# The membrane anchor of mammalian brain acetylcholinesterase consists of a single glycosylated protein of 22 kDa

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**Abstract Mammalian brain acetylcholinesterase (AChE; EC 3.1.1.7) is membrane-bound through a structural subunit of about 20 kDa. So far little is known about this anchor because it is only detectable after hydrophobic labelling. In the present study we demonstrate that the two bands migrating around 20 kDa on SDS-PAGE derive from the same protein containing the same N-terminal amino acid sequence. The difference in their mobility is due to different *N*-glycosidation. Radioalkylation of cysteine residues reveals that the anchor contains just the two cysteine residues involved in binding the catalytic subunits.**

*Key words:* Acetylcholinesterase; Hydrophobic label; Membrane anchor; Brain; Amino acid sequence

## 1. Introduction

Acetylcholinesterase (AChE) exists in multiple molecular forms differing in their subunit association and hydrodynamic properties (for reviews, see [1–4]). The majority of AChE comprises two types of catalytic subunits: hydrophobic lipid-linked (H-type) and hydrophilic tailed subunits (T-type) (for nomenclature, see [5]). These may exist as globular monomers ($G_1$-form), dimers ($G_2$-form), and oligomers. In dimers two monomers are covalently linked together by disulfide-bonds. Tetramers ($G_4$-form) are formed by hydrophobic assembly of two dimers. In case of AChE from motor endplates, oligomers of even higher numbers are built up by attachment of $G_4$-AChE through disulfide bonds to a triple-helical collagen-type subunit. This molecular assembly yields a highly asymmetric molecule containing 3 times 4 catalytic T-subunits called $A_{12}$-AChE.

In brain, AChE occurs mainly as a globular tetrameric enzyme ($G_4$-form) of which approximately 80% is amphiphilic membrane bound and about 20% is hydrophilic non-membrane bound. Both forms are made up of pairs of disulfide-bonded dimers of T-subunits. In the amphiphilic membrane bound $G_4$-form, one pair of catalytic T-subunits is disulfide-bonded to a structural subunit of approximately 20 kDa which serves as hydrophobic membrane anchor [6–9] and was denominated P-subunit [5]. Its analysis has been difficult because it is not detected on SDS-polyacrylamide gels stained with Coomassie blue and is barely visible by silver staining. The best way to detect the P-subunits is by autoradiography of SDS polyacrylamide gels after labelling AChE with the hydrophobic reagent 3-trifluoromethyl-3-(*m*-[$^{125}$I]iodophenyl)diazirine ([$^{125}$I]TID, described in [10]). This compound preferentially labels the hydrophobic membrane anchor of AChE with a few counts being incorporated into the catalytic subunits. Under reducing conditions SDS-PAGE of [$^{125}$I]TID labelled AChE shows a broad radiolabelled spot at around 20 kDa, which, as originally shown by Roberts et al. [8], consists of a doublet of closely spaced bands. The possibility thus exists that the membrane anchor of AChE from mammalian brain contains two different subunits one of which being the actual membrane spanning peptide and the other the link to the enzyme. Alternatively the band with the lower molecular mass could either have arisen from the one with the higher mass by proteolysis or by difference in glycosylation.

In order to answer these questions we isolated AChE from calf brain in amounts large enough to allow N-terminal sequencing of the membrane anchor and to investigate possible glycosylation. To test the current model of the subunit assembly of mammalian brain AChE we further estimated the content of Cys residues in the P-subunit using [$^{14}$C]iodoacetamide as label.

## 2. Materials and methods

Unless otherwise stated, all chemicals used were of analytical grade obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Sigma (St. Louis, MO, USA), or from Bio-Rad (Richmond, CA, USA).

### 2.1. Acetylcholinesterase

Detergent-soluble tetrameric AChE containing the P-subunit was purified from frozen calf brain essentially as described by Gennari and Brodbeck [11] using an affinity resin with trimethyl-ammonium *m*-phenylenediamine as affinity ligand [12]. All steps were performed at 4°C in presence of 20 mM EDTA and 2 mM benzamidine hydrochloride as protease inhibitors. With two consecutive affinity chromatography steps, a specific AChE activity of 5900 U/mg protein was routinely obtained.

### 2.2. Enzyme assay

AChE activity was measured at room temperature using the method of Ellman et al. [13] with 1 mM acetylthiocholine iodide and 0.25 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 100 mM sodium phosphate buffer pH 7.4, containing 0.1% Triton X-100. The increase in absorbance was followed spectrophotometrically at 412 nm.

### 2.3. Labelling of calf brain AChE with [$^{125}$I]TID

Hydrophobic labelling of detergent-soluble AChE from calf brain was performed as described by Heider and Brodbeck [9] using [$^{125}$I]TID (Amersham, Buckinhamshire, UK). The resulting [$^{125}$I]TID-labelled enzyme contained about 7500 cpm/U.

### 2.4. Labelling calf brain AChE with [$^{14}$C]iodoacetamide

Calf brain AChE (50 U in 50 μl) was added to 200 μl of a solution containing 0.1 M Tris pH 8.2, and 1-propanol (2:1 v/v) and reduction was carried out according to Rüegg et al. [18] by the addition of 1.2 μl

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*Abbreviations:* AChE, acetylcholinesterase; TID, 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirin e; PVDF, polyvinylidene difluoride.

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of tributylphosphine. The reaction was performed at room temperature in the dark for 3 h with constant shaking. All the steps were performed under nitrogen gas. Alkylation [18] was carried out by adding 15 μl of [$^{14}$C]iodoacetamide (1 mg/ml in $H_2O$) to the reduced AChE. The reaction was allowed to proceed by shaking the sample at room temperature in the dark for 1 h. Thereafter the sample was dialysed overnight against 10 mM phosphate buffer pH 7.4, containing 144 mM NaCl, 0.1% Triton X-100 and 0.05% $NaN_3$ with 3 changes of buffer in a microdialysis apparatus (Pierce, Rochford, IL, USA).

*2.5. SDS-PAGE*

SDS-PAGE was performed essentially according to the method described by Laemmli [14] using 5–15% polyacrylamide gradient gels. Samples were subjected to gel electrophoresis under reducing conditions. Protein was detected by silver stain according to Schoenle et al. [15], or by Coomassie Brilliant Blue R-250. Alternatively, in order to obtain optimal separation of low molecular weight proteins in the region of the 20 kDa membrane anchor, SDS-PAGE was also performed on 10% acrylamide Tricine gels that were casted according to Schägger and v. Jagow [16]. The gels were calibrated with Bio-Rad SDS-PAGE molecular weight standards myosin (200 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).

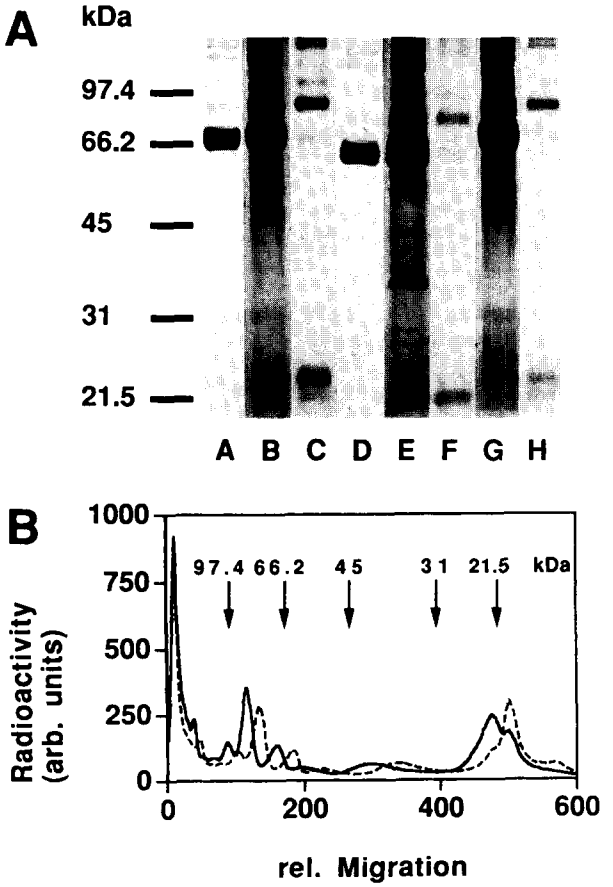



Fig. 1. (A) SDS-PAGE of [$^{125}$I]TID-labelled calf caudate nucleus AChE. Purified AChE was run in reducing conditions on 10% acrylamide Tricine gel before (lanes A–C) and after (lanes D–F) treatment with *N*-glycosidase F, visualized by Coomassie (lanes A, D), silver (lanes B, E), and autoradiography (lanes C, F). The additional band at 36 kDa on lane E is *N*-glycosidase F. Lanes G and H show silver stain and autoradiograph, respectively, of mock-treated AChE. The gel was calibrated as described in section 2. (B) Phosphorimage scan of lane C and F of Fig. 1A. [$^{125}$I]TID-labelled AChE before (solid line) and after treatment with *N*-glycosidase F (dotted line).

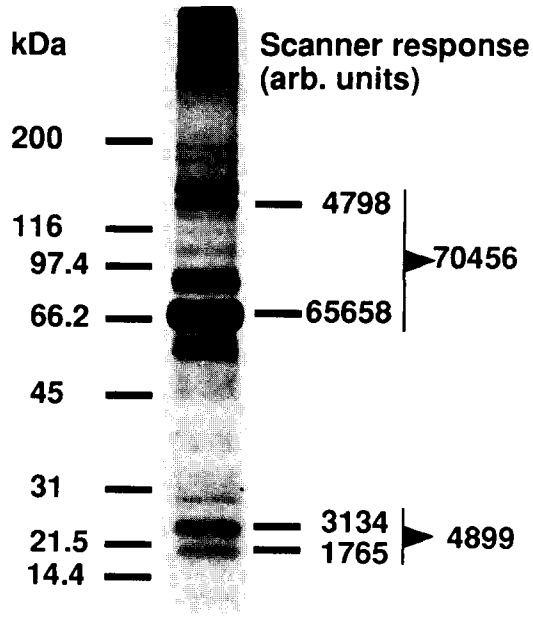



Fig. 2. SDS-PAGE (5–15% acrylamide gradient gel) of calf caudate nucleus AChE reduced with tributylphosphine and alkylated with [$^{14}$C]iodoacetamide. Radioactivity was quantified by counting the gel on a phosphor imager. Left: calibration of gels as described in section 2. Right: radioactivity incorporated into catalytic subunits not carrying the membrane anchor (light monomer and light dimer) and in membrane anchor (light and heavy P-subunit).

*2.6. Electroblotting on polyvinylidene difluoride membranes and staining*

After electrophoresis, proteins from acrylamide gels were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Pro Blot, Applied Biosystems, Foster City, CA, USA) in a tank-blot system (BioRad). Blotting was performed overnight at 30 V in a modified borate buffer containing 50 mM sodium borate buffer (pH 9.0) containing 20% (v/v) methanol, 0.05% (w/v) SDS and 1 mM thioglycolic acid. Blotted proteins were detected by staining with 0.1% amido black in 20% (v/v) aqueous methanol. Transfer efficiency for proteins smaller than 90 kDa was quantitative as no radioactive label was retained on the gel.

*2.7. Amino acid sequencing*

[$^{125}$I]TID-labelled protein bands detected by autoradiography of PVDF membranes were excised and subjected to automated Edman degradation on an Applied Biosystems sequencer model 477A according to the procedure described by Schaller et al. [17].

*2.8. N-Glycosidase F treatment*

AChE from calf brain (30 U) was dried under vacuum and redissolved in 15 μl of 50 mM sodium phosphate buffer pH 8.3, containing 10 mM EDTA, 0.1% SDS and 10 mM β-mercaptoethanol. For complete denaturation, the enzyme containing sample was incubated for 5 min at 95° C. After cooling to room temperature (20–25°C) β-D-octylglucoside to a final concentration of 1% and 0.4 U *N*-glycosidase F (Boehringer, Mannheim, Germany) were added. Incubation was carried out at 37° C for 20 h.

## 3. Results and discussion

To characterize the P-subunit of brain AChE, the enzyme was purified from calf brain and its subunits were separated by SDS-PAGE on 10% acrylamide Tricine gels [16] under reducing conditions. As seen in Fig. 1A, lane A, Coomassie staining gave essentially one band at 70 kDa corresponding to the monomer

Table 1
N-Terminal amino acid sequence of the P-subunit

| Sample | N-Terminal sequence | |
|---|---|---|
| 22 kDa band | EPQKSCSKYTD | |
| 20 kDa | EPQKSCSK | |
| Purified AChE: reduced and alkylated | EGPEDPELLV | (main sequence) |
| | EPQK | (minor sequence) |

Sequences of 22 and 20 kDa membrane anchor peptides (heavy and light P-subunits) were obtained after separation from the catalytic subunits by SDS-PAGE. Alternatively, the whole purified enzyme was sequenced resulting in a main sequence from the T- and in a minor sequence from the P-subunit. (The Cys residue in position 6 was detected by radioactivity after labelling of AChE with [$^{14}$C]iodoacetamide.)

of the catalytic subunit without membrane anchor (light monomer). The faint band appearing at 90 kDa is the catalytic subunit to which the structural subunit remained attached despite the presence of the reducing agent (heavy monomer). The faint bands at higher molecular masses represent dimers without anchor (light dimer) and with anchor (heavy dimers) as well as higher molecular mass aggregates (for assignment and nomenclature of bands see [19]). After staining the gel with silver several additional bands became visible (Fig. 1A, lane B). Those appearing at 22 and 20 kDa were also seen in the autoradiograph of [$^{125}$I]TID-labelled enzyme (Fig. 1A, lanes B and C) and represent two forms of the P subunit of brain AChE. The band at 22 kDa was assigned as heavy P-subunit, the one at 20 kDa as the light one.

To obtain information on the nature of the proteins appearing at 22 and 20 kDa, the two bands were electrophoretically transferred to PVDF membranes and subjected to N-terminal sequencing. As shown in Table 1, protein in both bands yielded the same N-terminal amino acid sequence showing that the bands at 22 and 20 kDa derive from the same polypeptide. The amino acid residue in position 6 could not be detected by HPLC. Since it may be a Cys residue AChE was labelled with [$^{14}$C]iodoacetamide and subjected to Edman degradation. Measuring the radioactivity in each fraction showed a peak (50% over background) at amino acid 6 proving the presence of Cys in position 6. In another approach reduced and alkylated AChE (consisting of the four catalytic subunits and one hydrophobic anchor) was dotted on a PVDF membrane and subjected to N-terminal sequencing. As expected the main sequence corresponded to the N-terminus of the catalytic subunit [20]. In addition four amino acids of a minor sequence could be clearly detected and they corresponded to the N-terminal sequence of the 22 and 20 kDa bands of the hydrophobic anchor (Table 1). Sequence comparison on protein databases (Swiss-Prot, Pir) showed no homology of the N-terminus of the hydrophobic membrane anchor of AChE to any known protein.

Since both the 22 and 20 kDa bands have the same N-terminus, the band with the lower molecular mass might be a proteolytic fragment of the other. Alternatively, differences in glycosylation might also account for the existence of the two bands. As shown previously the catalytic subunits of mammalian brain AChE contain N-linked carbohydrates [19,21,22]. We show now that treatment of the enzyme with *N*-glycosidase F not only decreased the molecular mass of the catalytic subunit (Fig. 1A, lane D) but also reduced the intensity of the 22 kDa band (Fig. 1A, lanes E and F). Control experiments showed that the band at 22 kDa only disappeared after treatment with *N*-glycosidase F and not by mere incubation of the enzyme thus excluding the possibility that the conversion was due to proteolysis during the 20 h incubation period (Fig. 1A, lanes G and H). To visualize the shift of the 22 kDa band to a lower molecular weight the gel was subjected to phosphorimage scanning. As seen from Fig. 1B the band at 22 kDa largely disappeared after deglycosylation while that at 20 kDa was not shifted to a lower molecular mass. From these results we conclude that the membrane anchor of mammalian brain AChE is *N*-glycosylated and that the heterogeneity seen on SDS-PAGE arises from differences in glycosylation.

The current model of subunit assembly of brain AChE predicts that two catalytic subunits are attached to one membrane anchor through two disulfide bonds [8]. Thus, the minimal number of Cys residues expected in the membrane anchor is two. From the primary structure of mammalian brain AChE [8,20] it is known that each of the four catalytic subunits contains 7 Cys residues, or 28 in total. In other words the expected maximum ratio of Cys residues between the four catalytic subunits and the membrane anchor would be 14. In order to test this model we fully reduced AChE and alkylated the free sulfhydryl groups with [$^{14}$C]iodoacetamide. Subsequently, the enzyme was subjected to SDS-PAGE under reducing conditions and the radioactivity recovered in the membrane anchor was compared to that detected in the light monomer and light dimer, i.e. in the catalytic subunits, from which the anchor had been released by disulfide reduction. As seen from Fig. 2, a signal of total 70,456 units was found in the light monomer and dimer while total 4899 units were in the heavy and light P-subunit. The resulting ratio of 14.4 is very close to the above calculated theoretical value of 14. This result shows that only those two Cys residues in the P-subunit could be labelled, to which two of the four catalytic subunits are attached. Since the amino acid residue in position 6 is a cysteine residue, it follows that the N-terminus of the anchor provides the attachment site for the catalytic subunits. It is noteworthy that no homology in the sequence of the N-terminus of the P-subunit was found to the collagenic Q-subunit from *Torpedo* AChE [23] nor to any other sequence known so far. Therefore the previous suggestion that P- and Q-subunits derive from the same gene by alternative splicing is unlikely.

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