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A mechanism for differential release of acrosomal enzymes during the acrosome reaction

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To study the organization of fertilization enzymes in the sperm acrosome, we isolated and characterized two physicochemically distinct acrosomal fractions of guinea-pig spermatozoa. A soluble fraction contained the 25000-M_r acrosomal autoantigen, AA1, and most of the acrosomal hyaluronidase and dipeptidyl peptidase II activity. A particulate fraction, designated acrosomal matrix (AM), consisted of membraneless crescent-shaped structures, and contained most of the acrosomal proacrosin. The AM also contained a 28000-M_r putative proacrosin-binding protein, and a very-high-M_r component which, on reduction, was dissociated into 48000-M_r and 67000-M_r subunits. Autoproteolytic dissolution of the AM correlated with proteolysis by acrosin of the 28000-M_r and 48000-M_r AM molecules. Components of both the AM and the soluble fraction were localized by immuno-electron microscopy to the electron-dense region of the guinea-pig sperm acrosome. We conclude that acrosomal molecules are segregated into soluble and matrix compartments. This segregation is a function of disulphide bonding and non-covalent interactions among the relatively few components of the AM. Association of acrosin with the AM may be the mechanism by which this enzyme's release from the spermatozoon during the acrosome reaction is delayed relative to the release of other acrosomal molecules.

INTRODUCTION

During fertilization, spermatozoa penetrate first the cumulus oophorus, then specifically bind and penetrate the zona pellucida of the egg. The acrosome-specific enzymes hyaluronidase (EC 3.2.1.35) and acrosin (EC 3.4.21.10) probably aid this process by hydrolysing substrates in the cumulus oophorus and zona pellucida respectively (reviewed in [1–3]). Models of fertilization which hypothesize functional roles for these enzymes must include a mechanism by which hyaluronidase would be released or exposed to act on its substrate in the cumulus oophorus, while acrosin is retained for interaction with the zona pellucida. Several satisfactory possible mechanisms can be envisioned. One hypothesis is that hyaluronidase diffuses from the spermatozoon at the time of membrane fusion during the acrosome reaction, while acrosin is retained by interaction with the sperm inner acrosomal membrane. Acrosin is not freely diffusible at the outset of the acrosome reaction [4,5], binds to anionic phospholipid vesicles [6], and is stabilized by detergents [7]. Proacrosin also binds to anionic phospholipid vesicles [8], and this interaction stimulates proacrosin autoactivation [9]. These observations have been interpreted as evidence that proacrosin and acrosin associate directly with the sperm inner acrosomal membrane [3,6–9]. However, the 'membrane hypothesis' does not account for the observation that ferritin-conjugated soybean trypsin inhibitor binds to a matrix which persists after the acrosome reaction, and not to the inner acrosomal membrane [10].

Huang *et al.* [11] isolated a particulate fraction comprised primarily of crescent-shaped structures originating from the acrosomes of epididymal guinea-pig spermatozoa. These structures, designated acrosomal matrix (AM), contain no visible membrane and remain intact at pH 5.2, but dissolve by virtue of endogenous proteolytic activity at alkaline pH [11]. We isolated the AM and a soluble fraction (SF) of the guinea-pig sperm acrosome, and surveyed the two fractions for the presence of several acrosomal macromolecules. The results indicate that at least two acrosomal compartments are distinguishable on the basis of the intermolecular associations, and therefore diffusibility, of the components in them. Proacrosin is a component of the AM. By immuno-electron microscopy we found that components of the two physicochemically defined compartments can, but do not necessarily, co-localize. These and other data suggest that acrosin remains attached to the heads of acrosome-reacted spermatozoa not by direct interaction with the inner acrosomal membrane, but through association with the AM. Furthermore, we find that the autolytic dissolution of the AM at alkaline pH probably involves acrosin-mediated proteolysis of its 48000-M_r and 28000-M_r subunits. We discuss some implications of these observations to acrosome function and sperm interaction with the zona pellucida.

EXPERIMENTAL

Materials

Unless otherwise specified, chemicals were reagent grade or purer. Acrylamide (electrophoresis grade) was obtained from Amresco (Solon, OH, U.S.A.). Other electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Nitrocellulose was purchased from Sartorius (Hayward, CA, U.S.A.), and guinea pigs were obtained from Charles River (Wilmington, MA, U.S.A.).

Isolation of sperm subcellular fractions

Epididymal spermatozoa were collected in 10 mM-Mes/HCl, pH 6.0, containing 0.264 M-sucrose and 0.5 mM-*p*-aminobenz-

Abbreviations used: AM, acrosomal matrix; SF, soluble fraction; AC, acrosomal contents; DPP II, dipeptidyl peptidase II; AA1, 25000-M_r acrosomal autoantigen.

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amidine [12], and the suspension was centrifuged at 600 *g* for 5 min at 25 °C. The supernatant fluid was re-centrifuged at 12000 *g* for 10 min at 4 °C; the supernatant solution thus obtained was designated 'sperm wash'. Sperm acrosomes were disrupted by shaking washed spermatozoa (600 *g* pellet, above) in 50 mM-sodium acetate, pH 5.2, containing 0.11 M-NaCl and 0.625 % Triton X-100; acrosome-less spermatozoa were removed by filtering the suspension through a column of glass beads [11]. The effluent, which contained both soluble and particulate materials originating from the acrosome, was centrifuged at 1000 *g* for 10 min at 4 °C. The pellet obtained was washed with 50 mM-sodium acetate, pH 5.2, containing 100 mM-NaCl by centrifugation at 15000 *g* for 5 min at 4 °C; this washed pellet was designated acrosomal matrix (AM). The supernatant fluid from the 1000 *g* centrifugation was re-centrifuged at 12000 *g* for 10 min at 4 °C; the supernatant solution obtained was designated soluble fraction (SF). Acrosomal contents (AC) were prepared by differential centrifugation of spermatozoa treated for 1 h at 37 °C with 10 μg of ionophore A23187/ml (from a 2 mg/ml stock in dimethyl sulphoxide) and Ca^{2+} [13–15]. Isolated sperm fractions were stored frozen at −20 °C.

Protein determinations

Protein concentrations were determined colorimetrically by using bicinchoninic acid (Pierce Chemical Co., Rockford, IL, U.S.A.) as described [16], with BSA as the standard.

Enzyme assays

Acrosin activity was determined spectrophotometrically with *N*-α-benzoylarginine ethyl ester as substrate [17], with modifications described previously [18]. Proacrosin activity was determined by calculating the difference between acrosin activities measured before and after complete autoactivation of the zymogen. Autoactivation was induced by mixing 3 vol. of autoactivation buffer (0.2 M-Tris/HCl, pH 8.0, 50 mM-$CaCl_2$) with 1 vol. of sperm subcellular fraction (final pH 8.0) and incubating at 25 °C for 20 min, by which time all proacrosin present had been activated. For acrosin and proacrosin assays, AM was dissolved in 10 mM-HCl; the other preparations were adjusted to pH 2–3 with 0.1 M-HCl to dissociate acrosin inhibitors before assay.

For hyaluronidase and dipeptidyl peptidase II (DPP II) assays, AM was suspended in 50 mM-sodium acetate, pH 5.2, containing 100 mM-NaCl, but the other preparations were used without further manipulation. Hyaluronidase activity was determined by measuring spectrophotometrically the *N*-acetylglucosamine end groups liberated during hydrolysis of hyaluronic acid [19]. DPP II activity was determined by measuring fluorimetrically [20] the methoxy-β-naphthylamine produced upon hydrolysis of the substrate lysyl-alanyl-methoxynaphthylamide (Enzyme Systems Products, Livermore, CA, U.S.A.); reaction conditions were 25 °C in 50 mM-Mes, pH 5.5, containing 1.0 mM substrate.

Electrophoresis

SDS/PAGE was performed as described by Laemmli [21]. Samples for SDS/PAGE were either applied to the gels without prior reduction or reduced by heating at 100 °C for 5 min with 5 % 2-mercaptoethanol. Coomassie-Blue-stained bands were quantified with a BioImage Visage 60 digital image analyser.

Proteases separated by SDS/PAGE in gels containing gelatin as the substrate were detected by activity staining [22], with the following modifications. After electrophoresis, gels were agitated sequentially at 25 °C in: 10 mM-Tris/HCl, pH 8.0, containing 1 % Triton X-100 (1 h); three changes of 10 mM-Tris/HCl, pH 8.0 (20 min each); 50 mM-Tris/HCl, pH 8.0 (1 h); 50 mM-Tris/HCl, pH 8.0, containing 5 mM-$CaCl_2$ (64 h).

Antisera

Monospecific heteroantisera to testicular proacrosin and to the 25000-M_r acrosomal autoantigen AA1 were those described in previous studies [15,18]. Monospecific heteroantisera to hyaluronidase were prepared by immunizing two female NZW rabbits with 210 μg each of purified [19] ovine sperm hyaluronidase (kindly provided by Dr. Robin A. P. Harrison, University of Cambridge, U.K.) in 0.7 ml of Freund's complete adjuvant, injected intramuscularly. Immune sera were collected 30 days after immunization and stored at −20 °C.

Western blots

Western blots were done as described previously [23,24]. Antisera (anti-proacrosin, anti-AA1 and anti-hyaluronidase) were diluted 400-fold. We used a horseradish peroxidase conjugate of affinity-purified goat anti-rabbit immunoglobulin (Antibodies Incorporated, Davis, CA, U.S.A.) diluted 1000-fold to detect bound primary antibody. Treated blots were developed at 25 °C, with 4-chloro-1-naphthol as substrate.

Immuno-electron microscopy

Proacrosin and AA1 were localized in guinea-pig spermatozoa by immunolabelling of ultrathin cryosections as described by Tokuyasu [25]. Fixed frozen sperm pellets were sectioned on a Reichert FC-4 ultracut microtome, and the sections were immunolabelled with specific antisera diluted 100- or 500-fold. The sections were then stained with oxalate/uranyl acetate and viewed in a JEOL 100 CX electron microscope.

RESULTS

To isolate the AM, suspensions of spermatozoa were disrupted by shaking with non-ionic detergent until 80–90 % of acrosomes had detached from the sperm heads (Fig. 1*a*). Acrosomal matrices obtained upon filtering the disrupted spermatozoa through a column of glass beads were virtually devoid of contaminating spermatozoa (Fig. 1*b*). In electron-microscopic views the AM appeared as a bilaminate structure, the shape and integrity of which was not a function of a delimiting membrane (Fig. 1*c*).

Initially, spermatozoa were washed at pH 7.4 with a phosphate-buffered saline solution which contained no added protease inhibitors. AM isolated from these cells was greatly enriched in acrosin activity, and proacrosin was not detectable. To ensure that the compositions of isolated sperm fractions were representative of the contents of intact spermatozoa, we subsequently isolated AM and SF from spermatozoa washed with a pH 6.0 buffer containing 0.264 M-sucrose and 0.5 mM-*p*-aminobenzamidine, conditions previously shown to preserve proacrosin activity [12]. The specific activities of hyaluronidase, DPP II, acrosin, and proacrosin in these sperm fractions are shown in Table 1. The AM contained proacrosin, which autoactivated at pH 8.0 with sigmoidal kinetics and a $t_{\frac{1}{2}}$ of 5 min. No acrosin activity was detectable in the AM before autoactivation. The specific activity of proacrosin was at least 24-fold higher in AM than in SF. Conversely, the specific activities of hyaluronidase and DPP II were 27-fold and 26-fold higher respectively in SF than in AM. Levels of the three enzymes were comparatively low in the materials washed from the spermatozoa before AM isolation (sperm wash).

The electrophoretic patterns of the sperm fractions differed (Fig. 2*a*). The pattern of SF was similar to that of AC released

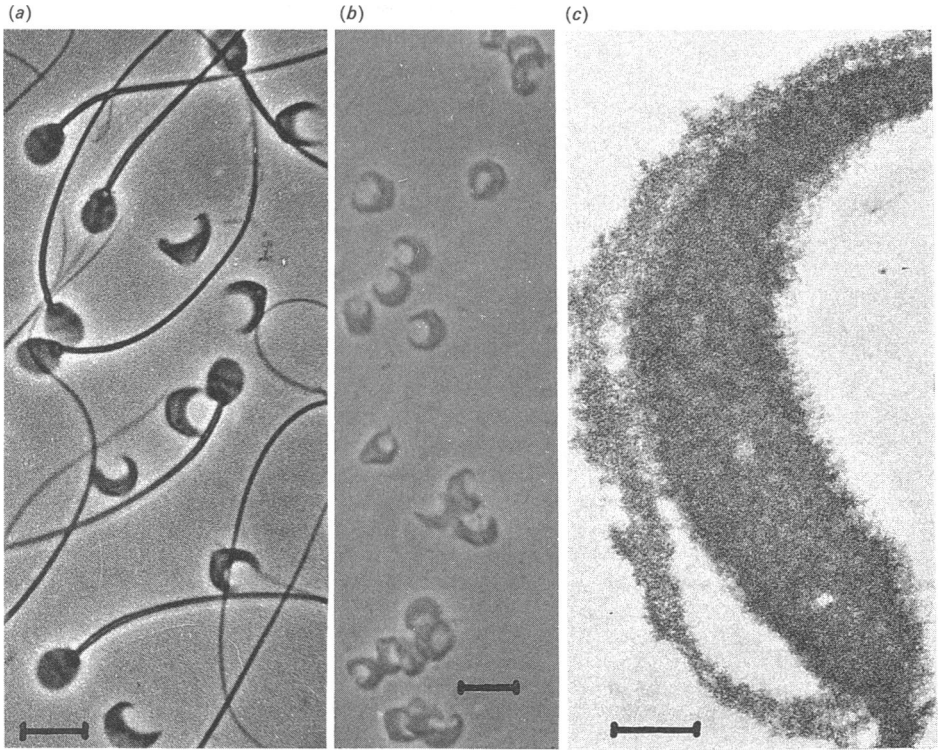

Fig. 1. Isolation of AM

(*a*) Phase-contrast image of washed, detergent-disrupted spermatozoa (bar = 10 μm). (*b*) Phase-contrast image of isolated AM (bar = 10 μm). (*c*) Transmission electron micrograph of isolated AM (bar = 1 μm).

Table 1. Specific activities of enzymes in acrosomal fractions

One unit of enzyme is the amount catalysing formation of 1 μmol of product/min. The results are means of three or more measurements of enzyme activities in a representative preparation of the three sperm fractions. We have obtained similar results with several other preparations of the sperm fractions, using both the same and other methods for assaying the enzymes.

Fraction	Acrosin (unit/mg)	Proacrosin (units/mg)	Hyaluronidase (m-units/mg)	DPP II (m-units/mg)
Sperm wash	< 0.1	< 0.1	11	31
SF	< 0.1	< 0.1	273	198
AM	< 0.1	2.4	10	7.6

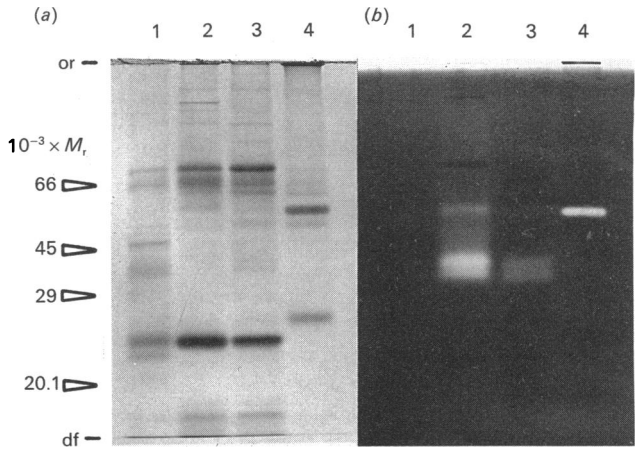

Fig. 2. SDS/PAGE (10% gel, samples not reduced) of sperm fractions

(*a*) 8 μg of protein/lane, gel stained with Coomassie Brilliant Blue R-250 for protein detection. (*b*) 9 μg of protein/lane, gel stained for proteolytic activity. Lanes: 1, sperm wash; 2, AC; 3, SF; 4, AM. Positions to which non-reduced M_r standards migrated are indicated on the left; or, origin, df, dye front.

from spermatozoa by treatment with ionophore A23187 and Ca^{2+}; these preparations shared prominent bands migrating with M_r 69000 and 25000. The 69000-M_r and 25000-M_r bands were also present in the sperm wash, but in lower quantity, owing to contamination by acrosomal materials from spermatozoa disrupted during washing. None of the prominent bands common to AC and SF were present in the AM. Rather, two AM bands migrated with M_r 56000 and 28000, and some AM materials were retained at the stacking-gel/resolving-gel interface. By digital image analysis of the stained bands, assuming equivalent dye binding by weight, we estimated the molar ratio of the 28000-M_r AM protein to the 56000-M_r protein to be 1.4 (mean of two experiments).

Proteolytic activity migrating with M_r 56000 was detected in the AM by activity staining (Fig. 2*b*). No proteolytic bands were detected in the sperm wash. Two proteolytic bands in the AC migrated with M_r 43000 and 40000, with a less prominent 56000-M_r protease also evident. The primary proteolytic activities in the soluble fraction co-migrated with the 43000-M_r and 40000-M_r activities in AC.

Since guinea-pig sperm proacrosin migrates with M_r 56000 in SDS/PAGE and is detectable by protease activity staining owing to its autoactivation to acrosin *in situ* [18], we tested by Western blotting whether the 56000-M_r AM protein was proacrosin. The

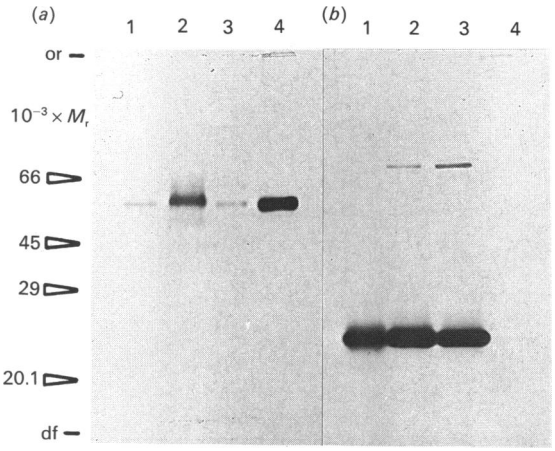

Fig. 3. Western blots of sperm fractions

(*a*) Antigens detected with antiserum to proacrosin. (*b*) Antigens detected with antiserum to AA1. Non-reduced samples (4 μg of protein/lane) were: lane 1, sperm wash; lane 2, AC; lane 3, SF; lane 4, AM. Migration of standards is indicated as for Fig. 2.

prominent 56000-M_r band in AM was strongly immunoreactive with a monospecific heteroantiserum to guinea pig testicular proacrosin (Fig. 3*a*). Immunoreactivity at M_r 56000 was also present in the sperm wash, AC and SF, but to a much lesser extent; these amounts of proacrosin were below the detection limit of the spectrophotometric assay (Table 1).

When an identical blot was probed with a monospecific heteroantiserum to the primary acrosomal autoantigen of guinea-pig sperm (AA1), the prominent 25000-M_r band present in SF and AC was strongly immunoreactive (Fig. 3*b*). No immunoreactivity at M_r 25000 was observed in the AM.

To identify hyaluronidase in the SF, we prepared polyclonal antisera to purified ovine hyaluronidase. Ovine sperm hyaluronidase monomers migrate with M_r 81000 and 89000 in SDS/PAGE, and form disulphide-bonded oligomers [19,26,27]. Sera from two immunized rabbits were strongly reactive with the immunogen (Fig. 4). Only one of these sera cross-reacted, on Western blots, with components of guinea-pig sperm SF; the major cross-reactive band migrated with M_r 69000, and a minor 140000-M_r immunoreactive band was also apparent (Fig. 4*b*).

Localization of AA1 and proacrosin by immunoelectron microscopy is shown in Figs. 5 and 6 respectively. Antibodies to both molecules bound antigen primarily in the electron-dense apical region of the acrosome, although some gold particles were also present in the electron-lucent dorsal bulge. Both antisera bound antigen also in the acrosome posterior to the apical region, adjacent to the sperm nucleus. Neither antigen localized to the equatorial segment; gold particles were present throughout the posterior acrosome, but the labelling ended abruptly at the equatorial segment. Nor did either antigen appear to be membrane-associated, as a majority of the gold particles were clearly distant from the visible lipid bilayer.

To evaluate the nature of the forces responsible for the integrity of the AM, we tested the effects of strongly acidic conditions and of disulphide-bond reduction on the solubility of the AM and on the electrophoretic mobilities of the AM components. At pH values less than 4.0, the AM dissolved. The electrophoretic patterns of acid-solubilized AM and of AM dissolved directly in SDS/PAGE sample buffer were nearly identical (Fig. 7). Proteins not solubilized at acidic pH accounted for only a small fraction of total AM protein, and may reflect presence of contaminating spermatozoa. Upon disulphide-bond reduction, a very large AM component (which without reduction had been retained at the origin) was dissociated into subunits which migrated with M_r 48000 and 67000 (compare Figs. 7*a* and 7*b*). Judging by the intensity of Coomassie Blue staining, these polypeptides appeared to constitute a large proportion of AM protein, with more weight of the 48000-M_r subunit present than of the 67000-M_r subunit.

Acrosin-mediated proteolysis of the components of the AM, initiated upon activation of proacrosin at alkaline pH, is illustrated in Fig. 8. When the products of the reaction were analysed by SDS/PAGE without prior reduction of disulphide bonds, hydrolysis of the 28000-M_r AM protein and appearance of several new bands (presumably fragments derived from the very-high-M_r material at the stacking-gel/resolving-gel interface) were observed (Fig. 8, lanes 1 and 2). To determine which of the subunits comprising the high-M_r AM component were hydro-

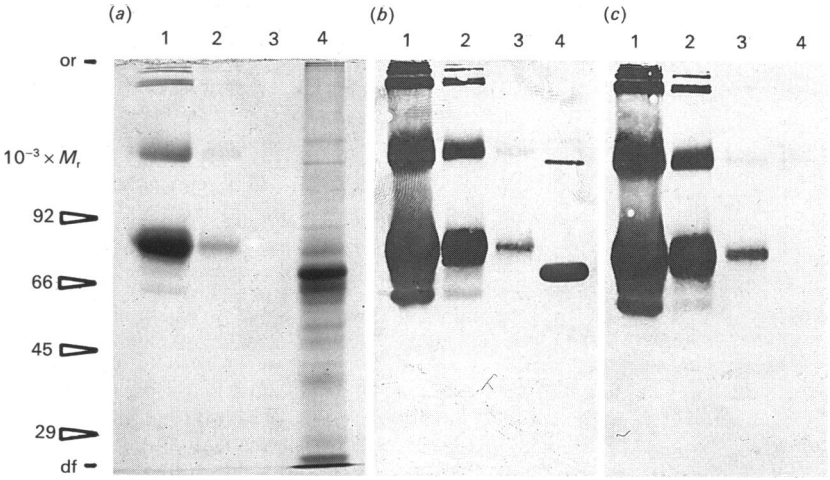

Fig. 4. Identification of guinea-pig sperm hyaluronidase by Western blotting

Protein samples were separated by SDS/PAGE (7.5% gel, samples not reduced). (*a*) Gel stained with Coomassie Brilliant Blue R-250. (*b*) Western blot, antigens detected with an antiserum to purified ovine hyaluronidase. (*c*) Western blot, antigens detected with a second antiserum to purified ovine hyaluronidase. Lanes: 1, 12 μg of purified ovine hyaluronidase; 2, 1.2 μg of purified ovine hyaluronidase; 3, 0.12 μg of purified ovine hyaluronidase; 4, 50 μg of guinea-pig sperm acrosomal SF. Migration of standards is indicated as for Fig. 2.

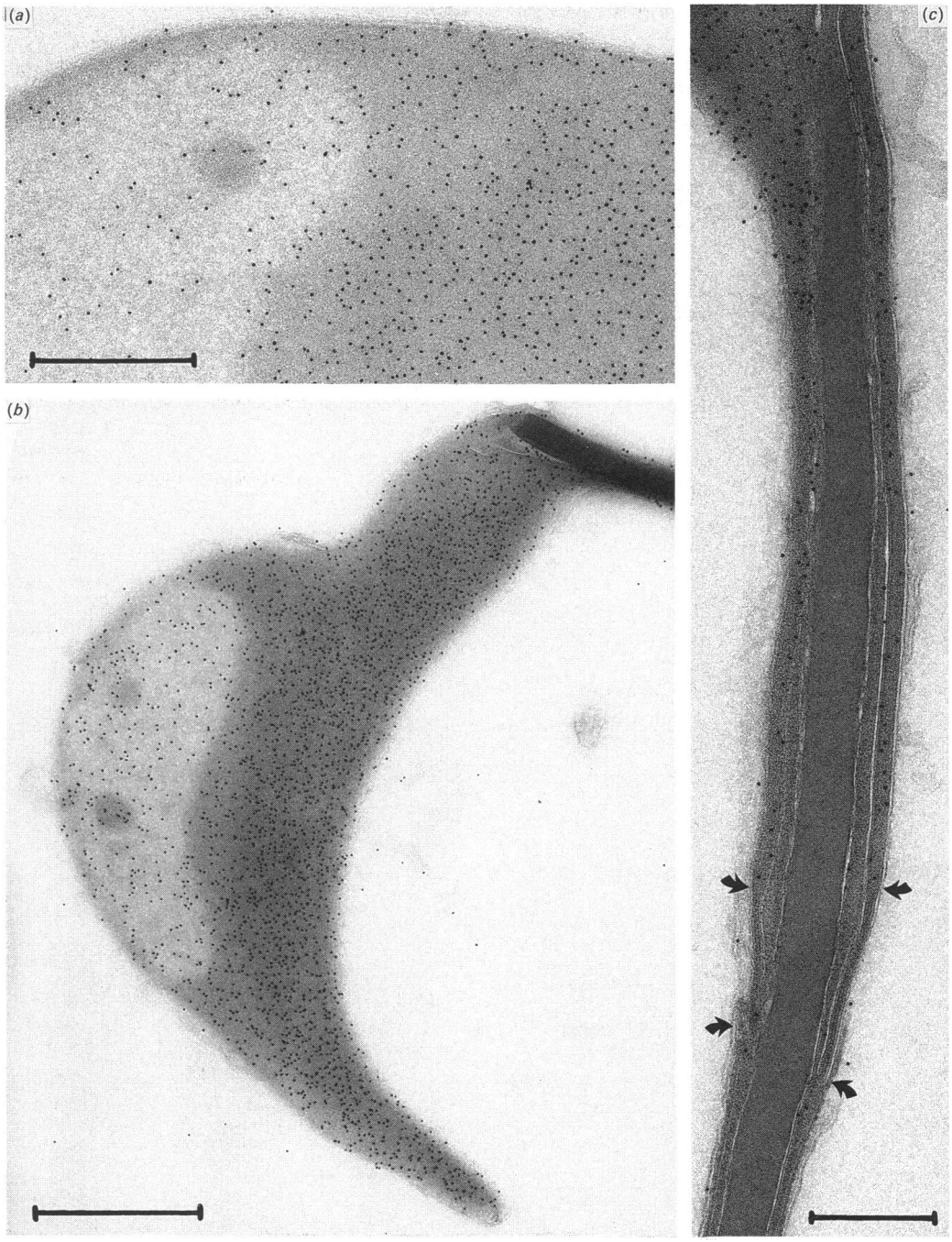

Fig. 5. Localization of AA1 by immuno-electron microscopy

(*b*) Sagittal section of apical acrosome (bar = 1.0 μm). (*a*) Higher-magnification view of boundary between electron-lucent and electron-dense region of the apical acrosome (bar = 0.5 μm). (*c*) Posterior acrosome and equatorial segment (bar = 0.5 μm). Arrows delimit the equatorial segment.

lysed, the products of an identical reaction were similarly analysed after reduction of disulphide bonds (Fig. 8, lanes 3 and 4). The 48000-M_r subunit was extensively proteolysed, and the 67000-M_r subunit was partially digested.

DISCUSSION

We conclude that proacrosin is a component of a particulate structure, designated acrosomal matrix (AM), which contains no visible lipid bilayer and comprises a subset of acrosomal macromolecules. Thus a majority of proacrosin, and consequently the acrosin formed from it, is not directly bound to the sperm inner acrosomal membrane. Rather, proacrosin and acrosin are attached to the inner acrosomal membrane indirectly, through their association with the AM. Since the specific activity of proacrosin in the AM was 2.4 units/mg, and purified guinea-pig proacrosin has a specific activity of 50 units/mg [18], proacrosin constitutes 5% of AM protein. Much of the balance of AM protein consisted of two subunits which migrated with M_r 67000 and 48000 in SDS/PAGE under reducing conditions. These molecules associated in a very-high-M_r multi-subunit complex stabilized by disulphide bonding. Proacrosin and a 28000-M_r

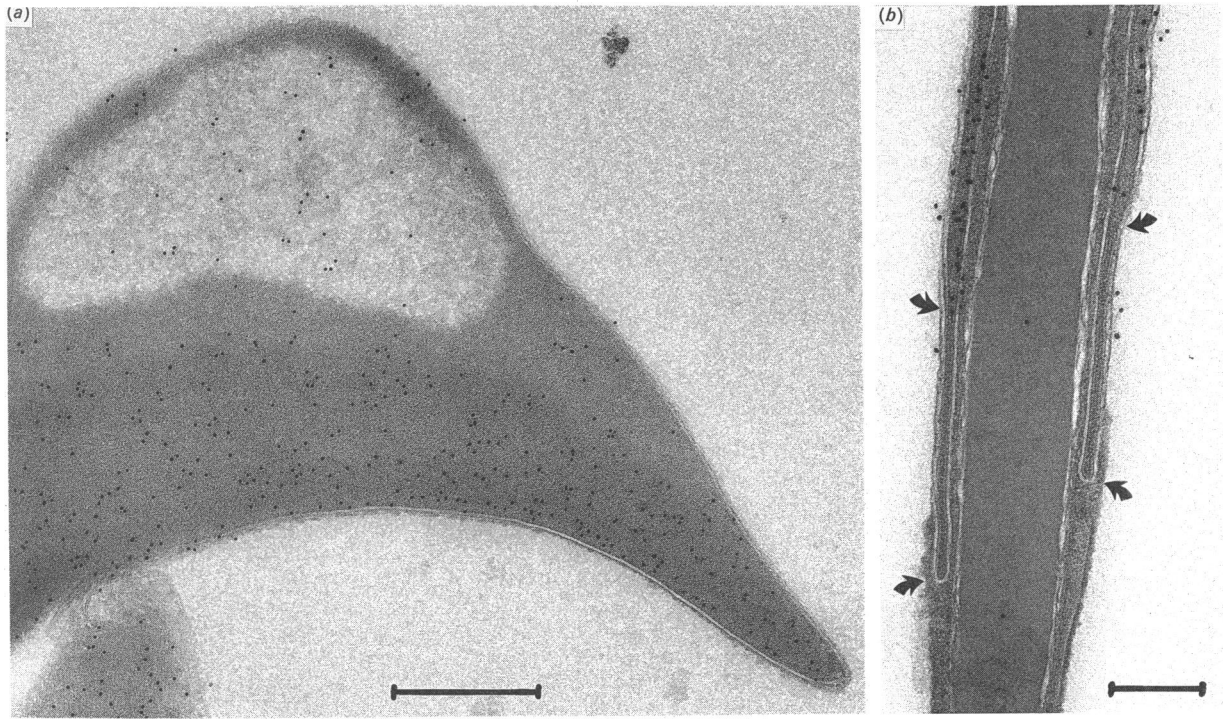

Fig. 6. Localization of proacrosin by immuno-electron microscopy

(*a*) Apical acrosome, showing electron-lucent and electron-dense regions (bar = 0.5 μm). Right panel: posterior acrosome and equatorial segment (bar = 0.2 μm). Arrows delimit the equatorial segment.

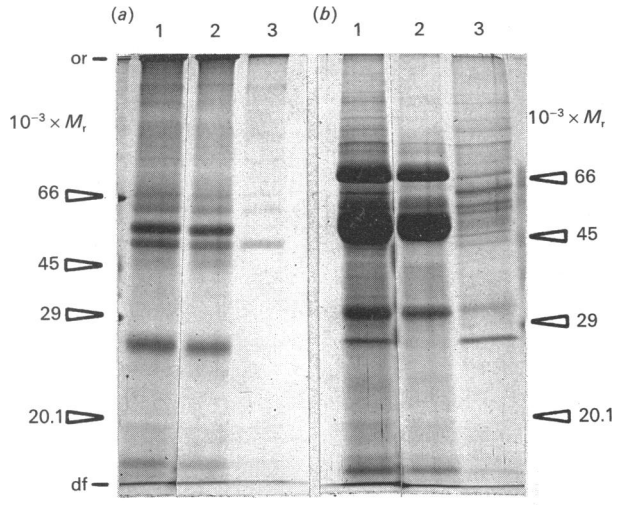

Fig. 7. Characterization of AM subunit composition and acid solubility

Shown are SDS/10%-polyacrylamide gels stained with Coomassie Brilliant Blue R-250. (*a*) Disulphide bonds not reduced. (*b*) Disulphide bonds reduced. Lanes: 1, 20 μg of AM dissolved directly in SDS/PAGE sample-preparation buffer; 2, soluble material obtained upon dissolving 20 μg of AM at pH 2.0; 3, residual AM insoluble at pH 2.0, dissolved in SDS/PAGE sample buffer. Positions to which non-reduced and reduced M_r standards migrated are indicated on the left and right sides respectively of the gels.

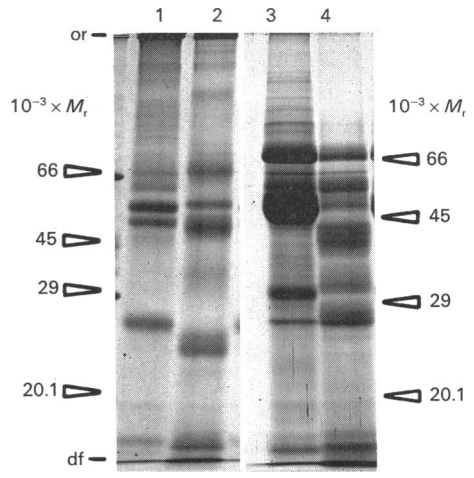

Fig. 8. Electrophoretic analysis (SDS/10%-polyacrylamide gels stained with Coomassie Brilliant Blue R-250) of AM proteolysis

Lanes: 1, 20 μg of AM, disulphide bonds not reduced; 2, 20 μg of AM incubated at pH 8.0 and 25 °C for 10 min, disulphide bonds not reduced; 3, 20 μg of AM, disulphide bonds reduced; 4, same as lane 2, disulphide bonds reduced. Migration of standards is indicated as for Fig. 7.

AM component were bound, in approximately equimolar amounts, to this high-M_r multi-subunit complex through non-covalent interactions which were not disrupted by non-ionic detergent, but were disrupted at acidic pH (4.0 or less) in the absence of detergent, or by treatment with SDS. At pH 8.0, proacrosin in the AM was rapidly ($t_{\frac{1}{2}}$ = 5 min) activated to acrosin, which extensively proteolysed both the 28000-M_r AM protein and the 48000-M_r subunit of the high-M_r AM complex, resulting in dissolution of the AM and release of soluble acrosin.

In contrast with the AM, a soluble fraction (SF) of the acrosome contains a 69000-M_r hyaluronidase and the 25000-M_r acrosomal autoantigen, AA1. The SF appears to constitute a majority of total acrosomal protein: its electrophoretic pattern was very similar to that of acrosomal contents (AC) released during the ionophore-induced acrosome reaction, which contains

both the SF and proteolytically solubilized AM. A proportion of guinea-pig sperm hyaluronidase may exist as a disulphide-bonded dimer (140000-M_r band, Fig. 4), similar to the ovine enzyme [19,26,27]. Despite this apparent similarity, the weak cross-reactivity of the anti-(ovine hyaluronidase) antisera with guinea-pig hyaluronidase, and the differences in the sizes of the enzymes, show that guinea-pig and ovine hyaluronidases differ substantially in structure.

Guinea-pig spermatozoa exhibit both electron-dense and electron-lucent acrosomal regions [4,5,13], suggesting that physicochemically distinct acrosomal compartments might exist. Although DPP II and AA1 were both present in the SF, a majority of AA1 localized to the electron-dense acrosome, whereas DPP II localized to the electron-lucent region [28]. Thus diffusible macromolecules are not confined solely to one or the other of these acrosomal regions. Proacrosin also localized primarily to the electron-dense acrosome. Since both diffusible (AA1) and non-diffusible (proacrosin) macromolecules co-localized, physicochemically distinct compartments can co-exist within a single morphologically defined acrosomal region. This conclusion is relevant to the generality of our results, as the acrosomal contents of spermatozoa from most species appear uniformly electron-dense [2].

Acrosin activity has previously been found associated with particulate acrosomal fractions of hamster [29,30] and guinea-pig [31] spermatozoa. The latter preparation differed from ours in that it contained membrane and some hyaluronidase activity [31]. Proacrosin activation in a similar preparation has been studied [32]; bivalent cations induced apparent conversion of proacrosin into a lower-M_r form (presumably acrosin), with continued association of the lower-M_r protein with the particulate fraction. This observation, made by electrophoretic methods, is consistent with an observation reported by Green [4] that proacrosin activation precedes by several minutes the release of soluble acrosin from the acrosome.

The identity of the 28000-M_r AM protein is uncertain. However, the SDS/PAGE pattern of AM (non-reduced) is strikingly similar to that of an acidic extract of denuded ovine sperm heads [33]. This extract contained primarily proacrosin and a 28500-M_r protein which binds to it [33]. Similarly, a 29000-M_r acid-soluble protein binds to and co-purifies with porcine proacrosin [34]. Since the 28000-M_r AM protein co-isolates with proacrosin through our procedure, is acid-soluble, and is present in the AM in approximately equimolar amounts as proacrosin, it may be the guinea-pig homologue of this previously identified proacrosin-binding protein. Until direct evidence of proacrosin binding is obtained, however, this identification is tentative.

Proacrosin appears not to be released in soluble form at any time during the acrosome reaction. Rather, acrosin is released from the sperm several minutes after the onset of the acrosome reaction. In studies using guinea-pig spermatozoa, the acrosome reaction (as judged by light microscopy) and proacrosin activation were both 50% complete less than 3 min after induction of synchronous acrosome reactions with ionophore A23187, but release of the acrosin formed was not 50% complete until 9 min after the acrosome reaction [4]. This release of soluble acrosin is further delayed by addition of trypsin inhibitors [4]. Trypsin-like enzymic activity associated with spermatozoa undergoing the acrosome reaction localizes to the residual electron-dense matrix [10]. Dissolution of the isolated AM is dependent on endogenous trypsin-like proteolytic activity [11]. We observed that AM isolated from sperm washed at pH 7.4 in the absence of added protease inhibitor contains acrosin. Collectively, these results show that acrosin remains associated with the AM, and hence the spermatozoon, for several minutes after proacrosin activation, and its release is dependent on endogenous trypsin-like proteolytic activity. Since acrosin, formed from proacrosin, was the only protease that we found associated with the AM, it is likely that acrosin controls its own release from the acrosome via proteolysis of one or more components of the AM. Both the 28000-M_r putative proacrosin-binding protein and the 48000-M_r subunit of the AM were acrosin substrates, so one or both of these molecules may be the physiologically relevant substrate for acrosin which, when hydrolysed, releases acrosin from the spermatozoon.

The results suggest a simple mechanism for the differential release of acrosomal enzymes during the acrosome reaction. Owing to their non-association with the AM, the protein AA1 and enzymes such as hyaluronidase and DPP II would be free to diffuse from the spermatozoon at the outset of the acrosome reaction. In contrast, since release of acrosin requires proteolysis of the AM, an inherently slower process than diffusion, acrosin would remain sperm-associated longer than enzymes not associated with the AM. The fact that AM proteolysis could theoretically be regulated *in vivo* by acrosin inhibitors suggests another possible element of control on the acrosin-release process.

Our results bear on the question of how the fertilizing spermatozoon penetrates the zona pellucida. Acrosin aids penetration of the zona pellucida by hydrolysing zona glycoproteins [3,35]. At the molecular level, attachment of acrosome-reacting spermatozoa to the zona pellucida appears to be via acrosin [36,37] binding to the zona glycoprotein ZP2 [38]. Since acrosin is immobilized on acrosome-reacting spermatozoa through its association with the AM, it is in the context of this matrix that the enzyme interacts with zona-pellucida glycoproteins during fertilization. One implication of this view of sperm–egg interaction is that acrosome-reacted spermatozoa which have lost all AM might not attach to the zona pellucida. Guinea-pig spermatozoa, which have relatively enormous quantities of AM, readily attach to the zona pellucida after the acrosome reaction [39], whereas mouse spermatozoa which have initiated acrosome reactions before reaching the zona pellucida do not [38,40]. It may be interesting to study the question of whether this species difference in fertilization can be explained by differences in the rate at which the AM is lost from spermatozoa during the acrosome reaction.

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