MOLECULAR FORMS OF ACETYLCHOLINESTERASE IN THE CHICK VISUAL SYSTEM

Collagenase-released 21.5 S and 16.5 S species

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1. Introduction

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is the enzyme thought to be responsible for the termination of the action of the neurotransmitter acetylcholine, at the cholinergic synapses [1]. Nevertheless, its presence is not limited to the cholinergic postsynaptic membrane, being widely distributed in nerve and muscle cell membranes, electrogenic tissue from electric fish, and erythrocyte membranes. This circumstance suggests the possibility that acetylcholinesterase may be involved in other membrane functions as well [1,2] and explains the difficulties observed when trying to use the enzyme as a marker for the presence of cholinergic synapses [3,4]. The identification of several structural forms of the enzyme, characterized by definite sedimentation coefficients [5–13], has opened the way for the assignment of different molecular forms to different localizations and/or potential functions [7,9,10,12]. Special attention has been paid to the so-called asymmetric or tailed forms of the enzyme composed of 1–3 tetramers of the catalytic subunit (mol. wt ~80 000) associated to a filamentous, collagen-like tail, probably implicated in the anchorage of the enzyme to the cell membrane matrix [12–15]. The specific association of a 16 S form of acetylcholinesterase to the muscle junctional membrane (the postsynaptic element of the neuromuscular cholinergic synapse) was detected [7]. Other fast-sedimenting forms of the enzyme, associated to cholinergic synapses, have been described in chick [9], mouse [16], and human [12] muscle, and in chick [9], rat [10,11] and bovine [13] ganglia. Furthermore, a direct dependence of the initial synthesis (induction) and/or maintenance of said molecular forms of acetylcholinesterase upon the integrity of the presynaptic component has been demonstrated in several cases [9,17–19]. However, it has not yet been possible to find a likely candidate for the synaptic form of the enzyme in the central nervous system [8,9,20].

It has been suggested [12,13] that the molecular and structural patterns of association of the acetylcholinesterase catalytic subunits (and tail proteins) may be very much the same for different species and tissues. This paper describes the release by collagenase of two molecular species of acetylcholinesterase of 21.5 S and 16.5 S, from the young chick visual system (both retina and optic tectum). It is postulated that these two collagenase-released enzyme forms derive from tailed native acetylcholinesterase species containing 3 and 2 tetrameric clusters of catalytic subunits, respectively (forms A_{12} and A_8 of [12,13]).

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2. Experimental

2.1. *General procedure*

White-Leghorn, Shaver strain chicks, 10–20 days, were used in this study. They were kept on a 12 h light/12 h dark schedule. The retinas and optic lobes were dissected over ice, and homogenized in 10 vol. 10 mM Tris–HCl (pH 7), 1% Triton X-100, 1 M NaCl, and 50 mM $MgCl_2$ (henceforth referred to as Tris–Triton–salt buffer, or homogenization buffer), at 4°C, in a glass–glass conical homogenizer (Kontes

Duall) [17]. This homogenate was centrifuged at 110 000 × *g*, for 30 min, at 2°C (Sorvall OTD-2 ultracentrifuge). The supernatant was set aside, and the pellet was rehomogenized as before, and centrifuged again. This second pellet, representing the material insoluble in homogenization buffer, was further digested with collagenase or pronase, as described below. The digest was, once again, centrifuged at 110 000 × *g*, and the supernatant was analyzed by sedimentation in linear sucrose gradients.

2.2. *Collagenase and pronase treatments*

For collagenase digestion, the material was resuspended in a buffer containing 10 mM Tris–HCl (pH 7), 0.1 NaCl, 50 mM $MgCl_2$, 5 mM $CaCl_2$, and collagenase (EC 3.4.24.3) at 100 units/ml (Sigma type VI, devoid of protease activity, 840 units/mg). Routinely, the incubation was performed at 37°C, for 30 min, in a shaking water bath. Digestion with pronase (final conc. 0.25 mg/ml) was carried out in 50 mM potassium phosphate buffer (pH 7) under the same conditions.

2.3. *Sedimentation analysis*

The proteins solubilized by the Tris–Triton–salt buffer, or by means of collagenase or pronase digestion, were analyzed by sedimentation through a 5–20% sucrose gradient, prepared in this Tris–Triton–salt homogenization buffer. The sedimentation coefficients were estimated according to [21], using *Escherichia coli* β-galactosidase (EC 3.2.1.23; 16 S), beef liver catalase (EC 1.11.1.6; 11.3 S) and *E. coli* alkaline phosphatase (EC 3.1.3.1; 6.1 S) as internal marker enzymes. The centrifugation was carried out in a SW40Ti (Beckman) rotor, at 154 000 × *g*, 4°C, for 18–20 h. Fractions (~0.4 ml) were collected and assayed for acetylcholinesterase and marker enzymes.

2.4. *Enzymatic assays*

Acetylcholinesterase was analyzed as in [22], using acetylthiocholine iodide as the substrate, 0.06 mM tetraisopropylpyrophosphoramide (Sigma) as a butyrylcholinesterase inhibitor (EC 3.1.1.8) and 0.01 mM 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284C51, Wellcome Lab., Beckenham) as an acetylcholinesterase inhibitor. For more precise, comparative measurements of some fractions, a radiometric method of assay [3] was employed, after localization of the peaks according to [22]. As for the marker enzymes, the procedures recommended in the technical bulletins accompanying the products (Sigma Chem. Co., St Louis) were followed in all experiments.

3. Results

When day 10 chick retinal or tectal tissues are extracted with the typical Tris–Triton–salt buffer, and the extract is analyzed by sedimentation in sucrose gradients, two peaks of acetylcholinesterase activity, with sedimentation coefficients of 11 S (retina, 11.02 ± 0.40; tectum, 10.84 ± 0.34) and 6 S (retina, 6.15 ± 0.18; tectum, 6.20 ± 0.34), can be observed (fig.1). This agrees closely with the results obtained by other authors working on chick brain [9,20].

In our experience, this buffer extract, in the case of the 10-day old chick, accounts for ~95% (retina) or 94% (tectum) of the total enzyme activity in the initial homogenate. To investigate the nature of the 5–6% not extracted by the Tris–Triton–salt buffer

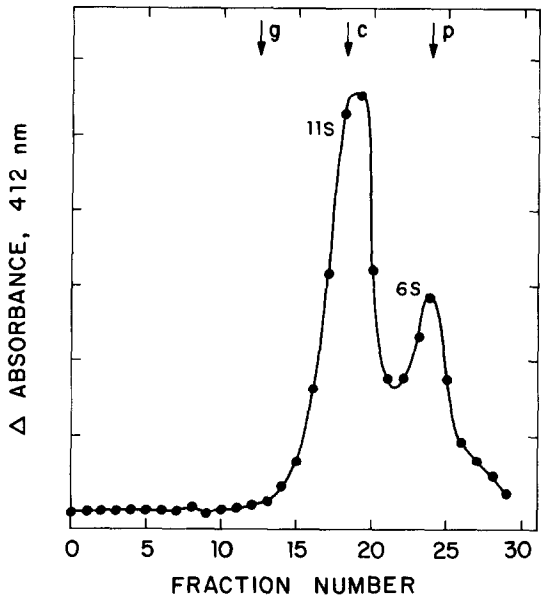

Fig.1. Sedimentation pattern of the molecular species of acetylcholinesterase solubilized, from 10 day chick retina, by means of a Tris–Triton–salt buffer. Sedimentation direction is to the left. The enzyme activity is given in arbitrary units, proportional to the ΔA_{412} [22]. The arrows point to the position of the markers in the gradient: g, β-galactosidase (16 S); c, catalase (11.3 S); p, alkaline phosphatase (6.1 S). 10 day chick tectum gives the same qualitative result.

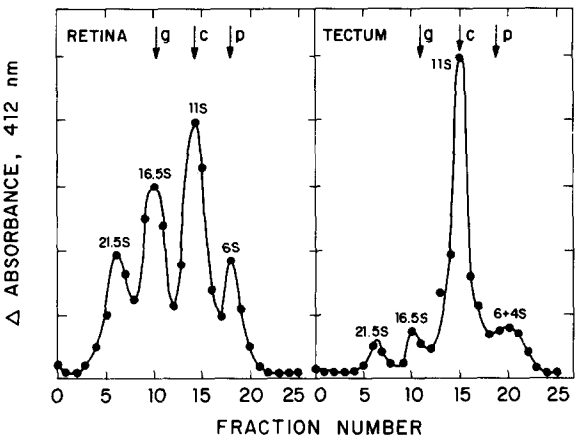

Fig.2. Sedimentation patterns of the molecular species of acetylcholinesterase released by collagenase from the Tris–Triton–salt-insoluble residue in 10 day chick retina (left), and optic tectum (right). Experimental details as in fig.1.

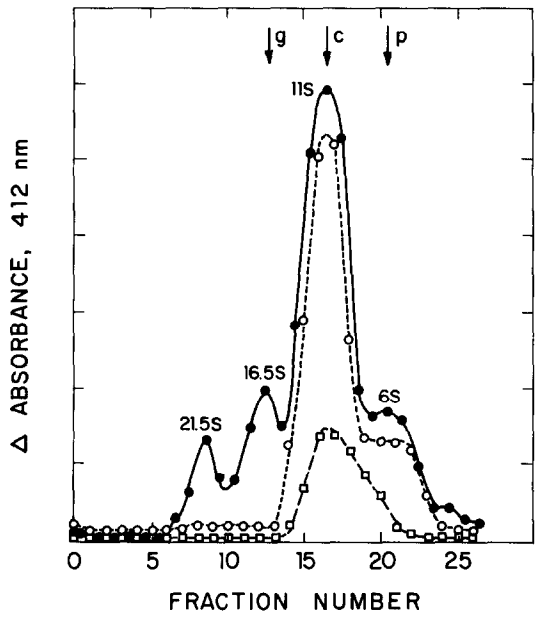

Fig.3. Specificity of the collagenase action. The residual pellet after extraction of 20 day chick tecta with Tris–Triton–salt buffer was divided into 3 parts: digested with collagenase (●——●); digested with pronase (□– – –□); and simply incubated at 37°C for the same time (○– – –○). After these treatments, the 3 samples were centrifuged at high speed, and the supernatants analyzed by sucrose gradient centrifugation. The 3 independent sedimentation runs have been matched to identical marker positions. Other details as in fig.1.

we subjected the residual pellet to digestion with highly purified, protease-free collagenase. This treatment released 50–70% of the remaining activity in that pellet. The sedimentation pattern of the molecular forms of acetylcholinesterase released by collagenase can be seen in fig.2. As the figure shows, two new peaks with acetylcholinesterase activity are now visible, both in retina and tectum. The apparent sedimentation coefficients for these two new forms of the enzyme are 21.5 S (retina, 21.80 ± 0.71; tectum, 21.55 ± 0.36) and 16.5 S (retina, 16.70 ± 0.14; tectum, 16.54 ± 0.30). In terms of activity they represent, in the case of the retina, a 0.66% (21.5 S), and 0.88% (16.5 S) of the total homogenate activity; as for the optic tectum, they constitute the 0.19% (21.5 S) and 0.25% (16.5 S) of the initial homogenate activity. Studies with specific inhibitors have shown that these peaks are ⩾85% true acetylcholinesterase. That the release of these two new molecular forms of acetylcholinesterase is due to the specific action of collagenase and not to the increased temperature during the digestion (37°C, in contrast with the 4°C maintained during the extraction with the Tris–Triton–salt buffer), is demonstrated in the control experiment illustrated in fig.3. We see that only collagenase, and not the higher temperature, or even pronase, can release the 21.5 S and 16.5 S forms of acetylcholinesterase from the Tris–Triton–salt-insoluble residue from chick tectum.

Next, we did some experiments to ascertain the individuality of, or the possible mutual relationship between, the 21.5 S and 16.5 S forms of acetylcholinesterase. First we varied the parameters relative to the collagenase digestion, namely incubation time and collagenase concentration. No qualitative or quantitative changes in the amount and relative proportion of the 21.5 S and 16.5 S forms were observed with digestions of 15–60 min, and 50–200 units collagenase/ml. To confirm the individuality of the two new molecular forms of acetylcholinesterase we took the fractions belonging to the 21.5 S and 16.5 S peaks in the retina gradient in fig.2, dialyzed them against homogenization buffer, and digested them again with collagenase (incubating a portion of the material at 37°C, without collagenase, as a control for the stability of the enzyme). The sedimentation patterns given in fig.4 show that collagenase just releases the 21.5 S and 16.5 S acetylcholinesterases from the Tris–Triton–salt-insoluble pellet, without further modifying them. On the other hand, pronase

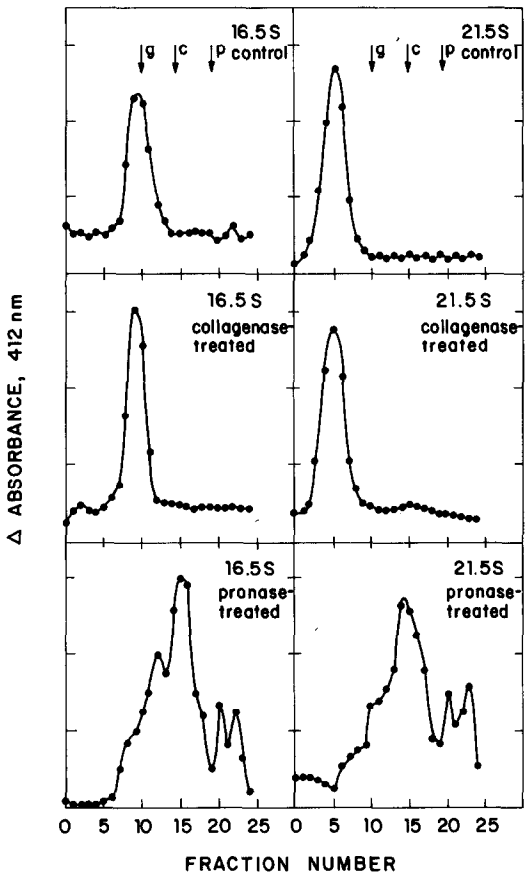

Fig.4. Stability of the 21.5 S and 16.5 S acetylcholinesterase molecules to further action of collagenase. The 21.5 S and 16.5 S peaks from 10 day chick retina (fig.2, left panel) were isolated and divided each into 3 parts: redigested with collagenase; redigested with pronase; and the control, was just incubated at 37°C for the duration of the enzymatic digestion. The ordinate scale in the pronase-treated peaks has been reduced for the sake of clarity, since in the pronase gradient only 35% of the activity (versus 100% in the collagenase and control gradients) was recovered. Other details as in fig.1.

(0.25 mg/ml), under the same incubation conditions, converts most of the 21.5 S and 16.5 S forms to the known 11 S and 6 S (and 4 S) species. While the treatment with collagenase did not affect the activity of the enzyme, after the pronase digestion only ~35% of the initial activity was recovered.

4. Discussion

We have described how collagenase can release two molecular species of acetylcholinesterase 21.5 S and 16.5 S, from the material insoluble after homogenizing chick retina and optic tectum in a Tris–Triton–salt buffer. These two forms seem to originate from independent tailed native forms, and are not affected by subsequent treatment with collagenase. However, limited exposure to pronase converts them, to a great extent, to the more common 11 S, 6 S and 4 S forms. In [23], the stepwise modification by collagenase of the tailed acetylcholinesterase forms of *Electrophorus* has been analyzed at 20°C and 37°C. While we have not been able to confirm, in the case of the chick enzyme, the final conversion by collagenase of the tailed enzyme forms to the 11 S species, at least under our experimental conditions, the apparently increased release of the 11 S form, upon incubation of the Tris–Triton–salt insoluble pellet at 37°C in the presence of collagenase (fig.3) suggests that the action of collagenase on the membrane-bound enzyme may be somewhat more complex than just releasing the 21.5 S and 16.5 S acetylcholinesterase forms.

For day 10-old chicks, the retinal fast-sedimenting forms account, together, for ~1.5% of the total activity in the initial retinal homogenate; in the case of tectum, this proportion is ~0.5%. This fact, and the potential difficulty in extracting them in their native form have, most likely, prevented their identification [9,20]. Nevertheless, using our procedure it is possible to detect them as early as day 8 of embryonic development (unpublished results).

What may be actually important about these two collagenase-released forms is that they bear out the existence, in the central nervous system, or multitetrameric tailed species of acetylcholinesterase, as with all other excitable tissues studied in depth. It is well known that collagenase, both in the electric fish enzyme forms, and in muscle, cleaves specifically the collagen-like tail without disorganizing the assembly of catalytic subunits (tetramers). This process results in a shift of the coefficient of sedimentation towards higher values since, in spite of the loss of protein mass, the increased symmetry of the tailless enzyme causes it to sediment faster than its native counterpart [13–16, 23–25]. Within this structural model, our 21.5 S form could derive from the brain equivalent of the 19.5 S native form (A_{12}) found [9] in the chick muscle and ciliary ganglion, and thought to be directly involved in cholinergic neurotransmission (we have observed two acetylcholinesterase species in embryonic chick muscle, extractable by the standard homogenization buffer, with sedimentation coefficients of

19.6 S and 15 S, giving rise to 21.5 S and 16.5 S forms upon collagenase treatment). In agreement with the general model [13], the 16.5 S and 21.5 S species would then correspond to the assemblies of 2 and 3 tetramers of catalytic subunits including, at least, the portion of the tail that keeps the tetramers together [15,23]. To confirm this working hypothesis it is, nevertheless, necessary to isolate and characterize the fully-tailed native enzyme molecular species. The fact that they are not releasable by the standard Tris–Triton–salt buffer suggests that they may interact with neuronal membranes in some different way from those proposed so far, namely hydrophobic interactions with specific lipid domains in the membrane [13,15,26] and/or ionic interactions with basal lamina-like structures [13,27]. We are working on these problems in the hope that all new developments on the structure and molecular properties of an enzyme so abundant and ubiquitous in the nervous system will be of decisive help, not only in understanding more deeply the mechanism of cholinergic neurotransmission, but also in gaining new insights on other possible roles of acetylcholinesterase in excitable membranes.

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