

Association of proacrosin with phospholipid membranes

J. William STRAUS and Kenneth L. POLAKOSKI

Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

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Proacrosin, the zymogen precursor of acrosin, was shown to associate with anionic phospholipid membranes through apparent electrostatic charge interactions. This association was diminished by elevated cation concentrations and was dependent on membrane composition, as shown both by direct binding assays and by following the phospholipid stimulation of conversion of proacrosin into acrosin.

Proacrosin is the zymogen precursor of acrosin (EC 3.4.21.10), a mammalian sperm proteinase that functions in the process of fertilization (Parrish & Polakoski, 1979). Current evidence has indicated that acrosin and, by implication, proacrosin are associated with membranes of the sperm acrosome (Brown & Hartree, 1976; Morton, 1977; Harrison & Brown, 1979), an intracellular organelle. However, the biochemical and physiological complexity of the sperm cell have limited meaningful characterizations of this enzyme system *in situ* (Zaneveld *et al.*, 1975). Studies with model membrane systems have shown that acrosin binds to liposome surfaces through salt-reversible electrostatic charge interactions (Straus *et al.*, 1981). This binding was dependent on the presence of anionic phospholipids and was diminished by increasing ionic strength, bivalent cations and acidity.

Anionic liposomes have also been shown to cause a marked acceleration in the spontaneous autolytic conversion of proacrosin into acrosin (Parrish *et al.*, 1978). Presumably this stimulation of zymogen conversion involves an electrostatic binding interaction between the zymogen and membrane surface similar to that observed with acrosin. If such binding interactions are involved, both zymogen-membrane binding and the resulting acceleration of conversion should be effected by ionic conditions and membrane composition, which also effect acrosin-liposome binding (Straus *et al.*, 1981).

Direct measurements of proacrosin-liposome binding are complicated by the extreme instability of the zymogen in the presence of anionic phospholipids (Parrish *et al.*, 1978). Accordingly, a sedimentation technique was developed to rapidly assess protein-liposome binding and was used in conjunction with benzamidine, an inhibitor of proacrosin conversion (Zahler & Polakoski, 1977; Polakoski & Parrish, 1977). In addition, liposome

stimulation of zymogen conversion was examined under conditions that effect electrostatic membrane binding.

Materials and methods

Highly purified proacrosin was prepared from acidic extracts of washed porcine spermatozoa by sequential pH 5.6, $(\text{NH}_4)_2\text{SO}_4$ and NaCl precipitations, urea denaturation and gel filtration (Polakoski & Parrish, 1977). Highly purified porcine m_β -acrosin was prepared as described previously (Parrish & Polakoski, 1977). Benzamidine and *N*- α -benzoyl-L-arginine ethyl ester were purchased from Sigma and phospholipids were from Avanti Polar Lipids (Birmingham, AL, U.S.A.).

Acrosin and proacrosin (after activation) were assayed by monitoring the enzymic hydrolysis of *N*- α -benzoyl-L-arginine ethyl ester spectrophotometrically as previously described (Polakoski & Parrish, 1977; Parrish *et al.*, 1978; Straus *et al.*, 1981). Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis was performed by the method of Laemmli (1970) as modified by Parrish *et al.* (1978).

Liposomes utilized for binding studies were prepared by dispersing equimolar mixtures of phosphatidylcholine and phosphatidylglycerol in 0.05 M-Tris/0.05 M-CaCl₂/0.02% NaN₃, pH 8.0, at 4°C. Dispersions were allowed to equilibrate for 1 h on ice and then dialysed against 4 × 2000 ml of 0.05 M-Tris/0.1 mM-EDTA/0.02% NaN₃, pH 8.0, at 4°C to remove Ca²⁺. Ca²⁺ has been shown to promote aggregation and fusion of anionic liposomes (Papa-hadjopoulos *et al.*, 1976), yielding large multilaminar vesicles that could be readily sedimented out of solution. Before binding experiments, the liposomes were centrifuged at 10000g for 2 min at 24°C, the supernatants discarded and the pelleted

phospholipids resuspended in fresh dialysis buffer. All other liposomes were prepared as described previously (Straus *et al.*, 1981). Phospholipids were quantified by phosphate analysis (Ames, 1966) and monitored for purity by t.l.c. (Straus *et al.*, 1981).

Binding experiments were performed by incubating proteins with $0.5\ \mu\text{M}$ phospholipid in $0.25\ \text{ml}$ of $0.05\ \text{M}$ -Tris/ $4\ \text{mM}$ -benzamidine/ $0.1\ \text{mM}$ -EDTA, pH 8.0, at 24°C for 2 min. Mixtures also contained NaCl or CaCl_2 as indicated below. The incubation mixtures were then centrifuged at $10000\ \text{g}$ for 2 min at 24°C . Immediately after centrifugation, a half volume ($0.125\ \text{ml}$) of the supernatant was withdrawn and the loosely packed pelleted phospholipid was resuspended in the remaining supernatant. Both the supernatant and resuspended pellet were diluted with $0.375\ \text{ml}$ of $0.5\ \text{M}$ -Tris, pH 8.0 (to dilute the benzamidine), incubated at 24°C for 20 min to promote proacrosin conversion into acrosin (Polakoski & Parrish, 1977) and assayed for acrosin activity. The activity associated with the pelleted phospholipid was determined by subtracting the activity present in the supernatant from the activity in the resuspended pellet/supernatant mixture. All experiments were repeated at least three times with different preparations of proacrosin, acrosin and liposomes.

Results and discussion

The ability of proacrosin to bind to anionic liposomes was determined by co-sedimentation of the zymogen with phospholipid. Initially proacrosin-liposome association was demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. As shown in Fig. 1, the proteins present in a partially purified proacrosin preparation (NaCl precipitate; see Polakoski & Parrish, 1977), when incubated with anionic liposomes (gel A), were differentially distributed between the supernatant (gel B) and pelleted phospholipid (gel C) fractions after centrifugation. Most of the proacrosin was present in

the pelleted fraction, whereas only a trace amount could be detected in the supernatant. Furthermore, this result shows that the zymogen was stable for the duration of the centrifugation procedure and that

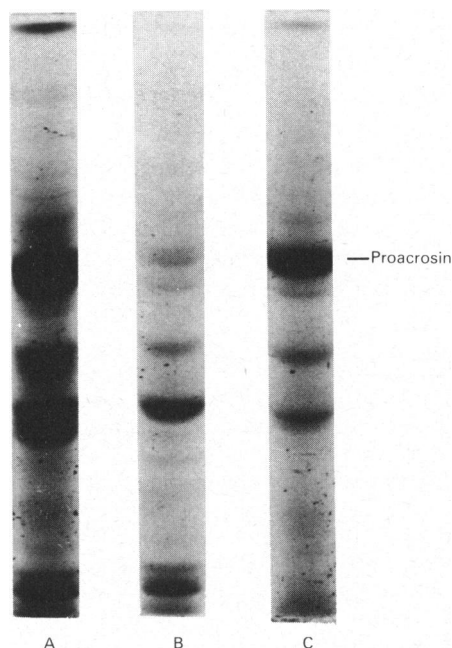


Fig. 1. *Proacrosin binding to anionic liposomes* Partially purified proacrosin ($160\ \mu\text{g}$ of protein) was incubated with liposomes in the presence of $4\ \text{mM}$ -benzamidine (gel A), as described in the Materials and methods section. After centrifugation, the supernatant was withdrawn and the pellet was washed once with $0.25\ \text{ml}$ of $0.05\ \text{M}$ -Tris/ $5\ \text{mM}$ -benzamidine, pH 8.0. The supernatant (gel B) and washed pellet (gel C) were incubated with 1% sodium dodecyl sulphate/ 1% mercaptoethanol for 2 min at 100°C and subjected to electrophoresis. The band containing proacrosin was determined by simultaneous electrophoresis of highly purified proacrosin (results not shown).

Table 1. *Binding of proacrosin to anionic liposomes*

Highly purified proacrosin ($44\ \mu\text{g}/\text{ml}$) and m_β -acrosin ($44\ \mu\text{g}/\text{ml}$) were incubated with liposomes, centrifuged and the pellet and supernatant fractions were assayed for activity. For details, see the Materials and methods section. Abbreviation used: Bz-Arg-OEt, *N*- α -benzoyl-L-arginine ethyl ester.

Protein	Salt	Enzymic activity (μmol of Bz-Arg-OEt/min per ml)		Bound (%)
		Supernatant	Pellet	
Proacrosin		0	9.1	>99
	NaCl (0.2 M)	8.9	0.2	2
	CaCl_2 (0.05 M)	9.2	0	<1
m_β -Acrosin		0.6	12.7	96
	NaCl (0.2 M)	12.3	0.8	6
	CaCl_2 (0.05 M)	12.4	0.4	3

non-specific trapping of protein was minimal, since several of the contaminating proteins were differentially distributed between the phospholipid and supernatant fractions.

The results in Table 1 show the degree of proacrosin/liposome association under several conditions. At low ionic strength, greater than 99% of the detectable highly purified proacrosin was associated with the sedimented phospholipid. The proacrosin-liposome association was substantially decreased when the ionic strength was elevated with NaCl or CaCl₂. Similar results were observed with *m*_β-acrosin under the same conditions. At low ionic strength, over 90% of the enzyme was associated with the phospholipid pellet, whereas increased ionic strength caused diminished association.

The results presented in Table 1 and Fig. 1 demonstrate that proacrosin is capable of binding to anionic liposomes and show that under identical experimental conditions, the zymogen and enzyme had similar liposome-binding properties. The ability of elevated cation levels to diminish binding suggests that the association involved electrostatic interactions (Gennis & Jonas, 1977). The effects of membrane composition on protein-liposome binding could not be tested with the above procedure, since liposomes with lower proportions of anionic phospholipid did not aggregate to the extent necessary for rapid sedimentation (results not shown).

Since proacrosin can bind to anionic liposomes, liposome stimulation of proacrosin conversion was examined as a function of membrane composition, a factor that affects acrosin binding (Straus *et al.*, 1981). The results in Fig. 2(a) show that when highly purified proacrosin was incubated with non-acidic liposomes (phosphatidylcholine), there was no apparent change in the rate of zymogen conversion. As the proportion of anionic phospholipid (phosphatidylglycerol) was increased, a concomitant increase in the rate of conversion was observed. The half-times for full conversion decreased from approx. 7 min in the absence of phospholipid to less than 1 min in the presence of equimolar phosphatidylcholine/phosphatidylglycerol liposomes. Similar results were observed when phosphatidylserine was used as the anionic constituent (Fig. 2b). Increasing proportions of phosphatidylserine in the phospholipid mixtures caused an increase in the rate of zymogen conversion.

The effects of various ionic conditions on liposome stimulation of proacrosin conversion could not be directly compared since changing ionic conditions also influence the rate of proacrosin conversion (Polakoski & Parrish, 1977). However, it was observed that at low salt concentrations (0.1M-NaCl), zymogen conversion half-times of less than 1 min were obtained with low concentrations (0.4 μM) of phospholipid (equimolar phosphatidyl-

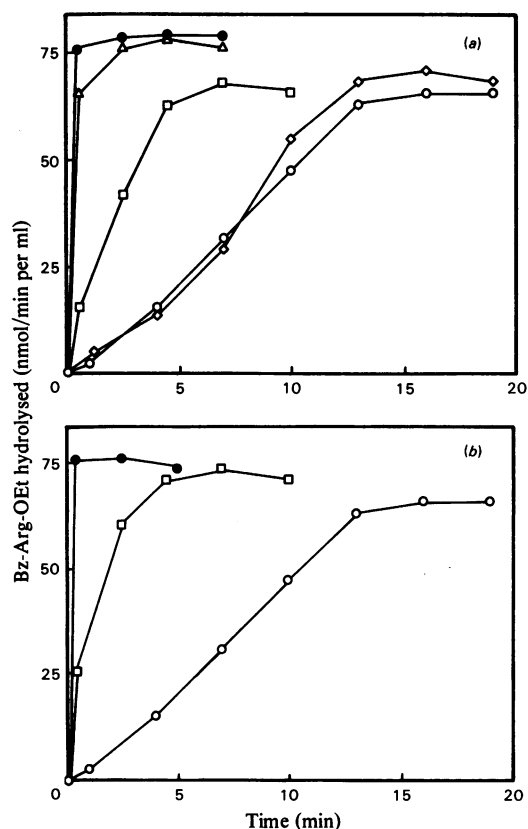


Fig. 2. Effect of membrane composition on liposome stimulation of proacrosin conversion

Highly purified proacrosin (3 μg) was incubated with phospholipid (0.4 μM) in 0.25 ml of 0.05 M-Tris/0.1 M-NaCl, pH 8.0, at 4°C. At the indicated intervals 10 μl aliquots were withdrawn and assayed for acrosin hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt). (a) Liposomes consisted of: ○, no phospholipid; ◇, phosphatidylcholine; □, phosphatidylcholine/phosphatidylglycerol at a 19:1 molar ratio; △, phosphatidylcholine/phosphatidylglycerol at a 7:3 molar ratio; ●, phosphatidylcholine/phosphatidylglycerol at a 1:1 molar ratio. (b) Liposomes consisted of: ○, no phospholipid; □, phosphatidylcholine/phosphatidylserine at a 19:1 molar ratio; ●, phosphatidylcholine/phosphatidylserine at a 1:1 molar ratio.

choline and phosphatidylglycerol). In contrast, phospholipid concentrations of 40–400 μM were required to obtain equivalent half-times at higher NaCl concentrations (0.15–0.2 M) (results not shown). Similarly, it has previously been shown that when proacrosin and anionic liposomes were incubated in the presence of 0.05 M-CaCl₂, the rate of conversion increased when the phospholipid concentrations were elevated (Parrish *et al.*, 1978). Since

elevated NaCl concentrations decrease proacrosin-liposome binding (Table 1), it follows that a decrease in liposome stimulation of zymogen conversion should also be observed. This conclusion can be indirectly inferred from the above observation since, as salt concentrations were increased, greater quantities of phospholipid were required to maintain a conversion half-time of less than 1 min.

In conclusion, the findings reported in the present paper have demonstrated the ability of proacrosin to associate with anionic phospholipid membranes through apparent electrostatic charge interactions. The effects of membrane composition on proacrosin-liposome binding can be inferred from acrosin-binding data and from the zymogen-conversion studies, since increasing proportions of anionic phospholipid caused an increase both in the extent of acrosin binding (Straus *et al.*, 1981) and in the rate of phospholipid stimulation of proacrosin conversion (Fig. 2). Increased ionic strength was shown to decrease the binding of proacrosin to anionic liposomes (Table 1) and was indirectly shown to reduce the rate of liposome stimulation of zymogen conversion. It is apparent from these data that there is a direct relationship between liposome binding and stimulation of zymogen conversion. The physiological significance of this phenomenon is not apparent, since the composition of relevant sperm membranes as well as other sperm components that may interact with proacrosin is presently unknown. An enhanced understanding of proacrosin-membrane interactions as well as the zymogen activation process is essential for elucidation of the complex biochemical events that culminate in fertilization.

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