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Heparin and the solubilization of asymmetric acetylcholinesterase

Ana Barat, Elena Escudero and Galo Ramírez+

Centro de Biología Molecular, CSIC-UAM, 28049 Madrid, Spain

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Heparin solubilizes asymmetric acetylcholinesterase, from chick skeletal muscle and retina, as a 24 S complex which is quantitatively converted to conventional asymmetric molecular forms of the enzyme (A_{12} and A_8, either class I or class II) upon exposure to high salt. The simultaneous presence of salt and heparin in the homogenization medium selectively prevents, however, the release of class II A-forms in both muscle and retina. Heparin may generally act by displacing native proteoglycans involved in the attachment of the enzyme tail to the extracellular matrix, or its neural equivalent, being in turn removed by salt to yield typical asymmetric enzyme forms. Heparin would also appear to displace some other molecules specifically involved in the EDTA-sensitive attachment of class II tailed forms, this effect being antagonized by salt.

Acetylcholinesterase *Heparin* *EDTA* *Proteoglycan* *(Chick muscle, Chick retina)*

1. INTRODUCTION

We have recently described the existence of 2 different classes of collagen-tailed, asymmetric molecular forms (A-forms) of acetylcholinesterase (AChE, EC 3.1.1.7) which appear to be present, in different relative proportions, in nearly all the cholinergic tissues and systems explored so far [1, 2]. The so-called class I and class II A-forms [1,2] have distinct solubilization properties (salt-soluble class I forms vs salt/EDTA-soluble class II forms) and, in the case of skeletal muscle, also show some differences in localization and neurogenic regulation [2].

Our previous work on the effect of complex carbohydrates on the solubilization of AChE (unpublished), and the findings of Inestrosa and coworkers [3,4] suggesting that heparin can release asymmetric AChE from muscle and nerve tissues, have prompted us to analyze the differential behavior of both classes of collagen-tailed AChE species in the presence of heparin. Special care has been exercised, however, to assess the effects of heparin per se, since in the work of Inestrosa et al. [3–5] the combined effects of heparin and salt (included in the gradient) were actually evaluated.

2. MATERIALS AND METHODS

Young chicks (10 ± 1 days) were used. Retinas and leg muscles were dissected and homogenized in a 10 mM Tris-HCl, pH 7, 1% Triton X-100, 2 mg/ml heparin TTH) solution (including 0.1 mg/ml bacitracin in muscle samples) and the homogenates were centrifuged at 110000 × *g* for 30 min. The detergent was omitted, at times, when processing retinal samples [6]. The pellets were rehomogenized in the above solution, and this cycle of extraction/centrifugation was repeated a minimum of 4 times. The material remaining insoluble after 4 homogenizations (or more) was, at times, re-extracted in other media as detailed in the text. The pooled extracts of each kind were analyzed for molecular forms of AChE by sucrose gradient centrifugation, with appropriate sedimentation markers, as described [6]. All operations were

+ To whom correspondence should be addressed

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carried out at 4°C. Additional experimental details are given in the figure and table legends.

3. RESULTS

3.1. *Skeletal muscle*

The sedimentation profile of the molecular forms of AChE released from chick leg muscle by a buffer-detergent solution containing 2 mg/ml heparin (TTH) is shown in fig.1. The left panel corresponds to a sedimentation run in which the sucrose gradients were prepared in the homogenization medium (TTH). The profile in the right panel was obtained when salt (1 M NaCl) was substituted for heparin in the sucrose gradient, as in the experiments of Inestrosa et al. [3,4]. The combined results in fig.1 suggest that heparin solubilizes asymmetric AChE as a 24 S complex (24.1 ± 1.1 in 10 independent runs) yielding the more usual A_{12} and A_8 AChE forms upon exposure of the complex to high salt. This is actually demonstrated in fig.2 where samples of the activity in the 24 S peak in fig.1 (left) were dialyzed against TTH buffer and sedimented again through sucrose

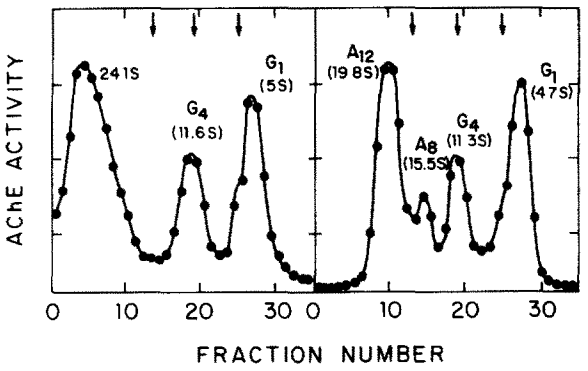

Fig.1. Extraction of chick skeletal muscle samples in heparin-containing solutions. 10-day chick leg muscles were homogenized repeatedly in buffer-detergent-heparin (2 mg/ml) solution (TTH), as described in section 2. The pooled extracts were run in sucrose gradients containing heparin (left) or salt (right), besides buffer and Triton X-100. The arrows point to the sedimentation markers, namely, from left to right, β-galactosidase (16 S), catalase (11.3 S) and alkaline phosphatase (6.1 S). Actual sedimentation coefficients of asymmetric (A) and globular (G) molecular forms of AChE are given in parentheses.

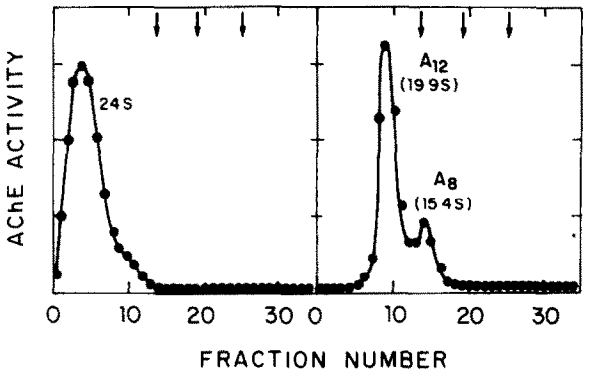

Fig.2. Conversion of the 245 S AChE complexes to conventional A-forms by exposure to 1 M NaCl. The 24 S peak fractions in fig.1, left, were pooled and dialyzed against TTH. Aliquots of the dialysate were centrifuged in sucrose gradients made up in either TTH (left) or TTS (1 M NaCl instead of heparin) (right). Other details as in fig.1.

gradients prepared in TTH (left) or Tris-Triton-1 M NaCl (TTS) (right).

A quantitative analysis of the peaks in fig.1 (right) furthermore suggests that both class I and class II A-forms are released from class-specific, heparin-extracted 24 S complexes, the total yield of A-forms in the experiment (56.1% of the total tissue AChE activity) being quite close to the value of 59% previously obtained for total A-forms (I + II) in 10-day chick leg muscle [2]. Further extraction of the pellet remaining after Tris-Triton-heparin (5 ×) in different solutions containing 1 M NaCl and 2 mM EDTA failed to release substantial amounts of additional A-forms. These results are summarized in table 1.

3.2. *Retina*

Chick retina contains class II A-forms almost exclusively [1,6], and can therefore be useful to ascertain the selective effects of heparin solutions on the solubilization of this specific class of asymmetric AChE. Fig.3, in a similar experimental set-up to that of fig.1, shows that heparin again solubilizes a 24 S complex (left) that is converted to A_{12} AChE when exposed to 1 M NaCl (right). The control experiment shown in fig.2 was repeated with the retinal 24 S complex with identical results. The indirect procedure of class II A_{12} solubilization from chick retina via the 24 S complex is

Table 1

Yield of A-forms in chick muscle and retina extracts obtained by different procedures

Tissue	First extraction solution	Extraction cycles	Yield (% of total tissue activity)		Second extraction solution	Extraction cycles	Yield (% of total tissue activity)	
			Total AChE	A-forms			Total AChE	A-forms
Muscle[a]	TTS	4	66	28[a]	TTS.EDTA	4	34	31[a]
	TTH	5	82	56	–	–	–	–
	TTS	5	65	27	TTHS	3	3	3
Retina[b]	TS.EDTA	4	35	10[b]	–	–	–	–
	TH	4	43	9	–	–	–	–

[ab] Results taken from experiments in [2] and [6], respectively

10-day chick samples (whole leg muscles and retinas) were extracted repeatedly in the solutions indicated above: T, Tris-HCl buffer (10 mM, pH 7); TT, Tris-Triton X-100 (1%); S, NaCl (1 M); H, heparin (2 mg/ml); EDTA, 2 mM. The different extracts were analyzed by sedimentation in sucrose gradients prepared in the respective extraction solutions, except that 1 M NaCl was substituted for heparin so as to obtain normally sedimenting A-forms. The presence or absence of detergent did not influende the yield of A-forms [6]

almost as efficient as the one-step extraction with buffered 1 M NaCl-2 mM EDTA, with or without detergent (table 1).

3.3. *Mechanism of conversion of the 24 S AChE complexes to A-forms*

We have carried out some preliminary experiments to explore the mechanism of conversion of the heparin-released 24 S complexes to conventional A-forms. Salt (1 M NaCl) was an absolute requirement in all cases. However, while running the heparin extracts in salt-containing gradients was sufficient to effect the conversion in retinal and dilute muscle samples, this simple procedure often gave rise to faster than usual A-forms (20–21.5 S instead of 19.5–20 S) when dealing with more concentrated muscle samples. Dilution and preincubation of the 24 S complexes with high salt, prior to sedimentation, facilitated the conversion. Dialysis against high salt, however, was more efficient (table 2). Thus, while a 1:1 dilution and 3 h of preincubation with 1 M NaCl reduced the sedimentation coefficient of A_{12} AChE from 21.3 to 20.3 S, dialysis (without dilution) against 1 M NaCl for 3 h resulted in normally sedimenting A_{12} forms (19.7 S; see table 2). This effect of dialysis, carried out in cellulose tubing of low M_r cutoff (~5000), which completely retains heparin, as confirmed in experiments using ^{35}S-heparin in the homogenization medium, suggests that some smaller molecule(s) might be involved in the attachment of proteoglycans to the enzyme tail.

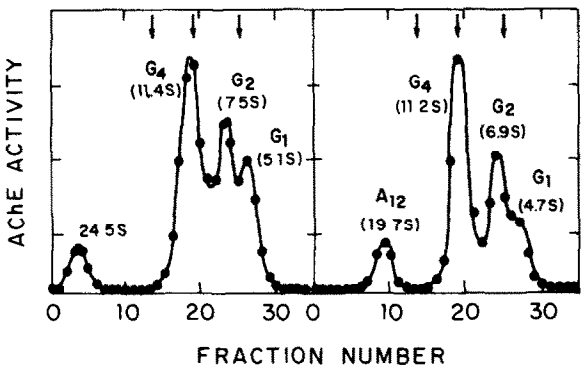

Fig.3. Extraction of chick retinal samples in heparin-containing solutions. 10-day chick retinas were homogenized repeatedly in TTH solution (see section 2 and fig.1). The pooled extracts were run in sucrose gradients containing heparin (left) or salt (right), besides buffer and detergent. Sedimentation markers as in fig.1.

3.4. *Effect of heparin-salt solutions on class II A-form solubilization*

When either muscle or retinal tissue is extracted with a buffer-detergent solution containing both heparin and 1 M NaCl, normally sedimenting A-forms are released instead of the intermediate 24 S complex. However, the yield of A-forms is

Table 2

Effects of dilution, preincubation with high salt and dialysis on the conversion of heparin-extracted 24 S AChE complexes to normally sedimenting AChE A-forms

Treatment	Time of preincubation or dialysis (min)	Sedimentation coefficients (S)	
		A_{12}	A_8
None (control)	-	21.3	17.4
Dilution (1:1)	-	20.8	17.0
Dilution and preincubation with high salt	30	20.6	16.8
	60	20.5	16.6
	120	20.4	16.3
	180	20.3	16.0
Dialysis	180	19.7	15.2

10-day chick leg muscles were extracted in the TTH solution described in section 2 and in table 1 (2 extraction cycles; one whole leg in a final volume of 15 ml). Samples of the TTH extract were diluted (1:1), diluted and made up to 1 M NaCl, or dialyzed against TTS (see table 1). The samples with added salt were preincubated at 4°C for 30–180 min. Dialysis was performed against 200 vols TTS buffer for 3 h (change of medium at 90 min)

substantially lower than in the 2-step experiments described in the foregoing sections. Apparently, the release of class II A-forms is selectively inhibited in the presence of both salt and heparin: in the case of muscle (table 1) heparin-salt does not solubilize more A-forms after previous extraction of the tissue with Tris-Triton-salt (which solubilizes only class I A-forms); even more to the point, heparin-salt solutions fail to solubilize A-forms from chick retina (where nearly all the asymmetric enzyme is class II), the effect being dependent on NaCl concentration (fig.4).

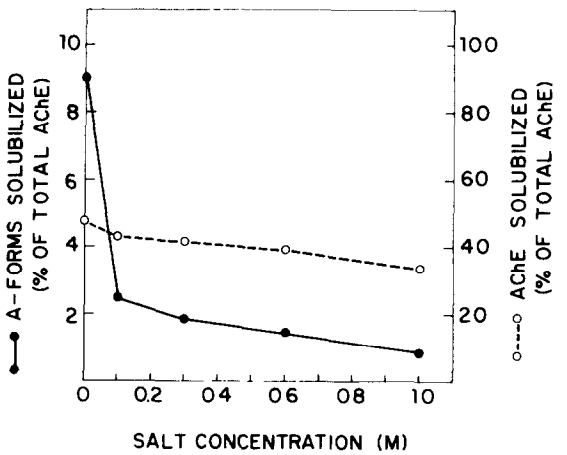

Fig.4. Extraction of retinal samples in salt/heparin-containing solutions. Retinal samples were extracted in TTH solution, supplemented with NaCl at different concentrations. The extracts were analyzed by centrifugation in sucrose gradients made up in Tris-Triton-salt solutions (1 M NaCl in all cases) so as to measure directly any A-forms released, as in figs 1 and 3, right panels.

This specific effect of heparin-salt solutions on the solubilization of class II A-forms is further confirmed in the experiments described in table 3: class II A-forms can still be solubilized, from both chick muscle and retina, by use of conventional salt-EDTA (but not salt alone) solutions [1,2,6], after extraction of class I species by heparin/salt-containing media. Moreover, the respective yields of class I and II A-forms in both tissues are in good agreement with previous estimates using our standard extraction procedures. The effects of heparin and heparin-salt solutions on the solubilization of both classes of asymmetric AChE are summarized in fig.5.

4. DISCUSSION

We have shown, in both chick skeletal muscle and retina, that heparin solubilizes asymmetric AChE as 24 S complexes which, upon further interaction with salt, are quantitatively converted into conventional class I or class II [1,2] A-forms (A_2 and A_8). This effect of heparin is independent of

Table 3

Delayed extraction of class II A-forms by salt-EDTA solutions

Tissue	First extraction solution	Extraction cycles	Yield (% of total tissue activity)		Second extraction solution	Extraction cycles	Yield (% of total tissue activity)	
			Total AChE	A-forms			Total AChE	A-forms
Muscle	TTSH	4	61	25	TTS.EDTA	3	37	32
Retina	TSH	4	34	0:8	TS.EDTA	3	11	6.5

10-day chick samples were first extracted repeatedly in buffered solutions containing both NaCl (1 M) and heparin (2 mg/ml), so as to inhibit the release of class II A-forms (see text). These were then solubilized by rehomogenization in a buffered solution containing NaCl (1 M) and EDTA (2 mM) [1,2,6]. Other details as in table 1

the presence or absence of detergent, as it has been shown to be the case with A-forms in different tissues [1,2,6]; in muscle samples, the presence of Triton X-100 is however desirable, as discussed in [2], while in retinal samples extracted without detergent there is a relative enrichment in A-forms in the primary extract that is usually beneficial for quantitative purposes [6].

The fast and full conversion of the 24 S AChE complex to A-forms is strictly dependent on the presence of salt. When dealing with retinal samples, running the heparin extracts in salt-containing gradients is enough to achieve the conversion. However, in the case of muscle, especially with concentrated samples, this procedure often gives rise to A_{12} forms sedimenting at 20–21 S, instead of the customary 19.5–20 S. This can be partly avoided by diluting or preincubating the heparin-containing samples with high salt, or, even more efficiently, by dialyzing against high salt. Our preliminary experiments with ^{35}S-heparin show that the effect of dialysis is not due to the loss of heparin itself, pointing to the involvement of some small molecule(s) in the stabilization of the 24 S complexes.

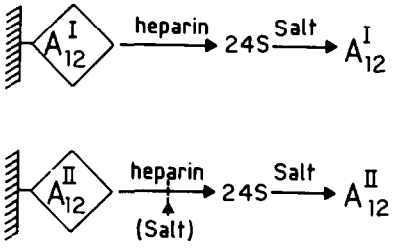

Fig.5. Diagram summarizing the effects of heparin and salt, used sequentially or simultaneously, on the release and solubilization of A-forms from chick muscle and retina. In the case of class II A-forms, salt (1 M NaCl) was found to inhibit the release by heparin of the 24 S AChE complex.

The specific interaction of heparin with the tail portions of AChE A-forms led Inestrosa and co-workers [3–5] to postulate the involvement of heparan sulfate proteoglycans in the attachment of A-forms to the extracellular matrix (or its neural equivalent in the case of retina). Furthermore, using heparinase digestion Vigny et al. [7] have provided additional evidence in support of such a hypothesis. Heparin would then act by competition with heparan sulfate in the interactions of this proteoglycan with the enzyme tail and/or with the extracellular matrix [8].

In addition to tailed enzyme molecules, the 24 S AChE complexes could then include some native proteoglycans partially or even totally replaced by heparin, and also some smaller molecule(s) involved in the proteoglycan-tail interactions, as evidenced by the dialysis experiments. The effect of high salt, both in the present work and in the routine extraction of tailed AChE with salt-containing solutions, could then be to break all interaction between the enzyme tail and the extracellular matrix proteoglycans. Interestingly, in the case of class II tailed forms heparin obviates the need for a chelating agent in addition to high salt, suggesting that heparin also displaces the molecules involved in the Ca^{2+} bridges postulated to explain the effect of EDTA and the attachment of class II A-forms. The antagonism between salt

and heparin, affecting only class II A-forms (figs 4,5 and tables 1,3) may be related to this particular effect of heparin. All these possibilities are being evaluated at present in our laboratory.

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