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A conformation-dependent monoclonal antibody against active chicken acetylcholinesterase

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We show that the C-131 monoclonal antibody, directed against chicken AChE, recognizes active chicken AChE, but not the SDS-denatured or heat-inactivated protein. Previous results indicated that C-131 only binds to the active enzyme, and not to inactive molecules which also occur in the embryonic chicken brain. In contrast with C-131, other monoclonal antibodies obtained in the same series, such as C-6 and C-54, also recognize denatured or inactive AChE. It is noteworthy that these antibodies all seem to react with a trypsin-sensitive peptide which is present in chicken but not in mammalian or *Torpedo* AChE, whereas the C-131 antibody binds trypsin-modified as well as intact molecules. These results show that C-131 is highly conformation-dependent, specific for active AChE. They confirm our previous conclusion that active and inactive molecules arise from different folding processes.

Acetylcholinesterase; Monoclonal antibody; Active conformation; Chicken

1. INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) plays a key role in cholinergic synapses, by rapidly hydrolyzing the extracellularly released neurotransmitter, acetylcholine. This enzyme is synthesized in the endoplasmic reticulum and is then exported towards the cellular surface, where its different molecular forms may be anchored in the plasma membrane (amphiphilic globular forms), attached to the basal lamina (asymmetric collagen-tailed forms) or secreted as soluble molecules (non amphiphilic globular forms) [1,2]. AChE is first synthesized as an inactive precursor, and acquires its catalytic activity in the rough endoplasmic reticulum after a lag of about 30 min, as shown by various experiments, in cultures of murine neuroblastoma T28 cells [3] and of primary chick muscle cells [4]. Rotundo and colleagues have shown that, in cultures of chick myotubes, about 80% of the synthesized AChE polypeptides do not become active, and are rapidly degraded in a non-lysosomal compartment [5]. We have recently reported that in chicken brain, in vivo, a significant proportion of AChE proteins (about 30%) is catalytically inactive, or possesses a much lower catalytic turnover than the normal active enzyme [6]. This protein, however, possesses an active serine which may react with organophosphate inhibitors, and co-sediments with active monomers (G_1) and dimers (G_2).

We presently describe a monoclonal antibody, C-131, which was raised against chicken AChE and seems to recognize only the active enzyme, but not the inactive component or denatured AChE. In addition, whereas the epitopes recognized by other antibodies seem to be located in a trypsin-sensitive region of chicken AChE, the binding of C-131 is not affected by a limited trypsin digestion.

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2. MATERIALS AND METHODS

2.1. *Materials*

Antiproteolytic agents (EDTA, benzamidine, bacitracin, aprotinin or Zymofren, pepstatin A, leupeptin), as well as trypsin and soybean trypsin inhibitor were obtained from Sigma (St. Louis, MO, USA).

2.2. *AChE activity*

AChE activity was determined by the colorimetric method of Ellman [7], using acetyltiocholine as a substrate, in the presence of dithio-bis-dinitrobenzoic acid. One Ellman unit (U) is defined as the amount of enzyme producing an increase in absorbance of 1 absorbance unit per minute in 1 ml of assay medium (1 cm pathlength), and corresponds to the hydrolysis of about 75 nmol of substrate per min. In the case of chicken AChE, 1 μg corresponds to approximately 28 Ellman units [8].

The purification of AChE was described previously [9,10]. Hybridomas and ascites fluids were prepared essentially as described by Grassi et al. (1988) [11].

2.3. *Solubilisation of AChE*

Brains of chickens were homogenized in a Potter glass–teflon homogenizer with 5 vols. of buffer containing 50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$ (buffer A), 1% Triton X-100, supplemented with 1/20

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of a stock solution of antiproteolytic agents (20 mM EDTA; 2 mM benzamidine; 2 mg/ml bacitracin; 50 U/ml aprotinin or Zymofren; 100 mg/ml pepstatin A; 100 mg/ml leupeptin). The homogenate was then centrifuged at 15,000 rpm for 30 min at 4°C.

2.4. *Sedimentation analyses of AChE molecular forms*

The tissue extract was centrifuged for 16 h in sucrose gradients (5–20% sucrose, w/v) in a Beckman SW41 rotor, at 4°C, 40,000 rev/min. The gradients were prepared in buffer A supplemented with 1% Triton X-100 or 1 M NaCl [12,13]. Sedimentation coefficients were determined by comparison with β-galactosidase from *E. coli* (16 S), catalase from beef liver (11.3 S), alkaline phosphatase from calf intestine (6.1 S), which were included as internal standards in the gradients.

2.5. *SDS-PAGE and Western blots*

Samples were denatured and reduced, in the presence of 0.25 M dithiothreitol, before electrophoresis in 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by electroblotting (0.6 mA during 3 h). The membrane was pre-incubated in buffer A containing 5% non-fat milk powder, and incubated overnight with a 1/500 dilution of ascites fluid, in the same solution. The membrane was then washed, and incubated with antimouse IgG antibodies coupled with peroxidase (Diagnostics Pasteur, France). Peroxidase activity was revealed with a reaction medium containing 50 mg diaminobenzidine, 0.1% H_2O_2, 50 mM Tris-HCl, pH 7.4.

2.6. *Digestion by trypsin*

The samples were treated for 30 min at 4°C with trypsin (0.3 mg/ml). The reaction was stopped by the addition of soybean trypsin inhibitor (0.2 mg/ml) and the tubes were kept on ice.

2.7. *Immunoradiometric assay of AChE protein*

AChE-immunoreactive protein was quantified by a two-site immunoradiometric assay, similar to that used previously by Brimijoin et al. [14] for rat AChE. The purified monoclonal antibodies C-6 or C-131 were adsorbed to the walls of plastic tubes (capture antibody), and the second ^{125}I-labelled antibody C-54 was used at saturating concentration (tracer antibody). The method will be described in greater detail elsewhere (Chatel et al., in preparation).

3. RESULTS

3.1. *Western blotting of chicken AChE with monoclonal antibodies*

We obtained a series of monoclonal antibodies which were selected according to their capacity to bind chicken AChE activity [6]. Most of these antibodies also recognize denatured AChE, in Western blots, staining AChE subunits and some degradation fragments (Fig. 1B). One of these antibodies, C-131, differed however from the others because it did not produce any labeling in Western blots, as shown in Fig. 1A, suggesting that it only recognized the native form of AChE.

3.2. *The monoclonal antibody C-131 does not recognize heat-denatured AChE*

The binding of AChE to antibodies C-131 and C-6 was monitored by a two-site immunoradiometric assay [6]. In this assay one monoclonal antibody (capture antibody), C-6 or C-131, was immobilized on plastic tubes, and the amount of AChE bound on the solid-phase was quantified with a second, ^{125}I-labeled antibody (tracer antibody), C-54. We analyzed the binding

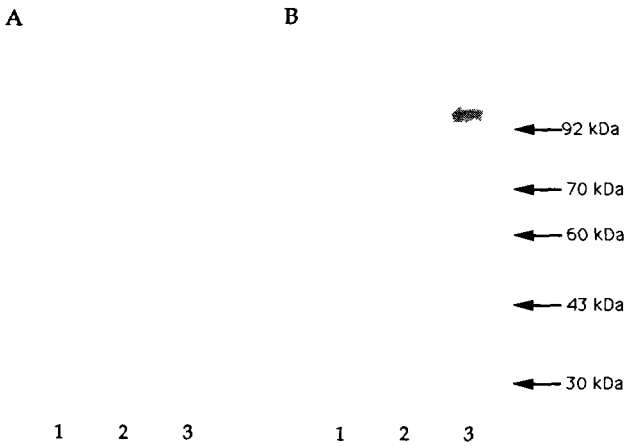

Fig. 1. Western blots, showing the staining of chicken AChE with monoclonal antibodies. A detergent extract from brains of 11-day chick embryos was subjected to SDS-PAGE, transferred onto a nylon membrane, and stained with the monoclonal antibody C-131 (A) and with a pool of anti-AChE monoclonal antibodies (B). Lanes 1, 2 and 3 correspond to samples containing 0.01, 0.02 and 0.03 Ellman units of AChE, respectively. Even at the lowest concentration, catalytic subunits of 100 and 110 kDa, corresponding to α and β alleles [16] as well as degradation fragments are clearly visible in B, but no stain can be detected with C-131, in A.

of chicken AChE, which had been submitted to heat denaturation at 54°C for various periods of time (Fig. 2). We found that activity disappeared within 15 min. This thermal denaturation is accompanied by a transient increase followed by a subsequent reduction of the immunoreactivity when the C-6/C-54* assay was used. These effects may result from a variation of the affinity of the enzyme for the immobilized or for the labeled antibody. We did not investigate this point further. The signal obtained with the fully denatured enzyme was, however, more than half of that obtained with the native extract, in agreement with the fact that both C-6 and C-54 antibodies recognize denatured AChE in Western blots. In contrast, the signal was totally abolished when C-131 was used as capture antibody, exactly in parallel with the loss of catalytic activity, demonstrating that this antibody recognizes only the native form of the enzyme.

3.3. *Effect of trypsin digestion of chicken AChE on the binding of C-6 and C-131*

A mild trypsin digestion modifies the sedimentation coefficients of all molecular forms of chicken AChE, probably by removing a peptidic region which is not involved in their catalytic activity or in their quaternary organization [15]. We characterized the binding of C-131 and other antibodies to intact and trypsin-modified forms of AChE, by analysing the capacity of the antibodies to shift their sedimentation in sucrose gradients. This is illustrated for C-131 and C-6 in Fig. 3. All antibodies displace the sedimentation of intact AChE peaks towards the bottom of the gradient (data not shown).

In contrast, trypsin-modified AChE was not affected by most of our monoclonal antibodies, except C-131 which clearly shifted the digested G_1, G_2 and G_4 forms (Fig. 3).

4. DISCUSSION

We showed that the monoclonal antibody C-131 does not bind detergent-denatured or heat-inactivated chicken AChE, in contrast with other monoclonal antibodies obtained in the same fusion, such as C-6 and C-54. These antibodies do not inhibit AChE activity, showing that they do not interact with the active site of the enzyme. In agreement with this, we found that the binding of these monoclonal antibodies, including C-131, is not affected by reaction of the active serine with the organophosphorus inhibitor *O*-ethyl-*S*^{2}-diisopropyl-aminoethylmethyl-phosphonothiotate (data not shown).

We have recently shown that the quantification of immunoreactive AChE protein with monoclonal antibodies C-6 and C-54 reveals that the specific activity (catalytic activity/immunoreactive protein) is higher for the G_4 form than for the G_2 and G_1 forms, in embryonic chicken brain [6]. Because purified AChE forms present an identical catalytic turnover per active site [8], this observation demonstrated the existence of inactive AChE molecules, co-sedimenting with G_2 and G_1. On the contrary, when we used C-131 instead of C-6 as immobilized antibody, with the same radio-labelled antibody C-54, the radioactivity profile obtained by assaying fractions from a sucrose gradient was superimposable with the profile of catalytic activity, indicating that C-131 retained only active molecules. In addition, the ratio between catalytic activity and radioactivity was identical with C-6 and C-131, in the case of the G_4 form, showing that this form consists only of active molecules.

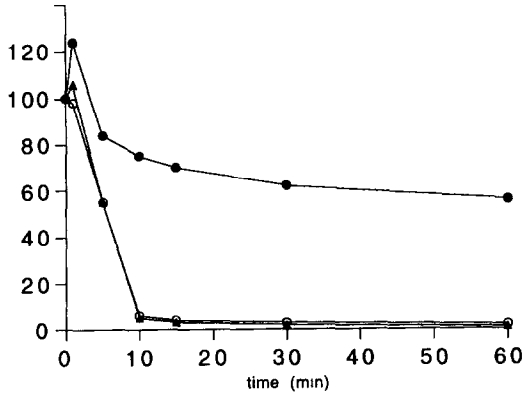

Fig. 2. Effect of thermal denaturation on the reactivity of AChE with monoclonal antibodies C-6 and C-131. A total extract of chicken brain was submitted to thermal denaturation at 54°C for the indicated times (abscissae): (-○-) enzymatic activity, (-●-) two-site immunoradiometric assay using C-6 as capture antibody, (-▲-) two-site immunoradiometric assay using C-131 as capture antibody. All values are normalized at 100% at 0 time. C-54 was used as the second ^{125}I-labelled tracer antibody.

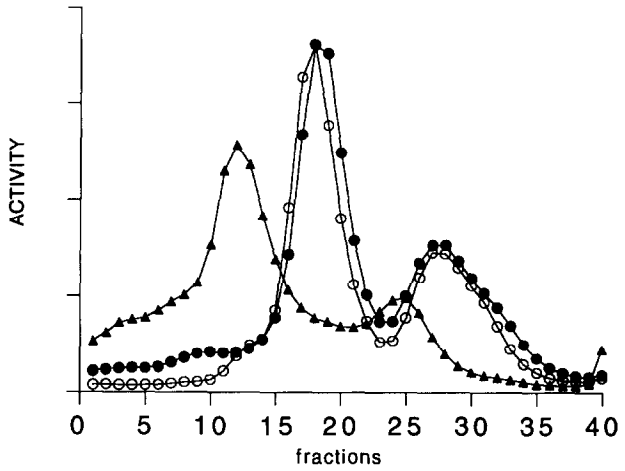

Fig. 3. The trypsin-modified AChE forms are recognized by the monoclonal antibody C-131, but not by C-6. A brain extract (cf. Fig. 1) was incubated for 1 h at 30°C with 0.3 mg/ml trypsin, and analyzed by sedimentation in a sucrose gradient, with or without addition of antibodies C-131 or C-6 (1/100). (○--○) Control: the trypsin-digested AChE forms sediment at 4 S (G_1), 6 S (G_2) and 10 S (G_4) instead of 5 S, 7 S and 11 S for the intact forms; (●--●) the sedimentation of these forms is not affected by C-6, in contrast to intact forms; (▲--▲) they are shifted towards the bottom of the gradient, showing that they form complexes with C-131. Note that a small fraction of the G_4 form remained intact after trypsin digestion, forming a shoulder on the left side of the main peak, and was shifted in the presence of C-6.

The present results confirm that C-131 is sensitive to the conformation of active AChE, since it has no affinity for denatured protein.

It is interesting that the epitopes recognized by the conformation-independent antibodies are associated with a trypsin-sensitive region of chicken AChE. The catalytic subunit of chicken AChE has a mass of 100–110 kDa [16], instead of 70–80 kDa for other vertebrate such as *Torpedo* and mammals [1]. The additional mass resides in the polypeptide chains themselves, and appears to be due to the presence of a peptidic domain of 235 amino acids, containing many glycines and prolines (75 and 17, respectively), which is encoded by an insertion in the AChE gene, around position 338 (*Torpedo* numbering) (Y. Maulet and M. Ballivet, unpublished results). All forms of chicken AChE thus sediment faster than their mammalian counterparts. A mild tryptic digestion, however, probably removes the additional peptide, because it reduces the sedimentation coefficients to the values observed in mammals, without modifying either the catalytic activity, or the quaternary organisation of the molecules; collagen-tailed and amphiphilic molecules retain their specific properties [15]. The antibodies C-6 and C-54, as well as all other conformation-independent antibodies, do not bind these modified AChE forms, whereas C-131 recognizes the intact and digested molecules equally well.

It would be extremely interesting to localize the region of the enzyme which binds C-131, since this might

give us some information on the difference between active and inactive molecules. Unfortunately this cannot be determined by classical epitope-mapping methods since only the complete, correctly folded polypeptide chain is likely to be recognized by this antibody. It is interesting that C-131 does not recognize the closely related quail AChE (Chatel et al., in preparation). When both coding sequences are available, a possible strategy for identifying residues involved in C-131 binding might be to construct chimeric proteins in which segments of chicken AChE would replace the corresponding quail peptides, hopefully in an active conformation so that they may bind the antibody.

The present results establish that the C-131 antibody is highly conformation-dependent, and recognizes only active AChE molecules. It does not, however, interact with the active site per se. Inactive and active AChE molecules co-sediment and probably result from similar but distinct folding processes, since they can be distinguished by this conformation-dependent antibody.

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REFERENCES

[1] Massoulié, J. and Bon, S. (1982) Annu. Rev. Neurosci. 5, 57–106.
[2] Massoulié, J., Pezzementi, L., Bon, S., Krejci, E. and Vallette, F.M. (1993) Progr. Brain Res. (in press).
[3] Lazar, M., Salmeron, E., Vigny, M. and Massoulié, J. (1984) J. Biol. Chem. 259, 3703–3713.
[4] Walker, C.R. and Wilson, B.W. (1976) Neuroscience 1, 509–513.
[5] Rotundo, R.L., Gomez, A.M., Fernandez-Valle, C. and Randall, W.L. (1988) Proc. Natl. Acad. Sci. USA 85, 7805–7809.
[6] Chatel, J.M., Grassi, J., Frobert, Y., Massoulié, J. and Vallette, F.M. (1993) Proc. Natl. Acad. Sci. USA (in press).
[7] Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95.
[8] Vigny, M., Bon, S., Massoulié, J. and Leterrier, F. (1978) Eur. J. Biochem. 85, 317–323.
[9] Vallette, F.M., Marsh, D.J., Muller, F., Massoulié, J., Marçot, B. and Viel, C. (1983) J. Chrom. 257, 285–296.
[10] Marsh, J.D., Grassi, J., Vigny, M. and Massoulié, J. (1984) J. Neurochem. 43, 204–214.
[11] Grassi, J., Frobert, Y., Lamourette, P. and Lagoutte, B. (1988) Anal. Biochem. 168, 436.
[12] Bon, S., Rosenberry, T.L. and Massoulié, J. (1991) Cell. Mol. Neurobiol. 11, 157–172.
[13] Vallette, F.M. and Massoulié, J. (1991) J. Neurochem. 56, 1518–1525.
[14] Brimijoin, S., Hammond, P. and Rackonczay, Z. (1987) J. Neurochem. 49, 555–562.
[15] Allemand, P., Bon, S., Massoulié, J. and Vigny, M. (1981) J. Neurochem. 36, 860–867.
[16] Rotundo, R.L. (1984) Proc. Natl. Acad. Sci. USA 81, 479–483.