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SOLUBILIZATION OF 20S ACETYLCHOLINESTERASE FROM CHICK RETINA

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SUMMARY

A 20S form of acetylcholinesterase has been solubilized from young chick retinas by means of a buffered salt-detergent solution containing EDTA. The release of this fast-sedimenting form of the enzyme is selectively blocked by the presence of even small amounts of Ca^{++} in the homogenization medium. The collagen-tailed nature of this molecular species of acetylcholinesterase has been ascertained by collagenase digestion. This finding suggests that the avian central nervous system contains asymmetric, collagen-tailed quaternary structural forms of acetylcholinesterase as is the case in skeletal muscle and cholinergic ganglia.

INTRODUCTION

Current structural models of vertebrate acetylcholinesterases (acetylcholine hydrolase, EC 3.1.1.7) assume the existence of a number of quaternary structural forms of the enzyme distributed in two main classes or groups: one class, the G forms (globular or symmetric forms) include monomers, dimers and tetramers (G_1,G_2 and G_4, respectively) of the catalytic subunit identified in a large number of cholinergic structures as a polypeptide chain with a molecular weight of approximately 80,000 daltons. The other class, the A forms (asymmetric or elongated forms),includes enzyme molecules containing 1,2 or 3 tetrameric clusters of the catalytic subunit (A_4, A_8 and A_{12}) linked to a three-stranded collagen-like tail (1-5). Both the G and A forms of acetylcholinesterase have been isolated, and characterized in terms of sedimentation and gel filtration behavior and subunit composition, from a number of cholinergic tissues such as the electrogenic tissue from electric fish, and the skeletal muscle, cholinergic ganglia and peripheral nerves of some

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mammals and birds (see 4,5 for reviews). However, attempts to solubilize any A forms from the vertebrate central nervous system have been unsuccessful and it has been contended that they may be totally absent from the central nervous system (4,5). The discovery that precisely the A_{12} form of acetylcholinesterase (6), or the A forms in general (5), may be preferentially associated with the postsynaptic membrane makes this absence rather striking.

We have been able to show recently that collagenase (an enzyme that cleaves specifically the tail protein of the A forms) can release small amounts (1-2% of the initial homogenate activity) of two fast-sedimenting molecular species of acetylcholinesterase from the residual pellet after extraction of chick retina and optic lobe tissues (i.e. central nervous system) with a buffered solution containing 1M NaCl, 1% Triton X-100 and 50mM $MgCl_2$ (7). Since these results pointed to the presumptive existence of collagen-tailed acetylcholinesterase molecules in the chick central nervous system, we have searched for a procedure to solubilize them intact, avoiding proteolytic agents, in order to confirm their existence and undertake their purification and characterization. We report below how the omission of $MgCl_2$ from the standard extraction solution (4,7), combined with the addition of EDTA to block endogenous divalent cations, results in the one-step solubilization of a substantial amount of 20S acetylcholinesterase (A_{12}) from chick retina.

EXPERIMENTAL

Retinas from 10-day chicks were homogenized in 10 volumes of a 1M NaCl, 1% Triton X-100 solution, buffered with 10mM Tris.HCl, pH 7, and supplemented or not with 1mM EDTA (K^+, pH 7), at 4°C. The homogenate was centrifuged, in each case, at 110,000g, for 30 minutes, at 2°C. The supernatants (extracts) were layered on top of 5-20% linear sucrose gradients, prepared in the respective homogenization solutions, and sedimented for 19 hours, at 154,000g, 4°C, in a Beckman SW40Ti rotor. About 35 fractions of 0.35ml were collected from each gradient and assayed for acetylcholinesterase and marker enzymes, as previously described (7-9). The apparent sedimentation coefficients were calculated, according to Martin and Ames (10), using *E.coli* β-galactosidase (EC 3.2.1.23; 16S), beef liver catalase (EC 1.11.1.6; 11.3S) and *E.coli* alkaline phosphatase (EC 3.1.3.1; 6.1S) as internal sedimentation markers.

Collagenase digestion of the 20S acetylcholinesterase peak was carried out, in the presence of 5mM $CaCl_2$, in homogenization buffer without EDTA, as described in a previous paper (7). Other experimental details are given in the legends to figures and tables.

RESULTS AND DISCUSSION

Fig. 1A shows the sedimentation pattern of the chick retinal acetylcholinesterase quaternary structural forms extracted by a 10mM Tris.HCl-buffered solution, at pH 7, containing 1M NaCl and 1% Triton X-100. In the case of Fig. 1B, 1mM EDTA (K^+, pH 7) has been added to the above extraction solution. Although both procedures solubilize up to 96% of the total tissue activity, we see,

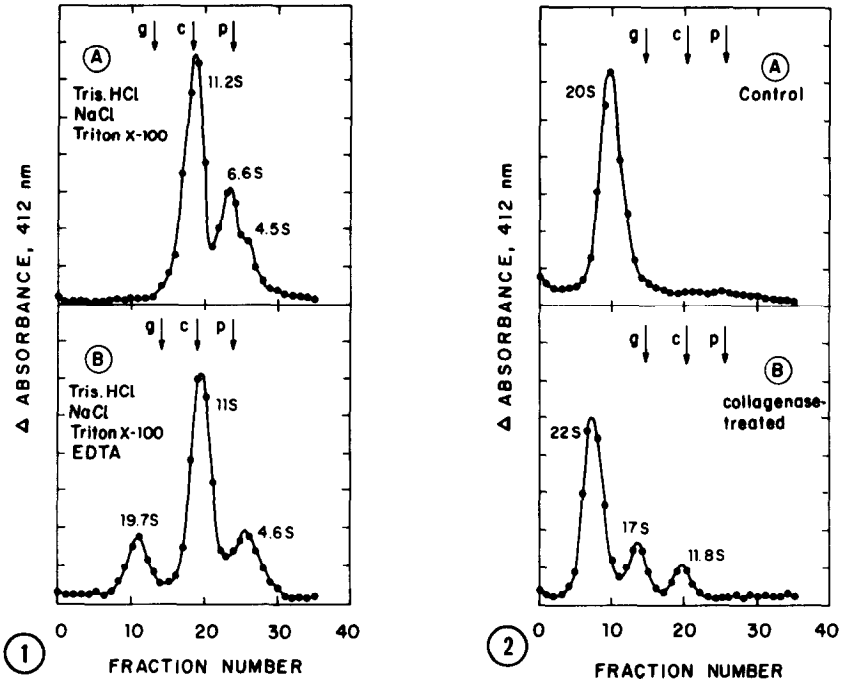

Fig. 1. Sedimentation behavior of the molecular species of acetylcholinesterase solubilized from chick retina by a buffered salt-detergent solution in the absence or presence of EDTA. Acetylcholinesterase activities are given in arbitrary units proportional to changes in absorbance at 412nm (8). The sedimentation direction is to the left. The arrows point to the position of the internal marker enzymes: g, β-galactosidase (16S); c, catalase (11.3S); and p, alkaline phosphatase (6.1S). A, acetylcholinesterase forms extracted by a buffered salt-detergent solution. B, acetylcholinesterase forms extracted by the same buffered salt-detergent solution containing 1mM EDTA (K^+, pH 7).

Fig. 2. Effect of collagenase on the retinal 20S acetylcholinesterase. The 20S peak from Fig. 1B was pooled, dialyzed against homogenization buffer (without EDTA), supplemented with $CaCl_2$ up to 5mM, and divided in two equal portions: one was digested with 100 units/ml of protease-free collagenase, at 37°C, for 30 minutes (7), and the other (control) was simply incubated at 37°C during the same time. At the end of the incubation both samples were analyzed by sucrose gradient centrifugation. A, control (undigested) sample. B, collagenase-digested sample.

in the case of Fig. 1B, a new 20S enzyme peak accounting for 8% of the total homogenate activity (Table 1). At the same time, changes in the distribution of the 4S and 6S acetylcholinesterase forms (G_1 and G_2 (5)) are usually observed associated with the presence of EDTA in the homogenization buffer.

When we then added increasing amounts of either Ca^{++} or Mg^{++} to the EDTA-containing homogenization solution we found out that while 1mM Ca^{++} completely blocked the release of acetylcholinesterase as 20S form, it was necessary to increase the Mg^{++} concentration to 10mM to decrease the 20S peak by half. We have thus tentatively concluded that Ca^{++} may be involved in the attachment of the 20S acetylcholinesterase to retinal membranes. On the other hand, the addition of Ca^{++} to the EDTA-released 20S acetylcholinesterase, either in the homogenate or in the 110,000g supernatant (extract), induces the aggregation of the 20S enzyme. This aggregation can be reversed by further addition of EDTA, in the presence of 1M NaCl and 1% Triton X-100. These results do not favor the alternative hypothesis that EDTA is not releasing 20S acetylcholinesterase but just preventing its immediate degradation by Ca^{++}-dependent free proteases.

To confirm the tailed nature of the newly solubilized 20S retinal acetylcholinesterase we subjected it to collagenase digestion, as illustrated in Fig. 2. The 20S peak was converted almost totally to the partially tail-less 22S form (21.8±0.3, corresponding to the 21.5S form described in (7)). The presence of small amounts of 17S and 11S forms in the collagenase-digested sample suggests that there may be a break point for collagenase, in a small subset of A_{12} molecules, beyond the point at which the tail strands separate, close to the attachment site of the tetramer, since this same batch of protease-free collagenase does not further degrade the isolated 22S peak (7). Thus, the retinal 20S acetylcholinesterase seems to be identical in sedimentation behavior and collagenase sensitivity to the A_{12} chick acetylcholinesterase previously described and characterized in the chick skeletal muscle and cholinergic ganglia (4,5,7,11-13). On the other hand, these results support the notion that the chick central nervous system does contain asymmetric, collagen-tailed forms of acetylcholinesterase, and that the validity of the general structural model of the enzyme outlined in the Introduction can possibly be extended to all vertebrate cholinergic tissues. The example of the chick retina may suggest that che-

Table 1. Distribution of chick retinal acetylcholinesterase activity in different molecular forms.

Extraction solution	% of homogenate activity solubilized	Activity in the different peaks			
		20S (19.8±0.2)	11S (11.1±0.2)	6-7S (6.5±0.3)	4-5S (4.6±0.1)
Tris/NaCl/ Triton X-100	96	-	49	47[a]	
Tris/NaCl/ Triton X-100/ EDTA	96	8	51	-	37

Data in the Table correspond to the quantitative analysis of 3 sets of experiments like the one described in Fig. 1. For precise quantitative estimation of enzyme activity the peaks in the gradient were first localized according to Ellman's procedure (8) and assayed subsequently by a radiochemical method based on the conversion of {1-^{14}C}acetylcholine to {1-^{14}C}acetate by the enzyme in the different fractions (9). Sedimentation coefficients are given as mean±standard deviation for the 3 runs. The activity in the different gradient peaks is given as % of the activity in the initial homogenate. The distribution of the experimental values never exceeded 5% of the mean values given in the Table.
[a]This value is the sum of the partially superimposed 6.6S and 4.5S peaks (Fig. 1).

lation of divalent cations would be the key for the solubilization of tailed acetylcholinesterase forms from all central nervous system areas and tissues, at least in avian species. However, when we attempted to apply the same solubilization schedule to other regions of the chick brain, namely the cerebral hemispheres, the optic lobes and the cerebellum, we were unsuccessful in spite of the fact that in the case of the optic lobe we had already isolated two collagenase-released fast-sedimenting forms of acetylcholinesterase (7). All this suggests that, although the asymmetric, tailed species of acetylcholinesterase may exist in all cholinergic tissues, including the central nervous system, the specific mechanism of attachment of the enzyme to the cell membranes (or to the extracellular basement membrane or related structures (5)) may be somewhat different for each tissue and each localization, and that no general solubilization procedure can be offered at this time which will be effective in all cases. Perhaps the only strict requirement defined so far is the necessity of a high salt concentration to prevent the aggregation of the tailed forms of the enzyme (5).

A final conclusion to be drawn from the experiments described in the present paper is that the qualitative and quantitative patterns of distribution of the acetylcholinesterase molecular forms described so far may not necessarily reflect the molecular organization of the enzyme in vivo. Thus, we see that both solubilization procedures presented in Fig. 1 release up to 96% of the acetylcholinesterase activity in the homogenate but only when EDTA is present do we see a 20S peak accounting for 8% of the homogenate activity (see Table 1). A possible explanation for this result is that we may not be dealing with completely independent pools of the different forms of the enzyme that are either extracted or left in the tissue but that, on the contrary, the pattern and percentual distribution of the quaternary acetylcholinesterase forms seems to be determined, at least in part, by and during the solubilization process. It should be, therefore, experimentally confirmed, in view of the present results, whether the native structural form of the membrane-bound enzyme is only one (perhaps the A_{12} form -the more complex- or even an unknown, larger precursor of it), or multiple and functionally specific (5,13).

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