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 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 FLPKLLSASETASAPCTC-SGPAHG, in agreement with the MS data and consistent with results from Edman protein sequencing. Dimerization of AChE polypeptides is mediated by intersubunit disulfide 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]Cacetamide, partial reduction and alkylation with iodo[14]Cacetamide 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 disulfide 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-6Manz1-2Manz1-6Manz1-4GlCN 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$_4$ and 10 mM H$_2$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$_4$, 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/ml (low specific radioactivity) by the procedure of Haas and Rosenberry [12]. Alternatively, purified bovine AChE was incubated with purified 1 μg/ml Bacillus thuringiensis phosphatidylinositol-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 (dHPC), at 25 °C for 2 h. PI-PLC-treated bovine AChE and purified human AChE were then reductively radiomethylated with NaCNBH$_3$ and 10 mM HCHO (NEN; 80 mCi/mmol), dialysed against 20 mM sodium phosphate, pH 7.0, and repurified by acridinum resin affinity chromatography. Specific radioactivities of the radiomethylated AChEs, determined by amino acid analysis, were 1670 ± 5 nmol 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 dCPC 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 with 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

[$^{14}$C]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 NH$_4$HCO$_3$, 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 was 970 μl of 20 mM sodium phosphate buffer (pH 8). Octyl-Sepharose (Burdick and Jackson) to enhance subsequent trypsin digestion slightly.

In another series of experiments, bovine AChE selectively reduced and alkylated with iodol[14]C]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 electrophloresed 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 electrophloresed 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 C$_{18}$ column) with a gradient of acetonitrile (Burdoc and Jackson) [0.05% trifluoroacetic acid (TFA) (Pierce)] in water (0.6% 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-GCGCTTCTGGAACCGTT (A526–F535) and TAGGAGCTTACGGCGTGAACCTC (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 MgCl$_2$, 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 Biosystem model 373A automated sequencer. Initial sequencing of a PCR product made with 1.5 mM MgCl$_2$, 250 μM dNTPs and DNA possibly mixed from more than one animal used primers A526–F535 and AGTGTAACCCCTCTTT (−27 to −11 Exon 5; also from O. Lockridge) and allowed design of an optimized sequencing primer CTCTCTCTCCTCCGGT–CCTGG (‘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 carboxymethylated 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. Radioiodinated lysine and [14C]carboxymethylated cysteine residues were identified by liquid-scintillation counting aliquots of effluent fractions. Amino acid analysis of radio iodinated amino acids and anchor amines was as described by Haas and Rosenberry [12].
Glycoinositol phospholipid anchor and C-terminus of acetylcholinesterase

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 ethanalamine, 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 × 10^6 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 (0.05% TFA) in water (0.05% TFA). (A) A_215 and (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).
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.
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 ¹⁴C-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 A₁₅₀ trace from cycles 8, 11 and 13. However, measurement of the radioactivity released in each sequencing cycle indicated when the PTH derivative of [¹⁴C]-carboxyamidomethylcysteine was released (Figure 5). For example, no radioactivity was released with the PTH derivative of the residue predicted by PCR analysis to be Glu⁵⁶ in cycle 8, but a sharp rise in radioactivity occurred at cycle 11 cor-

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 [¹⁴C]methylated lysine based on a discrete release of radioactive activity 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 is shown below.

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.

### Table 1 MS of peptide fragments, residues 1–13

<table>
<thead>
<tr>
<th>Residue</th>
<th>Amino acid</th>
<th>Mass</th>
<th>b₁⁺¹ Predicted</th>
<th>b₁⁺¹ Observed</th>
<th>b₁⁺² Observed</th>
<th>y₁⁺³ Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>148.2</td>
<td>148.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>L</td>
<td>113.2</td>
<td>261.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>P</td>
<td>97.1</td>
<td>358.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>K(Me)₂</td>
<td>156.2</td>
<td>514.7</td>
<td>514.2</td>
<td>(−0.5)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>L</td>
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<td>627.9</td>
<td>627.8</td>
<td>(−0.1)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>L</td>
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<td>741.0</td>
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</tr>
<tr>
<td>7</td>
<td>S</td>
<td>87.1</td>
<td>828.1</td>
<td>827.7</td>
<td>(−0.4)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>71.1</td>
<td>899.2</td>
<td>899.2</td>
<td>(0.0)*</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>T</td>
<td>101.1</td>
<td>1000.4</td>
<td>1000.9</td>
<td>(0.5)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>A</td>
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<td>1071.4</td>
<td>1068.2</td>
<td>(−3.2)*</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>S</td>
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<td>1158.5</td>
<td>1157.8</td>
<td>(−0.7)</td>
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<tr>
<td>12</td>
<td>E</td>
<td>129.1</td>
<td>1287.7</td>
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<td>71.1</td>
<td>1358.8</td>
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</tr>
</tbody>
</table>

* Observed peak matches more than one predicted peptide or glycan fragment.
data did not confirm the identity of Cys release of some of the radiolabelled cysteine from cycle 1, these
Edman reaction can result in a one- or two-cycle delay in the
persisted through cycles 12–14. Since incomplete cleavage in the
x
may

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, terminate after residue x, and C-terminal fragments y, include glycan and are initiated at residue
x + 1. Doubly cleaved fragments p, begin at Pro3 and terminate at residue x, and fragments q,
begin at Pro14 and terminate at residue x.

responding to Cys451. 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 Cys451 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 (b1+–b13+) and doubly charged
(b12+b132+) 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 b13+ and y13+ peaks in Figure 6, and the b13+,
y13+2 and y13+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 p3+1, p3+2, p3×3, p3×3 and p3×3 (not shown) and several
p3+4 and p3+5 peaks (in Table 2 below and Figure 6), and cleavage
at Pro14 gave at least two q1+ 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 MS of peptide fragments, residues 14–23

<table>
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<tr>
<th>Residue</th>
<th>Amino acid</th>
<th>Mass</th>
<th>Predicted b1+x</th>
<th>Observed peaks</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>b10, b11, b12</td>
<td>b1 x (Δ)</td>
</tr>
<tr>
<td>14</td>
<td>P</td>
<td>97.1</td>
<td>1455.9</td>
<td>808.6 (0.8)*</td>
</tr>
<tr>
<td>15</td>
<td>C(cm)</td>
<td>160.2</td>
<td>1616.0</td>
<td>679.5 (2.1)*</td>
</tr>
<tr>
<td>16</td>
<td>T</td>
<td>101.1</td>
<td>1717.2</td>
<td>1041.1 (0.7)†</td>
</tr>
<tr>
<td>17</td>
<td>(cm)</td>
<td>160.2</td>
<td>1877.3</td>
<td>908.8 (0.8)*</td>
</tr>
<tr>
<td>18</td>
<td>S</td>
<td>87.1</td>
<td>1964.1</td>
<td>567.7 (1.0)*</td>
</tr>
<tr>
<td>19</td>
<td>G</td>
<td>57.1</td>
<td>2021.5</td>
<td>606.5 (0.8)</td>
</tr>
<tr>
<td>20</td>
<td>P</td>
<td>97.1</td>
<td>2118.8</td>
<td>619.8 (0.7)§</td>
</tr>
<tr>
<td>21</td>
<td>A</td>
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<td>2189.7</td>
<td>832.0 (1.0)</td>
</tr>
<tr>
<td>22</td>
<td>H</td>
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<td>737.0 (1.4)*</td>
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<tr>
<td>23</td>
<td>G</td>
<td>57.1</td>
<td>2383.9</td>
<td>706.9 (0.7)†</td>
</tr>
</tbody>
</table>

* Observed peak matches more than one predicted peptide or glycan fragment.
† Observed peak matches two predicted fragments that both reflect cleavage at Gly22.
‡ Peak observed only in M+4 spectrum.
§ Peak observed in both M+3 and M+4 spectra.
¶ Also supported by y2×1 = 1472.9 (Δ = 1.7) and y2×3 = 491.4 (Δ = 1.0).
Table 3  Titration of partially reduced radiomethylated AChEs with iodo[14C]acetamide

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Monomer (%)</th>
<th>IAA monomer</th>
<th>IAA dimer</th>
<th>Monomer (%)</th>
<th>IAA monomer</th>
<th>IAA dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine AChE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-reduced control</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>No further reduction</td>
<td>21</td>
<td>0.88</td>
<td>0.10</td>
<td>46</td>
<td>0.83</td>
<td>0.12</td>
</tr>
<tr>
<td>After full reduction</td>
<td>72</td>
<td>0.28</td>
<td>0.11</td>
<td>95</td>
<td>0.38</td>
<td>0.47</td>
</tr>
<tr>
<td>Human AChE</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**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 Cys581 near the C-terminus [21]. The finding that the bovine AChE contains both Cys589 and Cys591 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 Cys589 and Cys591 were carboxymethylated (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 band (Table 3). This result was unexpected. The previous data suggested that both Cys589 and Cys591 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...
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 glycans were entirely consistent with the human GPI glycan, except that it contained an additional N-acetyllactosamine substituent on the core hexose adjacent to glucosamine. Derivatization at this position by N-acetyllactosamine (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 mannoside 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 iodol-14Cacetamide-labelled protein that was radioalkylated after selective reduction. Peptide 1 is four residues longer because methylation has rendered its Lys, residue susceptible 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 Ala83–0, 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 G human erythrocyte AChE [21] and G, 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 Cys83 and Cys85 available for disulphide bonding, but if 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 iodol-14Cacetamide-titratable free SH groups, after reduction, both bovine and control human AChE incorporated 0.83–0.88 mol of iodol-14Cacetamide/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. Cys83 is available to form the intersubunit disulphide in human AChE, and it seems unlikely that Cys83 in bovine AChE, only two amino acids away and distal to a helix-breaking proline residue, would be sterically inaccessible to iodol-14Cacetamide 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 Cys83 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 Cys83 and Cys85 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.

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REFERENCES


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