Hydrophobic labeling of the membrane binding domain of acetylcholinesterase from *Torpedo marmorata*

S. Stieger, U. Brodbeck, B. Reber* and J. Brunner*

Medizinisch-chemisches Institut der Universität Bern, Bühlstrasse 28, CH-3000 Bern 9 and **Laboratorium für Biochemie der ETH Zürich, Universitätsstrasse 16, CH-8092 Zürich, Switzerland*

Received 24 January 1984; revised version received 2 February 1984

Membrane-bound acetylcholinesterase (AChE) from the electric organ of *Torpedo marmorata* was labeled with the hydrophobic photoactivatable reagent 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). Labeling with [^{125}I]TID was restricted to the membranous polypeptide segment of AChE as shown upon conversion of the amphiphilic form to the hydrophilic one by limited digestion with proteinase K. The labeled membranous segment, which has an M_r of approx. 3000 was isolated by gel filtration on Sephadex LH-60 in ethanol/formic acid.

Acetylcholinesterase Torpedo marmorata *Membrane protein*
Hydrophobic labeling *Proteolytic digestion* *Anchor peptide*

1. INTRODUCTION

The electric organ of *Torpedo marmorata* contains a membrane-bound form of AChE, which can be solubilized with detergent and purified by affinity chromatography [1–3]. The pure enzyme has a subunit M_r of 67000. It can be reconstituted into phospholipid liposomes and requires interactions with amphiphilic molecules for the expression of catalytic activity [4]. By treatment with proteinase K AChE is converted to a catalytically active hydrophilic enzyme [2,5] which is only slightly smaller than the undigested enzyme as concluded from SDS-gel electrophoresis. The proteinase K-treated form is amphiphile independent and no longer interacts with detergents or lipids [4]. Thus the proteinase K-sensitive domain is apparently responsible for the membrane association of AChE.

The photoactivatable reagent [^{125}I]TID has been used to label specifically the hydrophobic domain of various membrane proteins [6–8]. [^{125}I]TID partitions nearly quantitatively into the hydrophobic phase, where the photogenerated carbene labels the intrinsic portion of a membrane protein. Here, [^{125}I]TID has been used to label selectively the membrane binding segment of AChE. This allowed easy detection of the small hydrophobic peptide and the development of a procedure for its isolation.

Abbreviations: AChE, acetylcholinesterase; [^{125}I]TID, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine

2. METHODS

Detergent-soluble AChE from the electric organ of *T. marmorata* was solubilized with Triton X-100 and purified by affinity chromatography as in [9]. Enzyme activity was assayed as in [10]. The purified enzyme (spec. act. 4500 IU/mg protein) was dialysed against 144 mM NaCl and 10 mM Tris–HCl (pH 7.4) until the concentration of Triton X-100 was decreased to 0.06% (A_{280} = 1.2). Just before photolabeling the enzyme (600 IU/ml) was dialysed against 10 mM phosphate buffer (pH 6.8). Then 8 μl [^{125}I]TID [6] (0.5 mCi) were added to 1 ml of enzyme solution. The sample was photolysed in a quartz cuvette at 4°C for 1 min. A 350 W medium-pressure mercury lamp (type

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

350-1008, Illumination Industries) was used as a light source. The beam was directed through filters of circulating water (30 mm) and a saturated solution of copper sulfate (20 mm) onto the center of the cuvette. Labeled AChE was adsorbed to a column of Bio-Gel HTP (0.5 ml) and washed with 10 mM phosphate buffer (pH 6.8) until no further radioactivity was eluted. Then the enzyme was eluted with 200 mM phosphate buffer (pH 7.4). The fractions containing enzyme activity were brought to 1% Triton X-100 and dialysed against 10 mM phosphate buffer (pH 6.8) with 1% Triton X-100. The chromatography on Bio-Gel HTP was then repeated except that all buffers contained 0.1% Triton X-100.

The [^{125}I]TID-labeled AChE was incubated with proteinase K (20 μg/ml) for 90 min at room temperature. The reaction was stopped by adding phenylmethylsulfonyl fluoride to a concentration of 1 mM. The digested enzyme was lyophilized and chromatographed on a Sephadex LH-60 column (1.0 × 40 cm) in ethanol/formic acid (90%) (2.8:1) [11].

SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out essentially as in [12] using gradient gels containing 10–20% polyacrylamide with 2% crosslinker. The gels were stained with silver [13]. For autoradiography gels were exposed to Kodak X-Omat films SO-282 at room temperature for at least 72 h.

3. RESULTS AND DISCUSSION

The pure amphiphilic form of AChE from the electric organ of *T. marmorata* was labeled in buffer containing Triton X-100 using [^{125}I]TID. Then the protein was separated from radioactively labeled detergent by repeated chromatography on Bio-Gel HTP. Firstly, the column was washed with buffer devoid of Triton X-100. Under this condition about 70% of the radioactivity could be separated from the protein. In the second chromatographic step another 29.5% of the radioactivity could be removed with buffer containing 0.1% Triton X-100 (fig.1). Thus under these conditions of labeling, approx. 0.5% of the radiolabel originally introduced was bound to the protein. In both steps, recovery of enzyme activity was 85%. By limited digestion with proteinase K [^{125}I]TID-labeled, amphiphilic AChE was converted to the hydrophilic form. Both native and proteinase K-treated [^{125}I]TID-labeled AChE were subjected to SDS–PAGE (fig.2) and stained for protein (lanes 1 and 3, respectively). Lanes 2 and 4 show the corresponding autoradiograph. In the native enzyme radioactivity was associated with the catalytic subunit with an apparent M_r of 67000. In contrast, the proteinase K-treated enzyme with an apparent M_r of 66000 had lost the radioactively labeled peptide, which appeared at about M_r 3000. This demonstrates that only the proteinase K-sensitive membrane binding domain of AChE was labeled by [^{125}I]TID. To isolate the hydrophobic segment, labeled AChE was treated with proteinase K, lyophilized and chromatographed on a column of Sephadex LH-60 in ethanol/formic acid (90%) (2.8:1) (fig.3). The labeled peptide released by proteinase K digestion eluted at 19.6 ml, undigested enzyme at 7.3 ml. The tailing of the peak of the labeled peptide is probably due to some radioactively labeled Triton X-100 which remained with the protein upon repeated chromatography on Bio-Gel HTP. By calibration of the Sephadex LH-60 column with marker proteins an M_r of 3100 was estimated for the hydrophobic anchor peptide.

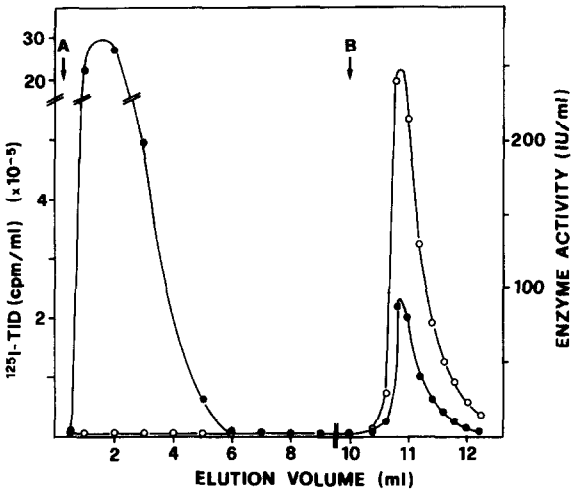

Fig.1. Separation of [^{125}I]TID-labeled AChE from [^{125}I]TID-labeled Triton X-100 by chromatography on Bio-Gel HTP. The column was washed with 10 mM phosphate (pH 6.8) containing 0.1% Triton X-100 (A), then the enzyme was eluted with 200 mM phosphate buffer (pH 7.4) containing 0.1% Triton X-100 (B). Elution profile of radioactivity (●) and enzyme activity (○).

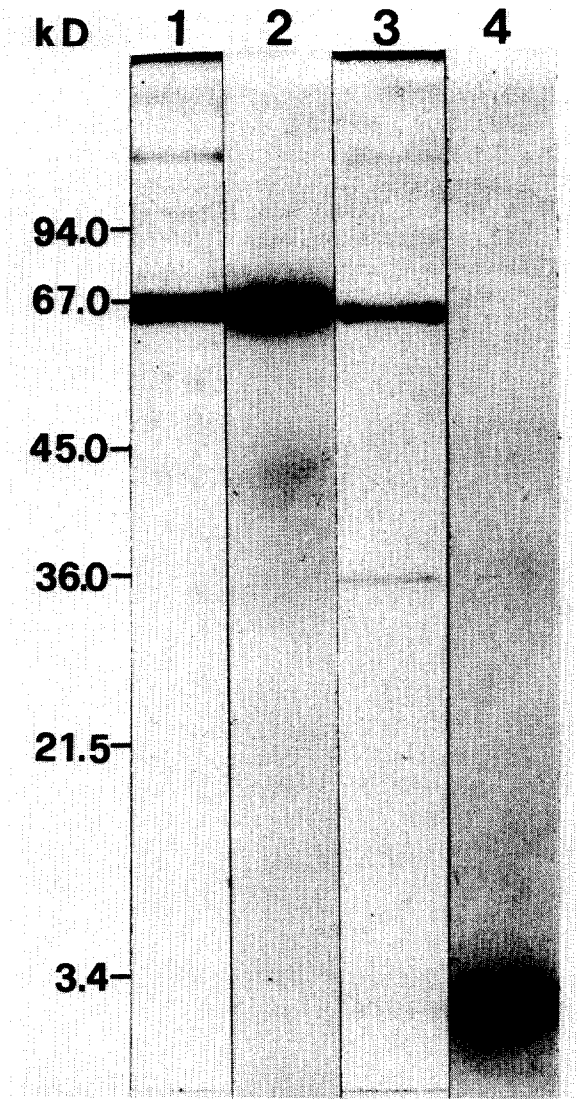

Fig.2. SDS–PAGE of [^{125}I]TID-labeled AChE. The samples contained [^{125}I]TID-labeled native (lanes 1 and 2) or proteinase K-treated (lanes 3 and 4) AChE (2 IU). The gel was stained for protein (lanes 1 and 3) and subjected to autoradiography (lanes 2 and 4). The gel was calibrated with phosphorylase b (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), proteinase K (M_r 36000), trypsin inhibitor (M_r 21500) and insulin (M_r 3400).

This value is remarkably consistent with that obtained by SDS–PAGE. Thus the hydrophobic domain seems to be somewhat larger than that estimated from the difference in electrophoretic mobility between native and proteinase K-treated

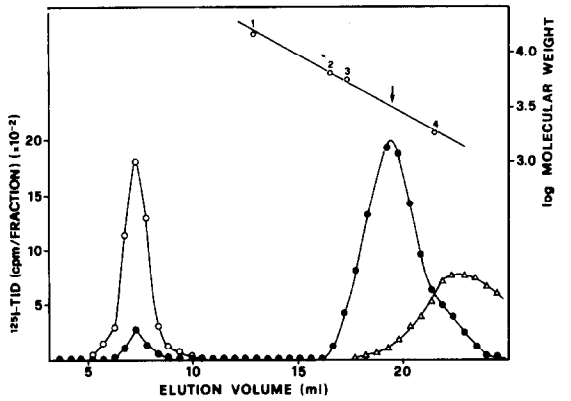

Fig.3. Sephadex LH-60 chromatography of the [^{125}I]TID-labeled peptide which was released by treatment with proteinase K (●). Elution profile of native [^{125}I]TID-labeled AChE (○) and [^{125}I]TID-labeled Triton X-100 (△). The standard proteins were lysozyme (M_r 14300) (1), aprotinin (M_r 6500) (2), insulin (M_r 5700) (3) and gramicidin (M_r 1800) (4).

AChE. Our results show that [^{125}I]TID only labeled a short hydrophobic peptide segment of AChE from *Torpedo* electric organ. This finding is in agreement with those obtained with AChE from human erythrocyte membranes [14] and with the observation that this peptide is essential for reconstitution of AChE into phospholipid vesicles [4]. These results taken together strongly suggest that AChE is anchored to the membrane through this short hydrophobic peptide. Assuming an α-helical structure of the peptide, it would span the membrane only once. With the peptide identified and a method for its purification available it is now possible to isolate the peptide in amounts sufficient for amino acid analysis and sequencing.

REFERENCES

[1] Viratelle, O.M. and Bernhard, S.A. (1980) Biochemistry 19, 4999–5007.
[2] Bon, S. and Massoulie, J. (1980) Proc. Natl. Acad. Sci. USA 77, 4464–4468.
[3] Lee, S.L., Camp, S.J. and Taylor, P. (1982) J. Biol. Chem. 257, 12302–12309.
[4] Brodbeck, U. and Ott, P. (1984) in: Cholinesterases – Fundamental and Applied Aspects, Proc. 2nd Int. Meet. on Cholinesterases (Brzin, M. et al. eds) De Gruyter, Berlin, in press.
[5] Li, Z.Y. and Bon, C. (1983) J. Neurochem. 40, 338–349.

[6] Brunner, J. and Semenza, G. (1981) Biochemistry 20, 7174–7182.
[7] Spiess, M., Brunner, J. and Semenza, G. (1982) J. Biol. Chem. 257, 2370–2377.
[8] Frielle, T., Brunner, J. and Curthoys, N.P. (1982) J. Biol. Chem. 257, 14979–14982.
[9] Brodbeck, U., Gentinetta, R. and Ott, P. (1981) in: Membrane Proteins (Azzi, A. et al. eds) pp.85–96, Springer-Verlag, Berlin.
[10] Ellmann, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95.
[11] Gerber, G.E., Anderegg, R.J., Herlihy, W.C., Gray, C.P., Biemann, K. and Khorana, H.G. (1979) Proc. Natl. Acad. Sci. USA 76, 227–231.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Merril, C.R., Goldmann, D. and Van Keuren, M.L. (1982) Electrophoresis 3, 17–23.
[14] Dutta-Choudhury, T.A. and Rosenberry, T.L. (1984) J. Biol. Chem., in press.