the FEBS Journal

The proline-rich tetramerization peptides in equine serum butyrylcholinesterase

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Keywords
mass spectrometry; polyproline peptide; serum butyrylcholinesterase

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(Received 20 February 2012, revised 27 July 2012, accepted 9 August 2012)

doi:10.1111/j.1742-4658.2012.08744.x

Soluble, tetrameric, plasma butyrylcholinesterase from horse has previously been shown to include a non-covalently attached polyproline peptide in its structure. The polyproline peptide matched the polyproline-rich region of human lamellipodin. Our goal was to examine the tetramer-organizing peptides of horse butyrylcholinesterase in more detail. Horse butyrylcholinesterase was denatured by boiling, thus releasing a set of polyproline peptides ranging in mass from 1173 to 2098 Da. The peptide sequences were determined by fragmentation in MALDI-TOF-TOF and linear ion trap quadrupole Orbitrap mass spectrometers. Twenty-seven polyproline peptides grouped into 13 families were identified. Peptides contained a minimum of 11 consecutive proline residues and as many as 21. Many of the peptides had a non-proline amino acid at the N-terminus. A search of the protein databanks matched peptides to nine proteins, although not all peptides matched a known protein. It is concluded that polyproline peptides of various lengths and sequences are included in the tetramer structure of horse butyrylcholinesterase. The function of these polyproline peptides is to serve as tetramer-organizing peptides.

Structured digital abstract

- http://mint.bio.uniroma2.it/mint/search/interaction.do?interactionAc=MINT-7260296
- **BChE** and **BChE** bind by comigration in non denaturing gel electrophoresis (View interaction)
- **BChE** and **BChE** bind by comigration in sds page (View interaction)

Introduction

Butyrylcholinesterase (BChE) is a serine hydrolase which has attracted attention because it hydrolyzes cocaine, heroin, aspirin, bambuterol and succinylcholine, and it scavenges organophosphorus pesticides and chemical warfare agents [1–6]. These properties endow BChE with therapeutic potential. The therapeutic effectiveness of BChE relies on its ability to stay in the circulation for a long time. The half-life of human plasma derived BChE is 12–14 days when injected into humans [7,8]. Longevity in the circulation requires BChE to be glycosylated and to have a tetrameric structure [9–12]. Therefore it is of interest to understand the factors that maintain the tetrameric structure of BChE.

In neuromuscular junctions and in the central nervous system, tetrameric forms of acetylcholinesterase (AChE) and BChE are anchored to the membrane. Attachment is via the proline-rich attachment domain of the collagen tail protein (ColQ) in the neuromuscular junction and via the proline-rich membrane anchor (PRiMA) in the central nervous system [13,14]. Both ColQ and PRiMA use unique proline-rich attachment domains near their N-termini to interact with AChE and BChE C-termini [15].

Abbreviations
AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ColQ, collagen tail protein; LTQ, linear ion trap quadrupole; MSMS, process of mass spectral fragmentation; NCBI, National Center for Biotechnology Information; PRiMA, proline-rich membrane anchor; α-CHCA, α-cyano-4-hydroxycinnamic acid.

Structurally, horse and human serum BChE are very similar. Alignment of their amino acid sequences (accession code Q9N1N9 for horse and P06276 for human BChE) shows 90% identity. Both have a 28 amino acid signal peptide followed by 574 amino acids per subunit. Horse BChE has eight and human BChE has nine Asn-linked glycans [16–18]. The glycans account for about 25% of the mass of the BChE protein. BChE has three intrachain and one interchain disulfide bonds per subunit [19]. The BChE in plasma is a soluble, globular tetramer with a molecular weight of 340 000. The tetramer is composed of four identical subunits in a dimer of dimers structure where a dimer is formed by a disulfide bond at Cys571. The catalytic triad residues are Ser198, Glu325 and His438 [20]. The 40 C-terminal residues of BChE constitute the tetramerization domain [21,22]. It was found by Li *et al.* [23] that a series of proline-rich peptides derived from lamellipodin function as tetramer-organizing peptides for both human and horse serum BChE. However, only the tetramer-organizing peptides from horse BChE with the most intense mass spectral signals were analyzed.

Cholinesterases are the only proteins known to employ proline-rich peptides to stabilize their tetrameric structure. A novel addition to this tetramerization scheme was made by the demonstration that polyproline peptides, presumably derived from lamellipodin, were employed by serum BChE. The unique nature of this process begs for confirmation and extension from additional studies. In the present study, our goal was to identify additional polyproline peptides from tetrameric horse BChE by examining the smaller peptides that were noted, in passing, in the earlier study by Li *et al.* We found a range of new polyproline peptides.

Highly purified horse BChE was boiled to denature the protein and thus release non-covalently bound peptides. Peptides were separated from the bulk proteins by centrifugation through a YM10 cut-off filter or by HPLC. Released peptides were analyzed by MALDI-TOF-TOF and linear ion trap quadrupole (LTQ) Orbitrap mass spectrometry. We found 13 types of polyproline peptides ranging in mass from 1173 to 2098 Da, of which nine could be matched to specific proteins. Only one of those peptides matched the human lamellipodin tetramer-organizing peptide reported by Li *et al.* [23].

Results

MS analysis

Peptides released by boiling were separated from BChE protein with a 10 000 Da cut-off centrifugal filter. The pass-through solution was collected and analyzed by MALDI-TOF mass spectrometry. Control BChE samples were treated identically except that they were not boiled. As shown in Fig. 1A,B, the pass-through solution from unboiled, native horse BChE sample (upper panels) contained virtually no ions, whereas the pass-through solution from boiled human BChE (lower panels) gave a series of signals which turned out to be proline-rich peptides. These ranged in mass from about 1200 to 2700 Da. Masses marked in the figure correspond to peptides for which complete amino acid sequences could be extracted. Details for these peptides are in Table 1. Most of the remaining signals yielded partial sequence information indicating that they also were proline-rich peptides.

We observed that freezing BChE in the absence of glycerol also caused release of proline-rich peptides. Separation of peptides from bulk protein was essential. If this step was omitted the protein suppressed ionization of peptides and no peptides were detected by MALDI-TOF mass spectrometry.

Peptide masses similar to those in Fig. 1 were found when HPLC was used to separate boiled BChE from peptides. HPLC purification reduced the complexity of the samples that were applied to the MALDI target thereby reducing signal suppression and improving the detection of peptides. Another advantage of HPLC purification was that it increased the concentration of purified peptides in the 0.5 μL aliquot analyzed by MALDI-TOF mass spectrometry. Better quality fragmentation spectra were obtained from the HPLC purified peptides.

The peptides observed with the MALDI mass spectrometer were typically in complex with a single sodium atom (sodium replacing the added proton that makes the peptide positively charged). In one instance, a peptide was detected that was in complex with two sodium atoms. We also observed ammonium and potassium adducts (Table 1).

Peptides released from horse BChE were also analyzed on the LTQ Orbitrap mass spectrometer where peptides were separated by liquid chromatography before their masses were determined (Fig. 2). Figure 2 shows the complete MS spectrum of a sample prepared by the filtration method. The annotated peaks indicate peptides for which complete sequence information could be obtained. Table 1 lists the sequences of peptides determined from collision-induced dissociation in the Orbitrap mass spectrometer. Masses observed in the Orbitrap mass spectrometer were all protonated; no metallized peptides were observed. Peaks that are not annotated in Fig. 2 yielded no

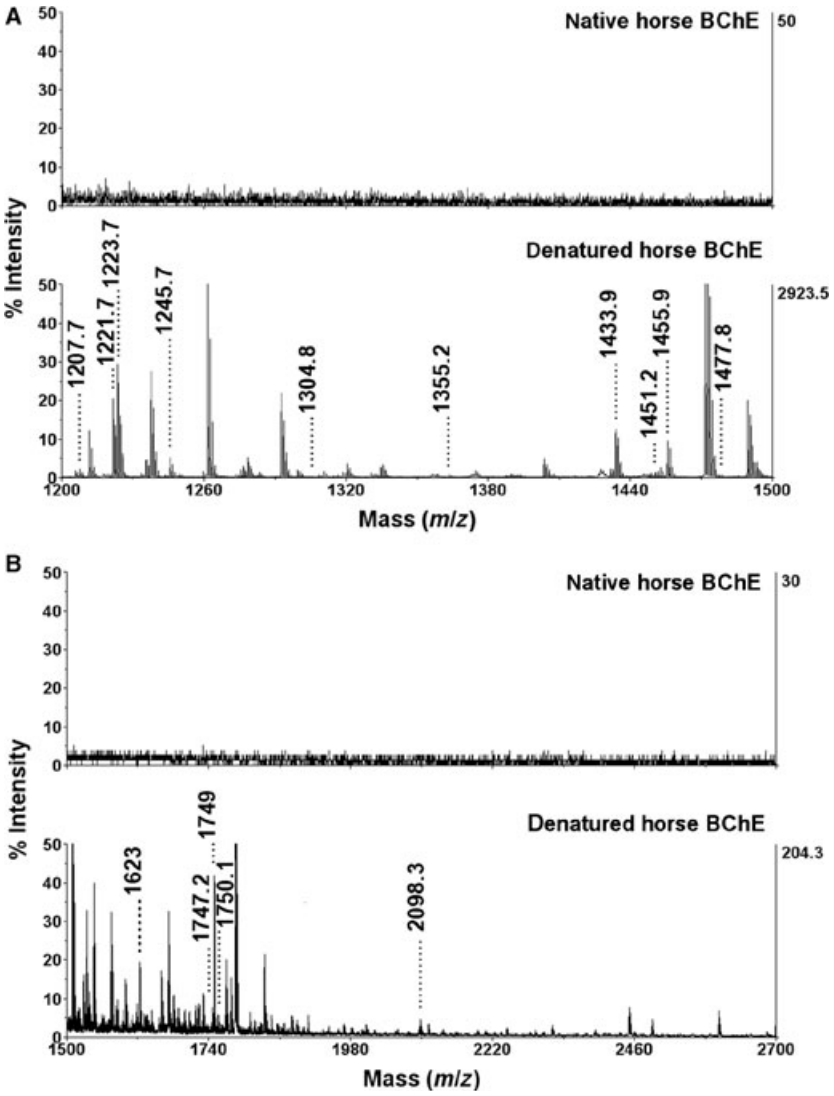

Fig. 1. MALDI-TOF mass spectra of peptides released from horse BChE by boiling followed by filtration to separate peptides from bulk protein. The mass range has been divided into two sections (A, B) to better resolve the signals and to emphasize low intensity signals: (A) masses from 1200 to 1500 Da; (B) masses from 1500 to 2700 Da. The top panel in each section shows that no peptides were released when BChE was not boiled. The bottom panel in each section shows peaks for the released peptides. The right-hand *y*-axes indicate the absolute signal intensity in counts per second. Labeled masses correspond to peptides for which complete amino acid sequences were extracted.

useful mass spectral fragmentation (MSMS) data. Peptides with charge states up to + 7 could be observed, but the MSMS spectra associated with charge states > +3 were typically not amenable to MSMS analysis. Occasionally, sequences of proline residues could be extracted from the peptides with higher charge states but complete sequences were not forthcoming.

Twenty-seven peptides representing 13 different families were extracted from the data in Figs 1 and 2. Assignment of a peptide to a family was based on the heteroatoms identified. Each family contains a unique set of heteroatoms, although in some instances one family might be considered to be a sub-set of another. For example, family 2 could be a sub-set of family 3, family 5 a sub-set of 6, family 10 a sub-set of 11, and family 11 a sub-set of 12 (but family 10 cannot be a sub-set of 12). These families of peptides are shown in Table 1. Family 1 is composed solely of proline residues (from 12 to 21 residues long). Family 2 has valine at the N-terminus of 11 or 12 prolines. Family 3 has leucine/isoleucine/hydroxyproline plus histidine and valine at the N-terminus of 14 prolines. Family 4 has serine at the N-terminus of 11 or 15 prolines. Family 5 has histidine at the N-terminus of 11 prolines. Family 6 has proline followed by leucine/isoleucine/hydroxyproline plus histidine or leucine/isoleucine/hydroxyproline plus histidine at the N-terminus. Family 7 has leucine/isoleucine/hydroxyproline at the N-terminus of 11–20 prolines. Family 8 has proline followed by three glycines at the N-terminus of 12 prolines. Family 9 has glutamate at the N-terminus of 15 prolines. Family 10 has alanine at the N-terminus of 12–17 prolines. Family 11 has proline and alanine

Table 1. Summary of the peptides released from horse BChE. Peptide sequences are written with the N-terminus on the left and the C-terminus on the right. Residues enclosed in parentheses indicate that separate signals for the masses of each residue were not resolved in the mass spectrum. However, masses of the fragments in parentheses exactly fit the indicated sequence. Leucine, isoleucine and hydroxyproline (L/I/Hyp) cannot be distinguished because they have the same nominal mass. The 'Series type' column lists the major fragment series observed in the MSMS spectrum of each peptide. 'Mass' is the monoisotopic mass for the singly charged peptide, i.e. $[M+H]^{+1}$ or $[M+metal]^{+1}$. Observed masses from the Orbitrap were not singly charged. Masses for such peptides have been transformed from the observed mass to their singly charged equivalent for presentation in this column.

Sequence	No. of residues	Series type	Instrument	Mass (Da)
Family 1				
(PP) PPP PPPPP (PP)	12	y- and b-ion series	Orbitrap	1183.6
PPPPP PPPPP PP potassium^{+}	12	y-ion series	MALDI	1221.1
(PPP) PP PPPPP PPPPP (PP)	17	y- and b-ion series	Orbitrap	1668.9
(PPPPP PPPPP) PPPPP PPPPP (P)	21	y-ion series	Orbitrap	2057.1
Family 2				
VPPPP PPPPP PP Na^{+}	12	y- and a-ion series	MALDI	1207.1
VPPPP PPPPP PPP Na^{+}	13	y- and a-ion series	MALDI	1304.8
Family 3				
L/I/Hyp (HV) PP PPPPP (PPPPP PP) Na^{+}	17	y-ion series	MALDI	1749.0
Family 4				
(SP) PPP PPPPP (PP)	12	y- and b-ion series	Orbitrap	1173.6
(SPP) PP PPPPP PPPP (P P)	16	y- and b-ion series	Orbitrap	1561.8
Family 5				
HPPPP PPPPP PP	12	y-ion series	MALDI	1223.2
HPPPP PPPPP PP Na^{+}	12	y-ion series	MALDI	1245.7
Family 6				
L/I/HypHPPP PPPPP PPPP	14	y- and b-ion series	MALDI and Orbitrap	1433.2
PL/I/HypHPP PPPPP PP (PP) Na^{+}	14	y-ion series	MALDI	1455.9
PL/I/HypHPP PPP (PP PPPP) $2Na^{+}$	14	y-ion series	MALDI	1477.8
(PL/I/Hyp) HPP PPPPP PPPPP (PP) Na^{+}	17	y- and b-ion series	MALDI	1747.2
Family 7				
(L/I/HypP) PPP (PP) PPP (PP)	12	y- and b-ion series	Orbitrap	1199.6
L/I/HypPPPP PP (PPP PP) Na^{+}	12	y-ion series	MALDI	1221.7
(L/I/HypP) PPP PPPPP PPP	13	y- and b-ion series	Orbitrap	1296.7
(L/I/HypP) PPP PP (PP) P PP (PP)	14	y- and b-ion series	Orbitrap	1393.8
(L/I/HypPPPP P) PPPP PPPP (P P)	16	y-ion series	Orbitrap	1587.9
(L/I/HypPPPP PP) PPP PPPPP (PP)	17	y-ion series	Orbitrap	1684.9
(L/I/HypPPPP PPPP) P PPPPP PPP (PP P)	21	y-ion series	Orbitrap	2073.1
Family 8				
PGGGP PPPPP PPPPP P Na^{+}	16	y- and b-ion series	MALDI	1473.8
Family 9				
(EPPPP P) PPPP PPPP (P P)	16	y- and b-ion series	Orbitrap	1603.9
Family 10				
(APP) PP PPPPP P (PP)	13	y-ion series	Orbitrap	1254.7
(APPP) P PPPPP PPPPP PP	17	y- and b-ion series	Orbitrap	1642.9
(APPPP P) PPPP PPPPP P (PP)	18	y-ion series	Orbitrap	1739.9
Family 11				
PAPPP PPPPP PPP Na^{+}	13	y- and x-ion series	MALDI	1276.7
Family 12				
(AP) PPP PPPPP PPPPP L/I/HypPPPP M NH^{+}_{4}	21	y-ion series	MALDI	2098.3
Family 13				
(TPPPP) PPPPP PPPPP L/I/HypT	17	x-ion series	Orbitrap	1692.9

at the N-terminus of 11 prolines. Family 12 has alanine at the N-terminus of 14 prolines followed by leucine/isoleucine/hydroxyproline, four more prolines and ending with methionine at the C-terminus. Family 13 has threonine at both the N- and C-termini and leucine/isoleucine/hydroxyproline near the C-terminus. Of these 13 families, only the peptides from family 7 are consistent with the lamellipodin sequence that Li *et al.* [23] reported for the tetramer-organizing peptides from human BChE.

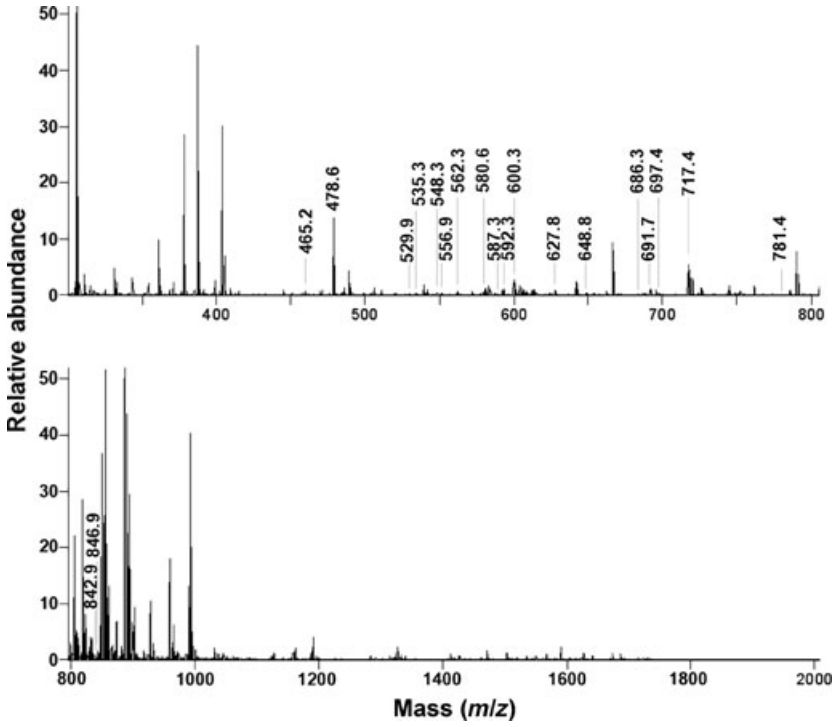

Fig. 2. LTQ Orbitrap mass spectra of peptides released from denatured horse BChE by boiling. The mass range has been divided into two panels to better resolve the signals and to emphasize low intensity signals at high mass. Labeled peaks indicate peptides for which complete sequence information was obtained (numbers are mass-to-charge ratios for doubly and triply charged ions). Intensity values are relative to the most intense peak in the spectrum. Peaks which are not marked gave either partial peptide sequence information or no sequence information.

Examples of MSMS spectra from these peptides are given in Figs 3–5. MALDI MSMS spectra generally yielded a-, b- and y-ion series. The intensity of the y-ion series was generally greater than that of the b-ion series. In addition, there were major fragments at high mass in MALDI MSMS spectra that were due to sodium promoted C-terminal fragmentation. Orbitrap MSMS spectra yielded b-ion and y-ion spectra and one x-ion series. Orbitrap MSMS spectra from triply charged peptides were complicated by prominent signals due to fragments for + 2 and + 3 charge states. In spectra from both instruments, it was common to find a series of fragments associated with internal fragmentation initiated by proline, as well as stretches of minor peaks which corresponded to prolines to which a formal series could not be assigned.

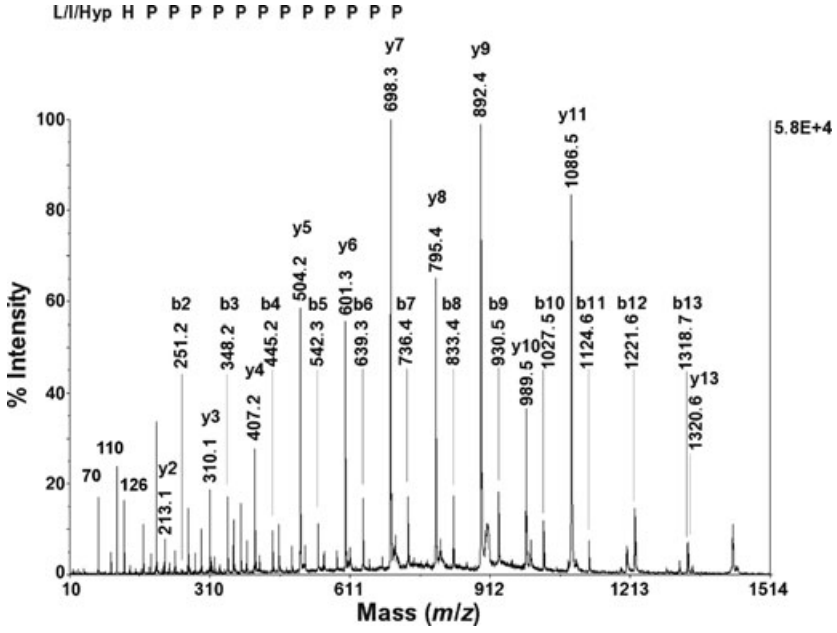

Fig. 3. MALDI MSMS spectrum of the 1433.2 Da peptide L/I/HypHPPP PPPPP PPPP that was isolated from boiled horse BChE by HPLC. Leucine, isoleucine and hydroxyproline cannot be distinguished because they have the same mass. Amino acid sequences are marked to show the y-ion series and b-ion series. The right-hand *y*-axis indicates the signal intensity in counts per second.

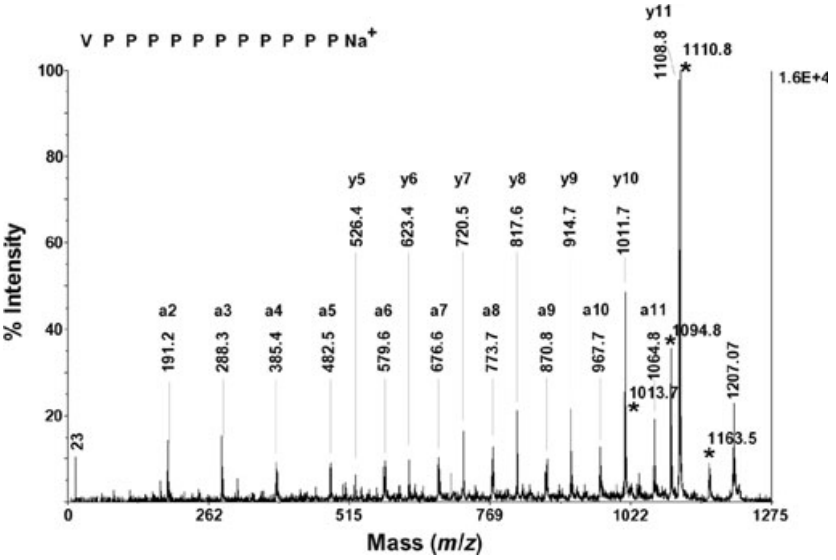

Fig. 4. MALDI MSMS spectrum of the 1207.07 Da peptide VPPPP PPPPP PP Na^+ that was isolated from boiled horse BChE by HPLC. Amino acid sequences are marked to show the y-ion and a-ion series. The sodium ion is associated with the N-terminal fragment in the a-ion series and with the C-terminal fragment in the y-ion series. The peaks marked with an asterisk (*) are a consequence of sodium ion promoted fragmentation. The presence of sodium is indicated by the peak at 23 Da. The right-hand *y*-axis indicates the absolute signal intensity in counts per second.

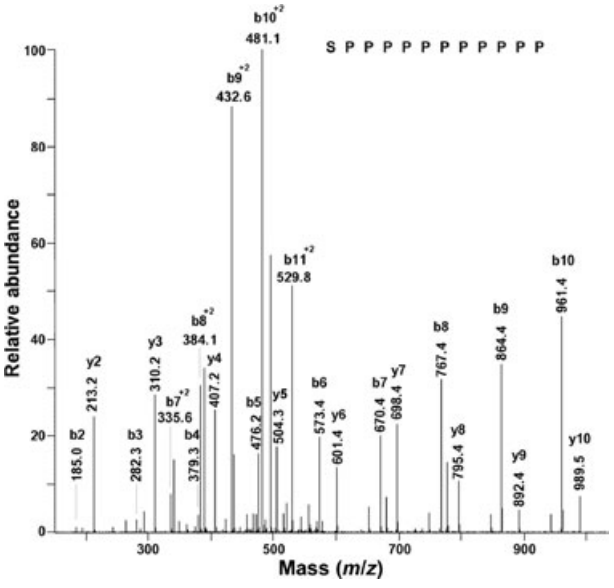

Fig. 5. Orbitrap MSMS spectrum of peptide SPPPP PPPPP PP isolated from boiled horse BChE by HPLC. The parent ion mass was doubly charged *m/z* 587.3 $[M + 2H]^{2+}$. Amino acid sequences are annotated to show a singly charged y-ion series, a singly charged b-ion series and a doubly charged b-ion series (marked with + 2). The *y*-axis is labeled in abundance, relative to the most intense peak in the spectrum.

MSMS fragmentation analysis

Figure 3 shows the MSMS spectrum for the 1433.2 Da parent ion, taken with the MALDI mass spectrometer. The same peptide was detected and sequenced with the Orbitrap mass spectrometer. The parent ion mass is consistent with 12 prolines, a histidine and either leucine, isoleucine or hydroxyproline. The spectrum shows both y-series and b-series ions for a peptide that contains 12 proline residues in a row with a Leu/Ile/Hyp His ion pair at the N-terminus. Starting at y11 there is an intense y-ion series wherein signals from alternating fragments, i.e. y11, y9 and y7, are more intense than signals from intervening fragments, i.e. y10, y8 and y6. This pattern is reminiscent of the polyproline fragmentation patterns described by Unnithan *et al.* [24]. In addition, there is a b-ion series. B-ions were not reported by Unnithan *et al.* [24]. They appear here in all likelihood because of the histidine residue at the b2 position. At low mass there are prominent signals for the immonium ion of histidine (110 Da), the immonium ion of proline (70 Da) and a characteristic proline fragment (126 Da) (listed on the Protein Prospector website; http://prospector.ucsf.edu). Additional prominent signals at low mass are due to internal polyproline fragments (not annotated). All major signals were annotated.

Figure 4 shows MSMS data for the 1207.1 Da peptide, taken with the MALDI mass spectrometer. The parent ion mass is consistent with the presence of 11 prolines, a valine and a sodium atom. The presence of the sodium atom was confirmed by the mass at 23 Da. The spectrum revealed both a y-ion series and an a-ion series for a peptide containing 11 proline residues in a row with a valine at the N-terminus. The sequence interval between 1108.8 Da and the parent ion at 1207.8 Da in the y-series is consistent with a valine residue at the N-terminus. The intensities of the

y-series do not follow the alternating pattern reported by Unnithan *et al.* [24]. The sodium atom remained associated with the N-terminal fragment in the a-ion series and with the C-terminal fragment in the y-ion series. Five residues in the low mass portion of the y-series gave no signals. However, the y5 mass (526.4 Da) is equal to the mass of a C-terminal proline (116 Da) plus the mass of four dehydro-prolines (4 × 97.05 Da) plus the mass of sodium (23 Da) and minus the mass of the proton displaced by the sodium (1 Da); results are consistent with the sequence PPPPP Na^+ for the y5 ion.

The a-ion series in Fig. 4 shows masses in which each a-ion is heavier by 22 Da than the masses in the absence of sodium ion. The combined information from the y-ion and a-ion series gives complete confidence that the peptide contains 12 residues with the sequence VPPPP PPPPP PP_{12}.

The most prominent peaks (marked with an asterisk) in Fig. 4 are at high mass and are due to sodium ion promoted fragmentation. They include the most intense signal (at 1110.8 Da) that is consistent with the loss of a C-terminal proline; a mass that is 44 Da smaller than the parent ion (at 1163.5) which is consistent with the loss of CO_2; a mass at 1094.8 Da that is 113 Da smaller than the parent ion; and a mass at 1013.7 Da that is consistent with loss of a second proline residue from the 1110.8 Da fragment.

Sodium ion promoted fragmentation is a unique form of mass spectral fragmentation that is promoted by complexes of peptides with metal ions such as sodium, lithium and potassium [25–29]. The principal fragmentation 'involves the transfer of the hydroxyl group from the C-terminus of the peptide to the adjacent amino acid and subsequent loss of the residue mass of the C-terminal amino acid [97 Da for proline] leading ultimately to the production of a new alkali cationized peptide lacking the original C-terminal residue' [29]. Additional fragmentation can occur for the newly formed alkali cationized peptide resulting in ions that are two and even three amino acids shorter than the original peptide. The signal intensity of the ion from the second fragmentation can be comparable with that from the first, but the signal intensity for the ion from the third fragmentation is generally much smaller [30]. Additional sodium promoted fragmentation can result in a prominent loss of 44 Da from the parent ion (probably due to loss of CO_2) [27]. Finally, for polyproline peptides, loss of 113 Da from the parent ion (equal to 97 Da plus 16) is typically seen (L.M. Schopfer, unpublished observations). All of these fragments were observed in the fragmentation spectrum for the 1207.7 Da peptide (Fig. 4).

Figure 5 shows MSMS data for a doubly charged peptide ($[M + 2H]^{2+}$ = 587.32 Da) taken with an LTQ Orbitrap mass spectrometer. The parent ion mass is consistent with the presence of a serine and 11 prolines. Singly charged peaks for both a y-ion series and a b-ion series are annotated. Doubly charged b-ion masses dominate the spectrum and singly charged b-ions yield more intense signals than singly charged y-ions. The N-terminal b-ion pair is not resolved, but the mass interval is consistent with the presence of a serine and a proline. This pattern of signals is completely different from that reported by Unnithan *et al.* [24] demonstrating the strong influence of heteroatoms (even uncharged heteroatoms) on the fragmentation of strings of prolines.

Polyacrylamide gel electrophoresis

Non-denaturing gel electrophoresis was used to determine the migration of BChE from which the polyproline peptides had been released by boiling. The activity stained portion of Fig. 6 (lanes 1–4) shows that human serum (lane 1) has fewer active forms of BChE than horse serum (lane 2). Human serum has four forms: a monomer, two types of dimer and a heavily staining tetramer. Horse serum has at least nine forms, six of which

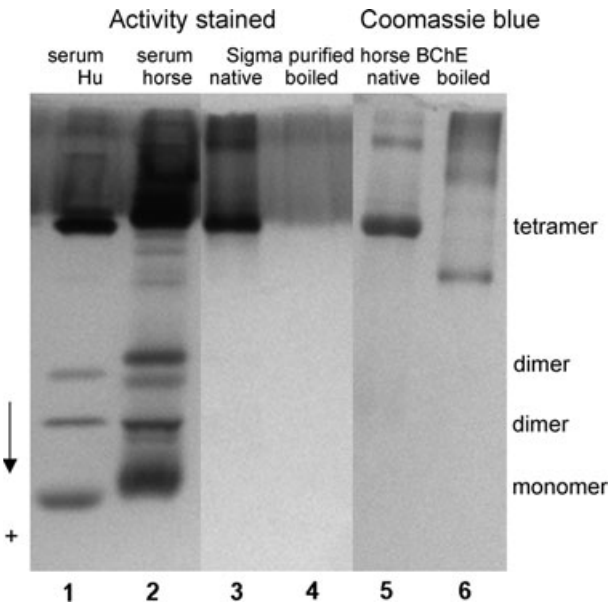

Fig. 6. BChE resolved by non-denaturing PAGE. Lanes 1–4 were stained for BChE activity. Lanes 5 and 6 were stained with Coomassie Blue R-250. Lane 1, human serum 5 μL; lane 2, horse serum 5 μL; lane 3, not boiled purified horse BChE (0.015 units = 0.02 μg); lane 4, boiled purified horse BChE 0.02 μg; lane 5, not boiled purified horse BChE 2 μg; lane 6, boiled purified horse BChE 2 μg. The arrow indicates the direction of migration towards the positive electrode.

correspond to the four bands in human serum: a monomer, a doublet at each dimer location and a tetramer. Several additional forms appear between the positions of the tetramer and dimer. Purified horse BChE from Sigma contained only the tetrameric form (lane 3). Boiled horse BChE did not show any activity (lane 4). All forms of non-denatured horse BChE migrate more slowly than the corresponding forms of human BChE. We propose that this is due to the fact that horse BChE has eight N-linked carbohydrates per subunit compared with nine in human BChE [16–18,31]. N-linked carbohydrates on BChE generally carry two sialic acids; therefore horse BChE would have two fewer negative charges per monomer than human BChE. The lower negative charge on horse BChE would explain a slower migration toward the positive electrode.

Coomassie Blue staining of purified horse BChE on a non-denaturing gel showed that the position of tetrameric horse BChE (not boiled, lane 5) was identical to the tetrameric BChE band in the gel stained for BChE activity (lane 3). Unexpectedly, the boiled form of horse BChE traveled at a position between tetramer and dimer, roughly at the position expected for a trimer (lane 6). Shape is an important determinant of migration on non-denaturing gels. We suggest that the boiled horse BChE dissociates into dimers and then rearranges to form aggregates such that normal dimeric migration of BChE is not seen. In support of this aggregation proposal, it can be seen that for the boiled sample in lane 6 new protein bands appear that migrate near the top of the gel, as expected for aggregates of BChE. Released polyproline peptides were not observed in the Coomassie stained gel.

To further examine the quaternary structure of horse BChE we performed SDS/PAGE in the presence and absence of dithiothreitol. Figure 7 shows that in the absence of dithiothreitol denatured horse BChE migrates primarily as a dimer (lane 1). Boiling did not change this pattern (lane 2) indicating that boiling did not cause fragmentation of the subunits. Addition of dithiothreitol followed by boiling caused the majority of the BChE to be converted into monomers (lane 3). Thus, like human BChE, tetrameric horse BChE consists of a dimer of cysteine cross-linked dimers. It is known that the disulfide bond that crosslinks two subunits is located at Cys571 in human BChE [19]. We propose that the interchain disulfide bond in horse BChE is similarly located at Cys571.

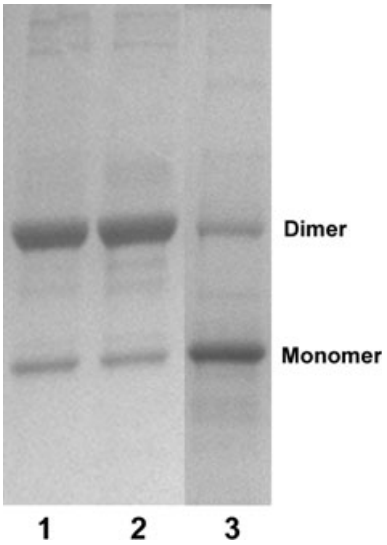

Fig. 7. The SDS/PAGE analysis of horse BChE. The gradient gel was stained with Coomassie Blue. Lane 1, horse BChE 2 μg; lane 2, boiled horse BChE 2 μg; lane 3, boiled horse BChE 2 μg with dithiothreitol.

BLAST search: the origin of proline-rich peptides

A total of 27 proline-rich peptides were released from horse BChE. These could be divided into 13 different families (Table 1). The longest representative from each family was submitted for a standard protein BLASTP search of the National Center for Biotechnology Information (NCBI) non-redundant protein sequence database using the Equus taxonomy (9789) or the mammalian taxonomy (40674). When the search was launched, it automatically switched to 'short input sequence' status. Parameters for that status were Expect Threshold = 200 000; Matrix = PAM30; Word Size = 2; GAP Costs = Existence 9, Extension 1; Compositional Adjustments = no adjustment; Filter = off; Masking = off; and 500 results requested. A perfect match was found for nine families. BLAST results are summarized in Table 2.

One peptide LPPPP PPPPP PPPPP PPPPP P_{21} in family 7 was consistent with the tetramer-organizing peptide from lamellipodin that was reported by Li *et al.* [23] for human BChE PSPPL PPPPP PPPPP PPPPP PPPPP LP_{27}. A second peptide VPPPP PPPPP PPP_{13} in family 2 was consistent with a lamellipodin sequence from *Rattus norvegicus*.

Four peptides were composed of only proline residues (family 1). A BLAST search of the *Equus caballus* taxonomy (9789) using the 21-residue proline peptide from this family found two matching proteins – 'Predicted: low quality protein: formin-like protein 2-like' and UDP-*N*-acetylglucosamine transferase subunit ALG13 homolog. Five other families were also matched with proteins from the equine proteome. When the L/I/Hyp PPPPP PPPPP PPPPP $PPPPP_{21}$ sequence in family 7 was searched using isoleucine or

Table 2. BLAST search results on the peptides released from horse BChE. Data were BLAST searched using the Equus taxonomy (9789) or the mammalian taxonomy (40674) in the NCBInr database. In the species column, if a matching sequence was not found in the equine proteome a sequence from another taxonomy was used. The GI number is the protein accession number in the NCBI database in PubMed. It should be emphasized that only peptides from family 7 are consistent with the sequence of the lamellipodin peptide reported by Li *et al.* [23] for the tetramer-organizing peptide from human BChE.

Family	Sequence	BLAST result	Species	GI number
1	$PPPPPPPPPPPPPPPPPPPP_{21}$	Predicted: low quality protein: formin-like protein 2-like and UDP-*N*-acetylglucosamine transferase subunit ALG13 homolog	*Equus caballus*	194222229 338729459
2	$VPPPPPPPPPPPP_{13}$	ras-associated and pleckstrin homology domain-containing protein 1 (i.e. lamellipodin)	*Rattus norvegicus*	341823648
3	$LHVPPPPPPPPPPPPPP_{17}$	No perfect match		
4	$SPPPPPPPPPPPPPPP_{16}$	Predicted: RIMS-binding protein 3A	*Equus caballus*	194214017
5	$HPPPPPPPPPPP_{12}$	R3H domain-containing protein 1	*Homo sapiens*	31543535
6	$PLHPPPPPPPPPPPPPP_{17}$	No perfect match		
7	$LPPPPPPPPPPPPPPPPPPPP_{21}$	Predicted: UDP-*N*-acetylglucosamine subunit ALG13 homolog and lamellipodin	*Equus caballus* *Homo sapiens*	338729459 82581557
8	$PGGGPPPPPPPPPPPP_{16}$	Predicted: low quality protein: hypothetical protein LOC100057058	*Equus caballus*	338729352
9	$EPPPPPPPPPPPPPPP_{16}$	Predicted: low quality protein: formin-binding protein 4-like	*Equus caballus*	194217903
10	$APPPPPPPPPPPPPPPPP_{18}$	Leiomodin-2	*Bos taurus*	157427900
11	$PAPPPPPPPPPPP_{13}$	Predicted: sal-like protein 2 and leimodin-2	*Equus caballus*	149692720 338724213
12	$APPPPPPPPPPPPPPPLPPPPM_{21}$	No perfect match		
13	$TPPPPPPPPPPPPPPLT_{17}$	No perfect match		

hydroxyproline, no matches were obtained. However, when it was searched using leucine, it matched to 'Predicted: UDP-*N*-acetylglucosamine subunit ALG13 homolog' in *Equus caballus* and lamellipodin in *Homo sapiens*. Consequently, we have assigned leucine as the N-terminal residue for this peptide.

Of the remaining families, peptides from three were matched with proteins from other taxonomies: one from *Rattus norvegicus*, one from *Homo sapiens* and one from *Bos taurus*. There was no perfect match from mammalian taxonomy for peptides in four families.

Discussion

Function of polyproline peptides is to organize subunits into tetramers

Native BChE in horse, mouse and human plasma is 98% tetrameric. Previous studies have shown that full-length BChE expressed in culture medium is predominantly monomeric and dimeric but that addition of poly(L-proline) to the culture medium or co-expression with the proline-rich N-terminus of ColQ increased the amount of tetrameric BChE to 70% [22,23]. Similarly, live mice transfected with adenovirus expressing mouse BChE had predominantly dimeric BChE in plasma. Addition of 100 μM poly(L-proline) to mouse plasma converted nearly 100% of the BChE into tetramers [32].

Conversely, native tetrameric BChE from horse and human plasma [23], as well as native tetrameric AChE from fetal bovine serum (K Biberoglu, LM Schopfer, A Saxena, O Tacal & O Lockridge, unpublished results), have been shown to release polyproline peptides following protein denaturation. This means that the polyproline peptides are part of the structure of soluble tetrameric BChE and AChE. We conclude that the function of polyproline peptides is to serve as tetramer-organizing peptides.

One polyproline peptide per tetramer

There has been no X-ray crystal structure showing how full-length BChE subunits associate with the proline-rich peptides. In the following discussion we will use amino acid composition analysis to argue that there is one polyproline peptide per BChE tetramer.

The amino acid sequences of human and horse plasma BChE have been determined. Both contain 574 amino acids per subunit [16–18]. There are 31 proline residues per subunit in the sequence of the horse BChE. However, amino acid composition analysis of tetrameric horse BChE [33] showed that there are 36.6 proline residues per subunit (Table 3). The number of observed prolines per subunit is 5.6 greater than expected. This means that there are 22 extra proline residues per tetramer. The longest proline-rich peptide that we observed for horse BChE contained 21 proline residues. The similarity in these two values strongly argues that there is one proline-rich peptide per tetramer for horse BChE.

The same argument can be drawn for human BChE, where the number of proline residues determined by amino acid composition analysis (36) is six greater than the number of proline residues in the sequence (30) [18]. The extra proline residues can be accounted for by the presence of one polyproline peptide (containing ~ 24 prolines) per tetramer. Polyproline peptides of this size were isolated from human BChE [23]. We conclude that soluble tetrameric BChE includes one polyproline peptide per tetramer.

Many of the peptides that we identified are shorter than the 22 residues projected by the amino acid composition analysis (some as small as 12 residues, see Table 1). On this basis, one might suspect that more than one polyproline peptide could be found in the tetramerization domain. However, Dvir *et al.* [15] have shown that the 15-residue proline-rich attachment domain peptide is more than sufficient to occupy the entire tetramerization domain of cholinesterase (see Fig. 8). Thus it is unlikely that two 12-residue peptides would be bound.

Table 3. Number of polyproline peptides per BChE tetramer.

Number of residues	Human BChE P06276	Horse BChE Q9N1N9
Number of residues per subunit	574[a]	574[b]
Number of prolines from subunit sequence	30[a]	31[b]
Number of prolines from amino acid composition of tetramer	36[a]	36.6[c]
Number of extra prolines per subunit	6	5.6
Number of extra prolines per tetramer	24	22
Calculated polyproline length	24	22
Number of polyproline peptides per BChE tetramer	1	1

[a] Taken from Lockridge *et al.* (1987) [19]. [b] Taken from Wierdl *et al.* (2000) [17]; Moorad *et al.* (1999) [16]. [c] Taken from Teng *et al.* (1976) [33].

Polyproline peptide interacts with the tetramerization domain

Assembly of BChE subunits into tetramers requires the presence of the 40 amino acids at the C-terminus [21]. Deletion of these residues yields monomeric BChE with full catalytic activity but reduced stability. The 40 C-terminal residues of BChE constitute the tetramerization domain. The tetramerization domain adopts an amphiphilic α-helical structure with seven conserved aromatic amino acids on one side of the helix. A similar tetramerization domain containing seven conserved aromatic amino acids is present in human AChE. Dvir *et al.* [15] solved the crystal structure of a synthesized peptide representing the tetramerization domain of human AChE in complex with a synthesized proline-rich peptide. The complex contained four parallel α-helical tetramerization peptides wrapped around a single antiparallel 15-residue proline-rich peptide LLTPP PPPLF PPPFF. The conformation of the proline-rich peptide was that of a left-handed polyproline II helix. The global structure was described as a

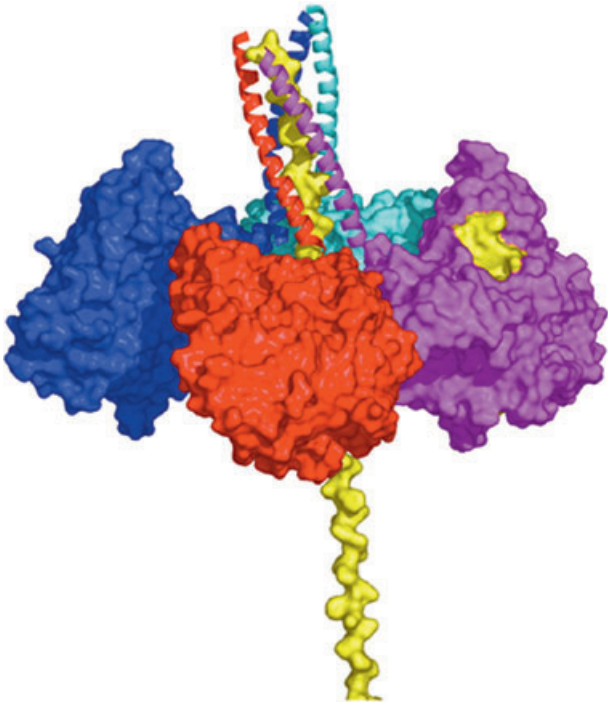

Fig. 8. Model of the AChE tetramer in the presence of the full-length ColQ protein. The figure is reproduced from [15]. AChE monomers are displayed as globular units with their tryptophan-rich amphiphilic α-helices protruding out of the top of each unit. The ColQ protein, or alternatively the tetramer-organizing polyproline peptide (yellow), projects through the middle of the AChE tetramer complex, occupying the core of the tetramerization domain on top and extending as a single chain on the bottom.

left-handed screw within a left-handed threaded hollow tube formed by the four tetramerization peptides [15].

Dvir *et al*. [15] produced a model of the AChE tetramer linked to the full-length ColQ protein through the proline-rich region at the N-terminus of ColQ. Figure 8 shows that the ColQ protein traverses the entire AChE tetramer, interacting with the tetramerization domain at its C-terminal end, passing through the center of the AChE tetramer, and extending its N-terminal end out the other side. This configuration will accommodate a variety of sizes of polyproline peptides. The structures of AChE and BChE are very similar so it is reasonable to propose that the polyproline peptides obtained from horse BChE (including the 21-residue polyproline peptide) will form a similar structure. Thus, we propose that the relative size and location of the organizing peptides in fully formed BChE tetramers are represented by the structure in Fig. 8.

Multiple origins of polyproline peptides

According to BLAST search results the tetramer-organizing, proline-rich peptides from horse BChE derive from at least nine different proteins. The well established tetramer-organizing proteins ColQ and PRiMA are not in that list of proteins (Table 2) and the proline-rich attachment domains from ColQ, LLTPP PPPLF $PPPFF_{15}$, and PRiMA, PPPPL PPPPP $PPPPP_{15}$, do not appear in the list of observed peptides (Table 1). These observations indicate that soluble BChE in horse serum is not generated by cleavage of BChE from its well characterized membrane anchored sites. Furthermore, it suggests that serum BChE may be associated with a variety of proteins at some time in its synthesis.

Because BChE appears to have associated with a variety of polyproline-containing proteins it is of interest to explore the nature of mammalian proteins that contain extended sequences of prolines. We therefore performed a BLASTP search of the NCBI non-redundant protein database for proteins containing at least 15 sequential prolines. A minimum of 15 sequential prolines was chosen because 10 of the tetramer-promoting polyproline families in our study contained sequences of 16–21 prolines. We excluded matches for entries that were translated from DNA without identifying the associated protein [i.e. entries from databases such as the Center for Genome Dynamics (CG), Kazusa cDNA sequencing project (KIAA) and the Japanese cDNA library (RIKEN) among others]. We included isoforms of a given protein when they appeared. Eighty-one matches were found (eight contained 25 contiguous prolines or more, 25 contained 20–24 prolines, while 48 contained 15–19). Twenty-eight of the matches were to taxonomy *Homo sapiens*; two were to *Equus caballus*. A quarter of the matches were to proteins involved in transcription. Another quarter were to proteins that bind to actin and/or were involved in cytokinesis, cell polarity or neurite formation. Six bound to RNA or DNA. Three were involved in cell–cell adhesion and two were associated with ubiquitin. There was no obvious tendency for these proteins to be membrane bound. For most proteins the polyproline sequence was located 50 or more residues from the nearest end of the protein.

To date soluble BChE and AChE tetramers are the only proteins reported in the literature that embed short polyproline peptides in their structure. The polyproline peptides fit no single precursor protein and therefore arise from multiple genes.

More heterogeneity in short polyproline sequences

Li *et al*. [23] reported on a group of proline-rich, tetramer-organizing peptides from human and horse BChE that matched a 39-residue sequence in lamellipodin PSPPL PPPPP PPPPP PPPPP PPPPP LPSQS APSAG $SAAP_{39}$. The peptides extracted from human BChE in that study ranged in mass from 2074 to 2878 Da, while those extracted from horse BChE ranged in mass from 2171 to 2878 Da. Additional peptides, at lower mass and of lower intensity, were visible in the mass spectra of both horse and human BChE extracts but they were not examined. In the current study, the peptides from horse BChE that we examined were in the mass range 1173–2098 Da. Larger peptides had much weaker intensities and did not yield productive sequence information. Only two of the peptides that we detected could be matched to lamellipodin. The obvious question is what differences exist between the two experiments that might explain the differences in observation.

The biggest technical difference between the experiments is in the lasers on the mass spectrometers that were used. This is an important consideration because polyproline is quite sensitive to the laser intensity during desorption/ionization in the MALDI mass spectrometer. That is, increasing laser intensity causes proline–proline fragmentation in the source (LM Schopfer, unpublished observation). The laser used by Li *et al*. (AB Sciex Voyager DE Pro from Applied Biosystems, Framingham, MA, USA) is arguably less intense than the one used in the current experiments (MALDI-TOF-TOF 4800 from Applied Biosystems). This conclusion is based on the following considerations. (a) The DE Pro uses a 337 nm nitrogen laser, while the 4800 uses a 355 nm Nd:YAG laser. The absorbance maximum for a thin film of dried

α-cyano-4-hydroxycinnamic acid (α-CHCA) matrix (which was used in both experiments) is at 371 nm [34]. Therefore the α-CHCA/sample mixture will absorb more light per pulse in the 4800 source than in the DE Pro source because the laser excitation wavelength is closer to the α-CHCA absorbance maximum in the 4800. (b) The laser pulse rate in the 4800 is 10-fold higher (200 Hz) than that in the DE Pro (20 Hz). Therefore, light will be delivered to the sample faster in the 4800 than in the DE Pro increasing the sample temperature to a greater extent. Higher temperatures should promote greater fragmentation. (c) The laser spot size is smaller in the 4800 than in the DE Pro. Therefore the laser energy will be concentrated on a smaller area of the sample in the 4800 than in the DE Pro, thereby increasing the laser intensity experienced by the sample. The predicted consequences of these factors would be increased fragmentation of the polyproline peptides in the source of the 4800, leading to a decrease in the size of the ions entering the mass spectrometer. This in turn would provide us with smaller peptides to study. Since the smaller peptides were not investigated by Li *et al.*, they will not have seen the same results that we have. The ultimate conclusion is that our results differ from those of Li *et al.* because we studied a different set of peptides.

It is noteworthy that the electrospray ionization source used on the Orbitrap mass spectrometer introduces the sample into the mass spectrometer more gently. The list of ions that we observed in the Orbitrap included four of the eight ions reported for horse BChE by Li *et al.* (2171, 2566, 2663 and 2878 Da). However, fragmentation of the latter three was unproductive. Fragmentation of the 2171 Da ion showed a string of 11 prolines but the complete sequence could not be determined.

Conclusion

Soluble, tetrameric BChE in plasma is an assembly of four identical 574-residue BChE subunits in complex with a heterogeneous group of non-covalently bound, short polyproline peptides.

Materials and methods

Materials

Highly purified BChE (EC 3.1.1.8) from equine serum (catalog number C1057), 5,5′-dithiobis-2-nitrobenzoic acid (catalog number D8130), SDS (electrophoresis grade, catalog number L3771), glycine (electrophoresis grade, G8898) and formic acid (puriss p.a. for mass spectrometry, catalog number 94318) were from Sigma (a member of the Sigma-Aldrich group, St Louis, MO, USA). α-CHCA (catalog number 70990) and butyrylthiocholine iodide (catalog number 20820) were from Fluka (a member of the Sigma-Aldrich group). Acetonitrile (DNA sequencing grade, catalog number BP-1170), dithiothreitol (electrophoresis grade, catalog number BP172), bromophenol blue (electrophoresis grade, catalog number BP114) and Coomassie Brilliant Blue R-250 (electrophoresis grade, catalog number BP101) were from Fisher Scientific (a member of the Thermo Fisher Scientific group, Waltham, MA, USA). Trifluoroacetic acid was of sequencing grade (> 99.9%) from Beckman (Brea, CA, USA; catalog number 290204). All other chemicals were of biochemical grade.

α-CHCA was prepared as a saturated solution (10 $mg \cdot mL^{-1}$) in 50% acetonitrile/water plus 0.3% trifluoroacetic acid (v/v).

BChE activity

BChE activity was assayed by recording the increase in absorbance at 412 nm of a 2 mL solution containing 1 mM butyrylthiocholine in 0.1 M potassium phosphate buffer pH 7.0 at 25 °C, in the presence of 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in a Gilford spectrophotometer interfaced to MacLab 200 (ADInstruments Pty Ltd, Castle Hill, Australia). The molar extinction coefficient for the product is 13 600 $M^{-1} \cdot cm^{-1}$ [35]. Units of activity are defined as micromoles of butyrylthiocholine hydrolyzed per minute.

The lyophilized horse BChE from Sigma contains ~ 30% protein and 70% buffer salts. A bottle of Sigma horse BChE (lot 084K7351) that contains 3325 mg solid and 1 000 000 Sigma units contains 326 mg BChE protein and 234 700 units as measured under our conditions. We estimated the Sigma horse BChE to be 81% pure based on our parameters for 100% pure BChE of 720 $units \cdot mg^{-1}$ where we measured units of activity with 1 mM butyrylthiocholine in 0.1 M potassium phosphate buffer pH 7 at 25 ºC, and we measured protein concentration by absorbance at 280 nm where a 1 $mg \cdot mL^{-1}$ solution has an absorbance of 1.8.

Filtration to separate tetramer-organizing peptides from horse BChE

Horse BChE was desalted by dialysis against 2 × 4 L of water. Twenty milliliters of desalted horse BChE (0.3 $mg \cdot mL^{-1}$ in water) was concentrated to 1.5 mL in an Amicon stirred cell using a YM 30 membrane 30 kDa cut-off (Millipore, Billerica, MA, USA). The 1.5 mL of the 4 $mg \cdot mL^{-1}$ sample was divided into two fractions of equal volume. One fraction was boiled for 5 min to denature the protein. Both fractions were filtered through Micron Ultracel YM 10 spin filters (Millipore) with a 10 000 Da cut-off to separate free peptides from residual protein. The solution that passed through the filter membrane was

recovered, concentrated to 0.3 mL in a vacuum centrifuge (Speedvac model sc100 from Thermo Fisher Scientific, Waltham, MA, USA) and saved for mass spectrometry. In the end, the samples (boiled and not boiled) contained peptides equivalent to 10 mg BChE·mL^{-1} (assuming no losses during handling) which is equivalent to 118 μM BChE monomer (M_r = 84 551). Assuming one peptide per tetramer this would predict 30 μM peptide.

HPLC purification of tetramer-organizing peptides

Lyophilized Sigma horse BChE, 29.8 mg containing 2.92 mg BChE protein, was dissolved in 2.92 mL water to make a 1 mg·mL^{-1} BChE solution. Non-covalently bound peptides were released by heating the BChE in a boiling water bath for 5 min. After boiling, 2 mL of the solution (equivalent to 2 mg BChE or 24 nmol) was filtered through a nylon 0.2 μm syringe filter to remove particulates and injected onto a Zorbax 300 SB C-18 reverse phase column (Agilent Technologies, Santa Clara, CA, USA) attached to a Waters 625 HPLC system (Milford, MA, USA). The HPLC was operated at room temperature (22 °C) at a flow rate of 0.5 mL·min^{-1}. Buffer A was 0.1% trifluoroacetic acid in water (v/v); buffer B was 0.09% trifluoroacetic acid in acetonitrile (v/v). Peptides were eluted with a gradient of 0–60% buffer B in 60 min. Absorbance was recorded at 210 nm. The HPLC eluent was collected in 1 min fractions (0.5 mL each). Each fraction was concentrated to 10–50 μL in a vacuum centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and saved at 4 °C for mass spectrometry. In the end, the fractions contained peptides equivalent to 2 mg of BChE (assuming no losses) or 2340–468 μM BChE monomer (M_r = 84 551). Assuming one peptide per tetramer this would predict 585–117 μM peptide.

MALDI-TOF-TOF mass spectrometry

Samples used for MALDI-TOF-TOF mass spectrometry were prepared by both the HPLC and the filtration methods. The filtered samples (boiled and not boiled) contained peptides equivalent to 10 mg BChE·mL^{-1} (assuming no losses during handling) or 30 μM peptide. The HPLC fractions contained peptides equivalent to 2 mg of BChE (assuming no losses during handling) in 0.01–0.05 mL or 585–117 μM peptide.

All MALDI-TOF-TOF experiments were performed on an Applied Biosystems MALDI TOF-TOF 4800 mass spectrometer equipped with a 355 nm Nd-YAD laser (Applied Biosystems). The samples (0.5 μL) were spotted on a MALDI target plate (Opti-TOF 384 well insert from Applied Biosystems), air-dried and overlaid with 0.5 μL of α-CHCA. Mass spectra were acquired in positive ion reflector mode, under delayed extraction conditions (500 ns), using an acceleration voltage of 20 kV, with laser intensity of 4000–6000 V, a mass range of 1000–4000 Da, a detector voltage multiplier of 0.75, with low mass gate on and low mass gate offset equal to zero. Mass spectra shown are the average of 500 laser shots collected from randomly selected locations on the target spot (50 pulses per location). Selected ions were fragmented by collision-induced dissociation, using a 1 kV method, with air as the collision gas at 2×10^{-6} Torr, a precursor mass window of ±1 Da, metastable suppression on, a detector voltage multiplier of 0.95, using factory calculated delayed extraction values (DE1 = 370 ns, DE2 = 37 271 ns) and with timed ion selector activated. The identity of the fragments in the MSMS spectra were assigned manually using the DATA EXPLORER software (version 4.9 from Applied Biosystems) with the aid of the PROTEOMICS TOOLKIT (http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html) and the MS Product algorithm in PROTEIN PROSPECTOR v 5.9.4 from the University of California, San Francisco, mass spectrometry facility (prospector.ucsf.edu/prospector/mshome.htm).

LTQ Orbitrap

Samples used for LTQ Orbitrap mass spectrometry were prepared by the filtration method. Filtered samples (boiled and not boiled) contained peptides equivalent to 10 mg BChE·mL^{-1} (assuming no losses during handling) or 30 μM peptide. A 3.5 μL aliquot of the filtered sample was diluted with 6.5 μL of 0.1% formic acid to make a 10 μM peptide solution.

Analysis by liquid chromatography/mass spectrometry including a peptide fragmentation stage was performed on an LTQ Orbitrap quadrupole mass spectrometer (Thermo Scientific, part of Thermo Fisher Scientific, Rockland, IL, USA) using electrospray ionization. Five microliters of sample were loaded onto a C18 reverse phase trap column (CapTrap Peptide from Michrom BioResources, Auburn, CA, USA; catalog # TRI/25109/32) and washed with 2% acetonitrile/water plus 0.1% formic acid (v/v). Peptides were transferred to a C18 reverse phase separation column (Picofrit BioBasics C-18 from New Objective, Woburn, MA, USA; catalog # PF 7515-100H052) and eluted with a 45 min linear gradient starting with 98% solvent A (2% acetonitrile/water plus 0.1% formic acid; v/v) and 2% solvent B (98% acetonitrile/water plus 0.1% formic acid; v/v) and ending with 35% solvent A and 65% solvent B. The flow rate was 250 nL·min^{-1}. The effluent was electrosprayed directly into the mass spectrometer. Data were collected in a data-dependent manner with each cycle of data collection consisting of one high-resolution mass spectrum (over a 300–2000 Da mass range) taken with the orbitrap and five MSMS fragmentation spectra taken with the LTQ ion trap. Collision-induced dissociation was used for fragmentation with helium as the collision gas (at 1×10^{-3} Torr) at a normalized collision energy of 35 (or about 35% of the maximum collision energy of 5 V). The activation time was 30 ms, and the activation Q was 0.25.

Parent ions of any charge state with a minimum signal intensity of 50 000 counts per second were accepted for fragmentation. After two data collections for a given parent ion mass were completed, that mass was excluded from further analysis for 60 s. Fragmentation spectra were analyzed manually using the Qual Browser feature of XCALIBUR software (v 4.9 from Thermo Scientific) with the aid of the PROTEOMICS TOOLKIT (http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html) and the MS Product algorithm in PROTEIN PROSPECTOR v 5.9.4 from the University of California, San Francisco, mass spectrometry facility (prospector.ucsf.edu/prospector/mshome.htm).

Non-denaturing gradient gel electrophoresis

Polyacrylamide gradient gels (11 × 18 cm, 4%–30%) with a 4% stacking gel, 0.75 mm thick, were prepared in a Hoefer SE6000 gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). The running gel was prepared in 375 mM Tris/HCl buffer pH 8.9. The stacking gel was prepared in 125 mM Tris/HCl buffer pH 6.8. The upper tank buffer was 20 mM Tris glycine pH 8.9, while the bottom tank buffer was 60 mM Tris/HCl pH 8.1. Electrophoresis was run at 208 V constant voltage for 24 h (5000 V h) at 4 °C. BChE samples (in 20 mM Tris/HCl, 1 mM EDTA, pH 7.5) were diluted with distilled water and mixed (1 : 1) with 50% (v/v) glycerol containing 0.004% (w/v) bromophenol blue, to give the final concentrations indicated in the following paragraphs. Some samples were boiled for 3 min (refer to Fig. 5 caption).

For total protein staining, the equivalent of 2 μg (1.44 units) of purified horse BChE was loaded per lane. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Protein staining requires 100 times more BChE per lane than activity staining.

For BChE activity staining, the equivalent of 5 μL of human plasma, 5 μL of horse serum or 5 μL of purified horse BChE (3 units·mL^{-1}) were loaded per lane. After electrophoresis, the gel was stained for BChE activity by the method of Karnovsky and Roots [36]. The staining solution contained 180 mL of 0.2 M sodium maleate pH 6.0, 15 mL of 0.1 M sodium citrate, 30 mL of 0.03 M cupric sulfate, 30 mL of 5 mM potassium ferricyanide and 0.18 g butyrylthiocholine iodide in a total volume of 300 mL. The gel was incubated, with gentle shaking, at room temperature for 3–5 h until brown bands of activity appeared. The reaction was stopped by washing the gel with water.

Denaturing gradient gel electrophoresis: SDS

Polyacrylamide gradient gels (11 × 18 cm, 4–30%) with a 4% stacking gel, 0.75 mm thick, were prepared as described for non-denaturing gels. The tank buffers were prepared as described for non-denaturing gels except that the upper tank buffer was supplemented with 0.1% SDS (w/v). Electrophoresis was run at 125 V constant voltage for 24 h (3000 V h) at 4 °C. BChE samples were diluted (1 : 1) with SDS buffer (62.5 mM Tris/HCl, 10 (v/v) glycerol, 2% SDS (w/v), 0.012% bromophenol blue (w/v), pH 7.2). Dithiothreitol (50 mM) was added to some samples; some samples were boiled for 3 min (refer to Fig. 6 caption). The equivalent of 2 μg of horse BChE was loaded per lane. Gels were stained with Coomassie Brilliant Blue R-250.

Acknowledgements

Mass spectra were obtained with the support of the Mass Spectrometry and Proteomics core facility at the University of Nebraska Medical Center. This work was supported by a TÜBITAK grant from the Scientific and Technological Research Council of Turkey to OT, a fellowship from Hacettepe University (to KB) and an NIH grant (P30CA36727, to the Eppley Cancer Center).

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