ANTIBODIES AGAINST THE MEMBRANE-BOUND ACETYLCHOLINESTERASE FROM ADULT RAT BRAIN

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1. Introduction

As in most other tissues [2–6,10,12,14,17,18, 21–23,25–27,30,32,36,37]; review [35], mammalian brain acetylcholinesterase exists in several molecular forms [1,7,15,16,19,20,24,29,31,33,34,38–40]. Depending on the method of extraction of the enzyme between 2 and 4 forms have been detected in the rat brain [1,15,24,29,31,34,38,40] either by gel filtration or sucrose gradient centrifugation or polyacrylamide gel electrophoresis. It is generally assumed that the various forms are different states of aggregation of a single monomer (review [39]) but no clear proof has been reported of this hypothesis for the rat brain enzyme. In [29], we compared by gel filtration and sucrose gradient centrifugation the membrane-bound molecular species of the rat brain AChE with the various 'soluble' species and reported that the structural relationships as well between the membrane-bound enzyme and the 3 soluble species as between the 3 soluble species are not evident. This paper describes the preparation of a monospecific antibody against the purified membrane-bound enzyme [28] which allows further progress in this research and permits the study of possible cross immunoreactivity between the soluble and the membrane-bound species of rat brain AChE.

2. Materials and methods

Separation of 'soluble' and 'membrane-bound' AChE: All operations were performed at 4°C. Adult rat brains were homogenized in 10 vol. 12.5 mM sodium phosphate buffer (pH 7.4) containing 0.4 M NaCl and the suspension was centrifuged for 2 h at 100 000 × *g*. The supernatant was used as the source of 'soluble' AChE. The pellet was dissolved in 100 initial vol. of the same buffer containing 0.5% Triton X-100 [28] and the suspension was centrifuged. This supernatant was used as the source of the 'membrane-bound' AChE.

The membrane-bound enzyme was purified by sequential affinity chromatography on con A–Sepharose (Pharmacia, Uppsala) and *m*-dimethylaminoethyl benzoic acid (DMAEBA) coupled to Sepharose 4B as in [28]. After DEAE-cellulose chromatography and a second affinity chromatography on DMAEBA–Sepharose, the membrane-bound enzyme was found to be pure and homogenous [28]. This material was used for immunizing the rabbit. The 'soluble' enzyme was partially purified using affinity chromatography on con A–Sepharose and DMAEBA–Sepharose in the absence of detergent. This enzyme was also used for immunodiffusion and immunoprecipitation.

Antibodies were raised in a rabbit by three successive injections at 15 day intervals of 200 μg protein antigen as follows:

- The antigen dissolved in 500 μl 0.5% Triton X-100 was mixed with 500 μl complete Freund's adjuvant and injected intradermically in the back of the rabbit;
- For the second injection, the same procedure was used except that incomplete Freund's adjuvant was added to the antigen;
- For the third injection, the antigen was adsorbed on alumina [8] and injected intravenously in the ear.

The serum obtained from the rabbit was partially purified using repetitive ammonium sulfate precipitations [9]. This fraction was called 'Ig fraction' and was used in all our studies.

The antiserum was tested against purified AChE by double immunodiffusion in 1% agarose (Industrie Biologique Française, Paris) in 70 mM Tris–barbitone buffer (pH 8.6) containing 0.01% (w/v) calcium lactate, 0.02% (w/v) sodium azide and 0.1% (w/v) Triton X-100. Diffusion was performed for ⩾5 days at 4°C. The gels were washed alternately with 0.5% Triton X-100 solution containing 0.4 M NaCl then water (10 times for 30 min) and stained either with Coomassie brillant blue R for protein or by the method in [13] for esterasic activity.

Crossed immunoelectrophoresis was performed under the same conditions: the antigen was run for 24 h in the first direction at 15 V/cm. Electrophoresis in the second direction in a gel containing a 1/60-fold dilution of the Ig fraction was performed for 72 h at 5 V/cm. Gels were washed and stained as above.

In vitro immunoprecipitation: For these experiments both 'crude' and 'partially purified', soluble and membrane-bound enzymes were used. A typical assay contained 100 μl enzyme in 12.5 mM sodium phosphate buffer (pH 7.2) containing 0.9% NaCl (PBS), 100 μl Triton X-100 (3% w/v) and 100 μl diluted Ig in PBS. The mixture was incubated for 24 h at 4°C and centrifuged for 1 h at 30 000 × g. Both supernatant and pellet were analysed for AChE activity as in [11] using 10^{-5} M 1,5-bis-(4-allyldimethylammonium phenyl) pentane-3-one dibromide (Sigma, St Louis MO) as specific inhibitor. In most cases, aliquots of the supernatant were incubated with 100 μl sheep anti-rabbit immunoglobulins (24 h at 4°C) and centrifuged as above. AChE was determined both in the pellet and the supernatant.

Blanks were performed using Ig obtained from rabbits not immunized with AChE or with anti-AChE Ig without enzyme. This blank is necessary for taking into account the low AChE activity present in the rabbit Ig fraction.

3. Results and discussion

As shown in fig.1, the antibody against the membrane-bound AChE gives only one precipitation line either with the purified or with the crude membrane-bound enzyme, either with a protein stain (fig.1a) or a specific enzyme stain (fig.1b). By crossed immunoelectrophoresis (fig.2), two peaks can be detected. However the slowly migrating one is found even if the second dimension is run in a gel not containing the

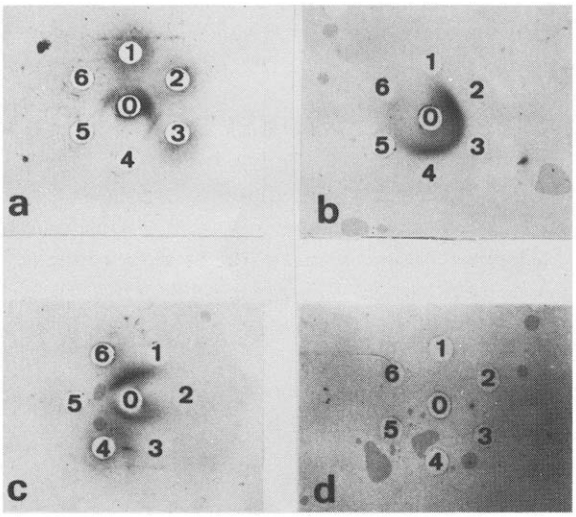

Fig.1. Double immunodiffusion test in the presence of Triton X-100 of the antibody raised against the membrane-bound rat brain acetylcholinesterase. (a) Coomassie brillant blue stain: the membrane-bound enzyme is placed in the centre well; various dilutions of the antibody are placed in wells 1,2, 3,6 and normal rabbit serum in wells 4,5.
(b) Enzyme stain: the membrane-bound enzyme is placed in centre well, dilutions of antibody in wells 2–4 and dilutions of non-immunized rabbit serum in wells 1,5,6.
(c) Enzyme stain [13]: the antibody is placed in the centre well. Well 6: purified membrane-bound AChE; well 4: total rat brain AChE; well 2: partially purified soluble AChE (same activity as in well 6).
(d) Enzyme stain [18]: the antibody is placed in the centre well. Well 1,2,3,4: dilution of the partially purified soluble AChE. Note the absence of any precipitation lines.

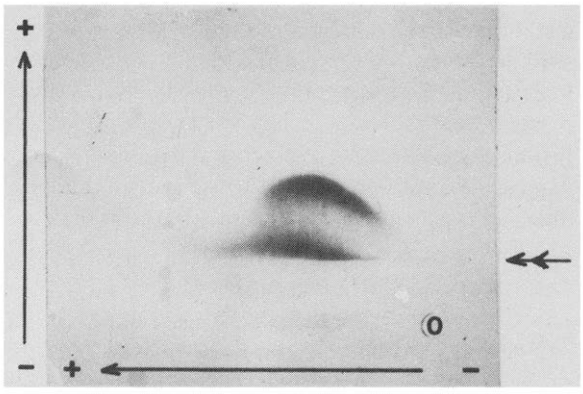

Fig.2. Crossed immunoelectrophoresis of the membrane-bound AChE. Horizontal first electrophoresis run in gel without antibody. Vertical: second run in gel containing the antibody. An artefactual precipitate is observed close to the limit of the two gels (double arrow).

antibody. This precipitation of a part of the enzyme at the boundary between the two agarose gels seems thus to be artefactual and may correspond to enzyme denaturation.

The pattern obtained with the soluble enzyme is completely different (fig.1c,1d). We never observed any precipitation lines between this material and our antibody against the membrane-bound enzyme even if the soluble antigen is run in the presence of Triton X-100. The absence of precipitation does not result from the presence of little antigen, since no precipitation occurs with the 'partially purified' enzyme (12 000-fold purification) when equivalent activities of soluble and membrane-bound enzymes are employed for the immunodiffusion test. This cannot be explained by the complete denaturation of the soluble enzyme during diffusion since enzyme activity is found around the corresponding wells when the immunodiffusion gels are not washed.

The antibody against the membrane-bound AChE precipitated the crude or the purified membrane-bound enzyme solubilized in the presence of Triton X-100 (fig.3a). No precipitation occurs with the serum of non-immunized rabbits under identical conditions. Addition of sheep anti-rabbit immunoglobulin increases the enzyme precipitation (fig.3a). When the partially purified soluble AChE or the crude soluble AChE from rat brain was treated under the same conditions (in presence of Triton X-100), no precipitation occurred (fig.3b).

As found with AChE from other tissues [1,16,23, 38], specific antibodies inhibited the activity of the membrane-bound AChE: up to 60% of the activity is lost during these experiments of precipitation with antibodies but not with sera of non-immunized rabbits. Such an inhibition does not occur with the soluble AChE (fig.3b).

These experiments indicate clearly that our antibody against the purified membrane-bound AChE from rat brain does not react on the species of AChE which are soluble in the absence of detergent [1,29]. The results in [38] where only 80% of the total AChE activity found in rat brain can be precipitated by antibodies against AChE prepared from mouse brain [1] can be interpreted by a lack of reactivity of a part of AChE. The lack of immunological reactivity could be intrepreted in two ways:

(i) The 'soluble' and 'membrane-bound' AChE from rat brain are completely different molecules;

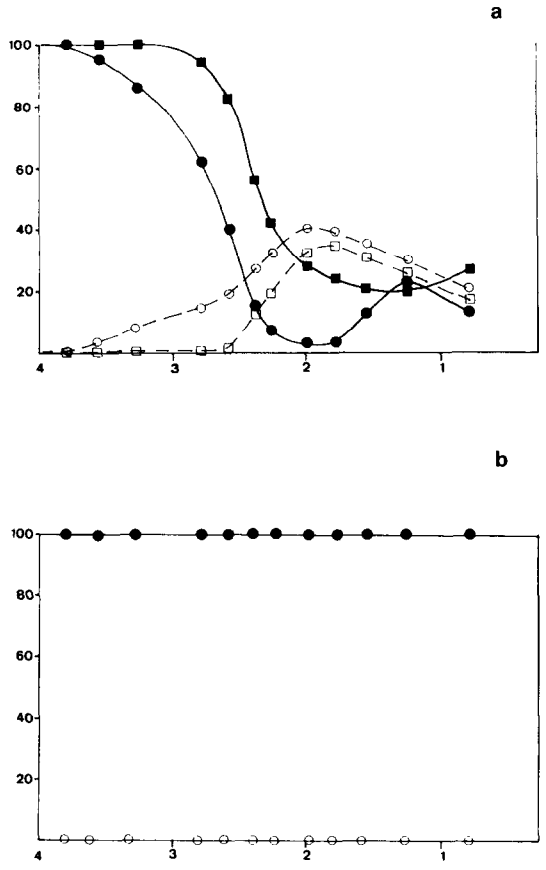

Fig.3. In vitro immunoprecipitation of the membrane-bound (fig.3a) and the 'soluble' (fig.3b) AChE from adult rat brain. Abscissae: –log (antibody concentration). Ordinates: percent of initial enzyme activity. Activity in the supernatant (■) and in the pellet (□) after incubation with anti-AChE. Activity in the supernatant (●) and in the pellet (○) after incubation with anti-AChE then anti-rabbit Ig.

(ii) These two kinds of AChE represent associations of the same monomer with very different conformations and (or) different degrees of association.

The findings in [29] that 'soluble' and membrane-bound AChE behave differently by filtration and sucrose gradient centrifugation and also differ in their behaviour with respect to reducing agents [29] might favour the first hypothesis but can not be taken as a proof of it. Work is now in progress to isolate the different species of soluble AChE [29] to see if the monomer corresponds to that of the membrane-bound enzyme [28].

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