Tetramerization domain of human butyrylcholinesterase is at the C-terminus

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Butyrylcholinesterase (BChE) in human serum consists predominantly of tetramers. Recombinant BChE, however, expressed in Chinese hamster ovary (CHO) cells, consists of approx. 55 % dimers, 10–30 % tetramers and 15–40 % monomers. To determine the origin of the monomer species we added the FLAG epitope (epitope tag, amino acid sequence DYKDDD-DK) to the C-terminus of the enzyme, and expressed BChE-FLAG in CHO cells. We found that secreted, active monomers had lost their FLAG epitope, suggesting that the monomers were made by proteolysis of dimers or tetramers at the C-terminus. To estimate the number of amino acids that could be deleted from the C-terminus without losing BChE activity, we expressed deletion mutants. We found that deletion of up to 50 amino acids from the C-terminus yielded active monomers, but that deletion of 51 amino acids destroyed BChE activity and caused the

inactive protein to remain within the cell. Deletion of eight or more amino acids from the N-terminus also resulted in inactive protein that remained inside the cell. Monomeric BChE had wild-type K_m and k_{cat} values (8 μ M and 24000 min⁻¹ for butyrylthiocholine) and showed substrate activation. The Cys-571→Ala mutant, though incapable of forming the interchain disulphide bond, had nearly the same amount of tetrameric BChE as recombinant wild-type BChE. These results support the conclusion that the tetramerization domain of BChE is at the C-terminus, within the terminal 50 amino acids, and that the interchain disulphide bond is not essential for tetramerization. Molecular modelling suggested that the tetramerization domain was a four-helix bundle, stabilized by interactions of seven conserved aromatic amino acids.

INTRODUCTION

Butyrylcholinesterase (BChE) (EC 3.1.1.8, acylcholine acylhydrolase, pseudocholinesterase, non-specific cholinesterase, human serum cholinesterase), isolated from human serum, is a globular, tetrameric molecule with a molecular mass of approx. 340 kDa [1]. Nine Asn-linked carbohydrate chains are found on each 574-amino-acid subunit [2].

Although the physiological function of BChE is unknown, the use of BChE has promising implications in the treatment of organophosphate poisoning. BChE purified from human serum and injected into mice shows a positive linear correlation between the blood level of human BChE and the extent of protection against the toxicity of nerve agents [3,4]. Although the wild-type BChE protects against organophosphate toxicity, it requires a one-to-one stoichiometry for this scavenger effect. It would be more efficacious to inject a BChE mutant that possessed organophosphate hydrolase activity, such as the recently reported G117H mutant of human BChE [5,6]. For therapeutic purposes a stable form of recombinant BChE is desirable. The most stable form of BChE is tetrameric. However, we found that the predominant form of recombinant BChE secreted from Chinese hamster ovary (CHO) cells was not the stable tetramer, but a mixture of relatively less stable dimers (50–55 %) and monomers (15–40 %) with few tetramers (10–30 %). Our goal was to characterize the recombinant BChE forms to identify which regions of BChE were responsible for tetramerization and which features lent stability to the tetrameric structure.

We report here that the monomeric form of recombinant wild-

type BChE resulted from proteolysis at the C-terminus. Deletion of the C-terminus of BChE by site-directed mutagenesis showed that removal of up to 50 amino acids resulted in the secretion of active monomers exclusively. The dimers of recombinant wild-type BChE were full-length proteins, but for some as-yet-unknown reason, were not assembled into the tetrameric form.

Removal of the interchain disulphide bond by site-directed mutagenesis of Cys-571 to Ala (C571A) resulted in the secretion of active BChE tetramers, dimers and monomers. We concluded that the interchain disulphide bond at Cys-571 was not essential for dimer or tetramer formation. Rather, dimers and tetramers arose by contact of approx. 40 C-terminal amino acids. The function of the interchain disulphide bond was to stabilize the dimeric structure. The C-terminal amino acids of BChE were predicted to form α -helices that might join to form a four-helix bundle constituting the tetramerization domain.

EXPERIMENTAL

Mutagenesis and expression of human recombinant BChE

The methods for PCR mutagenesis and expression in CHO cells were described previously [6]. Secreted recombinant BChE was collected for 4 days into serum-free and glutamine-free medium (UltraCULTURE[®] 12-725B; Bio Whittaker, Walkersville, MD, U.S.A.) containing 25 μ M methionine sulfoximine (Sigma, St. Louis, MO, U.S.A.), followed by collection into Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) 50:50 (v/v) mix without L-glutamine (Mediatech, 15-090-LM;

Abbreviations used: AChE, acetylcholinesterase; AMC, 7-amido-4-methylcoumarin hydrochloride; BChE, butyrylcholinesterase; BODIPY[®], fluorescent dye for protease assay; CHO, Chinese hamster ovary; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F12 medium; Endo H, endoglycosidase H; FLAG[®], epitope tag, amino acid sequence DYKDDDDK; M2, monoclonal antibody to FLAG epitope; PNGase F, peptide N-glycosidase F.

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Fisher Scientific, Fair Lawn, NJ, U.S.A.) with 25 μ M methionine sulfoximine for 3 days. CHO cells continued to secrete recombinant BChE at a consistently high level for 6 months without being fed serum. An absence of serum was desired because fetal-bovine serum is rich in the related enzyme acetylcholinesterase.

The codon for Cys-571 was replaced with a codon for Ala (GCT). A series of C-terminal deletion mutants was created by independently changing the codons for Gly-534, Leu-530, Lys-528, Pro-527, Phe-526, Phe-525, Ser-524, Thr-523, Trp-522 and Phe-521 to a stop codon (TAA). The FLAG tag (IBI-KODAK) of amino acid sequence DYKDDDDK was added to the C-terminus of recombinant wild-type BChE and to the C571A mutant immediately following Leu-574. A series of mutants replacing proteolytic-enzyme common targets, Arg and Lys, was created by replacing the codons for Lys-544, Arg-549, Lys-558, Lys-568 and Lys-569 with a codon for His (CAC).

An N-terminal deletion mutant, with BChE amino acids 4–62 removed (Δ 59), was made by first creating *EcoRV* and *HpaI* restriction sites in wild-type BChE, on either side of the area to be deleted. Digestion with the two enzymes resulted in two blunt ends which were ligated, thus removing the codons for the 59 amino acids from Ile-4 to Ala-62 inclusive. Two other N-terminal deletion mutants, Δ 8 and $\Delta\beta$ -2 β -1, were created using overlapping PCR reactions that deleted codons for the first eight amino acids and for 12 amino acids (Ile-4 to Gly-15 inclusive) respectively. The latter was named $\Delta\beta$ -2 β -1 because it lacked the entire β -2 strand and five out of eight amino acids of the β -1 strand, as described by Sussman et al. [7]. Another N-terminal deletion mutant, Δ 2N, was created by deleting codons for the first two amino acids of BChE.

Protease assay

The EnzCheck Protease Assay Kit red fluorescence (E-6639 from Molecular Probes, Eugene, OR, U.S.A.) was the primary assay for detection of protease activity in conditioned media. Protease activity was estimated from the fluorescence intensity of BODIPY-TR (BODIPY = fluorescent dye for protease assay), a dye released from covalent attachment to casein by proteolysis. Fluorescence was measured at excitation 590 nm, emission 620 nm, on a Perkin-Elmer 650-40 Fluorescence Spectrophotometer, temperature controlled at 25 °C. A second substrate for protease detection was L-arginine 7-amido-4-methylcoumarin hydrochloride (Arg-AMC) from Sigma (A-2027), which yielded a fluorescent product measured at excitation 370 nm, emission 440 nm.

The protease assays, in a total volume of 1.2 ml, contained 0.1 M Hepes, pH 7.0, 0.6 ml of conditioned medium and either 5 μ g/ml BODIPY-casein or 50 μ M Arg-AMC. Controls for background hydrolysis contained unused culture medium in place of conditioned medium. The following compounds were tested for ability to inhibit the protease activity in conditioned medium: di-isopropyl fluorophosphate, paraoxon, PMSF, anti-pain (Boehringer Mannheim), leupeptin (Boehringer Mannheim), *p*-chloromercuribenzoate, *N*-acetyl-leucyl-leucyl-norleucinal (Calpain inhibitor I, Calbiochem; stock solution in ethanol), iodoacetamide, *N*-ethylmaleimide, EDTA, EGTA and 1,10-phenanthroline (Molecular Probes). Inhibitors were purchased from Sigma unless indicated otherwise. Standard curves were made with known quantities of trypsin (T-8642, Sigma, from bovine pancreas; stock solution freshly prepared in 10 mM HCl) or chymotrypsin (C-4129; Sigma; from bovine pancreas; stock solution freshly prepared in 10 mM HCl). Samples were incubated for 24 h at 37 °C before fluorescence was read. Because of

the long incubation time in the assays, all reagents were prepared under sterile conditions.

Activity assays

Butyrylthiocholine iodide hydrolysis was measured by the Ellman method [8], in 0.1 M potassium phosphate, pH 7.0, at 25 °C, where $\epsilon = 13\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 412 nm. Benzoylcholine chloride hydrolysis was measured in 0.1 M potassium phosphate, pH 7.0, at 240 nm, where $\Delta\epsilon = 6700\text{ M}^{-1}\cdot\text{cm}^{-1}$. Hydrolysis of *o*-nitrophenyl butyrate was measured in 0.1 M potassium phosphate, pH 7.0, at 414 nm, where $\epsilon = 3190\text{ M}^{-1}\cdot\text{cm}^{-1}$ for *o*-nitrophenol. One unit was defined as the amount of BChE that hydrolysed 1 μ mol of substrate/min. The concentration of active subunits was determined by titrating with the inhibitor 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide [9].

Thermal inactivation

Samples of recombinant wild-type BChE (400 μ l) in protein-free DMEM/F12 were aliquoted into 500 μ l microfuge tubes and then incubated in a 55 °C water bath for varying amounts of time. At specified intervals, samples were removed from the water bath and placed on ice until they were assayed for activity with 50 μ M benzoylcholine chloride, as described above. Samples were later run on a PAGE gel.

Gels stained for activity

Precast, 4–20 % non-denaturing polyacrylamide gels (Bio-Rad) were electrophoresed at 200 V for 2 h at 4 °C. The 4–30 % gradient native polyacrylamide gels were poured using an SE600 apparatus (Hoefer Scientific) and electrophoresed at 100 V for 39 h at 4 °C. The gels were stained for BChE activity with 1 mM butyrylthiocholine iodide as substrate in the Karnovsky and Roots staining procedure [10].

Quantification of bands

Gel images were obtained by photographing them or the autoradiogram with a Kodak CCD (charged caption device) camera (BioImage 110S System[®]; Millipore). Images were transferred to a Sun SPARCstation 1 + computer and analysed using BioImage Whole Band[®] software and printed on a Seiko Instruments CH-5504 colour printer.

Antibodies

A polyclonal antibody against BChE, purified from pooled human serum, was custom made by HRP Inc. (now Covance Research Products, Denver, PA, U.S.A.). BChE was denatured in 0.1 % (w/v) SDS and boiled before it was injected into a rabbit. This antibody recognized both the active and denatured forms of recombinant and native BChE.

The monoclonal antibody to the FLAG epitope (M2) and the M2 affinity gel for the FLAG epitope were purchased from IBI-KODAK.

Secondary antibodies were peroxidase-linked sheep anti-mouse (NXA 931; Amersham) and peroxidase-linked goat anti-rabbit (NA 934; Amersham).

N-terminal sequencing

Recombinant wild-type BChE-FLAG was purified by binding to the M2 affinity gel consisting of monoclonal anti-FLAG, covalently attached to beads. The recombinant wild-type BChE-FLAG was eluted with approx. 1 ml aliquots of 0.1 M glycine, pH 3.5, that were immediately neutralized by the addition of

20 μ l of 1.0 M Tris/HCl, pH 8.0. Then 220 μ g of this recombinant wild-type BChE-FLAG was layered on top of a 5–20 % polyacrylamide/SDS reducing gel poured into an SE600 apparatus without a comb. The gel was electrophoresed at 70 V for 16 h and then the protein was transferred to PVDF (P15552; Millipore) membrane using a Bio-Rad Trans Blot apparatus at 480 mA for 1 h. The membrane was stained with Coomassie Blue, rinsed repeatedly with triple-distilled water and air dried. The recombinant wild-type BChE-FLAG appeared as a doublet of approximate molecular masses 85 kDa and 69 kDa. Both bands were cut from the membrane and subjected to N-terminal sequencing on an Applied Biosystems 477A Protein Sequencer, with an online 120A analyser.

HPLC separation of tetramers and monomers

A 100 μ l sample, containing 8.3 units of recombinant wild-type BChE, which had been purified by procainamide affinity chromatography and found to contain predominately tetramers and monomers but almost no dimers, was applied to a Toso Haas TSK-GEL G3000SW_{XL} size-sieving column. The flow rate was 0.5 ml/min with 0.1 M sodium phosphate buffer, pH 6.7, as the mobile phase. The Waters 486 tunable absorbance detector was set to 220 nm. Peak fractions were manually collected and run on PAGE gels.

C-terminal sequencing

The isolated tetramers and monomers of recombinant BChE, purified as described above, were treated with carboxypeptidase-Y (Sigma) followed by amino acid analysis of released amino acids.

Western blot analysis

Cell lysates were prepared by rinsing monolayers of CHO cells in 100 mm dishes three times with cold PBS, followed by lysis on ice with 500 μ l of PBS containing detergents [1.0 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate and 0.1 % SDS] and the protease inhibitors leupeptin (1 μ g/ml), aprotinin (5 μ g/ml), pepstatin (1 μ g/ml) and PMSF (2 mM) for 15 min. The cells were scraped loose, disrupted in a syringe with an 18-gauge needle, and transferred to a tube for centrifugation at 100 000 g for 30 min at 4 °C to pellet insoluble cellular debris.

Samples were applied to 5–20 % polyacrylamide/SDS non-reducing gels and electrophoresed at 70 V for 18 h, or to 4–20 % non-denaturing precast (Bio-Rad) mini gels and electrophoresed at 200 V for 2 h. The protein was then transferred to PVDF (Millipore) membrane using a Bio-Rad Trans Blot apparatus at 480 mA for 1 h. Blots were prehybridized with 5 % (w/w) instant non-fat dried milk in TBS [20 mM Tris/HCl (pH 7.4)/150 mM NaCl] containing 0.2 % (v/v) Tween 20, overnight at 4 °C. Blots were hybridized with primary antibody, either M2 monoclonal anti-FLAG (1:1000 dilution) or polyclonal anti-BChE (1:2000 dilution), for 3 h at 4 °C. The blots were washed in a series of washes with TBS alone, TBS with 0.2 % Tween (blocking buffer) and 3 % Tween, and blocking buffer with 5 % milk. Blots were then hybridized with secondary antibodies, either horseradish peroxidase-conjugated anti-mouse or horseradish peroxidase-conjugated anti-rabbit. Blots were incubated in a 1:2000 dilution for 1 h at 4 °C. The blots were washed again, as described above. The blots were treated with the LumiGLO[®] Chemiluminescent Substrate Kit from Kirkegaard and Perry Laboratories, and briefly exposed to X-ray film.

Deglycosylation of recombinant BChE

Recombinant BChE, composed of tetramer, dimer and monomer, was electrophoresed at 180 V for 17 h on a 4–30 % gradient non-denaturing polyacrylamide gel. One lane was cut from the gel and stained for BChE activity with butyrylthiocholine [10]. Using this as a guide to locate tetramers, dimers and monomers, horizontal strips of the gel containing only tetramers, only dimers or only monomers were cut out. Each strip was placed in 2 ml of water and incubated for 72 h at 4 °C, to elute the protein from the gel. A recovery of approx. 43 % of the 0.4 unit (benzoylcholine) of recombinant BChE initially loaded on the gel was realized. The volume of each sample was reduced to 450 μ l under vacuum. SDS was added to a final concentration of 0.1 %. β -Mercaptoethanol was added to a final concentration of 0.033 % (v/v), and the samples were boiled for 5 min. Each form of the enzyme was then independently treated with peptide N-glycosidase F (PNGase F), endoglycosidase H (Endo H) or neuraminidase for 16 h at 37 °C in the appropriate buffer (Boehringer Mannheim). After the addition of SDS reducing sample buffer, the samples were boiled again for 5 min. Samples were applied to a 5–20 % SDS/reducing PAGE gel and electrophoresed for 1100 V·h. The proteins were transferred from the gel to PVDF membrane and developed as described above, in the Western blot analysis section. The deglycosylation experiment was repeated on isolated tetramers and monomers that had been separated on a size-sieving HPLC column, as described above. The deglycosylation experiment was also repeated on a recombinant wild-type BChE-FLAG sample, which was collected in DMEM/F12 and concentrated 5-fold.

Pulse-chase analysis

Metabolic labelling of cells with radioactive amino acids

CHO cells were grown to 80–95 % confluence in 100 mm plastic dishes. After 30 min starvation in DMEM lacking cysteine and methionine, cells were pulse-labelled for 3 min with 5 ml of 306 μ Ci/ml L-[³⁵S]cysteine and L-[³⁵S]methionine (Expre³⁵S³⁵S, #NEG-072; New England Nuclear) in serum-free culture medium lacking cysteine and methionine. All pulse incubations were carried out at 37 °C in air/CO₂ (19:1) using culture media equilibrated to these conditions.

After the 3 min pulse, the labelling media were replaced with 5 ml of serum-free medium containing non-radioactive cysteine and methionine, and the cells were incubated for chase times ranging from 0 to 240 min. At the conclusion of the chase incubations, the cells were rinsed with PBS. Cells were lysed in 5 ml of PBS containing detergents (1.0 % Triton X-100, 0.5 % sodium deoxycholate and 0.1 % SDS), protease inhibitors (20 mM EDTA and 2 mM PMSF) and 50 mM iodoacetic acid [11] to alkylate any free thiol groups.

Immunoprecipitation of cell lysates

Recombinant wild-type BChE was immunoprecipitated with the polyclonal anti-BChE antibody in a final dilution of 1:1000. Immunocomplexes were precipitated with Protein A-Sepharose (Sigma) using 200 μ l of a 10 % (w/v) suspension of the beads per ml of culture fluid [11].

SDS/PAGE

The radiolabelled recombinant wild-type BChE that was adsorbed to the washed Protein A-Sepharose beads was eluted with

twice-concentrated SDS-gel sample buffer [0.125 M Tris/HCl/4 % SDS/20 % (v/v) glycerol (pH 6.8)] by boiling for 5 min. The eluates were applied to non-reducing SDS/PAGE gradient slab gels (5–20 %) that were run as previously described [11] in the Laemmli system [12]. To reveal the radioactive bands, the gels were immersed for 1 h in 150 ml of ENHANCE fluorography enhancer (DuPont–New England Nuclear), and rinsed in water and then in 2 % glycerol in water for 30 min. The gels were dried *in vacuo* on filter paper and exposed to X-ray film [11].

RESULTS

C-terminal FLAG-tagged recombinant wild-type BChE and C571A

The 8-amino-acid FLAG tag epitope was attached immediately after Leu-574 of recombinant wild-type BChE by PCR mutagenesis. This added the amino acids DYKDDDDK to the C-terminus of the full-length sequence to give 582 amino acids per subunit. Addition of the FLAG tag allowed us to easily detect the presence of the C-terminus of recombinant wild-type BChE–FLAG on Western blots.

On non-denaturing PAGE gels stained for activity with butyrylthiocholine, recombinant wild-type BChE–FLAG showed the same pattern of active bands as recombinant wild-type BChE without the FLAG tag, i.e. tetramers (10–30 %), dimers (50–55 %) and monomers (15–40 %) (Figure 1, lanes 1 and 10). In contrast, 63–90 % of native BChE from plasma was tetrameric (Figure 1, lane 9).

Western blot analysis of a non-denaturing gel, using the M2 anti-FLAG, showed tetramers and dimers of secreted recombinant wild-type BChE–FLAG but no FLAG-tagged monomers (results not shown), indicating that the monomers, though present and active, were missing a portion of their C-terminus.

A Western blot from an SDS non-reducing PAGE gel, using the monoclonal M2 anti-FLAG, showed two bands (one migrating as dimer and another as monomer) of recombinant wild-type BChE–FLAG from cell lysates (Figure 2, lane 3), while secreted recombinant wild-type BChE–FLAG appeared as a doublet, migrating at the dimer position (lane 1). The dimers in Figure 2 (lanes 1 and 3) result from covalent linkage of two subunits by a disulphide bond at Cys-571 [2]. The monomers in

Figure 2 Western blot analysis of cellular and secreted recombinant BChE

Bands on the non-reducing SDS/PAGE gel were revealed by Western blotting and hybridization with the monoclonal M2 anti-FLAG antibody and the peroxidase-conjugated anti-mouse. Lane 1, secreted recombinant wild-type (wt) BChE–FLAG; lane 2, secreted C571A–FLAG; lane 3, cell lysates of recombinant wild-type BChE–FLAG. C571A–FLAG was purified using the M2 affinity column, followed by treatment with Protein A–Sepharose to remove any M2 antibody from the sample. The faster-migrating monomer band in lane 2 is high-mannose-modified C571A, and the slower-migrating monomer band is C571A modified by complex sugars.

the cell lysate (Figure 2, lane 3) were assumed to be precursors that had not yet assembled into dimers. Secreted recombinant wild-type BChE–FLAG showed no FLAG-tagged monomer band in Figure 2, lane 1. This supported the proposal that the monomers of recombinant wild-type BChE–FLAG, present in conditioned media (Figure 1, lane 10), had been proteolytically cleaved so that the FLAG tag was removed and the secreted monomers were present but undetectable in the anti-FLAG Western blot (Figure 2, lane 1).

Tetramers and dimers included full-length subunits, since they had the FLAG tag. Following denaturation in SDS the tetramers and dimers migrated as dimers on the non-reducing gel in Figure 2, lane 1. The absence of FLAG-tagged monomers in Figure 2, lane 1, meant that the interchain disulphide bond was fully formed in all subunits of FLAG-tagged secreted tetramers and dimers.

Deletion of the C-terminus of BChE

Knowing that the enzymically active monomers of recombinant wild-type BChE had been cleaved, we wished to determine the exact number of amino acids that could be deleted from the C-terminus without loss of enzyme activity. The mutant Gly-534-stop had a stop at the last codon of exon 3, eliminating all of exon 4. This caused deletion of 41 C-terminal amino acids, eliminating the interchain disulphide bond at Cys-571, as well as seven conserved aromatic amino acids (three Trp, two Phe and two Tyr). Despite the deletion of the 41 C-terminal amino acids, k_{cat} was normal, and active BChE was secreted (Figure 1, lane 2). However, this deletion disrupted subunit association so that only monomers were made and secreted.

We placed a stop codon at each position from Phe-521 to Leu-530 and expressed the series of mutants in CHO cells. All mutants preserved the three intrachain disulphide bonds between Cys-65–Cys-92, Cys-252–Cys-263 and Cys-400–Cys-519, while Leu-530-stop preserved the entire α -helix H [7] at Ala-516 to Val-529. We found that 50 amino acids could be deleted (F525stop) (Figure 1, lane 7) without disrupting the secretion or the activity of BChE, but that deletion of 51 or more amino acids (Ser-524-

Figure 1 Activity-stained gel showing C-terminal deletion mutants of recombinant BChE

A 4–30 % native acrylamide gel was stained for BChE activity with butyrylthiocholine [10]. The lanes labelled recombinant wild BChE (rWild BChE), G534stop, L530stop, K528stop, P527stop, F526stop, F525stop and BChE–FLAG had 0.0005 unit of activity (assayed with benzoylcholine) per lane. The lane labelled S524stop had as much or more volume of conditioned media loaded as the lanes with activity. The secreted recombinant BChE was in serum-free culture medium. The lane labelled 'plasma' had 4 μ l of human plasma (approx. 0.004 unit of activity). G4, G2 and G1 refer to the tetramer, dimer and monomer forms of globular BChE respectively. The band between G4 and G2 in the plasma lane is a trimer, and the band between G2 and G1 is a heterodimer of albumin and BChE.

Figure 3 Estimation of protease activity level in conditioned culture medium

Serum-free culture medium harvested from CHO K1 cells expressing human recombinant BChE was assayed for protease activity in the BODIPY-casein assay. Fluorescence was measured at excitation wavelength 590 nm (Ex590), emission 620 nm (Em620). The protease activity of trypsin concentrations, 0, 0.01, 0.1, 1, 10 and 100 μ g/ml, was tested for comparison. The culture medium had protease activity equivalent to 30 μ g/ml trypsin.

stop, Thr-523-stop, Trp-522-stop, Phe-521-stop) resulted in an inactive form of BChE (Figure 1, lane 8), which was retained in the cell. Western blots showed that inactive Ser-524-stop BChE was present inside the cell, but not outside the cell. The stop mutants at Phe-525, Phe-526, Pro-527, Lys-528, Leu-530 and Gly-534 gave monomer bands, on native gels stained for activity, that migrated approximately the same distance as the monomers (that were missing the FLAG epitope) produced by recombinant wild-type BChE-FLAG (Figure 1). All nine carbohydrate attachment sites were present in the mutants in Figure 1, yielding a similar degree of charge heterogeneity in all mutants. The Gly-534 and Leu-530 stop mutant samples in Figure 1 (lanes 2 and 3) had been stored at 4 °C for a longer time than the others and migrated as broader bands, probably due to the loss of sialic acid residues. The complete absence of dimer and tetramer in the C-terminal deletion mutants showed that subunit association required the 40–50 amino acids at the C-terminus. These results supported the conclusion that the recombinant wild-type monomers present in culture media were due to proteolysis at the C-terminus.

Proteases in culture medium

Figure 3 shows the protease activity of conditioned medium compared with the protease activity of known amounts of trypsin in the BODIPY-casein assay. The protease activity of conditioned medium was equivalent to approx. 30 μ g/ml trypsin or 0.3 μ g/ml chymotrypsin in the BODIPY-casein assay and 100 μ g/ml trypsin in the Arg-AMC assay. Other samples of conditioned medium, where culture conditions were deliberately modified to minimize proteolysis, had as little as 1–10 % of the protease activity of the sample in Figure 3. The nature of the protease in the conditioned medium was tested with specific inhibitors (Table 1). Serine protease inhibitors had no significant effect, suggesting that the protease was not a serine protease. Antipain, leupeptin and the calpain inhibitor, *N*-acetyl-leucyl-leucyl-norleucinal, were not inhibitors either. However, *p*-chloromercuribenzoate, iodoacetamide and *N*-ethylmaleimide gave significant inhibition in the BODIPY assay, suggesting that the protease was a cysteine protease. The metal chelators EDTA, EGTA and 1,10-phenanthroline significantly inhibited protease

Table 1 Effects of protease inhibitors on cleavage of BODIPY-casein and Arg-AMC by proteases in conditioned serum-free culture medium harvested from CHO K1 cells

Activities had an error of 5%. Paraoxon and 1,10-phenanthroline inhibition could not be measured in the Arg-AMC assay because their absorbance overlapped with that of AMC. ALLN, *N*-acetyl-leucyl-leucyl-norleucinal.

Inhibitors	Activity in BODIPY assay (% of control)	Activity in Arg-AMC assay (% of control)
Serine protease inhibitors		
Di-isopropylfluorophosphate, 0.1 mM	83	100
Paraoxon, 0.1 mM	94	
PMSF, 0.1 mM	100	100
Serine/cysteine protease inhibitors		
Antipain, 0.1 mM	95	100
Leupeptin, 0.1 mM	100	100
Cysteine protease inhibitors		
<i>p</i> -Chloromercuribenzoate, 0.1 mM	51	90
ALLN, 0.1 mM	95	99
Iodoacetamide, 10 mM	66	74
<i>N</i> -Ethylmaleimide, 10 mM	34	25
Metalloprotease inhibitors		
EDTA, 10 mM	30	44
EGTA, 10 mM	31	95
1,10-Phenanthroline, 10 mM	32	

activity in the BODIPY assay, suggesting that the protease was a metalloprotease or a metal-requiring cysteine protease. In contrast, EGTA and *p*-chloromercuribenzoate were not inhibitors in the Arg-AMC assay, indicating that the protease that cleaved Arg-AMC was different from the protease that digested BODIPY-casein. In conclusion, the results in Table 1 suggest that there are several proteases in the culture medium and that these proteases may be metal-requiring cysteine proteases or a mixture of cysteine and metalloproteases.

How to minimize proteolysis

BChE monomers in culture media are artifacts resulting from proteolysis. The minimum amount of monomers was obtained by growing cells to no greater than 90 % confluence and collecting culture medium within 24 h of feeding with serum-free medium. These conditions also yielded the minimum level of protease activity in the culture medium. In contrast, a high proportion of monomers and a high level of protease activity were seen in culture medium when cells were grown under conditions that produced the highest amount of active recombinant BChE, i.e. when cells were grown to superconfluence and were fed every 3 or 4 days.

Mutation of common proteolytic enzyme targets in the C-terminus of BChE

We expressed the double mutant K568H/K569H, the triple mutant K558H/K568H/K569H and the quintuple mutant K544H/R549H/K558H/K568H/K569H in CHO cells. Whereas these mutants eliminated the common targets of proteases, Lys and Arg, in the susceptible C-terminus of BChE, there was no effect on the proportion of tetramers, dimers and monomers in the conditioned media (results not shown). This supports the notion that a protease with non-trypsin-like specificity was responsible for the cleavage at the C-terminus.

Table 2 Proportion of tetramers, dimers and monomers of secreted recombinant wild-type BChE varies with time

Samples of recombinant wild-type BChE collected from 90%-confluent CHO K1 cells 2–21 h after feeding with serum-free UltraCULTURE were subjected to non-denaturing PAGE and stained for activity. Bands were quantified using the BioImage® (as described in the Experimental section).

Time on cells (h)	BChE activity (BioImage IOD units)		
	Tetramers	Dimers	Monomers
2	0.3	1.2	Non-detectable
4	0.5	2.2	Non-detectable
6	0.9	3.5	0.4
21	2.9	9.4	7.3

Figure 4 Activity-stained gel of Cys-571-Ala

The non-denaturing PAGE gel was electrophoresed at 4 °C in order to preserve the activity of monomers and dimers, which disappeared when electrophoresis was at room temperature. Lanes 1 and 7 contained 4 μ l of human plasma (0.004 unit benzoylcholine activity) (as described in Figure 1). Lanes 2 (0.015 unit), 3 (0.0075 unit), 4 (0.001 unit) and 6 (0.0005 unit) contained secreted C571A-FLAG. Lane 5 (0.0005 unit) contained secreted Cys-571-Ala. Lane 8 contained (0.0005 unit) secreted recombinant wild-type BChE-FLAG.

Appearance of monomers

To determine whether cleaved monomers were secreted into the culture medium or created from secreted dimers and tetramers, we performed the following experiment. An 80–90%-confluent dish of CHO cells, expressing recombinant wild-type BChE, was washed with PBS and fed with serum-free UltraCULTURE. An aliquot of culture medium was collected at hourly intervals up to 24 h. The aliquots were stored frozen until electrophoresis on a non-denaturing gel. The gel stained for BChE activity showed that samples collected at 1–5 h contained only dimers and tetramers. Monomers first appeared in the 6 h collection and were present in all later samples. The experiment was repeated with collections at 2, 4, 6 and 21 h. The results are presented in Table 2. This result suggested that cells secreted primarily dimers and tetramers and that monomers resulted from proteolysis of dimers and tetramers.

Elimination of the interchain disulphide bond

The mutant Cys-571→Ala (C571A) was made to specifically eliminate the interchain disulphide bond of BChE while otherwise leaving the C-terminus of BChE intact. On a native gel stained for activity, C571A or C571A-FLAG each appeared as mainly monomers (70–95%), with a distinct tetrameric band (5–30%) (Figure 4, lanes 4–6). The level of dimers in the C571A-FLAG sample was so low (2–7%) that only following the concentration of the sample on the M2 anti-FLAG affinity column was it possible to see the active dimer band in addition to the tetramer and monomer bands (Figure 4, lanes 2 and 3). Electrophoresis was performed at 4 °C to prevent heat inactivation of the BChE

Figure 5 Heat inactivation of recombinant BChE

(A) Percentage residual activity of recombinant wild-type BChE assayed with 50 μ M benzoylcholine after heating the enzyme at 55 °C for 0–30 min. This sample of recombinant wild-type BChE contained 13% tetramers, 62% dimers and 25% monomers. (B) Percentage residual activity of Phe-525-stop and Cys-571-Ala after heating at 55 °C. Phe-525-stop consisted entirely of monomers. Cys-571-Ala had 13% tetramers, 2% dimers and 85% monomers. Phe-525-stop monomers had a half-time of 0.5 min at 55 °C. Tetramers of recombinant wild-type BChE and of Cys-571-Ala had a half-time at 55 °C of approx. 12 min. Residual activity at each time point was determined in triplicate.

monomer and dimer. Western blots of secreted C571A-FLAG run on non-denaturing gels showed tetramers, dimers and monomers that were full-length, based on the detection of the FLAG epitope placed at the C-terminus of BChE (results not shown). Thus the absence of the interchain disulphide bond resulted in the secretion of full-length monomers. This was in contrast with recombinant wild-type BChE, where all monomers appeared to be proteolysed.

A Western blot of a SDS non-reducing gel showed only monomers in the secreted C571A-FLAG (Figure 2, lane 2) and no dimers. The absence of the dimer band in Figure 2, lane 2, for the C571A-FLAG sample is consistent with the role of Cys-571 in the formation of the interchain disulphide bond [2]. The doublet band seen in secreted C571A-FLAG was due to the presence of two types of glycosylation, high-mannose and complex sugars, which modify recombinant BChE (see the Carbohydrates in recombinant BChE section below). All molecules of recombinant BChE in the cell lysates (Figure 2, lane 3) had high-mannose sugars attached and appeared as single bands at the monomer and dimer positions.

Heat inactivation of recombinant BChE

The heat stabilities of recombinant wild-type Cys-571-Ala and Phe-525-stop were measured at 55 °C. The total protein concentration in the samples was approx. 50 μ g/ml. Figure 5(A)

Table 3 Kinetic values for recombinant wild-type BChE (rWild-type BChE) and Phe-525-Stop

K_{ss} is the dissociation constant of the substrate/enzyme/substrate (SES) complex. The parameter b reflects the efficiency with which the ternary complex SES forms product. Substrate activation is observed when $b > 1$ [14,15].

Substrate	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{M}^{-1}$)	K_{ss} (mM)	b
rWild-type BChE					
Butyrylthiocholine	7 ± 0.7	24000 ± 10000	3.4×10^9	0.5 ± 0.1	4.4 ± 0.2
Benzoylcholine	8 ± 1	15000 ± 700	1.9×10^9		
<i>o</i> -Nitrophenylbutyrate	110 ± 20	36000 ± 5000	0.3×10^9		
Phe-525-Stop					
Butyrylthiocholine	9 ± 0.6	24000 ± 10000	2.7×10^9	0.4 ± 0.1	4 ± 0.2
Benzoylcholine	5 ± 0.6	13000 ± 600	2.6×10^9		
<i>o</i> -Nitrophenylbutyrate	80 ± 10	36000 ± 5000	0.5×10^9		

shows a biphasic decrease in activity with time of heating. The biphasic nature can be explained by assuming that the recombinant wild-type tetramers (15%) are more stable than the dimers (60%) and monomers (25%). Native tetramers purified from human serum are known to be very stable to heat [13]. The half-time of inactivation of the tetrameric recombinant wild-type, estimated from the slow phase (10–30 min time points), was 12 min. This measurement also yielded an estimate for the total amount of tetramer in the sample before heating: 10%, a value similar to the 13% calculated by BioImage® analysis and quantification of the activity-stained gel (as described in the Experimental section). When the tetramer contribution in the slow phase was subtracted from the rest of the curve in Figure 5(A), a straight line was obtained which had a half-time of approx. 1.8 min. That the fast portion was monophasic suggested that the heat stability of recombinant wild-type BChE dimers was about the same as the heat stability for recombinant wild-type monomers.

Figure 5(B) shows that the mutant C571A also had a non-linear dependence of activity on time of heating. This sample of the C571A mutant contained 13% tetramers, 2% dimers and approx. 85% monomers. The slow portion was fitted to a single phase with a half-time of approx. 12 min, comprising 10% of the total activity. This was consistent with the slow portion being due to the decay of the tetramer. After the tetramer contribution was subtracted, the half-time of the remaining monomeric sample was estimated to be 0.8 min. Mutant F525stop, which consisted entirely of monomers, had a half-time of heat inactivation of 0.5 min. These results supported the suggestion above that recombinant wild-type BChE dimers, containing the interchain disulphide bond, had a heat stability nearly as low as that of monomers. Thus the disulphide bond in the dimers did not add significantly to their heat stability.

The sensitivity of dimers and monomers to heat inactivation was evident on non-denaturing gels stained for activity. When gels were run at room temperature rather than 4 °C, the activity of the dimers and monomers disappeared, leaving tetramers as the only active BChE form (results not shown).

Monomers of recombinant BChE showed substrate activation

The K_m and k_{cat} values for Phe-525-Stop (monomeric) were similar to those for recombinant wild-type BChE (mostly dimeric) (Table 3) and native BChE (tetrameric). Native BChE had a K_m of $13 \pm 7 \mu\text{M}$ and a b value of 3.5 ± 0.5 (standard errors were determined using Sigma Plot software). The monomeric BChE had a b value of 4.0 ± 0.2 with the substrate butyrylthiocholine,

Figure 6 Western blot of deglycosylated recombinant wild-type BChE-FLAG from an SDS reducing gel

The Western blot was developed using the M2 anti-FLAG. Lane 1, recombinant wild-type BChE-FLAG treated with PNGase F; lane 2, untreated recombinant wild-type BChE-FLAG; lane 3, recombinant wild-type BChE-FLAG treated with Endo H. The doublet in lane 2 results from two types of glycosylation: the 85 kDa band has complex sugars, whereas the 69 kDa band has high-mannose sugars. Molecular-mass markers are shown on the left.

indicating that substrate activation at high substrate concentration was not due to allosteric effects between subunits.

Carbohydrates in recombinant BChE

On SDS reducing gels, secreted recombinant wild-type BChE-FLAG appeared as a doublet (Figure 6, lane 2). The two bands migrated at 85 kDa and 69 kDa. Secreted recombinant wild-type BChE, with or without the FLAG epitope, as well as all mutants examined to date, migrated as a doublet during SDS/PAGE. To determine whether the double band was due to differential glycosylation and to find out whether glycosylation was different in tetramers compared with dimers and monomers, we performed the following experiment. Tetramers were separated from dimers and monomers of recombinant wild-type BChE on a Toso Haas size-sieving column (as described in the Experimental section). Tetramers, dimers and monomers were also separated on a non-denaturing polyacrylamide gel and eluted. On SDS reducing gels, revealed by Western blotting with the anti-BChE antibody, isolated tetramers, dimers and monomers each ran as the 85 kDa and 69 kDa doublet band seen in Figure 6 (lane 2). Each form of the enzyme was treated with PNGase F, which removes all N-linked carbohydrates, with Endo H, which removes only high-mannose sugars, or with neuraminidase, which removes sialic acid residues from complex sugars. With each separated form of the enzyme, both the 85 kDa band and the 69 kDa band

Figure 7 Silver-stained gel of deglycosylated recombinant BChE tetramers and monomers

Isolated tetramers and monomers of recombinant wild-type BChE were treated with PNGase F and run on a 5–20% polyacrylamide/SDS reducing gel. The protein bands were revealed by silver staining. Deglycosylated tetramers of recombinant wild-type BChE migrated at 66 kDa (shown on the left), whereas monomers migrated at 64 kDa (shown on the right). This difference in size can be explained by proteolysis at the C-terminus of the monomers. The minor band in the tetramer lane just above the 66 kDa band may be due to incomplete deglycosylation of BChE. The band just below BChE was present in all lanes of the gel with or without sample loaded and can be explained as an artifact of the buffer. PNGase F, which was used to deglycosylate BChE, is also visible on the silver-stained gel and migrates at 34.6 kDa.

disappeared after treatment with PNGase F (Figure 6, lane 1). When either BChE tetramers or dimers were digested, the 69 kDa band migrated with an apparent molecular mass of 66 kDa following Endo H treatment, whereas the migration of the 85 kDa band was unaffected by this treatment (Figure 6, lane 3). The 85 kDa band shifted slightly when treated with sialidase but the 69 kDa band remained (results not shown). The deglycosylation experiment showed that tetramers, dimers and monomers of recombinant BChE were each mixtures of molecules having both complex sugars and high-mannose-containing sugars. In addition, the deglycosylation experiment showed that the proteins within the 85 kDa band had complex sugars including sialic acid, whereas the 69 kDa band had only high-mannose-containing sugars.

It also became apparent from SDS reducing gels that PNGase-F-treated tetramers and PNGase-F-treated monomers of recombinant wild-type BChE did not reduce to the same size. Subunits of the deglycosylated tetramers migrated at 66 kDa, whereas deglycosylated monomers migrated at 64 kDa, as detected by silver staining (Figure 7). This once again showed that the monomers had undergone proteolysis. In addition, it showed that recombinant wild-type BChE was cleaved as well as recombinant wild-type BChE-FLAG. This suggested that the FLAG epitope was not itself the site of the proteolysis.

Amino acid sequencing

N-terminal sequencing of both the 85 kDa and the 69 kDa bands (Figure 7, lane 2) of a highly purified sample of recombinant wild-type BChE-FLAG, separated on an SDS reducing gel, produced the sequence EDDIIIATKN. This sequence was identical with the first 10 amino acids of BChE derived from plasma. There was no evidence for proteolysis at the N-terminus of recombinant wild-type BChE-FLAG. This sample contained only tetramers and dimers because it had been purified using the M2 anti-FLAG column. Monomers did not stick to this column because the FLAG epitope was absent from the monomers. The

Figure 8 Pulse-chase of recombinant wild-type BChE in CHO K1 cells

A 3 min pulse with [³⁵S]Met and [³⁵S]Cys was followed by chase times of 0–240 min with non-radiolabelled media. From 0 to 60 min chase times, monomers of recombinant wild-type BChE were present. At 90 min, a trace of dimer was detected, and by 240 min, one-fourth to one-third of the BChE had been converted into dimer.

presence of the 85 and 69 kDa bands of BChE in this sample could be explained solely on the basis of the two types of glycosylation.

Carboxypeptidase Y treatment of tetramers released the expected Leu residue from the C-terminus, whereas carboxypeptidase Y treatment of monomers released a mixture of residues.

N-terminal deletion mutants

Four different N-terminal deletion mutants were expressed in CHO cells. Δ2N had nearly the same activity as recombinant wild-type BChE. However, no activity was detected in the conditioned media of cells producing Δ59, Δ8 or Δβ-2β-1. Western blots of cell lysates from CHO cells expressing Δ8 and Δβ-2β-1 (ΔIle-4-Gly-15) revealed that BChE protein was being made but retained by the cell (results not shown). No BChE protein was detected inside or outside cells transfected with Δ59, although PCR revealed that the CHO cells had been successfully transfected with the Δ59 mutant DNA. This demonstrates the importance of the N-terminus of BChE for proper folding and secretion.

Pulse-chase analysis

Following a 3 min pulse and chase times of 0–60 min, only monomers of recombinant wild-type BChE were detected in the CHO K1 cell lysates (Figure 8). By 90 min a trace of dimer was visible, and by 120 min nearly one-fourth of the labelled recombinant wild-type BChE was detected as dimer. At the 240-min-chase time point, one-third of the enzyme had assembled into dimers. This pulse-chase experiment demonstrated the progression from monomers to dimers of recombinant wild-type BChE in the cell and suggested that the half-time for assembly into disulphide-linked dimers was greater than 240 min. Western blot analysis of total cell lysates demonstrated that the majority of recombinant wild-type BChE was present as dimers (Figure 2, lane 3). The intracellular carbohydrates of dimer BChE possessed high-mannose-containing sugars. This implies that high-mannose-containing sugars do not interfere with assembly of BChE into dimers. This was also observed by Kerem et al. [16] for human acetylcholinesterase (AChE).

DISCUSSION

Monomers resulting from proteolysis

Pulse-chase studies (Figure 8) showed that recombinant BChE was assembled into dimers within 90–120 min from the time it was synthesized. This assembly occurred inside the cell. Early secretion products of recombinant BChE appeared as tetramers

Figure 9 Model for tetrameric BChE and α -helical structure of the tetramerization domain

(A) A proposed model for the tetrameric BChE. The four circles represent the globular portions of the subunits of tetrameric BChE. The C-terminal tetramerization domain is depicted as an antiparallel 4-helix bundle with interchain disulphide bonds at Cys-571. (B) Proposed α -helical structure of the C-terminus of BChE. Seven conserved aromatic amino acids are on one side of the helix shown in side view and top view. The structures were made with Sybyl (Tripos Inc.) software on a Silicon Graphics Workstation.

and dimers on non-denaturing gels. It was not until 5–6 h later that monomers appeared in the media collected from CHO cells producing recombinant wild-type BChE. Since secreted monomers were missing the FLAG tag from the C-terminus, whereas monomers inside the cell had the FLAG tag, it appears that monomers of secreted BChE arose from proteolysis of multimers.

The site of proteolysis was probably between Ser-524 and the C-terminus, producing a heterogeneous mixture of C-terminal residues. Deletions of amino acids up to the C-terminal side of Ser-524 produced active monomers, but deletion of any additional amino acids resulted in an inactive form of BChE, which was retained in the cell. Evidence that the C-terminus of monomers was a mixture of heterogeneous polypeptides was provided by C-terminal sequencing. Sequencing of tetrameric recombinant wild-type BChE gave the single expected residue, leucine, but sequencing of the monomeric BChE gave a mixture of residues.

Studies on AChE support our conclusion that the C-terminus is important for tetramer formation. Truncated *Torpedo* AChE, lacking the final 36 amino acids of the C-terminus, retains normal catalytic activity, but does not assemble into multimers when expressed in COS-7 cells [17]. HA-536, a truncated *Torpedo* AChE missing the 39 C-terminal amino acids, is inactive despite being secreted [18]. When the 39-amino-acid C-terminus was removed from the catalytic T-subunit of chicken AChE, the levels of multimers were severely reduced, but were nevertheless detected [19]. Monomers of mouse AChE, truncated by 37 amino acids (produced for crystallization), were active [20].

	524		574
human BChE	SFFPKVLEMTGNIDEAEEMWKAEGHWNINMMWKNQFNDYTSKKESCGL		
human AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
cat BChE	LFFPKVLEMTGNIDEAEEMWKAEGHWNINMMWKNQFNDYTSKKESCAGL		
cat AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
rabbit BChE	LFFPKVLEMTGNIDEAEQWKAEGHWNINMMWKNQFNDYTSKKERCAFL		
rabbit AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
mouse BChE	LFFPKVLEMTGDIETEQWKAEGHWNINMMWKNQFNDYTSKKESCAGL		
mouse AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
rat AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
bovine AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
chicken AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
<i>Torpedo</i> AChE	RFLPKLLSATDITLDEAEQWKAEGHWNINMMWKNQFNDYTSKKDRCSGL		
conserved aromatic	F	W	F
conserved other	P	K	L

Figure 10 Comparison of the C-termini of tetrameric AChE and tetrameric BChE among species

Sequences of domestic cat BChE and AChE were obtained from C. F. Bartels, University of Nebraska Medical Center. All other sequences were obtained from the Esther [wwwserver](http://meleze.ensam.inra.fr/cholinesterase/) <http://meleze.ensam.inra.fr/cholinesterase/> address.

Proteases in culture medium

In support of our conclusion that proteolysis was occurring in the culture medium, we demonstrated the presence of proteases in serum-free CHO-cell conditioned culture medium. The proteases were not serine proteases, but appeared to be metal-requiring cysteine proteases, or cysteine proteases and metalloproteases. Satoh et al. [21] have shown that CHO cells continuously secrete cysteine proteases. Velan et al. [22] and Bon and Massoulié [23] reported protease activity in culture medium of human kidney 293 cells and COS cells respectively.

Intracellular proteases

The possibility that proteolysis occurs not only in the culture medium but also inside the cell has not been excluded. We know that monomers are secreted, because full-length FLAG-tagged Cys-571–Ala monomers were found in the culture medium, and because the active truncated monomers were secreted into the culture medium in our series of C-terminal deletion mutants. Similarly, monomers of the Cys-580→Ala mutant of human AChE are secreted into the culture medium [16]. Several proteases have been reported in the endoplasmic reticulum, including the cysteine proteases ER-60 and ER-72 [24,25] and the multicatalytic proteasome [26]. Recombinant snake venom AChE provides an example of proteolysis inside the cell, which results in the secretion of a monomer. Cleavage of a basic 8-residue C-terminal fragment from snake venom AChE occurred inside the COS cells [27]. Thus while intracellular proteolysis is a possibility, our data point to the culture medium as the principal site of proteolysis.

Role of Cys-571 in subunit assembly

Earlier work with native human BChE purified from serum [13,28] and with bovine-brain AChE [29] led to the conclusion that the interchain disulphide bond was not required for tetramer formation. Native BChE was still a tetramer after the interchain disulphide bond between Cys-571 residues had been reduced and alkylated. However, the disulphide bond at Cys-571 contributed to heat stability of tetramers. Native tetramers had a half-life of 54 min at 55 °C, but tetramers in which the interchain disulphide bond had been reduced and alkylated had a half-life of 16 min [13]. Recombinant wild-type BChE tetramers had a half-life for inactivation of approx. 12 min, a value similar to that of native tetramers missing the interchain disulphide bond. However, recombinant wild-type tetramers had intact interchain disulphide bonds, so the reduced heat stability may be attributed to disorganization in non-covalent contacts at the C-terminus.

The C571A mutant consisted of approx. 10% tetramers, similar to recombinant wild-type. This result showed that the interchain disulphide bond was not essential for tetramerization. The role of the interchain disulphide bond in stabilizing the tetramer is illustrated in Figure 9(A), where two disulphide bonds are present in the tetramerization domain at the C-terminus.

Monomers of C571A-FLAG are found in the culture medium with the FLAG tag attached, whereas monomers of recombinant wild-type FLAG have no FLAG tag. The explanation for the presence of the FLAG tag on C571A-FLAG is that subunits of C571A do not assemble as efficiently and therefore are largely secreted as monomers. By contrast, recombinant wild-type BChE is primarily secreted as a dimer. Recombinant wild-type monomers arise by proteolysis. C571A-FLAG is secreted primarily as a monomer because assembly of C571A into dimers is not favoured due to the absence of the interchain disulphide bond.

Dimers

The mix of BChE forms secreted by the CHO cells expressing the C571A mutant included 2% dimers, whereas recombinant wild-type had approx. 50% dimers. This suggests that the disulphide bond is the major stabilizing influence in the dimer, though some stabilization from other factors must also exist. Velan et al. [22] also reported negligible levels of dimers in the human AChE mutant Cys-580→Ala. The high rate of failure of recombinant dimers to assemble into tetramers is puzzling. A possible explanation is that the dimer contacts at the C-terminus are incorrect. Even though the interchain disulphide bond is present, the aromatic contacts proposed in Figure 9 may be misaligned. Bon et al. [30] showed that addition of polyproline to the culture medium of COS cells expressing rat AChE resulted in a dose-dependent decrease of dimers and monomers, and a concomitant increase in tetramers. They noted that molecules of AChE solubilized from cell extracts were able to interact with polyproline to produce tetramers, whereas those secreted in the culture medium were not. Thus it appears that once dimers of cholinesterase are in the culture medium they are unable to oligomerize into tetramers even with the help of facilitators such as polyproline.

Glycosylation

Amino acid sequencing identified nine Asn-linked carbohydrate chains on native BChE [2]. Thus it was expected that recombinant BChE expressed in CHO cells would also contain complex glycans. However, the secreted recombinant BChE contained two types of oligosaccharides: complex carbohydrates on subunits of 85 kDa and high-mannose glycans on subunits of 69 kDa. High-mannose glycans are precursors of complex carbohydrates, so the 69 kDa size may be due to incomplete processing in the Golgi apparatus. High-mannose glycans, on secreted recombinant, human plasminogen and interferon- γ expressed by CHO cells, have been reported [31,32].

Since native BChE is present in serum predominantly as a tetramer, we had expected the recombinant BChE to be a tetramer. Contrary to our expectations, the recombinant BChE consisted of only 10% tetramers and approx. 50% dimers. To determine whether the failure of recombinant dimers to assemble into tetramers might be due to glycosylation differences, we measured the proportion of complex and high-mannose glycans in isolated tetramers and dimers. We found that complex and

high-mannose glycans were present in the same proportion in tetramers and dimers. This suggested that the presence of high-mannose structures was not the explanation for lack of assembly. This is a predictable conclusion, since protein folding and assembly occur in the endoplasmic reticulum at a time when only high-mannose-containing sugars are found. An alternative explanation for the low percentage of recombinant wild-type BChE tetramers may be an insufficient amount of chaperones or other cellular cofactors required for subunit assembly. This interpretation is supported by the finding of Bon et al. [30] that addition of polyproline increased the yield of secreted tetramers.

Tetramerization domain

The C-termini of tetrameric BChE and tetrameric AChE are highly conserved among species. Seven aromatic residues are conserved at positions 543–564 in the human, cat, rabbit, mouse, cow, rat, chicken and electric ray fish (*Torpedo*) cholinesterases (Figure 10), suggesting a particular importance for these residues [33,34]. Figure 9(B) shows the proposed α -helical structure for amino acids 543–564 of BChE. Based on helical wheel projections, all the aromatic residues are on one side of the helix, with hydrophilic residues on the other side.

We know that the tetramerization of BChE is dependent on the aromatic-rich region of the C-terminus. C-terminal deletion mutants form only monomers, suggesting that the C-terminus is necessary for tetramerization, whereas trypsinization results suggest the C-terminus is necessary for maintaining the tetrameric structure [28,35]. One AChE that exists exclusively as a monomer is the AChE in snake venom. This AChE does not have the conserved C-terminal sequence and is missing the aromatic amino acids, as well as the interchain disulphide Cys [27].

There are currently no structural data available for the tetramerization domain of BChE or AChE. No tetramers have been crystallized [36]. The dimer of *Torpedo* AChE crystallized by Sussman et al. [7] originated from dimeric glycolipid-anchored AChE. This is an alternatively spliced form of AChE that does not have the C-terminal amino acids belonging to the tetramerization domain. The *Torpedo* AChE dimer had a four-helix bundle involving amino acids 518–531 and 365–383. We propose that the four-helix bundle of the BChE tetramerization domain is formed by amino acid residues in the region 534–574.

Although most tetrameric proteins are held together by many interactions scattered throughout the primary structures of their subunits, a search of the Protein Data Bank revealed only three proteins that have a tetramerization domain at the C-terminus. These three are the lactose repressor of *Escherichia coli* (1TLF) [37], human p53 (1PES and 1PET) [38] and GCN4 of yeast (1GCL) [39]. In all three cases the C-terminal tetramerization domain consists of a four-helix bundle. The lactose repressor's tetramerization domain is an antiparallel four-helix bundle whose core has four Leu and one Val [37]. Wild-type GCN4 is a dimer, but substitution of eight amino acids created a parallel four-stranded coiled coil whose core had a heptad repeat of four Leu and four Ile forming a leucine zipper [39]. The tetramerization domain of p53 is an antiparallel four-helix bundle, having Phe-341 at the site of interaction between two helices. In addition, its helix core has one Met, three Leu and one Ala. The common features of these four-helix bundle tetramerization domains is a hydrophobic core. Molecular modelling suggests that the C-terminal domain of BChE may also form a four-helix bundle, with a novel type of core containing seven interacting aromatic residues. A model of this is shown in Figure 9(A). Support for our hypothesis that aromatic interactions are important in the intersubunit contacts of BChE

comes from the work of Masson and co-workers [34,40,41], who showed that, under pressures as high as 3.5 kbar (1 b), tetramers of BChE did not dissociate, whereas most other proteins do. It is known that stacking interactions between aromatic rings are stabilized by pressure, in contrast with uncharged aliphatic (e.g. Leu) and ionic contacts that are disrupted by pressure. Thus the behaviour of BChE under pressure supports the interpretation that tetramer formation of BChE is stabilized by aromatic interactions.

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