of BChE as compared to the wild-type (*10*). To confirm this observation in 293T cells, the C571A mutant was transiently expressed in these cells. We observed no active dimer and 2.3% active tetramers (Figure 3). To test the effect of the C571A mutation on the oligomeric composition of the aromatics-off BChE, we made the aromatics-off/C571A mutant. The secreted product of the aromatics-off/C571A BChE mutant contained only active monomers. These observations indicate that in both the wild-type and aromatics-off forms of BChE the dimer is stabilized by the intermolecular disulfide bridge, as has been previously suggested (*10*, *30*). However, the absence of the disulfide linkage did not preclude the formation of inactive dimers (see data below), indicating that the disulfide bond was not the only force responsible for the stabilization of dimers.

Interaction of BChE with Poly-L-proline Yields Tetramers. Bon et al. (*12*) reported that the addition of poly-L-proline

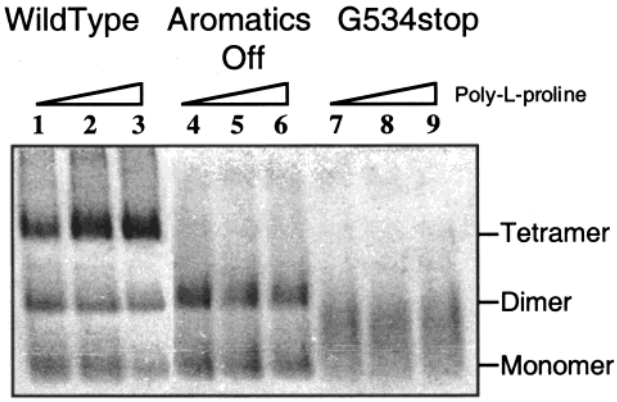

FIGURE 4: Poly-L-proline treatment of cells transfected with wild-type, aromatics-off, and G534stop. 293T cells transiently transfected with wild-type (lanes 1−3), aromatics-off (lanes 4−6), and G534stop (lanes 7−9) BChE were treated with poly-L-proline (10^{-7} M in lanes 2, 5, and 8; and 10^{-5} M in lanes 3, 6, and 9). Lanes 1, 4, and 7 are BChE from control cells untreated with poly-L-proline. Eight milliunits of BChE was loaded in each of the three lanes for the wild-type while 4 milliunits was loaded in each lane for the aromatics-off and G534stop mutants. 4−30% native polyacrylamide gels were electrophoresed at 120 V and 4 °C for 36 h. Gels were stained for activity with butyrylthiocholine iodide. Tetramer, dimer, and monomer positions are indicated.

to the culture medium of COS cells expressing AChE increased the proportion of AChE tetramers and proportionally decreased the amount of dimers and monomers. To investigate whether poly-L-proline could produce similar results with recombinant BChE, we expressed wild-type BChE in 293T cells in the presence of increasing concentrations of poly-L-proline up to 10^{-5} M. We found that the proportion of wild-type BChE tetramers in the culture medium increased from approximately 30% to about 65% with increased concentrations of poly-L-proline in the media (Figure 4). In addition, we observed a marked decrease in the proportion of dimers and monomers. Dimers were decreased from approximately 40% to about 20%, and monomers were decreased from approximately 30% to 15%. A higher molecular weight species was observed migrating above the tetramer. This species also increased with increasing poly-L-proline concentration. A similar high molecular weight species, characterized as an AChE octamer, was observed with higher concentrations of poly-L-proline in the experiments of Bon et al. (*12*).

To investigate whether the observed increase in the tetrameric form with the addition of poly-L-proline was the result of C-terminal interactions and, more specifically, interaction via the conserved aromatic residues, we expressed the G534stop and aromatics-off BChE mutants transiently in 293T cells in the presence of increasing concentrations of poly-L-proline up to 10^{-5} M. The G534stop mutant has amino acids 1−533 of BChE but is missing 41 amino acids from the C-terminus. With both of these mutants, the proportion of tetramers was not affected by the addition of poly-L-proline to the media; i.e., no tetramers were seen with either of these BChE mutants (Figure 4). These results indicated that the mechanism by which poly-L-proline increased the amount of tetramers in cell culture required the C-terminal residues of BChE and, particularly, the conserved aromatic residues in this region.

Interaction of Q_NPRAD with BChE Yields Tetramers. The rat collagen tail COLQ cDNA has been isolated and cloned

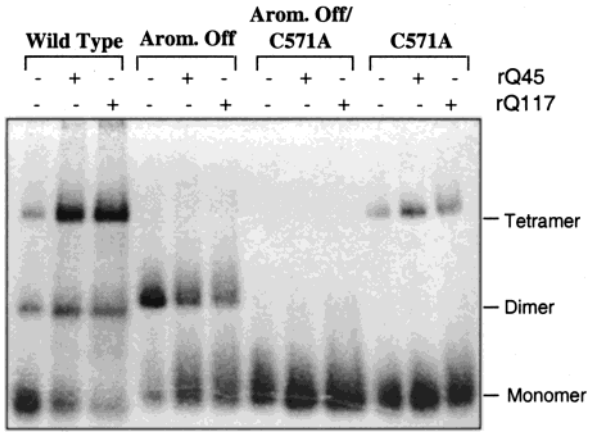

FIGURE 5: Coexpression of PRAD-containing peptide with wild-type and mutant BChE. Wild-type and mutant BChE were coexpressed with (+) and without (−) the 45 or 117 amino-terminal residues of rat COLQ including the PRAD peptide. Ten milliunits of BChE was loaded into each lane of a 4−30% native polyacrylamide gel. Electrophoresis was conducted at 120 V and 4 °C for 36 h. The gel was stained for activity with butyrylthiocholine iodide. Positions of tetramers, dimers, and monomers are indicated.

by Dr. Eric Krejci (*18*). To determine the effect of the noncollagenous N-terminal domain of COLQ containing PRAD (proline-rich attachment domain) on BChE tetramer production, plasmids rQ45 and rQ117 were transfected into CHO-K1 cells that stably expressed wild-type human BChE. Transfection with either of these PRAD-containing plasmids led to an increase in BChE tetramers from 10−15% to 60−70% (Figure 5). Therefore, the PRAD region of the collagen tail Q subunit induced BChE tetramer formation. A corresponding decrease in the proportion of dimers and monomers was also observed. Dimers decreased from 40−50% to 20−30%, and monomers decreased from 30−40% to 10−20%. The decrease in dimers and monomers was attributed to the formation of tetramers.

To determine whether the conserved aromatic residues were required for the interaction of the PRAD-containing peptides with BChE, we coexpressed rQ45 and rQ117 with the aromatics-off BChE in CHO-K1 cells. No tetramers were produced by the aromatics-off BChE in the presence of these PRAD-containing peptides. This indicated that the increase in tetramers observed when the wild-type BChE was coexpressed with PRAD-containing peptides was the result of the interaction of C-terminal aromatic residues with PRAD-containing peptides. When the aromatics-off/C571A mutant BChE was coexpressed with rQ45 and rQ117, active monomers were produced, but no active dimers or tetramers (Figure 5).

To determine whether the cysteine involved in intermolecular disulfide bonding was required for the observed increase in BChE tetramers with coexpression of Q_NPRAD, the C571A BChE mutant was coexpressed with rQ45 and rQ117 (Figure 5). Tetramers of this mutant were increased from 10% to approximately 20−30% in the presence of Q_N-PRAD peptides. This observation indicated that Cys 571 was not necessary for the interaction between PRAD and BChE to increase the proportion of tetramers. Our results agree with those of Simon et al. (*13*), who showed that the C-terminal cysteine is not necessary for association of rat AChE with PRAD.

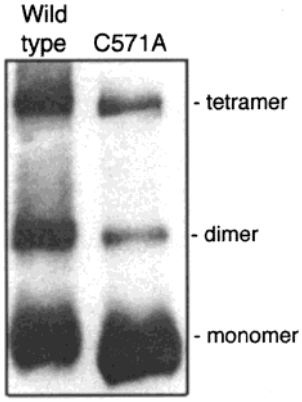

FIGURE 6: Western blot of wild-type and C571A BChE. Five nanograms of wild-type and 10 ng of C571A BChE stably expressed in CHO-K1 cells and secreted into culture medium were electrophoresed on 4−30% native polyacrylamide gels at 120 V, 4 °C, for 36 h. Blots were probed with polyclonal anti-BChE antibody and developed by electrochemiluminescence.

Inactive Dimers Form in the Absence of the Disulfide Bond. Western blots of wild-type and C571A BChE expressed in CHO-K1 cells were performed to determine whether BChE was capable of forming dimers in the absence of the disulfide linkage. Figure 6 shows that dimers as well as tetramers were formed in the absence of the disulfide linkage. This evidence suggested that the disulfide linkage was not the exclusive force mediating the interaction of dimers. The dimers appearing in the Western blot were inactive, as they did not appear in activity-stained gels (Figure 5). In addition, the proportion of dimers appearing in the Western blot of the C571A mutant (10% relative to other multimeric species) is lower than that of the wild-type (40% relative to all multimeric forms). Therefore, it was concluded that the disulfide bond assisted in the stabilization of dimeric interactions, along with other forces, and maintained the native conformation of dimer subunits to preserve their enzymatic activity.

PRAD-Containing Peptide Copurifies with BChE Tetramers. A stable CHO-K1 cell line expressing wild-type BChE in the presence of FLAG-tagged Q_NPRAD was established. The secreted BChE was purified from 16 L of culture medium on procainamide affinity gel and DE52 ion exchange. The resulting highly purified BChE consisted of 70−80% tetramers. To determine whether the rQ45 peptide with its FLAG tag was present in the purified BChE, 100 μg of BChE was denatured in SDS and mercaptoethanol and subjected to electrophoresis on a 30% polyacrylamide gel containing SDS. The proteins were transferred from the SDS gel to a PVDF membrane, and hybridized with a FLAG antibody. A small peptide approximately 5 kDa in size was detected by the FLAG antibody (data not shown).

To determine which form of BChE bound the PRAD-containing peptide, the purified BChE was separated into tetramers, dimers, and monomers on nondenaturing gel electrophoresis (Figure 7). After transfer of proteins to a PVDF membrane, the blot was hybridized with M2 anti-FLAG antibody. Figure 7 shows that the 5 kDa PRAD-containing peptide was bound exclusively to tetramers of BChE and not to dimers and monomers. The FLAG-tag on the Q_NPRAD peptide was visible on the Western blot only when large amounts of purified BChE, 5 μg, were loaded on the gel. By contrast, when the FLAG tag was on the

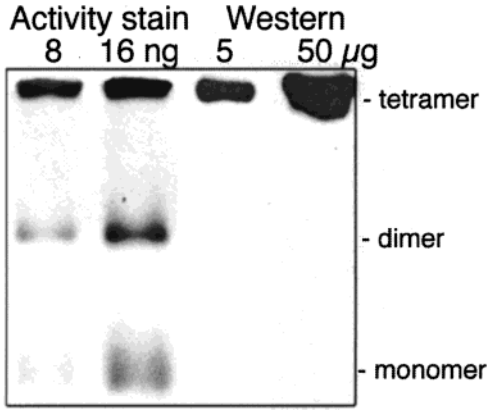

FIGURE 7: PRAD-containing peptide copurifies with recombinant BChE tetramers. BChE was purified from culture medium of a stable CHO cell line expressing both BChE and Q_NPRAD-FLAG. On a nondenaturing polyacrylamide gel, the purified BChE consisted primarily of tetramers, though dimers and monomers were also visible on an activity-stained gel. The M2 anti-FLAG antibody showed that the Q_NPRAD-FLAG peptide was associated only with tetramers of BChE.

C-terminus of BChE, 5 ng of BChE gave a strong signal. This 1000-fold difference in sensitivity suggests that the stoichiometry of 1 PRAD bound per 4 subunits (*13*) may have been reduced during the purification procedure. Alternatively, the FLAG epitope on the C-terminus of the 45 residue Q_NPRAD peptide may be unavailable for binding to antibody. We conclude that the PRAD-containing peptide that is responsible for tetramerization of BChE remains attached to BChE throughout the purification procedure and that only tetramers of BChE bind PRAD.

DISCUSSION

A Modular Domain of Quaternary Interaction for BChE. We have demonstrated in the current studies that the C-terminus of BChE is modular in function. Utilizing the yeast two-hybrid system, we showed that the C-terminal peptides of BChE interacted with one another, independently from the remainder of the molecule, and that they retained this function when fused to yeast proteins. In addition, by mutating the conserved C-terminal aromatic residues to alanine in the whole BChE molecule, we showed that tetramerization was specifically lost without destroying the catalytic activity of BChE. Additional evidence to support this idea is provided by experiments showing that deletion of the final 41 residues of the BChE subunit (G534stop mutant) preserved catalytic activity while abolishing the ability of BChE subunits to form tetramers (*10*). These studies demonstrate that the C-terminus of BChE is the tetramerization domain of the molecule. Loss of this domain affects only the quaternary interaction of BChE subunits but does not abolish BChE enzymatic activity.

The modular nature of the C-terminal domain of AChE has been demonstrated in a different way by Simon et al. (*13*). When the 40 residue C-terminal peptide of rat AChE was attached to green fluorescent protein and coexpressed with PRAD fused to exon 5 of ACHE, the green fluorescent protein was found on the cell surface (*13*). This indicated that green fluorescent protein had bound to PRAD through the C-terminal domain of AChE, illustrating that the C-terminal domain functions to bind PRAD regardless of whether it is attached to AChE or to a foreign protein such as green fluorescent protein.

Role of Aromatic Residues in Tetramer Formation. In vivo interaction between C-terminal peptides was greatly diminished in the absence of seven conserved aromatic residues. In addition, no tetramers appeared in the aromatics-off mutant. These findings suggested that the aromatic residues at the C-termini of BChE subunits provided the principal driving force facilitating tetramer formation. Masson and coworkers have found that BChE tetramers do not dissociate when subjected to ultrasound and high pressure (*17, 31*), suggesting that aromatic interactions are responsible for tetramerization. Weaker hydrophobic interactions between the aliphatic hydrocarbon side chains of Ala, Val, Leu, and Ile would dissociate upon application of high pressure (*17*). These data confirm the aromatic nature of the interactions in the tetramerization domain.

The aromatics-off and aromatics-off/C571A mutants of BChE were expressed at high levels in stable CHO-K1 cell lines, yielding up to 68 units/mL when assayed with butyrylthiocholine iodide. Wild-type BChE had an activity of 8 units/mL with the same substrate. The 68 units/mL is about 94 mg/L, comparable to the yield obtained from expression of *Bungaris fasciatus* snake acetylcholinesterase monomers (*32*). Mutations in the C-terminal domain of BChE do not affect k_{cat} (*10*). We conclude that the hydrophobic patch of seven aromatic amino acids at the C-terminus of BChE reduces the efficiency of secretion of BChE. This conclusion is consistent with the observation by Simon et al. (*13*) that the C-terminal peptide of AChE is retained intracellularly when it is expressed separately from the catalytic domain.

Role of C571 in Dimerization. The cysteine at position 571 is involved in intermolecular disulfide bonding between two BChE subunits in the dimeric species (*30*). This cysteine has minimal effect on tetramer formation, as (1) the C571A mutant of BChE is still capable of forming tetramers (ref *10* and Figure 5) and (2) tetramers of the native BChE remain intact upon chemical reduction and alkylation (*33*). We have investigated the effect of the disulfide bond on dimer formation in this study. In the two-hybrid system, the C571A mutant worked nearly as well as the wild-type (Figure 2), indicating that the C-terminal peptides of the dimer can interact without the disulfide linkage. Moreover, we found through Western blots that inactive dimers are present in the C571A mutant. This again suggests that the disulfide bond is not a prerequisite for dimer formation. However, because the dimers are inactive, we conclude that the disulfide bond contributes to the stability of the native conformation of dimers in order to preserve enzymatic activity.

Role of Poly-L-proline and Q_NPRAD in Tetramer Formation. Poly-L-proline as well as Q_NPRAD increased the amount of BChE tetramers. Without the key aromatic residues at the BChE C-terminus, the enhancement of the tetrameric population by the addition of poly-L-proline or Q_NPRAD was not possible. The finding that the PRAD-containing peptide copurified with recombinant BChE tetramers indicates that the hydrophobic patch of aromatic residues at the C-terminus of BChE interacts directly with PRAD. This result raises the question of whether native BChE tetramers isolated from human serum have a PRAD peptide attached to their C-terminus. Amino acid sequencing of native BChE tetram-

ers did not reveal a PRAD peptide (*30*, *34*). Lockridge et al. (*30*) specifically searched for a collagen-tail peptide by N-terminal sequencing of tetrameric BChE and by sequencing disulfide-linked peptides. No evidence for a PRAD peptide in native tetrameric BChE was found. If a PRAD peptide is present, it will have a blocked N-terminus and it will be noncovalently linked to BChE.

Model for Cholinesterase Tetramers. At present, there is no structural information on the conformation of the C-terminus of cholinesterases. Our data predict that the C-termini of four subunits are in close proximity, forming a tetramerization domain that is distinct from the catalytic domain. The tetramerization domain of BChE comprises residues 534−574, encoded by exon 4, while the catalytic domain comprises residues 1−533, encoded by exons 2 and 3. We propose that the tetramerization domain is exposed to solvent and protrudes away from the catalytic domains, based on the observations that the C-termini are readily accessible to proteases, reducing agents, and alkylating agents, unlike the remainder of the cholinesterase tetramer (*33*). The tetramerization domain is predicted to have α-helical character (*10*, *35*). The conserved aromatic residues would fall on one side of an α-helix to form a hydrophobic core. The PRAD peptide is attached to the tetramerization domain of recombinant BChE tetramers; however, there is no evidence that a PRAD peptide is present in native BChE tetramers.

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