Binding of the asymmetric forms of acetylcholinesterase to heparin

Enrique BRANDAN and Nibaldo C. INESTROSA*

Laboratory of Neurophysiology, Department of Cell Biology, Catholic University of Chile, P.O. Box 114-D, Santiago, Chile

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The interaction between acetylcholinesterase (EC 3.1.1.7) and heparin, a sulphated glycosaminoglycan, was studied by affinity chromatography. A specific binding of the asymmetric acetylcholinesterase to an agarose gel containing covalently bound heparin was demonstrated. This interaction required an intact collagenous tail, shown by the fact that the binding is abolished by pretreatment with collagenase. The globular forms did not bind to the column. Both total and intracellular asymmetric acetylcholinesterase forms isolated from the endplate region of the rat diaphragm muscle showed higher affinity for the heparin than did the enzyme from the non-endplate region. The binding to the resin was destabilized with 0.55M-NaCl, and, among the various glycosaminoglycans tested, only heparin was able to displace the acetylcholinesterase bound to the column. Our results added further support to the concept that the asymmetric acetylcholinesterase forms are immobilized on the synaptic basal lamina via interactions with heparin-like molecules, probably related to heparan sulphate proteoglycans.

Acetylcholinesterase (AChE) is highly concentrated at the neuromuscular junction, where it plays an essential role in synaptic transmission by rapidly removing, through hydrolysis, the acetylcholine released into the synaptic cleft from presynaptic nerve terminals (Kuffler & Nicholls, 1979). The different AChE molecules may be classified as globular forms (monomers G_1 , dimers G_2 and tetramers G_4) and asymmetric forms (containing one, two or three tetramers, plus a collagen-like tail: A_4 , A_8 and A_{12}) (Massoulié & Bon, 1982). In rat and mouse muscles the enzyme forms that are concentrated at the neuromuscular junction are the asymmetric forms of AChE (Hall, 1973; Inestrosa *et al.*, 1982). These forms would be anchored to the basal lamina by the collagen-like tail (McMahan *et al.*, 1978; Bon *et al.*, 1979; Inestrosa *et al.*, 1982). The basal lamina is an extracellular coat of collagen, glycoproteins and proteoglycans (Sanes, 1982; Yamada, 1983) that has been implicated in both pre- and post-synaptic differentiation (Sanes, 1983). This lamina sur-

rounds each muscle fibre, extending through the synaptic cleft (Sanes *et al.*, 1978). It has been suggested that the association of the asymmetric AChE with glycosaminoglycans and fibronectin may serve to bind the enzyme to the basal lamina (Massoulié & Bon, 1982). Recent studies from our laboratory have shown that heparin, a sulphated glycosaminoglycan, specifically solubilizes the collagen-tailed AChE from endplate regions of the rat muscle (Torres & Inestrosa, 1983) and from the rat peripheral nerve (Torres *et al.*, 1983).

We have studied the binding of the different AChE forms to heparin-agarose by means of affinity chromatography. Our studies show that only the asymmetric forms of AChE bind to the heparin column and that such binding is dependent on the tail structure. We also find a higher binding of asymmetric AChE obtained from the endplate region compared with that from the non-endplate region. Furthermore, we have characterized the properties of the binding using asymmetric AChE from *Discopyge* electric organ. The results indicated that the binding is strong and homogeneous and that among various glycosaminoglycans only heparin is able to displace the AChE bound to the column.

Abbreviations used: AChE, acetylcholinesterase (EC 3.1.1.7); Echothiophate, *S*-[2-(*NNN*-trimethylammonio)ethyl]-*OO*-diethylphosphorothioate iodide.

* To whom correspondence should be addressed.

Experimental

Animals

The experiments were carried out on male Sprague-Dawley rats (100–250g body wt.) and on *Discopyge tschudii*, a fish of the Torpedinidae Family (Inestrosa & Méndez, 1983).

Dissection of rat diaphragm muscle

Rat diaphragm muscle, together with attached ribs, was removed, washed in ice-cold saline (0.9% NaCl), rapidly cleaned of most of the connective tissue and then the muscle separated from the ribs. The diaphragm was divided into regions containing endplates and those without endplates, by cutting a 2–3mm strip of muscle alongside the nerve under a dissecting microscope (Hall, 1973; Inestrosa *et al.*, 1982; Torres & Inestrosa, 1983).

Extraction of AChE activity

Rat muscle diaphragm. The endplate and non-endplate regions were homogenized in 10mM-Tris/HCl buffer, pH7.4, containing 20 units of aprotinin/ml, 1mM-benzamidine and 5mM-EDTA. The homogenate was centrifuged in a Sorvall SS-34 rotor at 15000g for 15min at 4°C. This supernatant was the source of globular forms of AChE (Bon *et al.*, 1979). The pellet was resuspended in the same homogenization medium plus 0.5% (v/v) Triton X-100 and centrifuged again. The resulting pellet was resuspended in 10mM-Tris/HCl buffer, pH7.4, containing 20 units of aprotinin/ml, 1mM-benzamidine, 5mM-EDTA and 1.0M-NaCl and centrifuged at 15000g for 15min. The supernatant, containing the asymmetric forms of AChE, was dialysed against 1000vol. of 10mM-Tris/HCl buffer, pH7.4, containing 0.1M-NaCl for 3h at 4°C. The non-diffusible material was centrifuged as above. The pellet was resuspended in 10mM-Tris/HCl buffer, pH7.4, containing 0.2M-NaCl. This was the source of asymmetric forms of AChE.

Electric organ of *Discopyge*. Electric organ was dissected out and kept frozen at –80°C. The tissue was homogenized in 10mM-Tris/HCl buffer, pH7.4, containing 20 units of aprotinin/ml, 1mM-benzamidine, 5mM-EDTA, 0.5% (v/v) Triton X-100 and 0.1M-6-aminohexanoic acid. The homogenate was centrifuged in an SS-34 rotor at 15000g for 15min at 4°C. The pellet was rehomogenized in the same medium and centrifuged again. This step was repeated four times. The last pellet was resuspended in the same medium as above plus 1.0M-NaCl. The mixture was centrifuged as before and the supernatant contains a crude fraction of asymmetric forms of AChE.

Selective inhibition of external AChE from rat diaphragm muscle

In order to inactivate external AChE, the method described by Younkin *et al.* (1982) was used. In brief, diaphragms detached from the ribs were immersed in cold phosphate-buffered saline (150mM-NaCl/10mM-sodium phosphate buffer, pH7.4) containing 1.25μM-Echothiophate (an impermeable and irreversible inhibitor of the AChE) for 20min at 4°C. Then the muscle were rinsed several times with cold phosphate-buffered saline, and endplate and non-endplate regions were dissected out as described above.

Sedimentation analysis of AChE

The different molecular forms of AChE were separated by sedimentation on linear sucrose gradients (Inestrosa *et al.*, 1979, 1981). A Hoeffler gradient-former was used. Linear sucrose gradients (5–20%, w/v) in 50mM-Tris/HCl buffer, pH7.4, and containing 5mM-EDTA, 1.0M-NaCl and 0.5% (v/v) Triton X-100 were used. A 200μl sample was layered on the top of the gradient, and centrifugation was performed in a Beckman SW65 rotor at 215000g for 7.5h at 4°C in a Beckman L5-65 ultracentrifuge. Fractions were collected from the bottom of the gradient and assayed for AChE activity. Sedimentation coefficients for AChE forms were estimated by comparison with that for β-galactosidase (16.1S) measured as described by Craven *et al.* (1965).

Binding of AChE to heparin affinity column

The globular or asymmetric forms of AChE were applied to a heparin-agarose column (0.3cm × 2.0cm) containing 0.4ml of resin pre-equilibrated with 10mM-Tris/HCl buffer, pH7.4, containing 0.2M-NaCl. The columns were washed extensively with the same buffer, and the bound AChE was eluted with 10mM-Tris/HCl buffer, pH7.4, containing 1.0M-NaCl. When fractions obtained from the sucrose gradients were used, bovine serum albumin (2.0mg/ml) was included in all the solutions. In some experiments the bound AChE was eluted with different glycosaminoglycans, the conditions of which are specified in the legends to the corresponding Figures.

Treatment with collagenase and heparinase

Treatment with collagenase. Asymmetric forms of AChE were digested with highly purified collagenase from *Clostridium histolyticum* (Sigma type VI). In brief, asymmetric forms of AChE were incubated with 22 units of collagenase in the presence of 100mM-Tris/HCl buffer pH7.2, containing 7.5mM-CaCl₂ for 1h at 37°C. When EDTA was present the concentration used was 7.5mM.

Treatment with heparinase. Heparin-agarose resin was incubated for 20h with 1.2mg of heparinase/ml in 10mM-Tris/HCl buffer, pH 7.4, containing 0.15M-NaCl and 2.0mg of bovine serum albumin/ml at room temperature. Then the resin was washed with high-ionic-strength buffer, pre-equilibrated with the same buffer as above and the AChE-binding capacity evaluated.

AChE assay

AChE activity was measured by the method of Ellman *et al.* (1961). The determinations were done in a 1.0ml of reaction mixture containing 100mM-sodium phosphate buffer, pH 7.0, 0.75mM-acetylthiocholine iodide and 0.3mM-5,5'-dithiobis-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by the samples was decreased at least 95% by the addition of 10 μ M-compound BW284c51, a selective AChE inhibitor. One unit of AChE activity is equal to 1.0 μ mol of substrate breakdown/min at 37°C in the above conditions.

Reagents

The following chemicals were used: heparin, chondroitin sulphate, hyaluronic acid, collagenase, acetylthiocholine iodide were from Sigma Chemical Co.; heparin-agarose was from Bethesda Research Laboratories; immunoglobulin G-agarose was generously given by Dr. Alfredo De Ioannes from our Department; Echothiophate was a gift from Dr. Hugo Fernández (University of Kansas, Kansas City, KS, U.S.A.); compound BW284c51 was from Burroughs Wellcome Co. Crude heparinase was kindly given by Dr. Alfred Linker (Veterans Administration Hospital, Salt Lake City, UT, U.S.A.). Other reagents, where not otherwise specified, were obtained from commercial sources.

Statistical methods

Statistical significance was determined by Student's *t* test for paired samples.

Results

Binding of AChE from rat muscle to the heparin-agarose column

We examined whether rat skeletal-muscle AChE would interact with the sulphated glycosaminoglycan heparin. For this purpose, globular and asymmetric forms of AChE were tested by using affinity chromatography on different resins. No binding of asymmetric or globular enzyme preparations to Sepharose 4B nor immunoglobulin G-Sepharose was detected. However, 50% of the asymmetric form of AChE was bound to the heparin-agarose column. Most of the AChE activity from the globular preparation did not bind to the column.

Fig. 1 shows the chromatographic profiles of the globular (Fig. 1a) and asymmetric (Fig. 1b) preparations from the endplate regions of rat diaphragm muscle, eluted from an heparin-agarose affinity column. Most (80%) of the asymmetric forms bound to the column were released by heparin; the remaining 20% required high ionic strength (1.0M-NaCl) to be eluted. Of the total asymmetric AChE forms applied to the column, 50–60% of the activity was bound, and this value was not changed by increasing the column size 5-fold; moreover, when the unbound asymmetric AChE was re-applied to the column, no further binding was observed. This suggests that the asymmetric AChE forms can exist in different states, i.e. 'binding' and 'non-binding' species of the enzyme. In order to show the specificity of the column, a mixture of both globular and asymmet-

Fig. 1. Affinity chromatography of muscle AChE on an heparin-agarose resin column

Crude preparations of globular (4.5 munits) containing 2mg of protein (a) and asymmetric (7.7 munits) containing 1.7 mg of protein (b) forms of AChE were loaded on the column (0.4cm $\times$ 2cm) containing 0.8mg of heparin/ml of gel, as described in the Experimental section. Arrows marked Hep and NaCl indicate the beginning of the elution with heparin and NaCl respectively. Each fraction contains 180 μ l, and samples were assayed for AChE activity. The recovery of the esterase activity in (a) was 90% and in (b) was 79%.

ric AChE preparations was run through the heparin column, and both the applied enzyme and the AChE forms eluted by heparin were analysed in a linear 5–20% sucrose gradient. Fig. 2 shows the sedimentation pattern of the AChE molecular forms loaded on the column (Fig. 2a) and the forms released by heparin (Fig. 2b). It is clear that only the asymmetric A_{12} and A_8 forms were eluted by heparin from the heparin-agarose column.

The difference between asymmetric and globular AChE forms resides in the presence of a tail of collagenous nature in the asymmetric forms. In order to test whether the collagen tail is in fact required for the binding of the asymmetric AChE to the heparin-agarose column, we treated a

fraction enriched in asymmetric forms, obtained from rat diaphragm or electric organ from *Discopyge*, with purified clostridial collagenase and subsequently tested the mixtures for their binding capacity. As Table 1 illustrates, the treatment with collagenase abolished the interaction of the asymmetric forms from both sources with the heparin-agarose column. These data clearly suggest that the collagenous tail is required for the interaction between the asymmetric form of AChE and heparin. Table 1 also shows that when heparin is present in the column buffer no binding of AChE is detected.

Binding of total and internal asymmetric AChE species from rat muscle diaphragm to the heparin-agarose column

The asymmetric forms of AChE are concentrated at the endplate region of skeletal muscles (Hall, 1973). However, as established by Younkin *et al.* (1982), they are also found in the non-endplate regions. In order to compare the relative affinities of both pools of asymmetric forms for the heparin-agarose column, we tested fractions of endplate and non-endplate asymmetric forms for binding to the heparin-agarose column. Table 2 shows a higher relative affinity of the asymmetric AChE from the endplate region, as compared with that from non-endplate region, for the heparin-agarose. Because at the endplate region most of the asymmetric AChE corresponds to external AChE (Younkin *et al.*, 1982), we measured the binding capacity of the intracellular pool of AChE. For this purpose, we inactivated the external AChE from both the endplate and the non-endplate regions, using the impermeable inhibitor of AChE, Echothiophate (Inestrosa, 1984), and then worked with the residual asymmetric forms of AChE. No differences in binding capacity as compared with the total AChE was found, when the internal pool of AChE from both regions was applied to the column (Table 2). Since globular forms did not bind to the column, we wanted to know whether the differences in binding detected between endplate and non-endplate asymmetric forms was due to contamination of the non-endplate fraction with globular forms. Table 3 shows the distribution of the molecular forms of both samples; it is clear that the amount of globular AChE forms present in both regions is similar, and therefore we cannot attribute the differences observed in binding to contamination by globular forms of the non-endplate fractions. Therefore the only apparent difference between the endplate and non-endplate asymmetric enzymes, namely the different proportions of intra- and extra-cellular pools do not explain their observed differences in binding to the column.

Fig. 2. Sedimentation profiles of muscle AChE applied and specifically eluted from the heparin affinity column (a) corresponds to a mixture of the globular and asymmetric preparations loaded to the column of Fig. 1. (b) corresponds to the AChE activity specifically eluted by heparin from the column loaded with the mixture of AChE forms. Both samples were layered over 5–20% sucrose gradients in 50 mM-Tris/HCl buffer, pH 8.4, containing 1.0 M-NaCl and 0.5% Triton X-100. Gradients were fractionated into approximately 36 samples, and portions of each were assayed for AChE activity. The arrows indicate the sedimentation position of β -galactosidase (16.1 S).

Table 1. *Effect of collagenase and heparin on the binding of asymmetric AChE to heparin-agarose*

For the collagenase treatment, asymmetric forms from either endplate region of diaphragm or electric organ were digested with 22 units of purified collagenase in the presence of 100 mM-Tris/HCl buffer, pH 7.2, containing 7.5 mM-CaCl₂ for 1 h at 37°C. Controls were incubated in presence of 7.5 mM-EDTA and without CaCl₂; no effect on the binding was seen in either case. After the incubation, the mixture was loaded on the heparin-agarose column and the activity eluted was collected and measured as described in the text. For the heparin treatment, asymmetric forms were loaded in a column pre-equilibrated with 2.0 mg of heparin, and fractions were collected and AChE activity measured as indicated for the collagenase-treated functions. Binding is expressed as the percentage in each fraction of the total AChE activity recovered. Collagenase had no effect on AChE activity. The data are the mean values for two separate experiments. The total AChE activity recovered was more than 90% of the total activity applied to the column.

Elution	Treatment ...	Asymmetric forms of AChE (% of AChE recovered)					
		Diaphragm muscle			Electric organ		
		Control	Collagenase	Heparin	Control	Collagenase	Heparin
Tris/HCl (50 mM)		35.1	82.2	99.5	15.3	96.4	98.7
Heparin (2 mg/ml)		53.1	9.4	0.5	70.3	3.3	1.3
NaCl (1 M)		11.8	8.3	0.0	14.2	0.3	0.0

Table 2. *Binding of asymmetric forms of AChE from endplate and non-endplate regions of rat diaphragm to a heparin-agarose column*

AChE activity was extracted from endplate and non-endplate regions with high-salt solution as described in the Experimental sections. The activity solubilized by 1.0 M-NaCl was dialysed against 10 mM-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl for 3 h. The non-diffusible material was centrifuged in an SS-34 rotor at 15000g for 15 min at 4°C. The pellet was resuspended in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.2 M-NaCl and loaded on to a heparin-agarose column. The intracellular AChE was obtained as follows. Diaphragms from rat were immersed in 10 mM-Tris/HCl buffer, pH 7.4, containing 1.25 μ M-Ethothiophate for 20 min at 4°C. The residual AChE present in endplate and non-endplate regions was the intracellular enzyme. The activity unbound to the heparin-agarose column corresponded to the activity that was eluted with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.2 M-NaCl, and the bound activity to that which was eluted with 10 mM-Tris/HCl buffer, pH 7.4, containing 1.0 M-NaCl. The recoveries of the AChE activity were in the range 85–95%. Values shown in the Table are means $\pm$ S.E.M. for the numbers of observations indicated in parentheses. For the endplate region the total activity found in the homogenate was 0.95 ± 0.08 unit/g, and for the non-endplate region it was 0.43 ± 0.05 unit/g. The intracellular values were: endplate region, 0.22 ± 0.02 unit/g; non-endplate region, 0.17 ± 0.04 unit/g.

	AChE activity (%)	
	Non-endplate	Endplate
Total		
Unbound	50.8 ± 4.0 (5)	31.6 ± 3.1 (5)*
Bound	49.9 ± 4.0	68.4 ± 3.1
Intracellular		
Unbound	44.9 ± 5.0 (3)	36.9 ± 4.0 (4)
Bound	55.1 ± 5.0	63.1 ± 4.0

* $P < 0.001$.

Table 3. *Distribution of asymmetric forms of AChE from endplate and non-endplate regions*

Asymmetric forms of AChE obtained from the endplate and non-endplate regions of rat diaphragm muscle were isolated as indicated in Table 2 legend. Portions of these samples (total AChE) were loaded on a sucrose gradient (5–20%) in 50 mM-Tris/HCl buffer, pH 8.4, containing 5 mM-EDTA, 1.0 M-NaCl and 0.5% Triton X-100. The gradients were run at 215000g for 7.5 h in a Beckman SW65 rotor at 4°C. Gradients were fractionated and AChE assayed as indicated in Fig. 2 legend. The relative proportions of different AChE molecular forms in relation to total AChE recovered from the gradient (90% recovery) were evaluated by graphical integration of the area under each peak and under the total sedimentation profile. The data are the mean values for two separate experiments.

	AChE activity (%)	
	Endplate	Non-endplate
A ₁₂	45	41
A ₈	43	46
G ₄ + A ₄	12	14

Properties of the binding of AChE to heparin-agarose

In order to study some of the properties of the binding of AChE to the heparin-agarose column, we studied the binding capacity of the A₁₂ AChE from the electric organ of *Discopyge*.

The A₁₂ form was isolated from the peak of AChE activity (16S) obtained in a sucrose gradient loaded with a high-salt extract of the electric organ (results not shown). When this form of the enzyme was applied to an heparin-agarose column, more than 90% of the activity was bound, and all this activity was eluted with 1.0 M-NaCl. Since all the activity bound to the column was eluted with 1.0 M-

NaCl, we quantified the effect of ionic strength on the binding of the 16S AChE form to the heparin-agarose column. Fig. 3 shows the curve resulting when the AChE activity is loaded at different ionic strengths (Fig. 3, ●—●) or when activity loaded in 0.2M-NaCl was eluted with different ionic strengths (Fig. 3, ○—○). The results show that the binding of 16S AChE to the heparin-agarose column is strong. In fact, half of the AChE activity was still bound at 0.55M-NaCl. The Figure also shows that at 0.5M-NaCl 75% of the AChE was bound, but at 0.6M only 10% of the activity column remained bound. The fact that the destabilization of the binding of AChE to the heparin-agarose

Fig. 3. Effect of ionic strength on the binding of 16S AChE from *Discopyge* to heparin-agarose columns

Different columns were pre-equilibrated with 10mM-Tris/HCl buffer, pH 7.4, containing 2.0mg of bovine serum albumin/ml and NaCl at the concentrations indicated (●). A 0.14 unit portion of 16S AChE from *Discopyge* were loaded on each column and washed with 10 vol. of the same buffer. Then the columns were washed with 10 vol. of 10mM-Tris/HCl buffer, pH 7.4, containing 2.0mg of bovine serum albumin/ml and 1.0M-NaCl and the activities were assayed by the method of Ellman *et al.* (1961). Each point in the Figure was calculated as $100 \times$ the activity eluted by 1.0M-NaCl divided by the total activity recovered. In the curve (○), all the columns were pre-equilibrated with 10mM-Tris/HCl buffer, pH 7.4, containing 2.0mg of bovine serum albumin/ml and 0.2M-NaCl, and the columns were loaded with 0.14 unit of 16S AChE from *Discopyge* and washed with buffer containing NaCl at the indicated concentrations. After that, the columns were washed with 10mM-Tris/HCl buffer, pH 7.4, containing 2.0mg of bovine serum albumin/ml and 1.0M-NaCl in order to remove all the AChE still bound to the column. The activity was calculated as $100 \times$ the activity eluted by the buffer containing NaCl at different concentrations divided by the total AChE activity recovered. The recoveries in both experiments were in the range 78–90% of the loaded AChE activity.

column occurs over a rather narrow range of ionic strength indicates that probably there is only one binding site involved, or that, if more than one exists, then they behave in a very similar way.

It has been shown (Hayashi & Yamada, 1982) that fibronectin is able to bind heparin, and that such a binding is influenced by bivalent cations. In order to test if any effect of bivalent cations could be detected in the binding of the 16S AChE to heparin-agarose, we loaded different columns with electric-organ 16S AChE in presence of 10mM-MgCl₂, -CaCl₂ or -EDTA. In other experiments the enzyme was allowed to bind to the heparin-agarose column, and then it was eluted with the different cations or with EDTA. No effect on the binding or on the eluted activity was found in any of the conditions tested (results not shown).

As a control of the binding, we incubated the heparin-agarose with crude heparinase, in order to see how this treatment affects the binding of the enzyme to the column. The results are shown in the Table 4. The treatment of the resin with heparinase at the concentration used decreased the binding of the 16S AChE to the column to 30% of that obtained with the untreated resin. This result clearly indicates that the binding of the enzyme depends on the heparin attached to the agarose. Similar results were found when AChE from rat diaphragm was used.

Table 4. Effect of treatment of agarose-heparin with heparinase on the binding of asymmetric AChE from *Discopyge*

Heparin-agarose (0.3ml) was incubated for 20h, at the temperature indicated, in the presence of bovine serum albumin (2.0mg/ml). Heparinase, when present, was at a concentration of 1.2mg/ml. After the incubation the resin was poured into columns (0.4cm $\times$ 2.0cm) and washed with 20 vol. of 10mM-Tris/HCl buffer, pH 7.4, containing 2.0mg of bovine serum albumin/ml and 1.0M-NaCl. Then the columns were pre-equilibrated with 20 vol. of 10mM-Tris/HCl buffer, pH 7.4, containing 2.0mg of bovine serum albumin/ml and 0.2M-NaCl. Then 0.12 unit of 16S AChE from *Discopyge* was loaded on each column. The activity that was eluted in these conditions was the unbound AChE activity, and the bound activity corresponded to the activity that was eluted with 10mM-Tris/HCl buffer, pH 7.4, containing 2.0mg of bovine serum albumin/ml and 1.0M-NaCl. The recoveries of AChE activities were in the range 96–106%.

Treatment	...	AChE activity (%)		
		4°C for 20h	20°C for 20h	20°C for 20h + heparinase
Unbound		10.9	12.6	66.7
Bound		89.1	87.4	33.3

Heparin displaces the bound AChE from the heparin-agarose column

Since it has been shown that proteins bound to glycosaminoglycans can sometimes be displaced by other polysaccharides, we studied the effect of glycosaminoglycans in the elution buffer on the binding of the enzyme to the heparin-agarose column. For this, different columns were loaded with 16S AChE from *Discopyge*, and eluted with different concentrations of heparin, chondroitin sulphate or hyaluronic acid. The results are shown in Fig. 4(a). Only heparin was able to displace the AChE bound to the column. It may be noted that

Fig. 4. *Specific displacement of the 16S AChE bound to the heparin-agarose columns by heparin*

(a) Different columns were pre-equilibrated with 10mM-Tris/HCl buffer, pH 7.4, containing 2.6 mg of bovine serum albumin/ml and 0.2M-NaCl. A 0.12 unit portion of 16S AChE was loaded on each column, and washed with 2.0 ml of the same buffer. Each column was washed with the same buffer as above but containing heparin (●), chondroitin sulphate (○) or hyaluronic acid (Δ), at the concentrations indicated. The AChE activity was assayed in each fraction and was expressed as $100 \times$ the activity eluted by each concentration of glycosaminoglycan divided by the total activity loaded. (b) The percentage of activity eluted by each glycosaminoglycan at a concentration of 2.0 mg/ml is shown in (i). The remaining AChE activity bound to the column was eluted with 10mM-Tris/HCl buffer, pH 7.4, containing 2.0 mg of bovine serum albumin/ml and 1.0M-NaCl (ii). The recoveries of activity in (a) and (b) ranged between 83 and 105%. Key: Hep, heparin; CS, chondroitin sulphate; HA, hyaluronic acid.

the shape of the curve and the range of concentrations at which heparin displaced the enzyme from the column were similar to those found when heparin was used to release AChE from endplate region or electric-organ homogenates (Brandan *et al.*, 1983). No effect of the glycosaminoglycans employed on the AChE activity was involved, since almost all the bound AChE was recovered after elution with high-ionic-strength buffer (Fig. 4(b), compare (i) with (ii)).

Discussion

We have shown in the present work that the asymmetric forms, but not the globular forms, of AChE were able to bind to heparin-agarose columns. The asymmetric forms of AChE have a collagen-like tail and are sensitive to collagenase (Bon *et al.*, 1979). The binding was abolished when the asymmetric forms were treated with collagenase. This result suggests that the tail participates directly in the binding of the enzyme to the glycosaminoglycan. In view of our studies showing that heparin specifically solubilizes synaptic forms of AChE (Torres & Inestrosa, 1983) and the neural 16S AChE (Torres *et al.*, 1983), we have suggested that heparin might be competing with components of the muscle basal lamina, such as heparan sulphate proteoglycans, for binding sites on the collagenous tail of the asymmetric AChE. Furthermore, only heparin was able to release specifically the AChE bound to the column, for the enzyme obtained from both the endplate muscle regions and the electric organ. Therefore our studies strongly support the concept that a heparin sulphate proteoglycan is involved in the immobilization of the collagen-tailed AChE to the basal lamina of the neuromuscular junction.

Our results revealed that 90–95% of 16S AChE from electric organ is able to bind to heparin-agarose, compared with only a 65% of the asymmetric AChE of mammalian muscle. On the basis of such a difference we can offer some possible explanations: higher sensitivity to proteolytic damage of the muscle asymmetric forms as compared with the electric-organ enzyme. Although we used proteinase inhibitors in all the steps of the fractionation procedure, this possibility cannot be dismissed. Another possible explanation is the presence in the muscle extracts of other AChE-binding factors, which would decrease the actual amount of AChE available to bind to the column. A third possible explanation may be that the muscle contains a heterogeneous population of asymmetric forms of AChE with regard to their capacity to bind to the resin.

Another finding of this work that should be noted was the fact that the asymmetric AChE from

the endplate region showed higher affinity for the heparin-agarose resin than did the enzyme obtained from the non-endplate region. Since both preparations of enzyme contain similar proportions of globular forms, which do not bind to the column, then this difference should be related to some feature of the asymmetric pools of both regions.

Previous reports have strongly suggested that the asymmetric forms of AChE are concentrated at the neuromuscular junction in close association with the basal lamina (Bon *et al.*, 1979). However, it is not clear which are the molecules involved in the anchorage of the asymmetric AChE to the basal lamina. It has been suggested that the enzyme is associated with chondroitin sulphate (Bon *et al.*, 1978), fibronectin (Emmerling *et al.*, 1981), laminin, collagen type V and heparan sulphate (Vigny *et al.*, 1983). However, a recent report (Grassi *et al.*, 1983) has shown the absolute absence of binding of laminin, fibronectin and collagen type V to the asymmetric AChE. Unfortunately, the binding to heparan sulphate proteoglycans was not studied. However, in view of our previous work (Torres & Inestrosa, 1983; Brandan *et al.*, 1983) and the results given in the present paper, the relation between the asymmetric AChE and a heparin-like proteoglycan may turn out to be physiologically important.

Lipoprotein lipase is an enzyme that can be released from its anchorage sites by heparin (Olivecrona *et al.*, 1977), and it has been shown that it binds to heparin-Sepharose (Olivecrona *et al.*, 1971). Moreover, Williams *et al.* (1983) have shown that exogenous lipoprotein lipase can become bound to the endothelium cells by an interaction with heparan sulphate. Our results indicating both release by and binding to heparin of the asymmetric form of AChE strongly suggest the possibility that a similar binding mechanism might turn out to be involved in both cases.

In conclusion, a strong and reversible binding of asymmetric AChE to heparin, mediated by its collagen-like tail, has been demonstrated. Our results are very strong evidence supporting the concept that the asymmetric AChE is immobilized to the synaptic basal lamina via interaction with heparin-like macromolecules probably related to heparan sulphate proteoglycans, the most abundant proteoglycan species of the basement membranes (Farquhar, 1981).

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