Glycoinositol phospholipid anchor and protein C-terminus of bovine erythrocyte acetylcholinesterase: analysis by mass spectrometry and by protein and DNA sequencing

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Purified bovine erythrocyte acetylcholinesterase (AChE) was radiomethylated on its amine groups and incubated with bacterial phosphatidylinositol-specific phospholipase C to remove the lipid portion of the AChE glycoinositol phospholipid (GPI) anchor, and a C-terminal tryptic fragment that contained the residual GPI glycan was isolated by HPLC. Analysis by electrospray-ionization mass spectrometry revealed a parent ion of m/z 3798. The fragmentation patterns produced by collision-induced dissociation mass spectrometry of the +4 and +5 states of the parent ion indicated a 23-amino acid peptide in amide linkage to ethanolamine- PO_4 -Hex-Hex-Hex(PO_4 -ethanolamine) (HexNAc)-HexN(Me) $_2$ -inositol phosphate. The glycan structure is completely consistent with that obtained previously for the GPI anchor of human erythrocyte AChE except for the addition of the HexNAc substituent. A nearly complete peptide sequence was deduced from the fragmentation patterns, although four assignments were based only on single fragments of very low abundance. To resolve this uncertainty, a segment of bovine

genomic DNA corresponding to the C-terminal AChE sequence was amplified by PCR. DNA sequencing established the 23-amino acid peptide sequence to be FLPKLLSATASEAPCTC-SGPAHG, in agreement with the MS data and consistent with results from Edman protein sequencing. Dimerization of AChE polypeptides is mediated by intersubunit disulphide bonding in this C-terminal segment, but the bovine AChE contained two cysteine residues in a ...CTC... motif, in contrast with human AChE which contains only a single cysteine in this segment. Although bovine AChE contained no free thiol groups reactive with iodo[^{14}C]acetamide, partial reduction and alkylation with iodo[^{14}C]acetamide revealed that conversion into monomers occurred with an overall incorporation of only one alkyl group per monomer. An identical level of alkylation was observed when dimeric human AChE was converted into monomers by partial reduction. The question of whether the bovine AChE contains one or two intersubunit disulphide linkages is considered.

INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7) (AChE) is derived from a single mammalian gene but occurs physiologically in multiple forms produced through alternative mRNA splicing and distinct modes of subunit assembly or post-translational modification [1]. Extensive protein sequence homology exists among the catalytic subunits of bovine AChE from fetal serum and adult brain and erythrocytes [2]. However, the enzyme from these three tissues differs significantly in its quaternary structure. The brain enzyme is a tetramer (G_4) of catalytic subunits bound to the cell membrane by a non-catalytic subunit with a small peptide anchor [3,4]. Fetal serum AChE is also a G_4 tetramer, but it has no detectable non-catalytic subunit and is completely soluble. Erythrocyte AChEs are G_2 dimers, and their membrane anchor is a glycoinositol phospholipid (GPI) similar to those in a rapidly growing family of membrane proteins with such structures [5,6]. The GPI anchor of human AChE has been characterized by composition analysis and fast-atom-bombardment MS [7,8] and NMR (H. van Halbeek and T. L. Rosenberry, unpublished work). It consists of a core glycan identical with those observed in the GPI anchors of other proteins corresponding to ethanolamine- P -6Man α 1-2Man α 1-6Man α 1-4GlcN attached to an inositol phospholipid. It also contains one or two additional ethanolamine phosphate groups branching from the mannose residues. The phospholipid component of the GPI of bovine erythrocyte AChE

differs from that of human AChE by containing primarily an 18:0 rather than a 22:4, 22:5 or 22:6 acyl group at the C-2 position of glycerol [9], and by lacking the palmitoylation on inositol that confers resistance to phospholipase C cleavage on human erythrocyte AChE [10]. Protein acceptors of GPI anchors are initially translated with a C-terminal signal sequence that is cleaved as the GPI is transferred *en bloc* to the mature C-terminus (see Ferguson and Williams [11]), and the present work was initiated to examine the peptide structure of bovine erythrocyte AChE in this region of interest. In AChE the important cysteine residues responsible for protein dimerization are also located near the C-terminus. In this paper we have therefore analysed this C-terminal domain of bovine AChE by sequencing both the protein (by Edman and MS analysis) and the corresponding genomic DNA, and we have also obtained information about the glycan structure of the GPI anchor.

EXPERIMENTAL

AChE

AChE was purified from fresh bovine or outdated human erythrocytes solubilized with Triton X-100 using acridinium resin affinity chromatography [9]. Purified bovine AChE was reductively radiomethylated with 50 mM NaCNBH $_3$ and 10 mM H ^{14}CHO (ICN; 56 mCi/mmol) (high specific radioactivity) or

Abbreviations used: AChE, acetylcholinesterase; CID-MS, collision-induced dissociation mass spectrometry; diC $_7$ PC, diheptanoylphosphatidylcholine; ESI-MS, electrospray-ionization mass spectrometry; GPI, glycoinositol phospholipid; G_2 , globular dimers; G_4 , globular tetramers; PI-PLC, phosphatidylinositol-dependent phospholipase C; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

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after 1:20 dilution with unlabelled HCHO to 2.8 mCi/mmol (low specific radioactivity) by the procedure of Haas and Rosenberry [12]. Alternatively, purified bovine AChE was incubated with purified 1 µg/ml *Bacillus thuringiensis* phosphatidyl-inositol-dependent phospholipase C (PI-PLC) ([8]; J. A. Krall, P. J. Thomas, Q. W. Yang and T. L. Rosenberry, unpublished work) in 70 mM sodium phosphate, pH 7.0, containing 1.5 % Triton X-114 and 2 mM diheptanoylphosphatidylcholine (diC₇PC), at 25 °C for 2 h. PI-PLC-treated bovine AChE and purified human AChE were then reductively radiomethylated with NaCNBH₃ and 10 mM ³HCHO (NEN; 80 mCi/mmol), dialysed against 20 mM sodium phosphate, pH 7, and repurified by acridinium resin affinity chromatography. Specific radioactivities of the radiomethylated AChEs, determined by amino acid analysis, were 1670 ³H c.p.m./pmol of bovine AChE and 2180 ³H c.p.m./pmol of human AChE. This observed ratio of 0.77 is close to the ratio of 0.73 (8/11) predicted from the number of amine residues in each protein.

Octyl-Sepharose binding of AChE

Reductively methylated bovine AChE (7.1 nmol in 1.7 ml) was incubated with 1 µg/ml PI-PLC in 20 mM sodium phosphate (pH 7.0)/1 mM diC₇PC overnight at 25 °C. Aliquots of the incubation mixture (10 µl) and control undigested material were mixed with 100 µl of slurry of octyl-Sepharose in a total volume of 400 µl of 20 mM sodium phosphate buffer (pH 7.0)/0.01 % Triton X-100 and rocked for 1 h at 25 °C. The incubation supernatant was removed and the resin was washed three times by Microfuging in 1 ml of the same buffer. Resin-bound AChE was eluted by rocking the washed resin in 100 mM sodium phosphate (pH 7.0)/1 % Triton X-100 (total volume 800 µl) for 10 min at 25 °C. The unbound AChE activity in the incubation supernatant and the bound AChE eluted with 1 % Triton X-100 were expressed as percentages of initial activity.

Trypsin digestion and HPLC fractionation

[¹⁴C]Methylated bovine AChE was digested with PI-PLC as outlined in the previous section. A repurified sample (3.5 nmol in 1.7 ml) was selectively reduced with dithiothreitol (5 mM) in 20 mM sodium phosphate/50 mM Tris/HCl, pH 8.5, for 30 min at 25 °C and alkylated by the addition of iodoacetamide (to 40 mM) for 30 min at 25 °C in the dark. After dialysis against 50 mM NH₄HCO₃, pH 8.1, the 2.3 ml sample was denatured by addition of 970 µl of acetonitrile for 10 min at 25 °C. The acetonitrile was then nearly all removed (940 mg as acetonitrile and an acetonitrile/water azeotrope) by drying for eight cycles of 1–2 min on a Speed-Vac vacuum dryer (Savant) [13]. Residual acetonitrile is reported (Boehringer-Mannheim Biochemica product literature) to enhance subsequent trypsin digestion slightly.

In another series of experiments, bovine AChE selectively reduced and alkylated with iodo[¹⁴C]acetamide as outlined below was cleaved with 10 µg/ml PI-PLC in 10 mM Tris/HCl (pH 8)/1 % Triton X-100 at 37 °C for 1 h. The sample was subjected to electrophoresis in an SDS/polyacrylamide slab gel (7.5 %) and electroblotted on to nitrocellulose. The 70 kDa monomer band, visualized by staining with Ponceau S, was excised, blocked with polyvinylpyrrolidone 40, and digested with trypsin as previously described [2]. The supernatant from the trypsin digest of the 70 kDa band contained 9 % of the total ¹⁴C c.p.m. in the AChE applied to the gel.

Acetonitrile-denatured or electroblotted bovine AChE was digested with trypsin (Boehringer-Mannheim Biochemica; sequencing grade; 2 %, w/w; overnight at 37 °C; 5 % final acetonitrile, with 0.1 M added Tes/1.5 mM calcium acetate). The digest was

fractionated by HPLC (Beckman System Gold; with a Vydac C₁₈ column) with a gradient of acetonitrile (Burdick and Jackson) [0.05 % trifluoroacetic acid (TFA) (Pierce)] in water (0.06 % TFA).

Mass spectrometry

Electrospray-ionization mass spectrometry (ESI-MS) spectra were obtained on a triple-quadrupole model TSQ-700 (Finnigan-MAT Corp., San Jose, CA, U.S.A.) equipped with an electrospray ion source. Samples in aqueous solutions containing 50 % methanol and 0.5 % acetic acid were injected directly into the ESI chamber through a stainless-steel hypodermic needle at a rate of 1–2 µl/min. A –3.5 kV difference between the needle tip and source electrode results in the expulsion of charged droplets, and ions with one or more charges may be generated from a single molecular species. Collision spectra (MS–CID–MS) were obtained by selection of the multiply charged parent ion in the first quadrupole, collision-induced dissociation (CID) in the second cell, a curved octapole, and product-ion scanning in a second quadrupole. For collisions the energy was offset by –15 eV using argon as the collision gas.

DNA sequencing

DNA complementary to the region of interest was synthesized by PCR utilizing bovine single-animal genomic DNA (Clontech) and primers generated to the human AChE sequence. These primers, a gift from Dr. O. Lockridge (University of Nebraska Medical Center, Omaha, NE, U.S.A.), had the sequences CCAGGCCT-GCGCCTTCTGGAACCGCTT (A526–F535) and TAGGAG-CTCCAGCGGTGGAAGCTC (Y563–E556, complement reversed). The reaction mixture (100 µl) contained 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 0.01 % gelatin, 0.7 mM MgCl₂, 50 µM each dNTP, 2 mM dithiothreitol, 12.5 units of *Taq* DNA polymerase (Gibco/BRL), 1 µg of DNA and 1 µg of each oligonucleotide primer. The thermal profile involved 35 cycles of 1.25 min at 95 °C, 1 min at 60 °C and 3 min at 72 °C, followed by one cycle of 1 min at 94 °C, 1 min at 60 °C and 7 min at 72 °C. The DNA product was sequenced by Mr. Chuck Mountjoy of the Molecular Biology Core Laboratory of the University of Nebraska Medical Center, using fluorescent terminators, on an Applied Biosystems model 373A automated sequencer. Initial sequencing of a PCR product made with 1.5 mM MgCl₂, 250 µM dNTPs and DNA possibly mixed from more than one animal used primers A526–F535 and AGTGTAAACCCCTCTCTT (–27 to –11 Exon 5; also from O. Lockridge) and allowed design of an optimized sequencing primer CTCCTCCTCTCCCGGCT-CCTGCG (‘3006’, complement reversed) used to obtain residues 1–250 of the final sequence reported in Figure 1.

Protein sequencing and amino acid analysis

Fractions isolated by HPLC containing reduced and carboxyamidomethylated peptides were analysed on a pulsed-liquid sequencer (Applied Biosystems; model 477A) in the Case Western Reserve University Molecular Biology Core Laboratory. Sequences were also determined using a gas-phase sequencer (Applied Biosystems; model 470A) in the laboratory of Dr. Wolfgang Fischer at the Salk Institute, La Jolla, CA, U.S.A. Radiomethylated lysine and [¹⁴C]carboxyamidomethylated cysteine residues were identified by liquid-scintillation counting aliquots of effluent fractions. Amino acid analysis of radiomethylated amino acids and anchor amines was as described by Haas and Rosenberry [12].

Figure 1 Summary of bovine AChE DNA sequence data

The bovine DNA sequence obtained from sequencing a PCR product complementary to bovine genomic DNA (see the Experimental section) is aligned with the DNA sequences of human and mouse AChE [14]; (exon nomenclature as in [15]). Bovine code gives the amino acid sequence deduced from the DNA sequence. Identity between corresponding nucleotide sequences is indicated by :. Lower-case letters in the bovine sequence indicate less certain sequence assignments; lower-case letters in the human and mouse sequences indicate introns. Gaps introduced to preserve intersequence homology are indicated by — and = [14]. Underlines with arrowheads mark PCR primers: A526–F535 (synthesis and preliminary sequencing) ends at nucleotide 2; —27 to —11 exon 5 (preliminary sequencing) is at nucleotides 115–151; '3006' (final sequencing, complement reversed) at nucleotides 260–282; Y563–E556 (synthesis) is off the diagram in exon 6, beginning at about nucleotide 960.

Selective reduction and alkylation with iodo[¹⁴C]acetamide

AChE samples were selectively reduced with dithiothreitol and alkylated with iodo[¹⁴C]acetamide (Amersham Corp.; 56–58 mCi/mmol) essentially as previously described [2]. In brief, samples were incubated with dithiothreitol (0.05–1.0 mM) in 10–100 mM Tris/HCl (pH 8.0–8.5), containing 0.1% Triton

X-100 and 1–10 mM edrophonium chloride at 25 °C for 30 min. Iodo[¹⁴C]acetamide (in at least 4-fold molar excess of dithiothreitol) was then added for an additional 30 min at 25 °C in the dark.

RESULTS

Isolation of a tryptic peptide linked to the GPI anchor

Bovine erythrocyte AChE was reductively radiomethylated on free amine groups and treated with PI-PLC to remove the alkylacylglycerol component of the GPI anchor that tends to reduce recoveries of anchor peptide fragments. Assay by octyl-Sepharose binding (see the Experimental section) showed that PI-PLC cleavage was essentially complete: whereas 3% of control undigested AChE activity failed to bind to octyl-Sepharose and 89% was recovered after binding to the resin, 90% of the PI-PLC-treated enzyme remained unbound and only 4% was bound. The cleaved protein was subjected to trypsin digestion, and peptide fragments were resolved by microbore HPLC (Figure 2). Since reductive radiomethylation [16] labels the free ethanolamine and glucosamine residues in the AChE anchor, as well as the five lysine residues [17] and the N-terminus of the protein, the tryptic fragment bearing the delipidated anchor should be among the radiolabelled peaks from the digest. To identify this fragment, corresponding peak fractions from a parallel digest of AChE radiomethylated at high specific radioactivity were subjected to acid hydrolysis and analysed by cation-exchange chromatography on an amino acid analyser [12]. Fractions corresponding to the first major radioactive peak in Figure 2 (*) contained radiomethylated glucosamine and ethanolamine, and the major peptide in this peak was denoted peptide 1. Fractions in the second radioactive peak (near

Figure 2 HPLC profile of trypsin-digested bovine AChE

Bovine AChE was purified by acridinium resin affinity chromatography, ¹⁴C-labelled by reductive radiomethylation to low specific radioactivity (7.7 nmol; 2.9 × 10⁶ c.p.m.) and treated with PI-PLC as outlined in the Experimental section. The AChE sample was repurified by affinity chromatography, selectively reduced and alkylated, denatured with acetonitrile, and digested with trypsin (see the Experiment section). An aliquot of the digest (1.9 nmol) was subjected to HPLC using the indicated gradient of 5–65% acetonitrile (0.06% TFA) in water (0.05% TFA). (A) A₂₁₅; (B) radioactivity in 10 μl aliquots of 200 μl (1 min) fractions. Total recovery of radioactivity was 95%. The asterisk indicates the peak containing radiomethylated components of the GPI anchor (see the text).

Figure 3 MS of peptide-linked GPI and its deduced glycan structure

A peptide 1 sample was subjected to ESI-MS, and the collision spectrum (MS-CID-MS) obtained by selecting the m/z 3798 parent ion of $+4$ charge is shown. Mass values for the fragments are marked (e.g. doubly charged 966⁺² at m/z 483); (M)⁺⁴ denotes the quadruply charged 3798⁺⁴ parent ion at m/z 950. Asterisks mark major peptide peaks resulting from preferential cleavage before Pro₁₄: b₁₃⁺² at m/z 680, y₁₃⁺³ at m/z 813 and y₁₃⁺² at m/z 1220 (see Figure 6 and Table 1). The deduced glycan structure is indicated at the top, together with the molecular masses of fragments predicted from this structure which were found in the spectrum.

200 min), in contrast, did not have radiomethylated glucosamine, ethanolamine or lysine, identifying the corresponding peptide as the protein N-terminal fragment (results not shown). Throughout the text the positions of residues in peptide 1 and 2 sequences are denoted by subscripts, whereas those in the full-length protein sequence are denoted by superscripts.

Structural determination of the GPI glycan by MS

A peptide 1 sample was subjected to ESI-MS, and the CID-MS spectrum resulting from a m/z 3798 parent ion in the $+4$ state is shown in Figure 3. Characteristic glycan fragmentation peaks are marked, and the inferred glycan structure is shown at the top of the Figure. Most features are entirely consistent with the human AChE anchor structure (see the Introduction). As expected from the susceptibility of the anchor to PI-PLC, however, the inositol ring is not palmitoylated. One significant difference involves a glycan substituent: the hexose next to glucosamine bears an additional *N*-acetylhexosamine residue in the bovine anchor. Note that this finding is supported experimentally by two strong peaks in the spectrum, 3591⁺³ and 3591⁺⁴, which derive from the parent ion by loss of only *N*-acetylhexosamine. Glycans from several other anchors show this same variation in addition of *N*-acetylhexosamine (see the Discussion).

DNA Sequence Analysis

Human: 535 FLPKLLSAT ASEAPSTCPGFTHGEAA-R-RPGLPLPLLLLHQLLLFLSHLRRL*

Mouse: FLPKLLSAT ATEAPCTCPSPAHGEAAP--RPGPDALSLFFFLHSGLPWLK

Rat: FLPKLLSAT ATEVPCTCPSPAHGEAAP--RPGPALSLFFFLHSGLRWL*

Bovine: FLPKLLSAT//ASEAPCTCSGPAHGEAAPRPGLPLPLLLLFLHSLRLRL*

Bovine AChE Peptide Analysis

Peptide 1

Edman FLPKLLSAT ASEAPCTCSGPAHq

ESI-MS FLPKLLSAT ASEAPCTCSGPAHq

Peptide 2

Edman LLSAT ASEAPCTCSGPAHq

Figure 4 Summary of bovine AChE amino acid sequence data

Amino acid sequences for bovine AChE are aligned. Sequences are deduced from the DNA sequences as in Figure 1. The DNA splice junction is indicated by //; - denotes a gap introduced in the listing to preserve intersequence homology; * marks a stop codon. The sequence of peptide 1 was deduced from ESI-MS (Tables 1 and 2 and Figure 6) and Edman sequencing (400 pmol) and that of peptide 2 from Edman sequencing (Figure 5, repeated analyses of 50–200 pmol). Lower-case letters indicate ambiguity and underlines mark indefinite ESI-MS assignments (e.g. residues 1–3 inferred only from sum in fragment b₄ and fragmentation Pro₃, and Leu/Ile have the same mass). Note that peptide sequences end at Gly corresponding to residue 23 in peptide 1, the acceptor site for the GPI anchor, whereas DNA sequences continue for 25 or more residues in the C-terminal signal sequences removed during GPI transfer.

CID-MS spectra of the m/z 3798 parent ion in the $+4$ and particularly in the $+5$ ionization state also contained substantial information about the peptide portion of the anchor fragment. However, the interpretation here was much less straightforward than for the glycan, because of both methodological difficulties and peculiarities of the fragment itself. Analysis by protein and ultimately DNA sequencing was required to obtain the complete sequence shown in Figure 4 below. The latter experiments will be presented here first to provide context and comparison for the CID-MS data.

DNA sequencing of the bovine AChE gene

During the course of our experiments, DNA sequencing of the human [14] and nearly identical mouse [14] and rat [18] AChE genes was reported. The mammalian AChEs are derived from these genes by alternative RNA splicing of invariant exons 1–4 with either exon 5 or exon 6 (reviewed in [1]). Exon 5 encodes the C-terminus and the C-terminal signal sequence for the GPI-anchored form of AChE (Figure 1). The murine sequences are particularly intriguing because they contain cysteine residues at both residue 549 and residue 551 by the standard mammalian AChE sequence numbering [17], whereas the human enzyme has only one cysteine corresponding to residue 551 (Figure 1). To determine the C-terminal sequence of GPI-anchored bovine AChE, to confirm whether it contains two cysteine residues and also to gain new information about the nucleic acid structure of the bovine AChE gene, we undertook DNA sequencing of this region. Cross-species PCR was performed, using bovine genomic DNA and primers matching the homologous human gene, and the DNA sequence of the PCR product determined. Both PCR and sequencing proved technically somewhat challenging. The DNA sequence, which is compared with the human and mouse sequences in Figure 1, gave a predicted protein sequence for bovine AChE (Figure 4) containing both Cys⁵⁴⁹ and Cys⁵⁵¹ residues. It also indicated that this region crosses a splice site

Figure 5 Release of radioactivity during automated Edman sequencing of radioalkylated peptide 2 from bovine AChE

Purified AChE (4 nmol) was selectively reduced with 1 mM dithiothreitol, alkylated with 4 mM iodo[^{14}C]acetamide, and cleaved with PI-PLC, and the electroblotted AChE band was incubated with trypsin as outlined in the Experimental section. The supernatant from the trypsin digest (0.15 nmol of AChE) was fractionated by HPLC in a protocol similar to that in Figure 2. The fraction corresponding to the most prominent radiolabelled peak was subjected to automated Edman sequencing, and an aliquot of the effluent from each cycle was counted by liquid scintillation as shown. Asterisks indicate cycles in which an A_{270} peak corresponding to the PTH derivatives of Glu or (carboxyamidomethyl) Cys was observed.

between two exons (see the Discussion) and showed that, although the coding regions are highly conserved, the bovine and mouse genes have a longer intron than the human gene.

Edman sequencing of tryptic peptides

AChE peptides were also analysed by direct protein sequencing. Automated Edman analysis of 400 pmol of peptide 1, corresponding to the asterisked peak of Figure 2, yielded the sequence shown in Figure 4. Cycle 4 was assigned as [^{14}C]methylated lysine based on a discrete release of radioactivity in the sequencer effluent together with the appearance of a novel phenylthiohydantoin (PTH) derivative peak. Since trypsin cleavage does not occur at methylated lysine residues, the first four amino acids of peptide 1 were retained in the C-terminal peptide.

The sequence was not conclusive, however, as the yield of PTH derivative in cycle 23 was too low to permit assignment with confidence. Furthermore the PTH derivatives of (carboxyamidomethyl)cysteine and glutamate were coeluted in the HPLC system, so these two residues could not be distinguished in cycles 12, 15 and 17.

In a complementary approach, the C-terminal tryptic fragment of bovine erythrocyte AChE was isolated after selective reduction of intersubunit disulphides and radioalkylation of the reduced thiol groups with ^3H - or ^{14}C -labelled iodoacetamide. This approach offered a number of advantages. Most AChEs have no free thiol groups, and selective reduction under non-denaturing conditions has been shown to allow virtually exclusive radioalkylation of a cysteine residue near the C-terminus that is involved in intersubunit disulphide bonding ([2]; see the Discussion). The radiolabelled cysteine then provides a signal both to isolate the C-terminal tryptic peptide and to identify the cycle in which the label is released during Edman sequencing. HPLC fractionation of tryptic peptides obtained from selectively reduced and radioalkylated bovine AChE resulted in a single prominent radiolabelled peak (results not shown). GC-MS analysis [8] of acid hydrolysates of fractions from this peak revealed inositol, as expected for the C-terminal peptide conjugated to the GPI glycan. Repeated Edman sequencing of the peak fraction gave a single consensus sequence denoted peptide 2 in Figure 4. Peptide 2 began at the fifth residue in peptide 1, consistent with trypsin cleavage of the Lys-Leu bond in the radioalkylated AChE, and the remainder of the sequence was in agreement with peptide 1. Sequencing yields were too low to identify the expected histidine and glycine residues in cycles 18 and 19, and coelution of the PTH derivatives of (carboxyamidomethyl)cysteine and glutamate again prevented these two residues from being distinguished in the A_{270} trace from cycles 8, 11 and 13. However, measurement of the radioactivity released in each sequencing cycle indicated when the PTH derivative of [^{14}C]carboxyamidomethylcysteine was released (Figure 5). For example, no radioactivity was released with the PTH derivative of the residue predicted by PCR analysis to be Glu 546 in cycle 8, but a sharp rise in radioactivity occurred at cycle 11 cor-

Table 1 MS of peptide fragments, residues 1–13

The collision spectrum (MS–CID–MS) was obtained by selecting the m/z 3798 parent ion of +5 charge in a peptide 1 sample. Peak assignments tabulated here are for peptide residues 1–13. N-Terminal fragments b_x terminate after residue x , and C-terminal fragments y_x include glycan and are initiated at residue $x+1$. The predicted masses for singly charged b_i^{+1} fragments are shown, together with the mass of the corresponding observed peak and the difference (Δ = observed–predicted). Observed masses and differences are also shown for doubly charged b_i^{+2} and triply charged y_i^{+3} fragments.

Residue	Amino acid	Mass	b_i^{+1}			b_i^{+2}		y_i^{+3}	
			Predicted	Observed	(Δ)	Observed	(Δ)	Observed	(Δ)
1	F	148.2	148.2						
2	L	113.2	261.4						
3	P	97.1	358.5						
4	K(Me) $_2$	156.2	514.7	514.2	(–0.5)				
5	L	113.2	627.9	627.8	(–0.1)				
6	L	113.2	741.0	741.7	(0.7)*				
7	S	87.1	828.1	827.7	(–0.4)	413.5	(–0.6)		
8	A	71.1	899.2	899.2	(0.0)*	449.8	(0.2)*	966.3	(0.0)*
9	T	101.1	1000.4	1000.9	(0.5)	500.2	(0.0)	932.6	(0.1)*
10	A	71.1	1071.4	1068.2	(–3.2)*	534.8	(–0.9)*	909.7	(0.8)
11	S	87.1	1158.5	1157.8	(–0.7)	578.9	(–0.4)	880.3	(0.5)*
12	E	129.1	1287.7	1287.9	(0.2)	644.0	(0.2)	836.7	(–0.1)*
13	A	71.1	1358.8	1358.2	(–0.6)	679.5	(0.1)	813.3	(0.2)

* Observed peak matches more than one predicted peptide or glycan fragment.

Figure 6 MS and deduced C-terminal peptide structure

A peptide 1 sample was subjected to ESI-MS, and a portion of the CID mass spectrum of the m/z 3798 parent ion at +5 charge is shown. Peak nomenclature: N-terminal fragments b_x terminate after residue x , and C-terminal fragments y_x include glycan and are initiated at residue $x+1$. Doubly cleaved fragments p_x begin at Pro₃ and terminate at residue x , and fragments q_x begin at Pro₁₄ and terminate at residue x .

responding to Cys⁵⁴⁹. A lower shoulder of radioactivity release persisted through cycles 12–14. Since incomplete cleavage in the Edman reaction can result in a one- or two-cycle delay in the release of some of the radiolabelled cysteine from cycle 11, these data did not confirm the identity of Cys⁵⁵¹ at cycle 13.

Structural determination of peptide 1 by MS

CID-MS spectra permitted straightforward sequencing of the first 13 residues of peptide 1, as shown by the predicted and observed peptide peaks in Table 1. N-Terminal fragments that were both singly charged (b_4^{+1} – b_{13}^{+1}) and doubly charged

(b_7^{+2} – b_{13}^{+2}) were observed. Many complementary C-terminal fragments were also found as indicated in Table 1. A portion of the CID-MS spectrum of the m/z 3798 parent ion in the +5 state is shown in Figure 6, and several peaks listed in Table 1 are indicated.

Further sequencing was much more difficult, however, as several predicted peaks were not apparent above background noise. Much of the problem arose from the presence of three proline residues, at positions 3, 14 and 20 of the peptide. The exceptional affinity of the imino nitrogen for protons causes a peptide molecular ion to fragment preferentially before proline [19,20]. Fragmentation was particularly favoured before Pro₁₄: note the strong b_{13}^{+2} and y_{13}^{+3} peaks in Figure 6, and the b_{13}^{+2} , y_{13}^{+2} and y_{13}^{+3} peaks (*) dominating the M^{+4} peptide spectrum in Figure 3. Secondary cleavage of the resulting ion then yields a family of fragments that begin with proline. Such subfragmentation introduced much complexity here: cleavage at Pro₃ produced p_6^{+1} , p_7^{+1} , p_8^{+1} , p_{13}^{+1} and p_{13}^{+2} (not shown) and several p_i^{+2} and p_i^{+3} peaks (in Table 2 below and Figure 6), and cleavage at Pro₁₄ gave at least two q_i^{+1} peaks in Table 2 as well as up to 15 more glycan fragments with peak sizes comparable with if not larger than those of the peptide fragments (not shown). These proline cleavages also suppressed formation of informative fragments in the central region of the spectrum of greatest interest.

Table 2 itemizes fragments in the M^{+5} spectrum that partially resolve the sequence of residues 14–23 in peptide 1. Peaks in the spectrum that could arise from more than one fragment could not be assigned unambiguously, and these peaks are asterisked in Table 1 and 2. There is at least one unambiguous peak reflecting cleavage at each of the residues 16–18 and 20–22; ambiguity at residue 23 only concerns the fragmentation, not the origin of the peak. Although there is no identifiable peak derived from cleavage after Pro₁₄, at least two q_i peaks (Table 2) and up to 15 glycan fragments (not tabulated) appear that are attributable to cleavage before Pro₁₄. Thus, although the MS evidence is not simple to interpret, in the aggregate it provides strong support for the deduced sequence indicated in Figure 4.

Table 2 MS of peptide fragments, residues 14–23

The collision spectrum (MS–CID–MS) was obtained by selecting the m/z 3798 parent ion of +5 charge in a peptide 1 sample. Peak assignments tabulated here are for peptide residues 14–23. N-Terminal fragments b_x terminate after residue x , and C-terminal fragments y_x include glycan and are initiated at residue $x+1$. Doubly cleaved fragments p_x begin at Pro₃ and terminate at residue x (p_i^{+2} and p_i^{+3}), and fragments q_x begin at Pro₁₄ and terminate at residue x (q_i^{+1}). The predicted masses for singly charged b_i^{+1} fragments are shown, and the observed masses and the differences (Δ = observed – predicted) for fragments b_i^{+2} , b_i^{+3} , y_i^{+2} . C(cm), carboxyamidomethylcysteine.

Residue	Amino acid	Mass	Predicted b_i^{+1}	Observed peaks									
				b_i^{+2}	(Δ)	b_i^{+3}	(Δ)	p_i^{+2}	(Δ)	p_i^{+3}	(Δ)	q_i^{+1}	(Δ)
14	P	97.1	1455.9										
15	C(cm)	160.2	1616.0	808.8	(0.8)*			679.5	(2.1)*	449.8	(–1.8)*		
16	T	101.1	1717.2									1041.1	(0.7)‡
17	C(cm)	160.2	1877.3	938.5	(–0.2)	626.0	(0.2)	808.8	(0.8)*				
18	S	87.1	1964.4	982.3	(0.1)	655.8	(1.0)*			567.7	(0.0)*	606.5	(0.8)
19	G	57.1	2021.5					880.3	(0.2)*			664.7	(1.9)*
20	P	97.1	2118.6			705.2	(–1.0)	928.5	(–0.1)	619.8	(0.7)		
21	A	71.1	2189.7							644.0	(1.2)*	832.0	(1.0)
22	H	137.2	2326.8							687.5	(–1.0)		737.0
23	G	57.1	2383.9							706.9	(–0.7)†	1026.7	(1.4)*

* Observed peak matches more than one predicted peptide or glycan fragment.

† Observed peak matches two predicted fragments that both reflect cleavage at Gly₂₃.

‡ Peak observed only in M^{+4} spectrum.

§ Peak observed in both M^{+4} and M^{+5} spectra.

|| Also supported by y_{22}^{+1} = 1472.9 (Δ = 1.7) and y_{22}^{+3} = 491.4 (Δ = 1.0).

Table 3 Titration of partially reduced radiomethylated AChEs with iodo[¹⁴C]acetamide

Purified bovine and human AChEs were [³H]methylated and repurified by affinity chromatography as outlined in the Experimental section. Bovine AChE was partially reduced and alkylated with iodo[¹⁴C]acetamide as described in Figure 7, and a human AChE sample (0.2 nmol; 450 000 ³H c.p.m.) in 100 mM Tris/HCl (pH 8.5)/0.1% Triton X-100/1 mM edrophonium was partially reduced by addition of dithiothreitol (to 0.5 mM) at 0 °C for 1 h and alkylated by addition of iodo[¹⁴C]acetamide (to 2 mM) for another hour at 0 °C in the dark. Dialysed aliquots were either analysed directly by SDS/PAGE and gel slicing as in Figure 7 ('No further reduction') or treated with 40 mM dithiothreitol in SDS sample buffer (1 h at 25 °C) followed by alkylation in the dark with a 4-fold excess of unlabelled iodoacetamide (30 min; 25 °C) and then analysed by SDS/PAGE and gel slicing ('After full reduction'). Molar quantities of AChE and incorporated iodoacetamide in the dimer and monomer bands were calculated from the eluted radioactivity and the specific radioactivities of the proteins (see the Experimental section) and iodo[¹⁴C]acetamide (90.5 c.p.m./pmol [2]). Presented in the table are the percentage of monomer, determined from the ³H radioactivities, and the mol of iodo[¹⁴C]acetamide (IAA) incorporated/mol of protein. In an independent control experiment ('Non-reduced control'), non-reduced AChEs incorporated little radioactivity on incubation in the dark with iodo[¹⁴C]acetamide (0.4 mM at 25 °C for 1 h).

Treatment	Bovine AChE			Human AChE		
	Monomer (%)	IAA monomer	IAA dimer	Monomer (%)	IAA monomer	IAA dimer
Non-reduced control	0	0	0.01	0	0	0.01
No further reduction	21	0.88	0.10	46	0.83	0.12
After full reduction	72	0.28	0.11	95	0.38	0.47

Intersubunit disulphide bonding in bovine erythrocyte AChE

Human erythrocyte AChE is a homomeric dimer, with its two polypeptide chains linked by a single disulphide bond at Cys⁵⁵¹ near the C-terminus [21]. The finding that the bovine AChE contains both Cys⁵⁴⁹ and Cys⁵⁵¹ in this region prompted us to conduct a series of selective reduction and radioalkylation measurements to determine whether both of these residues participate in interchain disulphide bonding. Mammalian AChEs have six other cysteine residues in the catalytic subunit, but these are involved in three characteristic intrasubunit disulphide bonds and thus are unavailable for interaction with the two C-terminal cysteine residues (see the Discussion). Bovine AChE incorporated negligible radioactivity when a control was treated with 0.4 mM iodo[¹⁴C]acetamide before reduction (Table 3), indicating that all cysteines are either bound in disulphide linkage or are inaccessible to the alkylating reagent. Accessibility did not appear to be a major problem, since ESI-MS analysis of peptide 1, which was produced after selective reduction of radiomethylated bovine AChE with 5 mM dithiothreitol at pH 8.5 and alkylation with 40 mM iodoacetamide, clearly revealed that both Cys⁵⁴⁹ and Cys⁵⁵¹ were carboxyamidomethylated (Table 2).

To focus as exclusively as possible on cysteine residues involved in intersubunit disulphide bonding, we determined the minimum concentrations of dithiothreitol necessary to achieve partial dissociation of [³H]methylated bovine and human erythrocyte AChEs. Based on the appearance of a monomer band on SDS/PAGE gels, 20–50% dissociation was observed at 0.05 mM dithiothreitol for bovine AChE and 0.5 mM dithiothreitol for human AChE (results not shown). Alkylation of the partially reduced samples with 2 mM iodo[¹⁴C]acetamide permitted precise quantification of the titration of free thiol groups in the monomer and dimer bands from the ¹⁴C/³H ratio. An example of the titration of partial selectively reduced bovine AChE is shown in Figure 7, and quantification of the titrations of the bovine and human AChEs is given in Table 3. Figure 7 illustrates the good separation of monomers from dimers and the absence of unbound radioactivity at the front of the gel. The ³H labelling profile indicated that 21% of the AChE was converted into monomers. This conversion was accompanied by an increased extent of [¹⁴C]alkylation in the monomer band. On the basis of the ¹⁴C/³H ratio, 0.88 mol of iodo[¹⁴C]acetamide was incorporated/mol of catalytic subunit in the monomer band but only 0.10 in the dimer

Figure 7 Alkylation of partially reduced bovine erythrocyte AChE with iodoacetamide

Bovine AChE was cleaved with PI-PLC, reductively [³H]methylated, and repurified by affinity chromatography as described in the Experimental section. A sample (0.2 nmol; 280 000 ³H c.p.m.) in 100 mM Tris/HCl (pH 8.5)/1 mM edrophonium, was then partially reduced by mixing with a small volume of dithiothreitol (to 0.05 mM) at 0 °C for 1 h and alkylated by addition of iodo[¹⁴C]acetamide (to 2 mM) for another hour at 0 °C in the dark. Unincorporated radioactivity was removed by dialysis against 20 mM sodium phosphate (pH 7), and 40% of the sample was electrophoresed on an SDS/7.5% polyacrylamide gel. The gel was cut into 2 mm slices and extracted with Solvable (New England Nuclear) according to the manufacturer's instructions. ³H and ¹⁴C radioactivity was determined by dual-channel scintillation counting. Dimers (14 mm) were well separated from monomers (36 mm).

band (Table 3). This result was unexpected. The previous data suggested that both Cys⁵⁴⁹ and Cys⁵⁵¹ are involved in intersubunit disulphide bonding and led us to expect that reduction to monomers would result in exposure of two free thiol groups to radioalkylation. The human AChE control incorporated 0.83 mol of iodo[¹⁴C]acetamide/mol of catalytic subunit in the monomer band, a level virtually identical with that in the bovine enzyme (Table 3). Since human AChE catalytic subunits have only one cysteine involved in an intersubunit disulphide linkage, this suggests that reduction of a single bond is also sufficient to convert bovine AChE into a monomer. The conclusion was also supported by incorporation of the label in the dimer band, a comparable 0.10 for bovine and 0.12 for the human enzyme (Table 3). These small values presumably reflect a slight reduction

and alkylation of intrasubunit disulphides. If conversion of bovine AChE into monomers required breaking two disulphide bonds, some dimers containing only one reduced and alkylated disulphide should also arise and give significantly higher dimer incorporation values. In a final effort to detect such dimers, the partially reduced and radioalkylated samples were fully reduced and analysed by SDS/PAGE. This complete reduction should convert any partially reduced and labelled dimers into monomers and thus decrease the incorporation value in the residual, apparently irreversibly cross-linked dimer band. No such change was observed, however, in the incorporation value of 0.11 (Table 3). Thus no evidence for partially reduced and radioalkylated bovine dimers was obtained. In the parallel control with human AChE, virtually all the protein was fully reduced to monomers; the incorporation of 0.47 for the small amount of label at the dimer position probably just represents non-covalently associated reduced and radioalkylated monomers.

DISCUSSION

A striking experimental difference between bovine and human erythrocyte AChEs is the insusceptibility of the latter to PI-PLC. The difference was shown previously to reflect a structural divergence in their GPI anchors: only the human AChE GPI anchor is palmitoylated on the inositol ring [9,10]. In the present paper we have explored further differences in the structure of these anchors by analysing the bovine anchor glycan by MS (Figure 3). The bovine glycan was entirely consistent with the human GPI glycan, except that it contained an additional *N*-acetylhexosamine substituent on the core hexose adjacent to glucosamine. Derivatization at this position by *N*-acetylhexosamine (generally GalNAc) is a relatively common modification of mammalian GPI anchors, occurring also on those from *Torpedo* AChE [22], Thy-1 from brain [23] and thymocytes [24], and subpopulations of GPIs from bovine liver 5'-nucleotidase [25] and scrapie prion protein [26]. In certain subpopulations of prion GPI this GalNAc is the proximal residue of a unique extra side chain with the sequence sialic acid-Gal-GalNAc. The mannose adjacent to glucosamine is also the site of a branched-chain galactose modification occurring in the GPIs of trypanosomes [27]. Modelling studies indicate that the substituent at this position fulfils an important space-filling role in the physical structure of the GPI molecules [27].

Protein sequence data obtained in this study for the C-terminus of bovine AChE are summarized in Figure 4. We found, as have others [28,29], that multiple complementary methods were invaluable for building up an unambiguous picture. Two tryptic peptides radiolabelled with different reagents were isolated here: peptide 1 from the [¹⁴C]methylated protein and peptide 2 from the iodo[¹⁴C]acetamide-labelled protein that was radioalkylated after selective reduction. Peptide 1 is four residues longer because methylation has rendered its Lys₁ residue insusceptible to tryptic cleavage. Edman analysis of the two peptides yielded concordant sequences (Figure 4) but still left uncertainties at residues corresponding to 15, 17 and 23 in the 23-residue peptide 1. MS provided independent peptide sequence data in addition to the glycan structure. However, ESI-MS analysis was straightforward only for the first 13 residues (Table 1). The data for residues 14–23 confirm and support the final sequence (Table 2 and Figure 6), but the subfragmentations and lowered yield occasioned by the three proline residues make the ESI-MS data insufficient to establish a sequence. The third technique, DNA sequencing of genomic DNA amplified by PCR (Figure 1), provided protein sequence information by yet another method (as well as further information on the DNA, discussed below).

However, the time, cost and labour to optimize PCR primers and conditions made this a tedious protein-sequencing technique.

The bovine AChE sequence found here diverges from the previously reported C-terminal sequences of fetal bovine serum AChE [17] and bovine brain AChE [2] at Ala⁵⁴⁴, the beginning of the fifth exon in *Torpedo*, human and mouse AChE. This is consistent with the expectation that AChE exon boundaries are highly conserved and therefore easily recognized among various species. The DNA sequencing in Figure 1 also permitted us to compare exon 4, intron 4 and exon 5 (which encodes the GPI signal) in bovine and other mammalian AChEs. Interspecies homology is high, with identity at 16 of the last 23 residues of the mature protein. There is also nearly perfect homology for 11–12 nucleotides at each side of the splice junction. The introns, however, vary considerably: the 123-nucleotide bovine intron is 8 nucleotides longer than the mouse intron, and 46 longer than the human. Protein homology also continues, although at a somewhat lower level, past the C-terminus of the mature protein into the signal sequence that is cleaved on GPI addition. In all four AChEs there is an interesting proline- and arginine-rich stretch between the GPI acceptor site and the hydrophobic domain of the signal sequence, which perhaps has a role in promoting GPI attachment. The sequences conform to the attachment rules suggested by Udenfriend et al., [30] and Moran and Caras [31], with the GPI acceptor glycine being the first of three small amino acids (Gly-Glu-Ala).

An interesting although technically troublesome point in the analysis was to assess whether bovine AChE has more than one cysteine residue available near its C-terminus to mediate dimerization. Previous radioalkylation analysis of collagen-tailed A₁₂ eel electric organ AChE [32], GPI-anchored G₂ human erythrocyte AChE [21] and G₄ bovine brain AChE [2] in every case revealed only 1.0–1.5 selectively reducible thiol groups on the catalytic subunit. The sequence data in Figure 4 clearly indicate that bovine erythrocyte AChE, like murine erythrocyte AChEs, has both Cys⁵⁴⁹ and Cys⁵⁵¹ available for disulphide bonding, but it does not show whether two intersubunit disulphide bonds actually form. Our last series of experiments was an attempt to establish whether both of these cysteine residues are in fact involved in dimer formation. Quantitative analysis by double-label gel slicing showed that, whereas non-reduced bovine AChE had only a low amount of iodo[¹⁴C]acetamide-titratable free SH groups, after reduction, both bovine and control human AChE incorporated 0.83–0.88 mol of iodo[¹⁴C]acetamide/mol of monomeric protein. Thus, rather surprisingly, breakage of precisely one disulphide bond sufficed for monomerization to occur in both cases. X-ray crystallography of (*Torpedo*) AChE shows that the protein C-terminus forms a stem outside the globular core of the molecule [33], indicating that this region should be accessible to exogenous thiol-reactive reagents. Cys⁵⁵¹ is available to form the intersubunit disulphide in human AChE, and it seems unlikely that Cys⁵⁴⁹ in bovine AChE, only two amino acids away and distal to a helix-breaking proline residue, would be sterically inaccessible to iodo[¹⁴C]acetamide after reduction. Titration experiments with dithiothreitol (noted in the Results section) suggested that bovine AChE is somewhat more readily reducible than the human enzyme. *Ad hoc* explanations are possible, such as participation of Cys⁵⁴⁹ in a new intrachain bond present only in bovine AChE or in a disulphide linkage to a small undetected polypeptide. The most likely possibility, however, would seem to be that both Cys⁵⁴⁹ and Cys⁵⁵¹ participate in dimerization, but that because they are so closely spaced they can be reduced in a concerted reaction. Opening of one disulphide bond produces a free SH that may be capable of attacking the other bond, ultimately yielding one AChE monomer chain with two free SH

groups and the other with a cyclic disulphide, at a final stoichiometry of one free SH per chain. Full verification of this model, e.g. by model compounds, mutant proteins or charge-shift gel assay of tryptic peptides, appears beyond the scope of the present study.

This work was supported by grant NS-16577 from the National Institutes of Health and grants from the Muscular Dystrophy Association. We thank Jean Eastman and John Stein for preparation of acetylcholinesterase, Dr. Wolfgang Fischer for DNA sequencing, Dr. Oksana Lockridge for primers, Christopher Eckman and Dr. Steven and Dr. Linda Younkin for much help with PCR and Mr. Chuck Mountjoy for DNA sequencing.

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