Polyproline-rich peptides associated with *Torpedo californica* acetylcholinesterase tetramers

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ABSTRACT

Acetylcholinesterase (AChE) terminates cholinergic neurotransmission by hydrolyzing acetylcholine. The collagen-tailed AChE tetramer is a product of 2 genes, ACHE and ColQ. The AChE tetramer consists of 4 identical AChE subunits and one polyproline-rich peptide, whose function is to hold the 4 AChE subunits together. Our goal was to determine the amino acid sequence of the polyproline-rich peptide(s) in *Torpedo californica* AChE (*Tc*AChE) tetramers to aid in the analysis of images that will be acquired by cryo-EM. Collagen-tailed AChE was solubilized from *Torpedo californica* electric organ, converted to 300 kDa tetramers by digestion with trypsin, and purified by affinity chromatography. Polyproline-rich peptides were released by denaturing the *Tc*AChE tetramers in a boiling water bath, and reducing disulfide bonds with dithiothreitol. Carbamidomethylated peptides were separated from *Tc*AChE protein on a spin filter before they were analyzed by liquid chromatography tandem mass spectrometry on a high resolution Orbitrap Fusion Lumos mass spectrometer. Of the 64 identified collagen-tail (ColQ) peptides, 60 were from the polyproline-rich region near the N-terminus of ColQ. The most abundant proline-rich peptides were SVNKCCLLTPPPPPMFPPPFFTETNILQE, at 40% of total mass-spectral signal intensity, and SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK, at 27% of total intensity. The high abundance of these 2 peptides makes them candidates for the principal form of the polyproline-rich peptide in the trypsin-treated *Tc*AChE tetramers.

1. Introduction

Soluble AChE tetramers for crystal structure studies of native AChE tetramers were prepared from *Torpedo californica* electric organ. Membrane-bound collagen-tailed asymmetric *Torpedo californica* AChE (*Tc*AChE) was solubilized. and converted to soluble *Tc*AChE tetramers by tryptic digestion (Taylor et al., 1974). A schematic model of the collagen-tailed asymmetric AChE in Fig. 1 shows the triple ColQ helix attached to 3 AChE tetramers. Two AChE subunits (accession # CAA27169) are disulfide linked to each other through Cys 593 while two other AChE subunits are disulfide linked to ColQ via two adjacent cysteine residues: Cys 593 of AChE to Cys 70 of ColQ, and Cys593 of AChE to Cys 71 of ColQ (accession # Q03637) [1–5]. The sedimentation coefficients of the 14S and 18S species of collagen-tailed AChE are unaltered by specific reduction of the Cys 593 disulfide bonds [6].

Assembly into tetramers or into collagen-tailed forms does not require these disulfide bonds [6,7]. The critical components for tetramer assembly are a string of proline residues in ColQ, and the presence of conserved aromatic residues in the WAT tetramerization domain of AChE [8].

Our goal was to identify the amino acid sequence of the polyproline-rich peptides in the protease-digested *Tc*AChE tetramers. The digestion step was expected to have trimmed ColQ at both the N- and C- termini, but to have left untouched the critical polyproline-rich region that is protected by the catalytic subunits that assemble around it. Peptides associated with highly purified *Tc*AChE tetramers were released by denaturing the enzyme in a boiling water bath, and reducing the disulfide bonds with dithiothreitol. Carbamidomethylated peptides were separated from the *Tc*AChE protein, and identified by liquid chromatography tandem mass spectrometry.

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2. Materials and methods

2.1. Materials

Soluble *Tc*AChE tetramers from the electric organ of *Torpedo californica* were generated by digesting membrane bound collagen-tailed AChE with trypsin, as described in section 2.2.1 [11]. The tetramers were purified by affinity chromatography [11] followed by size exclusion chromatography on a HiPrep Sephacryl 16/60 S-200 HR column. AChE in fetal bovine serum (Gibco) was used as a standard for AChE tetramers on activity stained gels. Pure human butyrylcholinesterase (HuBChE) [12] was used as a standard for BChE tetramers on activity stained gels. YM-10 centrifugal filters (10,000 NMWL, Merck Millipore, cat# MRCPRT010) were used to separate peptides from intact protein.

2.2. Methods

2.2.1. Extraction of AChE from Torpedo californica electric organ and purification of AChE tetramers

A batch of 350 g of *Torpedo californica* electric organ tissue (Aquatic Research Consultants, San Pedro, CA), which had been stored at −70 °C, was thawed, and cut into small cubes. The tissue was homogenized in two volumes of 0.04 M MgCl$_2$/0.1 M NaCl/0.01 M Tris, pH 8.0, at 4 °C, for 90 s, followed by a 90 s interval, and again for 90 s. The homogenate was centrifuged, in a ty35 rotor for 40 min, at 30,000 rpm, in a Beckman Coulter Optima XE-90 refrigerated ultracentrifuge. The supernatant was discarded, and the pellet resuspended in 250 mL of 0.04 M MgCl$_2$/0.1 M NaCl/0.01 M sodium bicarbonate, pH 7.8 (bicarbonate buffer). The suspension was warmed to 25 °C, and 250 μL of
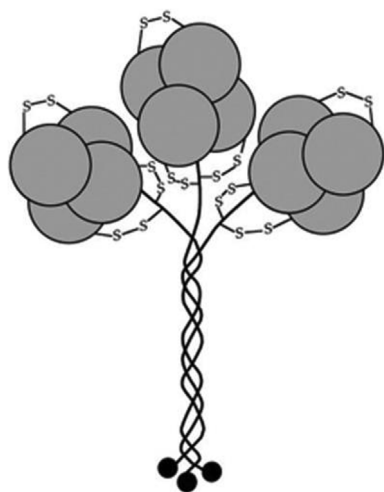

Fig. 1. Schematic diagram of the structure of collagen-tailed asymmetric AChE at the neuromuscular junction. The collagen tail has a polyproline-rich region near its N-terminus whose function is to organize 4 subunits of AChE into a tetramer [9]. The polyproline-rich region of ColQ is not seen in this figure because it is embedded within the tetramers. Stoichiometry experiments demonstrated that one polyproline-rich peptide associates with 4 AChE subunits [10] to make a stable AChE tetramer. Figure taken from Ref. [10].

5 mg/mL bovine pancreatic trypsin (Sigma), in 1 mM HCl, pH 3.6, were added to the homogenate to yield a final concentration of 5 μg/mL. After incubation for 7 min, digestion was terminated by adding 250 μL of a solution of soybean trypsin inhibitor (Sigma), 10 mg/mL in water, and immediately cooling to 4 °C in an ice bath. The tryptic digest was then centrifuged for 1 h, at 33,000 rpm, in the same rotor and centrifuged as above. The pellet was discarded, and the supernatant diluted 4-fold with 10 mM Tris, pH 8.0, to reduce the ionic strength. The diluted supernatant was passed over a 4-mL column of an affinity resin in which [*N*-(6′-aminohexanoyl-6-aminohexanoyl)-*m*-aminophenyl]trimethylammonium was coupled to cyanogen bromide-activated Sepharose 4B [13], as described earlier [14], at a rate of 60 mL/h. The affinity column was washed with 50 vol of the 4-fold diluted bicarbonate buffer, followed by 5 vol of the undiluted buffer. The purified *Tc*AChE was eluted with 20 mM decamethonium bromide (Sigma-Aldrich) in the undiluted buffer. To ensure the preparation consisted exclusively of AChE tetramers, it was subjected to size exclusion chromatography on a HiPrep Sephacryl 16/60 S-200 HR column, with a yield of 2 mg AChE tetramers.

2.2.2. Nondenaturing gel electrophoresis stained for AChE activity

Polyacrylamide 4–22% gradient gels with a 4% stacking gel were prepared in the absence of detergent, and run in Tris-glycine buffer, pH 8.9, without detergent, for 16 h at 320 v and 4 °C. Gels were stained for AChE activity in a solution containing 90 mL of 0.2 M maleic acid adjusted to pH 6.0 with sodium hydroxide, 7.5 mL of 0.1 M sodium citrate, 15 mL of 0.030 M CuSO$_4$ pentahydrate, 15 mL of 5 mM K$_3$Fe(CN)$_6$, water to a total of 150 mL, and 150 mg of solid acetylthiocholine iodide [15]. Reddish brown bands of activity developed in about 1 h.

2.2.3. SDS gels

4–22% gradient polyacrylamide SDS gels with a 4% stacking gel were prepared in an SE600 Hoefer apparatus and run for 15 h at 200 v, at room temperature. Gels were stained with Coomassie blue.

2.2.4. Release of polyproline-rich peptides

A 40 μL aliquot containing 0.2 mg of *Tc*AChE was treated with 10 mM dithiothreitol in a boiling water bath for 3 min to release disulfide-bound peptides. Free sulfhydryls were carbamidomethylated with 25 mM iodoacetamide. Carbamidomethylated peptides were separated from denatured *Tc*AChE protein by centrifugation through a pre-wetted YM-10 spin filter. The flow through had a volume of 25 μL, representing 50% of the 50 μL that had been applied. Polyproline peptide concentration was estimated based on the assumption of 1 mol polyproline peptide per AChE tetramer, and no loss of peptide concentration during handling. This was calculated to be 12 pmole of polyproline-rich peptide per μL in the 25 μL sample that was prepared for mass spectrometry.

2.2.5. Liquid chromatography tandem mass spectrometry

The polyproline-rich peptide fraction was desalted on an Acclaim PepMAP 100C18 trap column; 3 μm, 100 Å, 75 μm × 2 cm (Thermo Scientific #164535), using an UHPLC Ultimate 3000 RSLC nano System (Dionex/Thermo Scientific), and separated on a PepMAP RSLC C18 separation column; 2 μm, 100 Å, 75 μm × 50 cm (Thermo Scientific

#ES803A). The 2 μL peptide sample (24 pmole) was loaded onto the desalting column and washed with 100% solvent A (0.1% formic acid in water) for 10 min at 4 μL per min before the sample was pumped onto the separation column. Elution was at a flow rate of 0.3 μL/min. Solvent B was 80% acetonitrile/20% water/0.1% formic acid. The separation column was washed for 10 min with 9% solvent B followed by a 9–70% gradient of solvent B in 60 min. The column was stripped with 100% solvent B for 10 min, and re-equilibrated with 9% solvent B for 20 min for a total run time of 100 min per sample. The trap column was left in the flow path for 100 min. The elution gradient described above gave the best results. Additional runs with a shorter gradient were not satisfactory.

Peptides eluting from the UHPLC column were electrosprayed directly into an Orbitrap Lumos Fusion mass spectrometer (Thermo Fisher Scientific). MS data dependent acquisition parameters included Orbitrap resolution 120,000, Orbitrap detector, scan range 200–1800 m/z, AGC target 4e5, maximum injection time 100 ms, positive polarity, charge state 2–6, dynamic exclusion after 1 s for 3 s, mass tolerance ± 10 ppm, and intensity threshold 2.5e4. The parameters for acquisition of MS/MS data included quadrupole isolation mode, HCD activation type, collision energy fixed at 35%, Orbitrap detector, auto scan range, Orbitrap resolution 30,000 and AGC target 5e4.

Orbitrap data files were converted from raw to mgf using MS Convert from Source Forge. Files in mgf format were searched for ColQ peptides with the Batch-Tag program in Protein Prospector (University of California San Francisco Mass Spectrometry Facility, directed by Dr. Alma Burlingame). Search parameters included User database = ColQ for *Torpedo marmorata* (Q03637); Digest = no enzyme; parent tolerance = 20 ppm; fragment tolerance = 30 ppm; constant modification = carbamidomethyl Cys; variable modification = oxidized Met; Instrument = ESI Q TOF; crosslinking link search type = no link. The sequence of ColQ from *Torpedo marmorata* was used as the user database because the sequence for *Torpedo californica* is not available. It was important to search for peptides with the search parameter set to "no enzyme" for digestion.

Manual evaluation of MS/MS fragmentation spectra was performed with Xcalibur/Qual Browser (Thermo Scientific ver. 4.2.47). The elution time for the desired peptide was taken from the Protein Prospector/Batch-Tag/Search Compare output. The MS/MS spectrum for a particular elution time was found in the chromatogram file of Qual Browser. Peaks in the MS/MS fragmentation spectrum were manually assigned with the aid of the expected sequence from Batch-Tag. In the event that more than one spectrum for the desired peptide was present, the spectrum with the most intense signal was used.

3. Results

3.1. Tetramer status of TcAChE

Soluble tetramers of *Tc*AChE were isolated by affinity chromatography, followed by size exclusion chromatography on a HiPrep Sephacryl 16/60 S-200 HR column (GE Healthcare, cat# 17-1166-01), using an AKTA pure chromatography system (GE Healthcare Life Sciences) powered by Unicorn software version 5.11. The peak at 9.67 mL in Fig. 2 contains pure *Tc*AChE tetramers.

The tetrameric status of the pure *Tc*AChE preparation was confirmed by the activity stained gel in Fig. 3 on which it migrated to the same position as tetrameric AChE from fetal bovine serum.

3.2. Purity of TcAChE

The Coomassie-stained SDS polyacrylamide gel in Fig. 4 shows an intense band for reduced *Tc*AChE monomers at 75 kDa. A very weak band for a non-reducible *Tc*AChE dimer is barely visible at 150 kDa. No contaminating proteins were visible in Fig. 4. Purity of the *Tc*AChE was confirmed by mass spectrometry, which identified no proteins other

than *Tc*AChE and ColQ in the preparation.

3.3. ColQ peptides identified by mass spectrometry

Boxed residues in Fig. 5 show the combined sequences from 60 polyproline-rich peptides that were released from *Tc*AChE tetramers, and identified by mass spectrometry (see Table 1). Residues 64–112 include the polyproline-rich region of ColQ and the cysteine residues that make a covalent bond with the C-terminal sequence of *Tc*AChE. The polyproline-rich region of ColQ intercalates with the C-terminal tetramerization domain of AChE. The function of the polyproline-rich region is to assemble 4 subunits into a tetramer.

Fig. 5 has a second boxed region for residues 307–341 of ColQ. These residues represent the combined sequences from 4 peptides produced during the digestion step that released *Tc*AChE tetramers from the collagen tail. The purification protocol washed off most extraneous peptides but retained these 4. The 4 non-polyproline peptides account for 1% of the total mass-spectral signal intensity in Table 1.

Of the 60 proline-rich peptides in Table 1, 38 contained the CysCys amino acid pair. These peptides represent 90% of total intensity. Overall, the mass errors for the 60 proline-rich peptides were around 1.5 ppm, making the peptide assignments robust. The most abundant proline-rich peptides were SVNKCCLLTPPPPPMFPPPFFTETNILQE, at 40% of total intensity, with a mass error of 0.45 ppm, and SVNKCCL-LTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK, at 27% of total intensity, with a mass error of − 0.45 ppm. The high abundance and low mass errors of these 2 peptides make them candidates for the principal form of the polyproline-rich peptide in the trypsin-treated *Tc*AChE preparation.

Many peptides in Table 1 terminate in an amino acid that is neither Lys nor Arg. Thus the trypsin sample used for converting collagen-tailed *Tc*AChE into tetramers included non-specific protease activity.

The shortest polyproline-rich peptide in Table 1 that could function as a tetramer organizing peptide is the 16-residue CCLLTPPPPPMFP-PPF. A synthetic 15-residue LLTPPPPPLFPPPFF peptide was successfully used by Dvir et al. to form a complex with the AChE tetramerization domain [16]. Massoulie and coworkers demonstrated that the 16-residue PRAD peptide remained functional when reduced to six residues followed by a string of 10 prolines (Glu-Ser-Thr-Gly3-Pro10) [17]. Furthermore, synthetic polyproline added to the culture medium of live transfected cells or to cell extracts served as an efficient
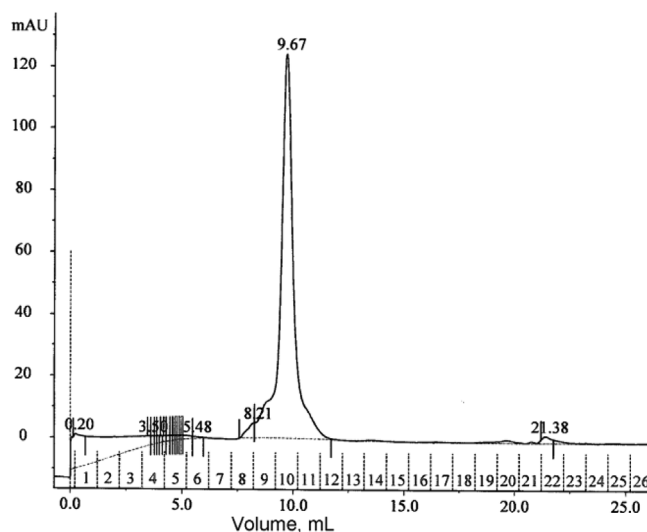

Fig. 2. Isolation of *Tc*AChE tetramers by size exclusion chromatography (SEC). Fractions eluting from the SEC column were monitored at 280 nm. The figure was generated with UNICORN 5.11 (Build 407) software. The peak at 9.67 mL contains tetrameric *Tc*AChE.
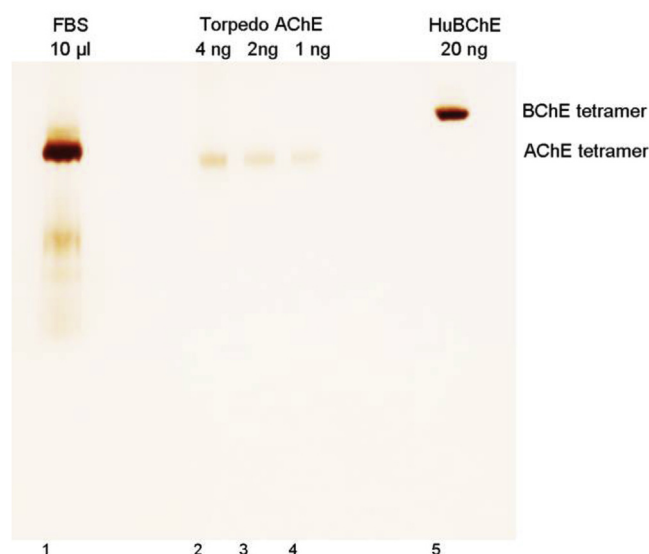
Fig. 3. Non-denaturing polyacrylamide gel stained for activity with acetylthiocholine. Lane 1) Fetal bovine serum (Gibco) is a rich source of tetrameric AChE. Lanes 2, 3, 4) The *Tc*AChE sample migrates to the same position as the fetal bovine serum tetrameric AChE; Lane 5) HuBChE tetramers hydrolyze acetylthiocholine, but migrate to a position different from that of the AChE tetramers. The gel thus confirms that the *Tc*AChE sample is a tetramer.
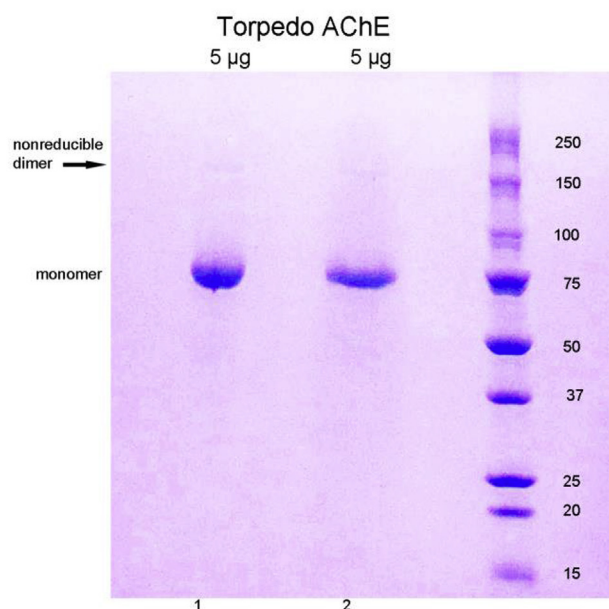

Fig. 4. SDS gel stained with Coomassie blue. The highly purified *Tc*AChE sample displays an intense band at 75 kDa corresponding to the denatured, reduced monomer, and a barely detectable band for a non-reducible dimer at 150 kDa.

tetramer-organizing peptide [17].

The MS/MS fragmentation spectrum for the polyproline-rich peptide SVNKCCLLTPPPPPMFPPPF is shown in Fig. 6. The mass to charge ratio, m/z, for the $[M+H]^{+2}$ parent ion was 1148.57. Both cysteines were carbamidomethylated. The amino acid sequence was confirmed by a series of 13 b-ions and 12 y-ions that overlapped at the central PPPP sequence.

4. Discussion

4.1. Stoichiometry: one polyproline-rich peptide assembles 4 AChE subunits into a tetramer

The study of Simon and coworkers [10] determined the stoichiometric ratio of polyproline-rich peptides, known as polyproline-rich attachment domains (PRAD), and AChE catalytic subunits. The relative quantities of AChE tetramers and of other forms were assessed by sucrose density centrifugation. It was found that virtually all AChE subunits assembled into tetramers when the ratio of AChE subunits to PRAD was four-to-one. Additional experiments identified the important features for assembly into AChE tetramers. The requirements were the presence of the 40-amino acid COOH-terminal tetramerization domain of AChE, also known as the tryptophan amphiphilic tetramerization domain (WAT), and a peptide with a high proline content, the proline-rich attachment domain (PRAD). The catalytic domain of AChE was not essential for tetramer assembly.

The stoichiometry of one polyproline peptide per 4 subunits was confirmed in the cryo-EM structure of human butyrylcholinesterase (HuBChE) tetramers [18,19]. Fig. 7 shows one polyproline peptide in the center of the superhelix formed by the C-terminal tetramerization (WAT) domains of 4 HuBChE subunits. The polyproline peptide adopts the polyproline II helix conformation and runs antiparallel to the WAT superhelix. In contrast to collagen-tailed *Tc*AChE, the HuBChE tetramers are not linked to PRAD via disulfide bonds.

Near-atomic resolution (3–4 Å) is now possible by cryo-EM [20]. The exact sequence of the polyproline-rich peptide will be helpful if the cryo-EM structure of the AChE tetramer is in the near-atomic resolution range.

4.2. Limited sources of native soluble AChE tetramers

Cryo-EM studies on the tetrameric structure of soluble AChE require a substantial amount of the AChE tetramer. Soluble AChE in human plasma is a monomer [21], and has a concentration of 8 ng/mL [22]. AChE tetramers have not been detected in human blood, but are present in fetal bovine serum. Use of AChE tetramers from fetal bovine serum (FBS) was ruled out because of the high cost of fetal bovine serum. Thus, it has been shown that 250 L of fetal bovine serum are required to obtain 29 mg of AChE tetramers [23]. Adult bovine blood and newborn bovine blood have insignificant concentrations of AChE [24]. Consequently, a trypsinized form of the collagen-tailed AChE from *Torpedo* electric organ was selected as the source of soluble AChE tetramers.

4.3. Origin of polyproline-rich peptides in soluble AChE and BChE tetramers

The polyproline peptides in collagen-tailed *Tc*AChE originate from

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  1  mlgillqkat atlasglnss ragmfpialg lllqlffdha laestfldka fslqaallpm
 61  ehkkrsvnkc clltpppppm fpppfftetn ilqevdlnnl pleikpteps ckitciigpp
121  gpsgpqgpqg iqgimgpkge igeigrpgrk grpgvrgprg mpgspcspgp igprgekgdi
181  gltglpgarg pmgpkgltgq kgekgiigek gqqgikgemg vmglpgmlgq kgemgpkgvs
241  gapghrgpvg rpgkrgktgl kgdigppgim gpsgppgpsg lpvmsgsghl mvgpkgergl
301  pgpvgrcdcn lpqtvvnpsy nkfptlinpp qvpaifvvds edeleklnte nalafrkdqk
361  slyyrdtvgw lpiqiapiqq mrqnptgfcg deivqvenge ecddgnrivt dscinckqay
421  cgdgylqsgl eecdgkdfgy htcksylpgs ygelkctsyc yidstgcryf t
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Fig. 5. ColQ sequence accession number Q03637, showing peptides originating from *Tc*AChE tetramers. Boxed residues 64–112 include the polyproline-rich region whose function is to assemble 4 subunits into a tetramer. Boxed residues 307–341 are from the non-polyproline region of ColQ.

Table 1
ColQ peptides associated with TcAChE tetramers.

M + H	*m/z*	z	Intensity	ppm	Peptide
718.4137	359.7105	2	2.47e+6	0.56	LTPPPPP
826.5035	413.7554	2	1.05e+6	0.22	NLPLEIK
947.4687	474.2380	2	894312	0.70	TETNILQE
948.5513	474.7793	2	5.54e+6	0.090	LINPPQVPA
1158.6011	579.8042	2	1.31e+6	0.89	TNILQEVDLN
1173.5833	587.2953	2	1.76e+6	1.1	LEIKPTEPSC
1267.7269	634.3671	2	470082	0.97	VDLNNLPLEIK
1301.6783	434.5643	3	1.05e+6	1.1	LEIKPTEPSCK
1321.6687	661.3380	2	457269	2.9	TPPPPPMFPPPF
1434.7513	717.8793	2	2.78e+6	1.6	LTPPPPPMFPPPF
1450.7457	725.8765	2	1.32e+6	1.3	LTPPPPPMFPPPF
1497.7627	749.3850	2	5.39e+7	0.66	NLPLEIKPTEPSC
1503.6579	752.3326	2	5.57e+6	0.91	CDCNLPQTVVNPS
1511.8145	504.6097	3	264060	0.58	LPLEIKPTEPSCK
1563.8283	782.4178	2	334700	0.28	LLTPPPPPMFPPPF
1625.8568	542.6238	3	2.18e+7	0.15	NLPLEIKPTEPSCK
1679.8315	840.4194	2	1.03e+6	−0.049	FPPPFFTETNILQE
1778.9557	889.9815	2	1.31e+6	1.3	VDLNNLPLEIKPTEPS
1867.8963	934.4518	2	324647	1.1	CCLLTPPPPPMFPPPF
1908.8587	954.9330	2	961953	0.44	CDCNLPQTVVNPSYNK
1938.9839	969.9956	2	6.86e+6	−0.088	VDLNNLPLEIKPTEPSC
2083.1133	1042.0603	2	393870	1.1	FPTLINPPQVPAIFVVDSE
2102.0667	1051.5370	2	1.87e+6	0.72	FTETNILQEVDLNNLPLE
2296.1355	1148.5714	2	2.55e+7	1.2	SVNKCCLLTPPPPPMFPPPF
2312.1333	1156.5703	2	7.71e+6	2.5	SVNKCCLLTPPPPPMFPPPF
2452.2380	818.0842	3	2.70e+6	1.8	RSVNKCCLLTPPPPPMFPPPF
2468.2319	823.4155	3	805672	1.3	RSVNKCCLLTPPPPPMFPPPF
2544.2569	1272.6321	2	1.48e+6	3.2	SVNKCCLLTPPPPPMFPPPFFT
2550.3344	850.7830	3	1.95e+6	−5.4	ILQEVDLNNLPLEIKPTEPSCK
2673.2969	891.7705	3	2.42e+7	2.1	SVNKCCLLTPPPPPMFPPPFFTE
2687.3621	896.4662	3	412454	0.64	FPPPFFTETNILQEVDLNNLPLE
2689.2835	1345.1454	2	5.91e+6	−1.0	SVNKCCLLTPPPPPMFPPPFFTE
2888.3840	963.4662	3	1.18e+6	0.76	SVNKCCLLTPPPPPMFPPPFFTETN
3071.5130	1024.5092	3	1.18e+6	1.6	KCCLLTPPPPPMFPPPFFTETNILQE
3114.5557	1557.7815	2	2.69e+6	1.8	SVNKCCLLTPPPPPMFPPPFFTETNIL
3242.6183	1081.5443	3	1.75e+6	3.0	SVNKCCLLTPPPPPMFPPPFFTETNILQ
3270.6557	1090.8901	3	3.37e+6	1.4	RSVNKCCLLTPPPPPMFPPPFFTETNIL
3371.6528	1124.5558	3	4.75e+8	0.45	SVNKCCLLTPPPPPMFPPPFFTETNILQE
3386.6807	1129.5651	3	1.16e+7	−11	PFFTETNILQEVDLNNLPLEIKPTEPSCK
3387.6511	1694.3292	2	2.47e+6	1.5	SVNKCCLLTPPPPPMFPPPFFTETNILQE
3398.7149	1133.5765	3	889169	1.5	RSVNKCCLLTPPPPPMFPPPFFTETNILQ
3470.7229	1735.8651	2	1.04e+6	0.93	SVNKCCLLTPPPPPMFPPPFFTETNILQEV
3486.7181	1162.9109	3	2.26e+6	1.0	SVNKCCLLTPPPPPMFPPPFFTETNILQEV
3527.7566	882.6946	4	1.32e+7	1.2	RSVNKCCLLTPPPPPMFPPPFFTETNILQE
3543.7530	886.6937	4	3.11e+6	1.6	RSVNKCCLLTPPPPPMFPPPFFTETNILQE
3585.7505	1793.3789	2	523467	1.1	SVNKCCLLTPPPPPMFPPPFFTETNILQEVD
3655.8521	1219.2889	3	3.57e+6	1.3	KRSVNKCCLLTPPPPPMFPPPFFTETNILQE
3671.8438	918.7164	4	2.95e+6	0.44	KRSVNKCCLLTPPPPPMFPPPFFTETNILQE
3698.8370	925.4647	4	603244	1.7	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDL
3812.8805	1271.6317	3	4.63e+7	1.8	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLN
3828.8771	1276.9639	3	4.48e+6	2.2	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLN
3869.9022	968.2310	4	3.77e+7	−11	KRSVNKCCLLTPPPPPMFPPPFFTETNILQEVD
3968.9802	993.0005	4	4.78e+6	1.4	RSVNKCCLLTPPPPPMFPPPFFTETNILQEVDLN
4069.0379	1357.0175	3	581474	1.0	PPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK
4097.0750	1025.0242	4	3.31e+6	1.3	KRSVNKCCLLTPPPPPMFPPPFFTETNILQEVDLN
4395.1769	1465.7305	3	2.46e+6	0.43	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLE
5291.6198	1764.5448	3	4.54e+6	0.43	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSC
5307.6178	1327.6599	4	4.97e+6	1.0	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSC
5332.7044	1067.3467	5	8.34e+6	4.5	VNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK
5419.7102	1355.6830	4	3.15e+8	−0.45	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK
5435.7189	1087.9496	5	6.89e+6	2.1	SVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK
5447.7086	1362.6826	4	6.46e+6	−1.8	RSVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSC
5575.8162	1394.7095	4	4.08e+6	0.44	RSVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK
5703.9126	951.4915	6	1.77e+7	0.72	KRSVNKCCLLTPPPPPMFPPPFFTETNILQEVDLNNLPLEIKPTEPSCK

M + H is the theoretical, singly-protonated mass in Da. m/z is the observed peptide mass. Z is the observed peptide charge state. Intensity is the relative signal intensity observed for the peptide in the mass spectrometer. PPM is the mass error between the observed mass for the peptide and the theoretical mass of the peptide sequence shown in the peptide column, in parts-per-million. Peptide is the sequence of the peptide that best fits the observed mass and charge state. Note that cysteine residues in Table 1 are carbamidomethylated. Some peptides contain oxidized methionine. The presence of the oxidized methionine explains why two identical sequences are listed with masses that differ by 16 Da, for example SVNKCCLLTPPPPPMFPPPF.
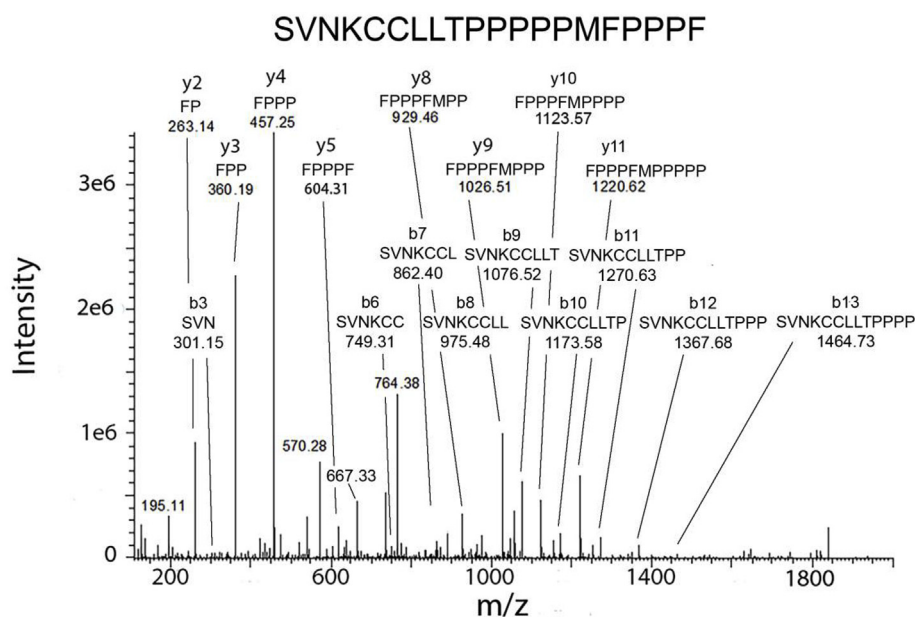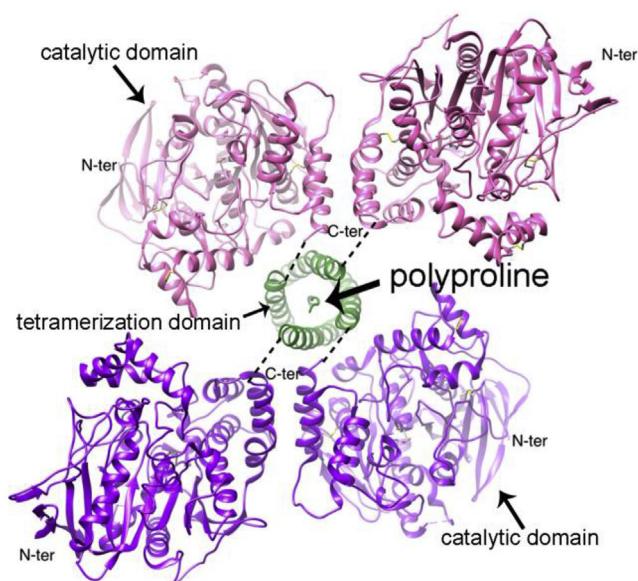
SVNKCCLLTPPPPPMFPPPF

Fig. 7. Cryo-EM structure of soluble HuBChE tetramers. One polyproline peptide organizes 4 subunits into a stable tetramer by non-covalent interactions with tryptophan residues in the tetramerization domain. PDB code 6I2T. Figure taken from Ref. [18].

the single ColQ gene [9]. In contrast, soluble AChE tetramers in fetal bovine serum, soluble BChE tetramers in human serum, equine serum, and porcine milk contain polyproline-rich peptides that originate from a variety of genes [25–29]. Chinese Hamster Ovary (CHO) cells secrete recombinant human BChE tetramers containing polyproline-rich peptides derived from 60 different proteins [30]. The proteins that donate their polyproline-rich peptides to AChE and BChE tetramers reside in the nucleus, cytoplasm and endoplasmic reticulum. They are not secreted. Furthermore there is no consistent set of polyproline-rich peptides in the tetramers. For example, lamellipodin is a source of polyproline-rich peptides for serum AChE [27], serum BChE [25,26], and BChE secreted by CHO cells [30], but is absent in milk BChE [28]. We conclude that polyproline-rich peptides are not specifically synthesized to function as tetramer organizers of AChE and BChE. The polyproline-rich peptides are degradation products of a variety of cellular proteins. The high affinity of the AChE and BChE tetramerization domains for

polyproline peptides allows the newly synthesized AChE and BChE proteins to scavenge polyproline peptides as they become available through turnover of proteins.

An example of the scavenging activity of the AChE protein is provided by work from the Rotundo laboratory [31]. Newly synthesized AChE molecules were rescued from intracellular degradation by taking up synthetic polyproline peptides from the culture medium. The peptides enhanced AChE folding resulting in increased secretion of AChE tetramers. The scavenging activity of BChE molecules was demonstrated in the following example. Treatment of mice with an adenovirus that expresses mouse BChE resulted in a 3000-fold increase in BChE activity in mouse plasma [32]. The BChE was a dimer. Incubation of mouse plasma with synthetic polyproline peptides converted all the dimers to BChE tetramers. These observations support the conclusion that the soluble AChE and BChE tetramers scavenge polyproline peptides as they become available through turnover of proteins that have sequences rich in polyproline sequences.

4.4. ColQ PRAD sequence comparison

A BLAST search for proteins in the NCBI non-redundant database that contain the 29 residue amino acid sequence svnkccllttpppppmfppppfftetnilqe from the *Torpedo* AChE ColQ protein identified a 100% match in only one species, *Torpedo marmorata*. The next best match was for elephant fish and whale shark with 23 identical amino acids out of 29. A search for the 17 residue PRAD sequence cclltppppppMfpppff yielded identical PRAD sequences in only 3 species: *Torpedo marmorata*, *Monodelphis domestica* (grey short-tailed opossum), and *Phascolaretos cinereus* (koala). In contrast the 17-residue PRAD peptide containing L in place of M, cclltppppppLfpppff, was found in ColQ from a variety of species including human, whale, dog, cat, cow, dolphin, rat, armadillo, goat, horse, pig, bat, hamster, sea lion, water buffalo, cheetah, lynx, giant panda, African elephant, monkey, rabbit, llama, squirrel, camel, baboon, rhinoceros, white-tail deer, scaly anteater, manatee, and mouse.

4.5. Significance

Soluble AChE and BChE enzymes in blood are efficient bioscavengers of nerve agents and organophosphorus pesticides. Animals pretreated with pure enzymes [33–35] or with a viral vector that encodes BChE and PRAD, and consequently produces soluble BChE

tetramers [36], are protected from the toxicity of nerve agents. AChE and BChE tetramers have a longer residence time in the circulation than dimers and monomers, making tetramers more valuable for therapeutic purposes. Thus, native human BChE tetramers have a half-life of about two weeks [37], but dimers and monomers are cleared in less than 1 h.

Information on the critical role of polyproline-rich peptides for tetramer organization is applicable to production of AChE and BChE tetramers by recombinant DNA techniques. Expression of recombinant AChE yields monomers and dimers, but only very low amounts of tetramers. Coexpression of AChE with PRAD or with a polyproline-rich peptide enhances AChE tetramer expression [17,38,39]. This finding supports the conclusion that assembly into tetramers requires a polyproline-rich peptide. The polyproline-rich peptide can have a specific sequence as in PRAD, or it can consist exclusively of consecutive proline residues [17].

Approximately 80% of the AChE synthesized within a cell is degraded within the cell and is not secreted [31]. The driver for secretion is PRAD. Co-expression of AChE with PRAD increased the level of secreted AChE up to 400% [31]. Secreted AChE was tetrameric. Addition of polyproline peptides to the culture medium also promoted secretion of tetrameric AChE. It was thus suggested that administration of PRAD peptides could be a therapeutic strategy for organophosphate-poisoned individuals because the peptides would greatly increase the level of bioscavenger AChE [31].

CRediT authorship contribution statement

Lilly Toker: Methodology. **Israel Silman:** Writing - review & editing, Supervision, Project administration. **Tzviya Zeev-Ben-Mordehai:** Conceptualization, Writing - review & editing. **Joel L. Sussman:** Conceptualization. **Lawrence M. Schopfer:** Investigation, Writing - review & editing. **Oksana Lockridge:** Investigation, Formal analysis, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2020.109007.

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