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Polyproline-rich Peptides Organize 4 Cholinesterase Subunits into A Tetramer; BChE and AChE Scavenge Polyproline Peptides Released during Metabolic Turnover

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Abstract: The genes for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) encode the proteins responsible for enzyme activity. Additional gene products, PRiMA and ColQ, anchor AChE and BChE proteins into membranes. Soluble AChE and BChE tetramers are composed of 4 identical subunits plus one polyproline-rich peptide. Dilution does not release the polyproline-rich peptide from tetramers. However, protein denaturation, for example heating in a boiling water bath, dissociates the polyproline-rich peptide. Using mass spectrometry to sequence peptides released from soluble AChE and BChE tetramers, we find sequences that correspond to proline-rich regions from a variety of proteins. A typical peptide sequence contains 20 consecutive prolines in a 23-residue peptide LPPPPPPPPPPPPPPPPPPPPLP. There is no single, common consensus sequence i.e., no specific gene appears to be responsible for the polyproline-rich peptides found in soluble AChE and BChE tetramers. We propose that during metabolic turnover, protein fragments containing polyproline-rich sequences are scavenged by AChE and BChE dimers, to make stable AChE and BChE tetramers. The 40-residue, alpha-helical C-terminus of AChE or BChE is the tetramerization domain that binds the polyproline-rich peptide. Four parallel alpha helices wrap around a single antiparallel polyproline peptide to lock the tetramer in place. This organization was established by classical X-ray crystallography for isolated C-termini in complex with a proline-rich peptide. The organization was confirmed for intact, tetrameric human BChE using cryoelectron microscopy. When 40 amino acids are deleted from the carboxy terminus, monomeric enzymes are created that retain full enzymatic activity.

Keywords: polyproline; tetramer; polyproline peptide scavenger; mass spectrometry

1. Introduction

Butyrylcholinesterase (P06276) in human plasma is stable in the circulation with a half-life of 11 days [1]. Its stability is attributed to several factors including a) its large size of 340 kDa, b) the fact that it is sugar coated with 36 N-linked glycans per tetramer [2,3], c) it is resistant to proteolysis, and d) it is a tetramer. The focus of this review is the tetramer organization of butyrylcholinesterase (BChE). Soluble BChE and acetylcholinesterase (AChE) are assembled into tetramers through interaction of 4 tetramerization domains with one polyproline-rich peptide [4,5]. This motif for tetramerization is unique for the cholinesterases as of the year 2020, but future studies may find it in other protein tetramers.

2. Tetramers are the product of more than one gene

The coding sequence for the 85 kDa monomer of human BChE (P06276) is on chromosome 3q26 [6] and for the 70 kDa monomer of human AChE (P22303) on chromosome 7q22 [7]. Monomeric proteins with these sequences have full enzyme activity, but they are unstable in the circulation because they are not tetramers. Assembly into tetramers requires additional gene products. The membrane bound forms of BChE and AChE use polyproline-rich regions of ColQ and PRiMA to assemble into tetramers. The tail end of these polyproline-rich proteins anchor BChE and AChE into the basal lamina at neuromuscular junctions or to membranes in the brain [8,9]. In contrast, no specific gene encodes the polyproline-rich peptides found in soluble BChE and AChE tetramers. The soluble BChE and AChE tetramers assemble around any polyproline-rich peptide, regardless of its origin or length as long as the peptide has at least 12 residues. An example is the 15 residue LLTPPPPPLFPPPFF of ColQ [10]. Polyproline peptides purchased from Sigma-Aldrich with molecular weights from 2000 to 5000 convert recombinant BChE monomers and dimers into tetramers [11,12].

3. Tetramerization domain

The tetramerization domain of soluble BChE and AChE tetramers is located at the C-terminus and is encoded by a separate exon. The sequence of the 40-residue BChE tetramerization domain is NIDEAE**W**E**W**KAGFHR**W**NNYMMD**W**LNQFNDYTSKKESC$_{571}$VGL. The tetramerization domain forms an alpha helix [4,13]. Two alpha helices are linked through a disulfide bond at Cysteine 599 (C571 in the mature secreted BChE). This disulfide bond is the only disulfide bond between subunits [14]. The BChE tetramer is a dimer of two disulfide-linked dimers containing a 4-helix bundle at the interface between 2 monomers [4]. Four tetramerization domains assemble in a superhelical, coiled-coil structure around a central polyproline II helix, as in Figure 1. The polyproline peptide is tightly bound via hydrophobic stacking with tryptophans and by hydrogen bonds [4,13] .

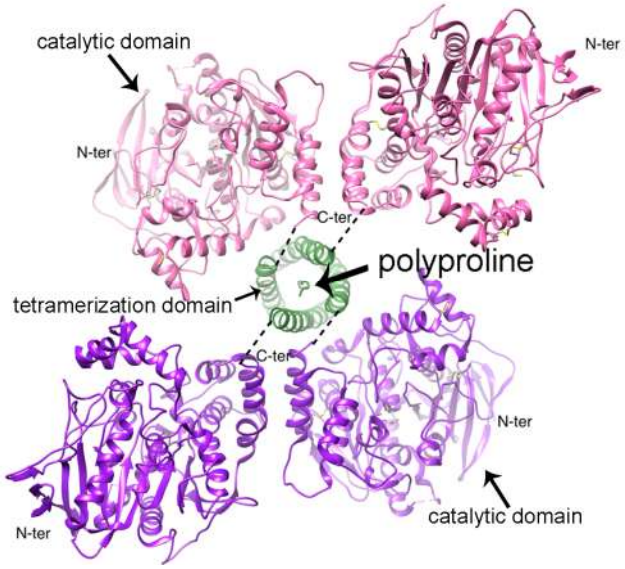

Figure 1. Cryo-EM structure of the BChE tetramer purified from human plasma. PDB code 6i2t. Figure from reference [4]. Four identical subunits, each composed of 574 amino acids and 9 N-linked glycans, assemble into a tetramer in the presence of a polyproline-rich peptide. Assembly into tetramers does not occur when polyproline peptides are unavailable.

4. Mass spectrometry identification of tetramer organizing peptides

We have identified polyproline-rich peptides in BChE tetramers isolated from human plasma, equine plasma, porcine milk, and from recombinant human BChE expressed in Chinese Hamster Ovary Cells [15-19]. In all cases the polyproline peptides were bound noncovalently. Polyproline

peptides remained tightly bound in dilute protein solutions, but were released when the proteins were denatured in a boiling water bath. The sequences of the released polyproline peptides were determined by mass spectrometry. Figure 2 shows the masses and sequences of 10 polyproline-rich peptides released from human BChE tetramers.

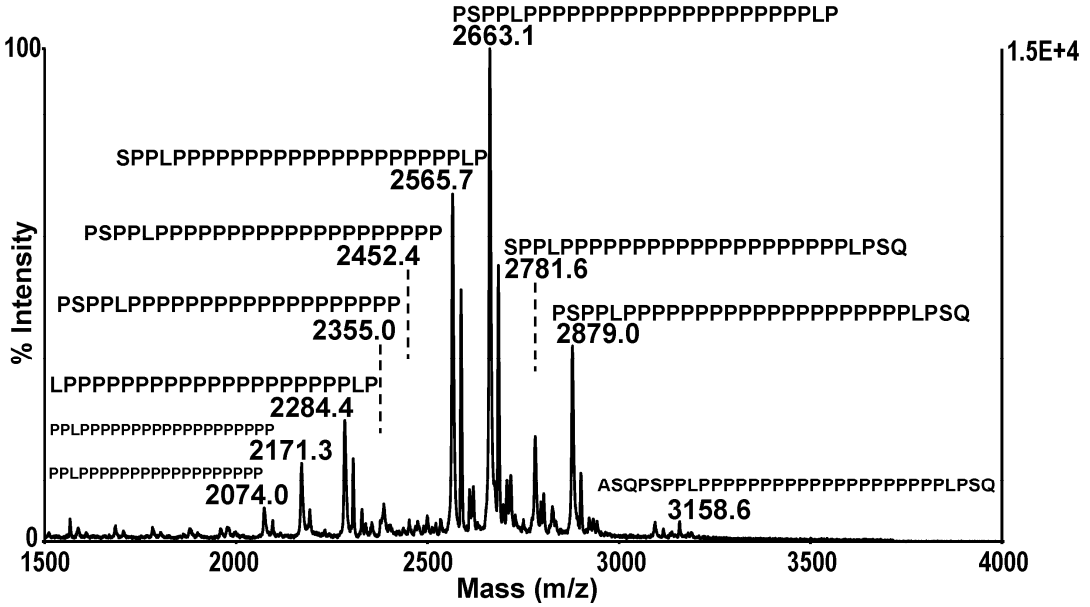

Figure 2. MALDI-TOF spectrum of polyproline-rich peptides released from human plasma BChE tetramers by denaturing the pure BChE protein in a boiling water bath. All ten peptides match human lamellipodin (Q70E73). Reproduced from [18].

Peptides were separated by high pressure liquid chromatography followed by electrospray ionization mass spectrometry (LC-MS/MS). Fragmentation of the 29-residue lamellipodin peptide in the 5600 Triple-TOF mass spectrometer yielded the MS/MS spectrum in Figure 3. Masses of the b-ion and y-ion series support the amino acid sequence PSPPL PPPPP PPPPP PPPPP PPPPP LPSQ. Peptides released from equine plasma BChE tetramers, porcine milk BChE tetramers, fetal bovine serum AChE, and recombinant human BChE tetramers expressed in Chinese Hamster Ovary cells were also separated and sequenced by LC-MS/MS in the 5600 Triple-TOF mass spectrometer.

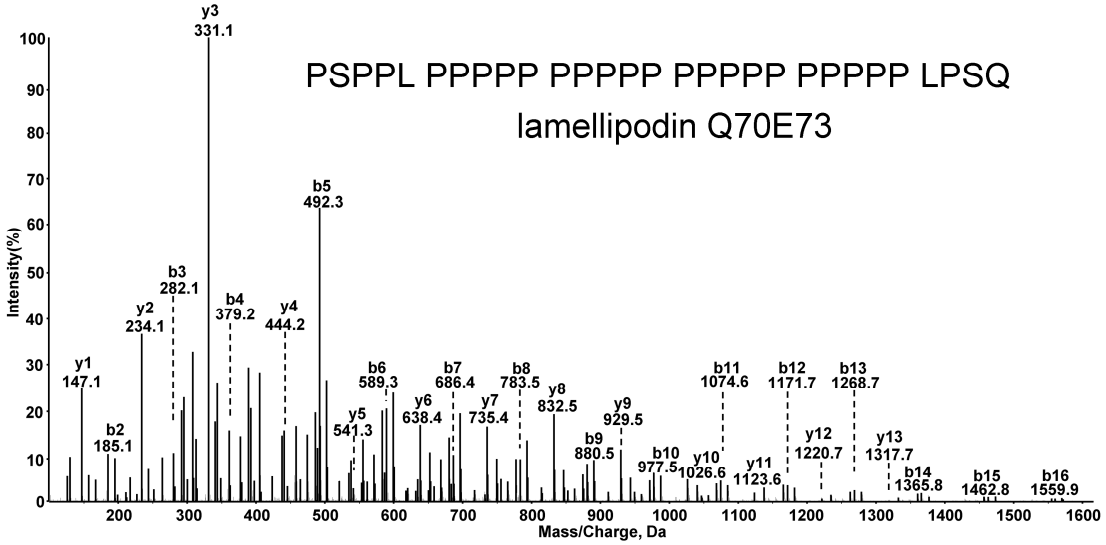

Figure 3. MS/MS fragmentation spectrum of the 29-residue peptide PSPPL PPPPP PPPPP PPPPP PPPPP LPSQ released from the human plasma BChE tetramer. The quadruply-charged parent ion

92 has a mass of 719.9/m/z. The protein donor of this peptide is human lamellipodin Q70E73.
93 Reproduced from [18].

94 **5. Polyproline-rich peptides in soluble BChE tetramers.**

95 Table 1 shows that the polyproline peptides in human plasma BChE tetramers originate from 13
96 different proteins [18]. Lamellipodin contributes 70% of the polyproline peptides. Lamellipodin
97 donates 39 polyproline peptides, ranging in length from 11 to 29 residues, to human plasma BChE
98 tetramers. Short peptides can be derived from longer peptides by losing amino acids through the
99 action of N- and C-terminal aminopeptidases and carboxypeptidase [20]. The longest observed
100 peptide associated with a specific donor protein is listed in Table 1. In some cases polyproline
101 peptides could be matched to more than one donor protein. The short LPPPP PPPPP P peptide was
102 matched to 27 different proteins. Polyproline-rich peptides consist predominantly of prolines and
103 often include leucine, alanine, serine or glutamine, but never tryptophan.

104 **Table 1.** Human protein donors for polyproline-rich peptides released from serum BChE [A]. A) Data
105 from [18]. B) The longest observed peptide in a family of related peptides is listed for each protein
106 donor. Two peptides are listed when a protein has two different polyproline-rich regions. C) Pept#
107 is the number of different observed peptides that match to fragments from the Observed Peptide. D)
108 Spectral Count is the total number of times that polyproline peptides from this Protein Donor
109 appeared in the mass spectral data. E) Prot Match indicates the number of proteins that contain the
110 Observed Peptide.

Protein Donor	Swiss Prot Accession #	Observed Peptide [B]	Pept #[C]	Spectral Count[D]	Prot[E] Match
Lamellipodin	Q70E73	PSPPL PPPPP PPPPP PPPPP PPPPP LPSQ	39	1937	1
UDP-*N*-acetylglucosamine transferase and deubiquitinase ALG13 isoform 1	Q9NP73	LPPPP PPPPP PPPPP PPPPP P	17	239	3
Synaptopodin	Q8N3V7	APPPP PPPPP PPP	4	183	5
Leiomodin-2	Q6P5Q4	LPPPP PPPPP P and TPPPP PPPPP PPPP	2 4	180	1
Acetylcholinesterase membrane anchor precursor PRiMA variant II	Q86XR5	LPPPP PPPPP P	2	121	27
Formin-like protein 1	O95466	LPPPP PPPPP PP	4	67	2
Zinc finger protein ZIC5	Q96T25	SPPPP PPPPP PP and LPPPP PPPPP PPPPP P	2 10	61	1
Homeobox protein Hox-B4	P17483	GPPPP PPPPP PPP	4	47	2
Zinc finger CCCH domain- containing protein 4	Q9UPT8	GPPPP PPPPP PPP	4	47	2
Diaphanous 1	O60610	STTPP PPPPP PPPPP P	5	38	1
Zinc finger homeobox protein 4	Q86UP3	TPPPP PPPPP PPPPP PPPPP PSA	10	23	1

Protein piccolo	Q9Y6V0	PPPPP PPPPP PPPPP PPPPP PL and QPPPP PPPPP PPPPP P	11 5	17	1
Formin binding protein 4	Q8N3X1	EPPPP PPPPP PP	2	36	3

Polyproline peptides in equine plasma BChE tetramers originate from 12 proteins, of which 8 proteins have a match in the mammalian taxonomy, but 4 have no perfect match [16]. Some polyproline sequences could be matched to more than one protein. For example a string of 21 contiguous prolines fits both UDP-N-acetylglucosamine transferase subunit ALG13 homolog and formin-like protein 2-like in the Equus caballus taxonomy. Polyproline peptides originating from lamellipodin were present in both equine and human plasma BChE tetramers.

Human plasma BChE and equine plasma BChE tetramers have 4 polyproline peptide donor proteins in common: UDP-N-acetylglucosamine transferase subunit ALG13 homolog, lamellipodin, leimodin-2, and formin-binding protein 4.

Table 2 lists 12 proteins that donate polyproline peptides to BChE tetramers in porcine milk [19,21]. The most frequent donors are lysine-specific demethylase 6B, acrosin, proline-rich protein 12, and homeobox protein hox-B4. No polyproline peptides from lamellipodin were found in BChE tetramers of porcine milk. The protein donors of polyproline-rich peptides in BChE tetramers from porcine milk are not identical to those in BChE tetramers from human plasma, though 3 protein donors appear in both Tables 1 and 2. They are homeobox protein HoxB4, Zinc finger homeobox protein 4, and Zinc finger CCCH domain-containing protein 4.

Table 2. Protein donors for polyproline-rich peptides released from porcine milk BChE [A]. A) Data from [19]. B) A composite of observed peptides from a family of related peptides for each protein donor. Two peptides are listed when two different proline-rich peptides appear in one protein. C) Pept# is the number of different peptides that match to fragments from the Observed Peptide. D) Spectral Count is the total number of times that polyproline peptides associated with this Protein Donor appeared in the mass spectral data.

Protein Donor	**Accession #**	**Composite Peptide [B]**	**Pept#[C]**	**Spect [D] Count**
Lysine-specific demethylase 6B	XP_005657086	PLPPP PLPPP PPPPP PPPPP PPLPG LAT	23	210
Acrosin	P08001	PAPPP APPPP PPPPP PPPPP PPPPP QQ	25	138
Proline-rich protein 12	XP_003127395	APPPP PPPPP PPPAS EPK and LPPPP PPPPP PPPPP PPPPP	5 11	123
Homeobox protein Hox-B4	XP_003131596	RDPGP PPPPP PPPPP PPPPG L	11	116
proline-rich membrane anchor 1	XP_003482358	PPPPL PPPPP PPPPP R	7	107
Zinc finger homeobox protein 4	XP_005663076	TPPPP PPPPP PPPPP PPPPP SA and TPPPP PPPPP PPPPP SSL	8 4	70 29
Zinc finger CCCH domain - containing protein 4	XP_005664683	GGPPP PPPPP PPPPG PPQM	4	33
Disabled homolog 2-interacting protein-like isoform 1	XP_003353684	IDQPP PPPPP PPPAP R	1	12
FH2 domain-containing protein 1	XP_005666867	PPPPS PPPPP PPPP	4	10

WAS/WASL-interacting protein family member isoform X1	NP_001231241	MPIPP PPPPP PGPPP PPTF	2	6
Protein FAM171A2	XP_005668832	AAAPP PPPPP PPAPP R	1	4
Proline-rich protein 16	XP_005655053	PNPPP PPPR	1	1

Recombinant human BChE tetramers expressed in Chinese Hamster Ovary cells (*Cricetulus griseus*) were purified and analyzed for polyproline peptides. The goal was to determine whether polyproline peptide sequences are specific to the BChE protein or to the cells that synthesize BChE. We identified 60 protein donors of the polyproline peptides in recombinant BChE tetramers [15]. The 60 donor proteins are all Chinese Hamster Ovary (*Cricetulus griseus*) proteins. Despite their origin from a nonhuman species, the polyproline peptides were incorporated into recombinant human BChE. Five donor proteins from Chinese Hamster Ovary cells were also donor proteins for human plasma BChE synthesized in the liver. The names and accession numbers of the 5 common donor proteins are listed in Table 3.

Table 3. Five donor proteins in common between recombinant human BChE tetramers expressed in Chinese Hamster Ovary cells (*Cricetulus griseus*) and human plasma BChE tetramers synthesized in human liver.

Donor protein	Accession number
Lamellipodin	(EGW06139 *Cricetulus griseus)*
Zinc finger homeobox protein 4	(ERE85184 *Cricetulus griseus*)
Leiomodin-2	(ERE89074 *Cricetulus griseus*)
Homeobox protein Hox-B4	(NP_034589 *Mus musculus*)
Zinc finger CCCH domain-containing protein 4	(Q6ZPZ3 *Mus musculus*)

From [15]. Two proteins have accession numbers for *Mus musculus* because the *Cricetulus griseus* database is incomplete.

No donor protein contributed the majority of polyproline-rich peptides to recombinant human BChE tetramers expressed in Chinese Hamster Ovary cells. This contrasts with BChE tetramers purified from human plasma, where 70% of the tetramer-organizing peptides were traced to lamellipodin. It was concluded that polyproline peptide sequences in human BChE tetramers are specific to the cells that synthesize BChE and are not specific to the BChE protein.

6. Polyproline-rich peptides in soluble AChE tetramers.

Purified fetal bovine serum AChE tetramers released polyproline-rich peptides [22] from the 5 donor proteins listed in Table 4. All 5 of these proteins are also donors for the peptides in human plasma BChE tetramers.

Table 4. 5 proteins donate polyproline-rich peptides to AChE tetramers in fetal bovine serum.

Donor protein	Accession number
Lamellipodin	Q70E73 (*Homo sapiens*)
Zinc finger homeobox protein 4	NP_001180156 (*Bos Taurus*)
Leiomodin-2	NP_001098857 (*Bos Taurus*)
UDP-N-acetyl glucosamine transferase ALG13 subunit homolog	NP_001093392 (*Homo sapiens*)
Protein Piccolo	Q9Y6V0 (*Homo sapiens*)

From [22]. Accession numbers for *Homo sapiens* proteins are listed because the *Bos Taurus* database is incomplete.

7. BChE and AChE scavenge polyproline peptides released from proteins in the cytoplasm, nucleus, endoplasmic reticulum, extracellular space, and cell membrane

Tetramer-organizing polyproline-rich peptides derive from a large number of proteins that reside in a variety of cell compartments including the cytoplasm, nucleus, endoplasmic reticulum, extracellular space, and cell membrane. For example, lamellipodin resides on the cytoplasm side of the cell membrane. Homeobox protein Hox-B4 resides in the nucleus. BChE is secreted through the Golgi apparatus and is never in the cytoplasm or the nucleus. Another fact to consider is that human BChE dimers are converted to human BChE tetramers upon addition of polyproline peptides from Sigma-Aldrich [23]. This was demonstrated for mouse plasma. The human BChE dimers had been produced in mouse plasma by injecting mice with an adenovirus vector encoding human BChE [23]. Exogenously added polyproline peptides became incorporated to form BChE tetramers.

The AChE tetramer in fetal bovine serum, like the BChE tetramer in human serum, incorporates polyproline peptides from a variety of protein donors. These observations lead to the conclusion that polyproline peptides are released from cellular proteins during metabolic turnover. The peptides circulate in the blood. Before the peptides reach the kidney they are taken up by newly synthesized BChE and AChE subunits. This process defines a new function for BChE and AChE, that of scavenging polyproline-rich peptides.

8. Conclusions

Soluble BChE and AChE are peptide scavengers. They scavenge polyproline-rich peptides that are released during cell degradation. This is a newly defined function of soluble BChE and AChE. If excess polyproline-rich peptides are toxic to cells, then scavenging activity protects the cells.

Polyproline-rich peptides in BChE and AChE tetramers originate from a variety of proteins that reside in the cytoplasm, nucleus, endoplasmic reticulum, and cell membrane. Secreted BChE and AChE have no access to proteins in the cytoplasm and nucleus. During cell degradation peptides are released to the circulation, where they are scavenged by newly synthesized BChE and AChE monomers.

Soluble BChE and AChE tetramers are not degradation products of membrane bound BChE and AChE. The evidence for this statement is that their polyproline peptides derive primarily from lamellipodin and not from ColQ and PRiMA polyproline peptides.

The BChE tetramer incorporates not only short polyproline-rich peptides, but also long protein fragments that contain a polyproline-rich region. An example is the C5 variant of human BChE whose tetrameric structure includes a 60 kDa lamellipodin fragment [24]. The ability of BChE monomers to assemble into stable, long-lived tetramers by binding the polyproline-rich region of a protein, suggests that BChE could serve as a delivery vehicle for any protein that has been engineered to include a polyproline-rich peptide tag.

AChE and BChE have non-cholinergic functions in bone development [25]. A possible explanation for their non-cholinergic function is that AChE and BChE tetramers serve as carriers of proteins that confer the non-cholinergic function.

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