DETECTION OF HYDROXYPROLINE IN PREPARATIONS OF ACETYLCHOLINESTERASE FROM THE ELECTRIC ORGAN OF THE ELECTRIC EEL

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1. Introduction

Acetylcholinesterase (AChE; Acetylcholine hydrolase; EC 3.1.1.7) extracted from fresh electric organ tissue of the electric eel, *Electrophorus electricus*, is a complex molecular structure in which a multi-subunit head is connected to an elongated tail [1,2]. The head is believed to contain the catalytic subunits of the enzyme, while the role and composition of the tail are as yet unknown. The major 'native' form of AChE has a sedimentation coefficient of 18 S, and two other species, of sedimentation coefficients 14 S and 8 S, are also observed [3,4]. Proteolytic digestion or autolysis lead to detachment of the tail from all three forms and to their conversion to an 11 S globular tetramer [5,6].

Collagenase and other proteases detach the nerve-endings from muscle endplates by digestion of the basement membrane, and this process is accompanied by release of AChE [7–9]. Since collagen is a major constituent of the basement membrane, the possibility exists that AChE is attached to this structure [9], and that the tail of the 'native' enzyme is of a collagenous nature [1,10,11]. As hydroxyproline (Hyp) is a characteristic component of collagen and collagen-like proteins [12], the Hyp content of 'native' 14 S + 18 S electric eel AChE was examined. Detection of Hyp in amino acid hydrolysates is difficult owing to its low ninhydrin colour yield and since it migrates close to aspartic acid even in a chromatographic system especially developed to separate these two components [13]. The Hyp content of AChE was, therefore, determined by a specific colorimetric procedure based on oxidation of Hyp to pyrrole and colour formation with p-dimethylaminobenzaldehyde, as described by Neuman and Logan [14] and modified for small quantities by Bondjers and Björkerud [15].

Abbreviations: AChE, acetylcholinesterase; Hyp, hydroxyproline; Clq, subcomponent Clq of the first component of complement.

2. Materials and methods

Trypsin and bacterial protease were purchased from Worthington Biochemical Corp. (Freehold, N. J., USA), lysozyme, bovine serum albumin and ovalbumin from Sigma Chemical Co. (St. Louis, Mo., USA) and calf skin gelatin from Eastman Organic Chemicals (Rochester, N.Y., USA). Rat tail tendon collagen, purified acetylcholine receptor from *Torpedo californica* and Dolichos biflorus lectin were gifts from Daniela Kleinberg, Dr Rebecca Tarrab-Hazdai, and Dr W. G. Carter, respectively.

Chloramine-T (reagent grade) was from BDH Chemicals Ltd. (Poole, Dorset, UK). 4-dimethyl-aminobenzaldehyde (puriss.) was from Fluka A. G. (Buchs S. G., Switzerland). Hyp, obtained from N.B.C. (Cleveland, Ohio, USA), was recrystallized from ethanol/water before use.

14 S + 18 S AChE and 11 S AChE were purified by affinity chromatography as described previously [16].

Samples for amino acid analysis (400-600 μg) were hydrolysed for 22 h at 110°C in 6 N HCl in vacuo. Half of the total hydrolysate was taken for amino acid analysis and half reserved for Hyp deter-

mination. Amino acid analysis was performed by the method of Spackman et al. [17]. Hyp was determined according to the colorimetric procedure of Bondjers and Björkerud [15], with the following modifications: Since our hydrolysates contained quite large amounts of acidic salts, they were dissolved in 0.3 ml of water and neutralized to pH 5.0 by addition of 1 N NaOH from a microsyringe biuret. The total volume for colorimetric determination was 2 ml; amounts of all reagents were, therefore, scaled up accordingly as compared to the original procedure.

Absorbance of the chromophore at 558 nm was measured in a Gilford 2400 S spectrophotometer, while absorption spectra were recorded using a Cary 14 spectrophotometer.

Hyp content was calculated from the absorbance at 558 nm, in the colorimetric reaction, using a standard Hyp calibration curve. Total protein content of the AChE samples was calculated from the amino acid analysis data and from the known amino acid compositions of 14 S + 18 S AChE and 11 S AChE [6,18].

3. Results

Acid hydrolysates of 14 S + 18 S AChE were analysed for Hyp content by the colorimetric procedure of Bondjers and Björkerud [15]. Figure 1 shows a typical absorption spectrum thus obtained for 14 S + 18 S AChE, and, for comparison, spectra obtained similarly with calf skin gelatin, Hyp, 11 S AChE and ovalbumin. The spectrum obtained with 14 S + 18 S AChE closely resembles those for Hyp and gelatin, with a maximum at 558 nm and a shoulder at 530 nm. Even with the large excess of ovalbumin taken, no significant colour development was observed in this region of the spectrum, but a small peak was obtained with 11 S AChE.

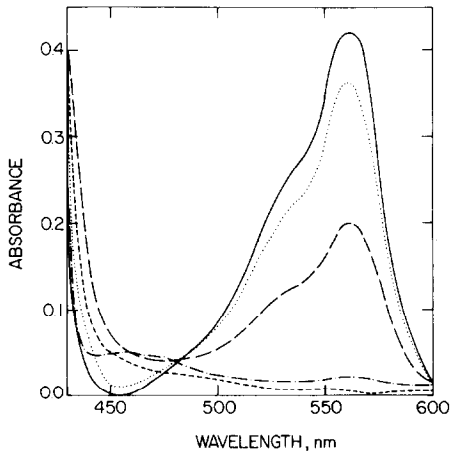

Fig.1. Absorption spectra obtained in the colorimetric procedure for determination of hydroxyproline in acetylcholinesterase. 14 S + 18 S AChE, 100 μg (– – – –); 11 S AChE, 105 μg (----); Calf skin gelatin, 14 μg (. . . . .); Ovalbumin, 970 μg (———); Hydroxyproline, 2 μg (————).

By use of a standard calibration curve the Hyp content of 14 S + 18 S AChE was measured and compared with several other proteins (table 1). It can

Table 1
Hydroxyproline content of acetylcholinesterase and other proteins

Protein	No. of determinations	Hydroxyproline % (w/w)
14 S + 18 S AChE	8	0.83 ± 0.13[a]
11 S AChE	6	0.10 ± 0.06
Acetylcholine receptor	3	0.03 ± 0.02
Bovine serum albumin	4	0.04 ± 0.04
Ovalbumin	3	not detectable
Trypsin	3	not detectable
Lysozyme	1	not detectable
Bacterial protease	1	not detectable
Dolichos biflorus lectin	1	0.01
Rat tail tendon collagen	3	12.0 ± 0.2
Calf skin gelatin	5	14.2 ± 1.4

[a] Mean value ± SEM

be seen that samples of 14 S + 18 S AChE contain ∿0.8% Hyp (w/w), while the 11 S form of this enzyme contains at most ∿0.1%. The Hyp contents of the other proteins analysed confirm the general observation that Hyp is a characteristic component of collagen not usually found in globular proteins [19]. It should be noted that acetylcholine receptor, also purified from electrogenic tissue, contains negligible amounts of Hyp.

Confirmation that the colorimetric procedure was measuring Hyp, rather than some other unidentified component, was obtained by passing aliquots of an acid hydrolysate of 14 S + 18 S AChE through an amino acid analyser and determining Hyp colorimetrically in the effluent fraction emerging at the position corresponding to aspartic acid and the putative Hyp. In control runs authentic Hyp was found to be recovered above 95% in this procedure, and Hyp contents of 0.99% and 0.91% were obtained for two samples of 14 S + 18 S AChE examined.

Proteolytic digestion converts the 'native' forms of AChE into the 11 S species with detachment of the tail [1,2]. 14 S + 18 S AChE was, therefore, subjected to digestion by trypsin under conditions leading to complete conversion to active 11 S AChE. The 11 S AChE thus produced was then adsorbed on the phenyltrimethylammonium-Sepharose conjugate routinely used for purification of this molecular form [6]. Hyp was determined both in the effluent and in the 11 S AChE subsequently eluted specifically with the quaternary AChE inhibitor decamethonium. Eighty-five percent of the total Hyp content of the sample of 14 S + 18 S AChE taken passed straight through the column; less than fifteen percent was adsorbed together with the 11 S enzyme and subsequently eluted with decamethonium.

4. Discussion

The results presented above demonstrate that 14 S + 18 S AChE, purified from electric organ tissue of the electric eel, contains significant amounts of the amino acid Hyp which is typically found only in collagen-like proteins. The 11 S AChE clearly contains much smaller amounts of Hyp than 14 S + 18 S AChE. This was shown both by direct determination of the amino acid and by the experiment in which 11 S AChE was generated directly from 14 S + 18 S AChE and then adsorbed onto an affinity column with concomitant removal of most of the Hyp present in the 'native' enzyme. Although the colour yields at 560 nm are on the average higher than for various control proteins, they are variable and close to the limits of sensitivity under our assay conditions. We do not feel, therefore, that they provide proof for the presence of Hyp in 11 S AChE, and this point will demand further investigation.

It is most unlikely that the Hyp detected in 14 S + 18 S AChE is the result of adventitious contamination with collagenous material rather than an intrinsic component of the purified AChE. All samples of AChE analysed were purified by two cycles of affinity chromatography performed at high ionic strength, each involving selective adsorption on the affinity resin and specific elution with a quaternary AChE inhibitor [16]; they were consistently found to have Hyp contents of over 0.7%. In contrast, acetylcholine receptor from *Torpedo californica,* purified by a procedure involving only one affinity chromatography step, contained insignificant amounts of Hyp.

If it is assumed that the tail structure of 'native' AChE is collagenous, one can calculate the amount of Hyp which might be expected from the dimensions of the tail observed in the electron microscope and from the known Hyp content of collagen. In the triple helical structure of collagen there are about 100 amino acid residues in a 100 Å segment [19]. Since the tail of 'native' AChE is ∿500 Å long [1,2], it would correspond to a segment of collagen triple helix with an overall molecular weight of ∿50 000. As 14 S and 18 S AChE have molecular weights of ∿750 000 and 1 100 000 respectively [2,20], such a collagenous constituent would thus comprise 4.5–7% of the total mass of the protein. Since the Hyp content of basement membrane collagens is about 15% [21], values of 0.7–1% Hyp for the purified enzyme would thus be expected, and are indeed in good agreement with the values we report.

The occurrence of a functional protein in which globular regions are linked to collagen-like sequences associated in triple-helical structures has already been suggested in the case of subcomponent Clq from complement [22]. Recent electron micrographs of preparations of 18 S AChE [23] strongly suggest that

this molecule contains 3 sets of subunit tetramers, and that the tail of the enzyme is composed of 3 individual filaments, each associated with one tetramer, which are intertwined in the distal half of the strand. The quaternary structure of 'native' AChE, although differing in detail from that of Clq, may thus be similar in its overall features.

The observation of significant amounts of Hyp in the 'native' 14 S + 18 S AChE lends support to the hypothesis referred to in the introduction that the tail of the enzyme is collagenous and that electric organ AChE is indeed a 'basement membrane enzyme' associated with the collagenous matrix within the synaptic cleft.

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References

[1] Rieger, F., Bon, S., Massoulié, J. and Cartaud, J. (1973) Eur. J. Biochem. 34, 539–547.
[2] Dudai, Y., Herzberg, M. and Silman, I. (1973) Proc. Natl. Acad. Sci. USA 70, 2473–2476.
[3] Massoulié, J. and Rieger, F. (1969) Eur. J. Biochem. 11, 441–455.
[4] Dudai, Y., Silman, I., Shinitzky, M. and Blumberg, S. (1972) Proc. Natl. Acad. Sci. USA 69, 2400–2403.
[5] Massoulié, J., Rieger, F. and Tsuji, S. (1970) Eur. J. Biochem. 14, 430–439.
[6] Dudai, Y., Silman, I., Kalderon, N. and Blumberg, S. (1972) Biochim. Biophys. Acta 268, 138–157.
[7] Hall, Z. W. and Kelly, R. B. (1971) Nature New Biol. 232, 62–63.
[8] Betz, W. J. and Sakmann, B. (1971) Nature New Biol. 232, 94–95.
[9] Betz, W. J. and Sakmann, B. (1973) J. Physiol. 230, 673–688.
[10] Rieger, F., Bon, S. and Massoulié, J. (1973) FEBS Lett. 36, 12–16.
[11] Dudai, Y. and Silman, I. (1974) J. Neurochem. 23, 1177–1187.
[12] Piez, K. A. and Gross, J. (1959) Biochim. Biophys. Acta 34, 24–39.
[13] Piez, K. A. and Morris, L. (1970) Anal. Biochem. 1, 187–201.
[14] Neuman, R. E. and Logan, M. A. (1950) J. Biol. Chem. 184, 299–306.
[15] Bondjers, G. and Björkerud, S. (1973) Anal. Biochem. 52, 496–504.
[16] Dudai, Y. and Silman, I. (1974) Methods Enzymol. 34, 571–580.
[17] Spackman, D. H., Stein, W. H. and Moore, S. (1958) Anal. Chem. 30, 1190–1206.
[18] Dudai, Y. (1974) D. Phil. Thesis, The Weizmann Institute of Science.
[19] Traub, W. and Piez, K. A. (1971) Advan. Protein Chem. 25, 243–352.
[20] Bon, S., Rieger, F. and Massoulié, J. (1973) Eur. J. Biochem. 35, 372–379.
[21] Kefalides, N. A. (1973) Int. Rev. Conn. Tissue Res. 6, 63–104.
[22] Reid, K. B. M. and Porter, R. R. (1975) in: Contemporary Topics in Molecular Immunology (Inman, F. P. and Mandy, W. J., eds) Vol. 4, pp. 1–22, Plenum Press, New York.
[23] Cartaud, J., Rieger, F., Bon, S. and Massoulié, J. (1975) Brain Research 88, 127–130.