Interaction of zona pellucida glycoproteins, sulphated carbohydrates and synthetic polymers with proacrosin, the putative egg-binding protein from mammalian spermatozoa

ROY JONES

Department of Molecular Embryology, AFRC Institute of Animal Physiology & Genetics Research, Babraham, Cambridge CB2 4AT, UK

Summary

Fertilization in mammals is a unique cell-cell recognition event that involves specific receptors on the surface of each gamete. Previous work has shown that proacrosin, a protein found within the acrosome of mammalian spermatozoa, binds non-enzymatically to zona pellucida glycoproteins (ZPGPs) that surround the egg and that this binding can be inhibited by sulphated polysaccharides such as fucoidan. The mechanism of this interaction has been investigated using 125I-ZPGPs and 125I-fucoidan as probes. Results show that it involves poly(sulphate) groups on zona glycoproteins that bind with high affinity (\(K_d = 1.2 \text{ to } 5.0 \times 10^{-8} \text{M}\)) to complementary 'docking' sites on proacrosin. The spatial orientation of these sulphates, together with the tertiary structure of the target protein, determines the selectivity of polymer binding. Thus, dextran sulphate and poly(vinyl sulphate) are strong inhibitors of the above probes whereas dextran, chondroitin sulphates A and C and poly(vinyl phosphate) are ineffective. Proacrosin, therefore, has properties analogous to those described for 'bindin', the egg adhesion protein found within the acrosomal vesicle of sea urchin spermatozoa.

Key words: fertilization, sulphate binding, fucoidan, proacrosin, zona pellucida.

Introduction

Proacrosin is the zymogen form of the serine proteinase acrosin (EC 3.4.21.10) that is found exclusively within the acrosome of mammalian spermatozoa (reviewed by Hedrick et al. 1988). It has been purified and characterized extensively from several species and recently the genes for human and porcine proacrosin have been identified and sequenced (Adham et al. 1989; Baba et al. 1989a,b). The protein is synthesized postmeiotically in round spermatids in the testis (Arboleda and Gerton, 1987; Adham et al. 1989) and is subsequently processed during late spermatogenesis and epididymal maturation to produce the mature molecule characteristic of fully formed spermatozoa (Hedrick et al. 1988; Arboleda and Gerton, 1988). It is found mostly within the acrosomal matrix where it is complexed to a binding protein and a naturally occurring inhibitor (Hedrick et al. 1988). Unusually for a serine proteinase, it autoactivates by modification of both N-terminal and C-terminal ends. At the N terminus there is site-specific cleavage between amino acids 23 and 24 to produce a 2-chain molecule consisting of a 'light' chain (relative molecular mass 4.2\(\times\)10^3) cross-linked by disulphide bridges to a 'heavy' chain (Fock-Nüzel et al. 1984) while at the C terminus there is removal of a proline-rich domain, 72 to 75 amino acids long (Baba et al. 1989a).

From a functional standpoint, the enzyme has long been presumed to facilitate penetration of spermatozoa through the zona pellucida, the extracellular matrix that surrounds mammalian eggs. Whilst this possibility cannot be discounted, doubts about it have been raised on several occasions (Bedford and Cross, 1978), especially in light of recent findings on the ability of proacrosin/acrosin to bind non-enzymatically to zona glycoproteins (Brown and Jones, 1987; Jones and Williams, 1990). Such an hypothesis correlates with earlier observations that the inhibitory effects of sulphated polymers on fertilization in vitro are directed more against spermatozoa than eggs (Huang et al. 1982; Boldt et al. 1989).

In this communication, we have investigated the structural properties of sulphated polymers and homologous zona glycoproteins that are critical for me-
diating their recognition and binding to proacrosin from
boar spermatozoa. Results indicate that it is the density
and stereochemical alignment of poly(sulphate) groups
along the polymer chain that are the important
parameters for interaction with basic residues on the
surface of proacrosin. This work provides direct
biochemical evidence that mammalian proacrosin has
properties analogous to bindin, the egg adhesion
protein that is found within the acrosomal granule of
sea urchin spermatozoa (Vaqquier, 1986). Similar
conclusions have been reached in a parallel study to this

Materials and methods

Chemicals
All routine chemicals were of the highest purity available
commercially and were purchased from Sigma, British Drug
Houses or Pharmacia. Poly(vinyl phosphate) and poly-
(styrene sulphonate) (sodium salt) were supplied by Poly-
sciences, galactan (from larch) by Kodak Laboratories and
xylan (from Trichum spelta) and poly(vinyl sulphate),
(potassium salt) by Sigma.

Preparation and purification of 125I-fucoidan and 125I-
 zona pellucida glycoprotein probes
Crude fucoidan (Sigma) from Fucus vesiculosus was purified
by β-elimination and extensive pronase digestion (DeAngels
and Glabe, 1987). It had an average relative molecular mass
(Mr) of 100x10^3 as determined by gel filtration on Sephadex
G-75 and did not contain detectable amounts of protein.
A compositional analysis after acid hydrolysis revealed 7.4 %
fucose, 8% xylose, 5% galactose, 1% mannose, 1%
arabinose and 7% uronic acid (R. M. Williams and R. Jones,
personal communication). These values are close to those
reported originally by Medcalf and Larsen (1977) for fucans
from this source. The purified fucoidan was conjugated with
fluoresceinamine and iodinated with 125 I-Na and 1,3,4,6-
tetrachloro-3,6-diphenylglycouril ('lodogen' Pierce) as
reported originally by Medcalf and Larsen (1977) for fucans
et al. (1983). Fucoidan was estimated colorimetrically with orcinol/H2SO4 reagent (Vasseur, 1948)
using bovine serum albumin (BSA) as standard. Electrophoresis
was either stored frozen at —20°C or dialysed against 1 mM
phosphate-buffered saline pH7.2 containing lmM
but to nitrocellulose membranes (0.45 μm) by electroblotting
(Towbin et al. 1979) or stained with 1 % Coomassie Brilliant
Blue R-250 in methanol:acetic acid:water (40 %:7 %:53 %,
respectively). Blots were blocked with 5 % BSA in phos-
phate-buffered saline pH7.2 containing 1 mM
pBA for 3h at 23°C. Strips were rinsed in PBS and blocked
with one of three different probes; (a) a rabbit anti-boar
proacrosin antibody for identification purposes (Jones and
Brown, 1987); (b) 125I-fucoidan (200 000 cts min^-1 ml^-1
in PBS/pBA); (c) 125I-ZPGPs (550 000 cts min^-1 ml^-1
in PBS/ pBA). For b and c, blots were incubated for 1 h, unbound
probe removed by washing in PBS (3 times for 5min with
shaking) and bound probe detected by autoradiography with
X-ray film (Fuji) at —80°C. Relative molecular masses were
calculated by reference to the mobility of known protein
standards (Pharmacia 'Rainbow™' markers).

Electrophoresis and western blotting
Proteins were separated in one dimension by reducing or non-
reducing SDS–PAGE (Laemmli, 1970) and either transferred
to nitrocellulose membranes (0.45 μm) by electroblotting
(Focke et al. 1984). Binding of CMC-protease-digested
proteins was investigated using a solid phase assay (see later). Equal amounts
(~300 000 cts min^-1 in 100 μl) of 125I-ZPGPs were incubated
alone (control) or in the presence of CMC-protease for 1 h,
centrifuged to remove beads and recovered supernatants
made 10 mm with p-aminobenzamidine. Equal amounts were
then used to probe immobilized proacrosin and specific binding calculated.

Extraction and purification of sperm proacrosin
Proacrosin was extracted from washed ejaculated boar
spermatozoa into 0.25 M sucrose/50 mM benzamidine HCl
titrated to pH 3 with 0.1 n HCl (Jones et al. 1988). Proacrosin
was purified from these acid extracts by the method of Fock-
Nüzel et al. (1984) or, in later experiments, by electrophoresis
from gels after non-reducing SDS–PAGE. Purified protein
was either stored frozen at —20°C or dialysed against 1 mM
HCl pH 3 and lyophilized.

Assay for binding of 125I-fucoidan and 125I-ZPGPs to
proacrosin
A solid phase competition assay was developed to measure
quantitatively the avidity of the interaction between proacrosin
and 125I-fucoidan and proacrosin and 125I-ZPGPs. 100
pmoles of purified proacrosin was immobilized onto strips of
nitrocellulose (1 cm²) and blocked with 5 % BSA in PBS/
pBA for 3 h at 23°C. Strips were rinsed in PBS and blocked
for a second time for 1 h with various concentrations (see Results) of saccharides, polysaccharides and synthetic
polymers in 0.1 ml PBS/pBA. Samples were then incubated and
probed with 100 000 cts min^-1 of either 125I-fucoidan (0.4 x
10^6 cts min^-1 μg^-1) or 125I-ZPGPs (1.4 x 10^5 cts min^-1 μg^-1) in

proacrosin antibodies (Jones and Brown, 1987) and (b) proacrosin on the basis of (a) its recognition by anti-
proacrosin antibodies (Jones and Brown, 1987) and (b) N-terminal sequence analysis which yields Arg-Asp-
Asn-Ala-Thr-Cys-Asp-Gly-. This is identical to that reported by Fock-Niizel et al. (1987). Asn-Ala-Thr-Cys-
Asp-Gly-.

**Results**

**Recognition of proacrosin by 125I-fucoidan and 125I-ZPGP probes**

As reported previously and as shown in Fig. 1, 125I-fucoidan and 125I-ZPGPs bind strongly to two proteins at $M_r$ 67 x 10^3 and $M_r$ 53 x 10^3 on western blots of pH 3 extracts of boar spermatozoa (Jones and Brown, 1987; Töpfer-Petersen and Henschen, 1987; Jones, 1989). The $M_r$ 67 x 10^3 protein has not been positively identified but the doublet at $M_r$ 53 x 10^3 has been shown to represent proacrosin on the basis of (a) its recognition by anti-
proacrosin antibodies (Jones and Brown, 1987) and (b) N-terminal sequence analysis which yields Arg-Asp-
Asn-Ala-Thr-Cys-Asp-Gly-. This is identical to that reported by Fock-Niizel et al. (1984) for acrosin light chain. The purified preparation of proacrosin was >90% homogenous as judged by staining with Coomassie Blue (Fig. 1) and bound both 125I-fucoidan and

125I-ZPGPs in a linear fashion over the range 10–200 pmol target protein in the presence of excess probe (Fig. 2A).

**Effects of protease digestion on binding of 125I-ZPGPs probe to proacrosin**

Since the 125I-fucoidan probe had been subjected to extensive pronase digestion followed by dialysis and gel filtration on Sephadex G-75, its interaction with proacrosin was presumed to involve reactive groups on the polysaccharide chain and not contaminating glycopeptides. To investigate if binding of 125I-zona probe to proacrosin was mediated via its protein or carbohydrate moiety, 125I-ZPGPs were treated with Streptomyces griseus protease and the resultant glycopeptides then used as probes in the solid-phase binding assay. As shown in Fig. 2B insert, protease digestion for 120 min caused considerable breakdown of ZPGPs to lower $M_r$ components ranging from approx. 5 x 10^3 to 40 x 10^3. These 125I-zona glycopeptides, however, still bound to immobilized proacrosin (Fig. 2B), suggesting that integrity of the protein backbone of ZPGPs is less critical for recognition than reactive groups on the carbohydrate moiety.
To determine if binding of the whole $^{125}$I-ZPGP probe to proacrosin was mediated by one or several of its component glycoproteins, purified $M_r$ 90×10$^3$ and $55\alpha+55\beta\times10^5$ glycoproteins were iodinated and used in solid phase assay described above. As shown in Fig. 2C, both the $M_r$ 90×10$^3$ and $55\alpha+55\beta\times10^5$ preparations bound to proacrosin in a linear fashion when increasing amounts of target protein were incubated with excess probe. Consistently higher binding was obtained with the $M_r$ 55$\alpha$+55$\beta\times10^5$ glycoproteins that the $M_r$ 90×10$^3$ component. However, saturation kinetics with these purified ZPGPs proved difficult, suggesting that all 3 may be co-operatively involved in binding to proacrosin. Therefore, in subsequent experiments on the mechanism of proacrosin recognition, we used the whole $^{125}$I-ZPGPs probe as this is closer to the biological situation at the cellular level.

**Kinetics of binding of $^{125}$I-fucoidan and $^{125}$I-ZPGPs to proacrosin**

The binding of both $^{125}$I-fucoidan and whole $^{125}$I-ZPGPs to proacrosin approaches saturation under the conditions described for the solid phase binding assay. 0.5 µg of proacrosin bound a maximum of 32 ng of fucoidan and 0.7 ng of zona glycoproteins (results not shown). Scatchard plot analysis of the binding data yielded a slightly curved function for $^{125}$I-fucoidan suggesting the presence on the target protein of several binding sites with different affinities. The apparent $K_d$ for fucoidan was $5.0\times10^{-8}$ M and for ZPGPs it was $1.2\times10^{-9}$ M.

**Inhibition of binding of $^{125}$I-fucoidan and $^{125}$I-ZPGPs by polysaccharides and synthetic polymers**

Preliminary studies using western blotting techniques have suggested that binding of $^{125}$I-fucoidan to sperm proacrosin displays some specificity, in the sense that certain polysaccharides can effectively compete with the probe whereas other closely related polymers are unable to do so (Jones and Williams, 1990). To assess quantitatively, under defined conditions, the critical features of the polymer (size, composition, charge density, etc.) that are important for mediating binding of $^{125}$I-fucoidan and $^{125}$I-ZPGPs to proacrosin, inhibition experiments were carried out with a variety of saccharides, polysaccharides and synthetic polymers. As shown in Fig. 3 and summarized in Table 1, binding of $^{125}$I-ZPGPs to proacrosin was inhibited by ‘cold’ (i.e. non-radioactive) zona glycoproteins, fucoidan, poly(vinyl sulphate), poly(styrene sulphonic acid) and dextran sulphate ($M_r$ 500×10$^3$ and 5×10$^3$) but not by chondroitin sulphates A or C, hyaluronic acid, dextran ($M_r$ 500×10$^3$), glucosamine hydrochloride, poly(vinyl phosphate), glucose-6-sulphate, glucosamine-2,
3-disulphate or any monosaccharide (D(+) glucose, D(+) fucose, L(−) fucose, D(+) mannose, D(+) galactose) or disaccharide (lactose, sucrose) or trisaccharide (raffinose, fucosyllactose) tested. Heparin gave inconclusive results in that although 50% inhibition could be achieved with as little as 2 μM, a reliable dose–response relationship was difficult to obtain. The importance of sulphation and chain length for binding is demonstrated clearly by the dextran/dextran sulphate series. Dextran sulphate Mr 5×10⁵ was nearly 10 times less inhibitory than Mr 500×10³ dextran while Mr 5×10³ was nearly 10 times more inhibitory than Mr 500×10³ dextran sulphate (29.9 μM versus 2.24 μM). Sodium sulphate (0.2 M) had no inhibitory ability. It should be noted at this point that both the D(+) fucoidan and D(+)ZPGPs probes are sulphated (DeAngelis and Glabe, 1987, 1988; 1990). As shown in Fig. 4, at low ionic strength (20 mM sodium phosphate pH 7.2), both probes showed appreciable

The above experiments were repeated using total pH 3 extracted proteins immobilized on nitrocellulose in place of purified proacrosin. Identical results were obtained to those shown in Fig. 3 in terms of the relative potency of competing agents (results not shown). Assuming that proacrosin contributes to at least 50% of total binding in crude extracts, these results indicate that its purification had not had a deleterious effect on its fucoidan- or zona-binding properties. Furthermore, when qualitative assays were performed on western blots taken from SDS gels containing total pH 3 extracted proteins, binding of D(+)fucoidan and D(+)ZPGPs to both the Mr 67×10³ and Mr 53×10³ components were blocked only by ‘cold’ fucoidan, poly(vinyl sulphate), dextran sulphate (Mr 500×10³ and 5×10³), xylan and galactan; Mr 67×10³ dextran, chondroitin sulphates A and C and hyaluronic acid were ineffective competitors. These results also reinforce the hypothesis (Jones et al. 1988) that the Mr 67×10³ protein may be related to proacrosin.

Effects of ionic strength on binding of D(+)fucoidan and D(+)ZPGPs to proacrosin

Since the above results suggest that the presence and number of negatively charged sulphate groups on fucoidan and ZPGPs are important parameters for binding to proacrosin, the ability of high salt concentrations to perturb the interaction was investigated. It is known that binding of many polyanions to charged matrices is primarily electrostatic in nature, the strength of which is dependent on the ionic strength of the medium (DeAngelis and Glabe, 1987, 1988; 1990). As shown in Fig. 4, at low ionic strength (20 mM sodium phosphate pH 7.2), both probes showed appreciable

---

**Table 1. Concentration of competitors for 50% inhibition (IC₅₀) of binding of D(+)fucoidan and D(+)ZPGPs to proacrosin**

<table>
<thead>
<tr>
<th>Competitor (Mr)</th>
<th>125I-Fucoidan IC₅₀</th>
<th>125I-ZPGPs IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucoidan (-100K)</td>
<td>0.1 μM</td>
<td>0.35 μM</td>
</tr>
<tr>
<td>Dextran SO₄ (500K)</td>
<td>0.03 μM</td>
<td>1.4 μM</td>
</tr>
<tr>
<td>Dextran SO₄ (5K)</td>
<td>8.9 μM</td>
<td>12.0 μM</td>
</tr>
<tr>
<td>Dextran (500K)</td>
<td>&gt;160 μM $^\dagger$</td>
<td>&gt;160 μM $^\dagger$</td>
</tr>
<tr>
<td>Galactan (-100K)</td>
<td>22.4 μM</td>
<td>&gt;80 μM</td>
</tr>
<tr>
<td>Xylan (-10K)</td>
<td>4.3 mM</td>
<td></td>
</tr>
<tr>
<td>Poly(vinyl sulphate) (-100K)</td>
<td>29.9 μM</td>
<td>7.9 μM</td>
</tr>
<tr>
<td>Poly(styrene sulphonate) (-75K)</td>
<td>22.4 μM</td>
<td>12.6 μM</td>
</tr>
<tr>
<td>Poly(vinyl phosphate) (-75K)</td>
<td>80.0 μM</td>
<td>&gt;80 μM $^\dagger$</td>
</tr>
<tr>
<td>Chondroitin SO₄ A (-30-50K)</td>
<td>&gt;400 μM $^\dagger$</td>
<td>&gt;400 μM $^\dagger$</td>
</tr>
<tr>
<td>Chondroitin SO₄ B (-20-50K)</td>
<td>&gt;400 μM $^\dagger$</td>
<td>&gt;400 μM $^\dagger$</td>
</tr>
<tr>
<td>Chondroitin SO₄ C (-30-50K)</td>
<td>&gt;400 μM $^\dagger$</td>
<td>&gt;400 μM $^\dagger$</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>&gt;2 mg ml⁻¹ $^\ddagger$</td>
<td>&gt;2 mg ml⁻¹ $^\ddagger$</td>
</tr>
<tr>
<td>ZPGPs</td>
<td>&gt;1.6 mg ml⁻¹ $^\ddagger$</td>
<td>35.0 μg ml⁻¹ $^\ddagger$</td>
</tr>
<tr>
<td>Monosaccharides*</td>
<td>&gt;400 μM $^\dagger$</td>
<td>&gt;400 μM $^\dagger$</td>
</tr>
<tr>
<td>Oligosaccharides†</td>
<td>&gt;400 μM $^\dagger$</td>
<td>&gt;400 μM $^\dagger$</td>
</tr>
<tr>
<td>Fucoxylactose</td>
<td>&gt;80 μM</td>
<td>&gt;80 μM</td>
</tr>
<tr>
<td>Amino sugars‡</td>
<td>&gt;400 μM</td>
<td>&gt;400 μM</td>
</tr>
<tr>
<td>Glucose-6-SO₄</td>
<td>&gt;400 μM</td>
<td>&gt;400 μM</td>
</tr>
<tr>
<td>Glucosamine-2,3-di SO₄</td>
<td>&gt;400 μM $^\dagger$</td>
<td>&gt;400 μM $^\dagger$</td>
</tr>
<tr>
<td>Sodium Sulphate</td>
<td>&gt;200 μM</td>
<td>&gt;200 μM</td>
</tr>
</tbody>
</table>

*D(+)glucose, L(−) fucose, D(+) mannose, D(+)galactose.
† raffinose, sucrose, lactose.
‡ glucosamine, N-acetylgalactosamine, galactosamine.
§ where 50% inhibition could not be achieved the values shown are the highest concentration tested.

$^K \times 10^3$. 

---

Fig. 3. Inhibition of specific binding of 125I-fucoidan or 125I-ZPGPs to proacrosin by sulphated and non-sulphated polymers. Percentage maximum specific binding is plotted against log₁₀ concentration of competitor. Fucoidan (○); poly(vinyl sulphate) (•); galactan (△); ZPGPs (+); dextran sulphate Mr 500×10³ (□); dextran sulphate Mr 5×10³ (■); dextran Mr 500×10³ (◇).
Fig. 4. Effects of ionic strength on specific binding of $^{125}$I-fucoidan and $^{125}$I-ZPGPs to proacrosin. Iodinated probes were suspended in 20 mM sodium phosphate pH 7.2 to which various concentrations of NaCl were added. Specific binding was measured and expressed as a percentage of the maximum recorded. $^{125}$I-fucoidan ($\Delta$); $^{125}$I-ZPGPs ($\triangle$).

binding but this increased rapidly to reach a maximum between 150 mM and 200 mM added NaCl and then decreased progressively to low levels at 1.0 M NaCl. $^{125}$I-fucoidan binding to proacrosin was half-maximal at 490 mM added NaCl and $^{125}$I-ZPGPs binding half-maximal at 595 mM added NaCl. However, if $^{125}$I-fucoidan and $^{125}$I-ZPGPs were first allowed to bind to proacrosin, then incubation in 1 M NaCl for 30 min displaced only 38%–48% of bound probes (results not shown). Between 25% and 40% displacement of bound $^{125}$I-fucoidan was also obtained with 10 $\mu$M 'cold' fucoidan or 3 $\mu$M dextran sulphate ($M_r$ 500 x $10^3$) or 6 M urea but not 160 $\mu$M dextran ($M_r$ 500 x $10^3$) or distilled water. In total, these results suggest that although electrostatic forces may be involved in the binding of $^{125}$I-fucoidan and $^{125}$I-ZPGPs to proacrosin other types of bonding must also participate to stabilize the interaction.

Cytochemical labelling of spermatozoa with FITC-ZPGPs
Since the previous experiments had been carried out on proteins immobilized on solid supports, it was important to investigate if FITC-ZPGPs would bind to spermatozoa in suspension and if the interaction was sensitive to inhibition by sulphated polymers. When freshly washed boar spermatozoa were stained with FITC-ZPGPs, fluorescence was observed over the acrosomal domain of only 10%–20% of cells. These spermatozoa were also positive on the acrosome with Rh-PNA; intact spermatozoa (negative with Rh-PNA) did not bind FITC-ZPGPs (Fig. 5). However, if spermatozoa were first permeabilized by cold shock and then stained, >95% showed fluorescence over the acrosomal domain with both FITC-ZPGPs and Rh-PNA (Fig. 5). No staining was associated with the tail under these conditions. Uptake of the FITC-ZPGPs by permeabilized spermatozoa was reduced to background levels by 10 $\mu$M fucoidan or 3 $\mu$M dextran sulphate ($M_r$ 500 x $10^3$) or 790 $\mu$M poly(vinyl sulphate) but not by 160 $\mu$M dextran ($M_r$ 500 x $10^3$) or 0.25 M L(-) fucose or 0.25 M D(+)-galactose or 0.25 M lactose or 0.25 M glucose-6-sulphate. Thus, cytochemically, binding of zona glycoproteins to the acrosome of permeabilized spermatozoa takes place in a similar fashion to that found on the solid phase assay system for purified proacrosin.

Discussion
This work has shown that the density and orientation of negatively charged sulphate groups on fucoidan and ZPGPs are critical parameters in mediating recognition and high-affinity binding to boar sperm proacrosin. Superimposed upon these requirements are restrictions of tertiary structure and complementarity with binding sites on the target protein. Therefore, together with its cellular location, these properties strongly support the hypothesis that mammalian sperm proacrosin is biochemically and functionally analogous to bindin from sea urchin spermatozoa. The apparent low specificity of recognition by both proteins would be tolerated by virtue of their intracellular location (within the acrosome) and the fact that they are targeted to their site of action (surface of the egg). Hence, spurious interactions with other cell types are avoided. Species specificity could be imparted on the system by the
requirement for an exact stereochemical ‘fit’ between aligned sulphate groups on zona glycoproteins and complementary binding sites on homologous proacrosin.

The zona pellucida of mammalian eggs is known to consist of relatively few species of glycoproteins e.g. 3 in the mouse (Bliel and Wassarman, 1980) and 3–4 in the pig and guinea-pig (Hedrick and Wardrip, 1987; Jones and Williams, 1990). In the mouse only one (ZP3) has sperm receptor activity, apparently mediated by terminal galactose or fucose residues on O-linked carbohydrate chains (Bliel and Wassarman, 1987). The nature of the complementary ligand(s) on the surface membrane of mouse spermatozoa remains problematic but Leyton and Saling (1989) have provided evidence for involvement of tyrosine kinases and Bliel and Wassarman (1990) have detected an Mr 56 × 10^3 glycoprotein from photoaffinity crosslinking studies. This type of binding is clearly different from what we have observed here. First, proacrosin is normally contained within the acrosomal vesicle and only exposed during the acrosome reaction. Thus, it is unlikely to be operative as a primary zona ligand. Second, all 3 of the pig ZPGPs inhibit sperm-zona binding in vitro (Berger et al. 1989) indicating that receptor activity is not confined to one specific glycoprotein. This may or may not be related to our finding that all 3 ZPGPs also have affinity for proacrosin. The latter point concurs with our previous observations (Brown and Jones, 1987) and the recent data by Nakano et al. (1990) that both the Mr 55α+55β×10^3 and Mr 90×10^3 ZPGP families are sulphated. Nakano et al. (1990) have also shown that 60–90% of bound sulphate can be released from pig ZPGPs by endo-β-galactosidase digestion confirming our view that the carbohydrate moiety forms the ‘backbone’ structure for poly(sulphate) groups.

The involvement of poly(sulphate) groups in the binding process is shown clearly by the inability of high relative molecular mass (Mr 500×10^3) dextran to inhibit binding of 125I-ZPGPs or 125I-fucoidan probes to proacrosin whereas its sulphated form is very potent in this respect. Since the composition of the competing polymers is very different (e.g. dextran sulphate is a branched polymer of repeating α 1–6 linked glucose units whereas galactan consists of β 1–3 and α 1–4 linked galactose disaccharides; Percival, 1970), it indicates that constituent sugars do not play a direct role in the binding process, except insofar as they provide a repeating polymeric framework. This is demonstrated most clearly by poly(vinyl sulphate), a polymer that lacks any saccharide structure. In the case of ZPGPs, the protease digestion experiments suggest that the polymeric framework is provided primarily by the carbohydrate moiety although the involvement of sulphated amino acids cannot be excluded. Therefore, it might be more accurate to refer to proacrosin as a poly(sulphate) binding protein and not as a fucose- or carbohydrate-binding protein. In passing, it is noteworthy that the IC_{50 %} for fucoidan against either probe is close to that for inhibition of sperm binding to pig eggs in vitro (i.e. 0.1 μm fucoidan gave 73 % inhibition; Jones et al. 1988).

However, it is also apparent that binding is not simply a function of the presence or absence of sulphate groups (glucose-6-sulphate and glucosamine-2,3-disulphate are ineffective competitors), or charge density (chondroitin sulphates, heparin and hyaluronic acid have charge densities equal to or greater than fucoidan; DeAngelis and Glabe, 1987, 1988) or polymer size (although chain length is relevant, e.g. Mr 500×10^3 versus 5×10^3 dextran sulphate). Rather, the above parameters appear secondary to the position and alignment of the sulphate groups. A useful precedent illustrating the subtlety of such a recognition system is the binding of heparin to anti-thrombin III (reviewed by Lindahl et al. 1986). Heparin consists of an alternating backbone of hexuronic acid (α-glucuronic or τ-iduronic acids) and β-glucosamine units joined by 1,4, glycosidic linkages. The anti-thrombin III binding region resides in an internal pentasaccharide containing four-O-sulphates and two-N-sulphates. At least four of these sulphates are required for recognition, especially the O-sulphate on C3 of the central glucosamine. Absence of a sulphate group at this position results in low affinity binding heparin. If a similar situation applies to the interaction of fucoidan and ZPGPs with proacrosin, then it would explain why glycosaminoglycans such as chondroitin sulphates show low affinity binding; their projecting sulphate groups are simply not in the correct spatial configuration.

The role of these charged sulphate groups in the recognition process raises the question of the nature of the primary adhesive forces involved. From a detailed study of the binding of sulphated fucans to bindin, DeAngelis and Glabe (1987, 1988, 1990) concluded that the mechanism was not a simple electrostatic one but required coordinated hydrogen bonding between guanido moieties on arginine residues in the protein and the 3 oxygen atoms of co-planar sulphate groups. The degree of ionization seems important as phosphate groups, whose oxygen atoms have a similar tetrahedral geometry to sulphate, were not inhibitory. Such a mechanism, which would be highly sterically dependent, has been described for the sulphate binding protein from Salmonella typhimurium (Pflugarth and Quiocio, 1985) and would explain much of the data for proacrosin. The greater sensitivity to salt inhibition shown by the fucoidan binding to proacrosin relative to bindin (IC_{50}=0.5 M NaCl and 1.2 M NaCl respectively) may be due to the participation of basic residues such as lysines and histidines rather than arginines. It has been shown for example, that fucoidan binding to polylysine or polyyhistidine is half-maximal at 0.5 m to 0.75 m NaCl (DeAngelis and Glabe, 1988). The greater resistance to salt shown in the bindin system may be related to the fact that fertilization in sea urchins takes place in sea water (0.5 m–0.6 m NaCl) whereas in mammals oviduct fluid contains only 0.1 m–0.12 m NaCl. Interestingly, there is less than 17 % linear sequence homology between boar proacrosin and sea urchin bindin (Adham et al. 1989; Gao et al. 1986) suggesting that they have
evolved their poly(sulphate) recognition capacities independently.

Allied to the above problem of the type of binding forces involves, is the number and distribution of sulphate binding sites on the target protein. The finding that 'cold' ZPGPs are poor inhibitors of $^{125}$I-fucoidan binding suggests that ZPGP binding sites on proacrosin are more restricted in distribution than fucoidan binding sites, but that the latter overlap the former. Another possibility is that fucoidan inhibits ZPGP recognition by some kind of allosteric mechanism and that the binding sites are completely distinct from each other, bearing in mind that both probes are large molecules. However, the presence of multiple binding sites on proacrosin for fucoidan is supported by Scatchard plot data and by the observation that fucoidan inhibits both amidase and protease activities of $\beta$-acrosin (Urch and Hedrick, 1988). These results lend weight to the hypothesis that binding sites for fucoidan are distributed over the surface of the molecule whereas the site(s) for ZPGPs are localized and are more dependent on protein folding. Many examples of the latter phenomenon are known, e.g. substrate and drug binding sites on enzymes. It is difficult to predict where a ZPGP-sulphate 'docking' site might lie but it would seem not to be on the N-terminal 23 amino acid peptide that constitutes the light chain (Töpfer-Petersen and Henshen, 1987) or on the 75 amino acid proline-rich domain at the C terminus. During activation to $\beta$-acrosin the proline-rich domain is cleaved by endoproteolysis, yet $\beta$-acrosin retains its ability to bind ZPGPs (Urch and Patel, 1991). Latest evidence indicated that ZPGP binding activity is associated with the N-terminal peptide of the heavy chain (Töpfer-Petersen et al. 1990). Again an instructive analogy is the heparin binding site on antithrombin III. Fragmentation analysis has shown that the heparin docking site is associated with two S-S linked peptides comprising residues 89–96 and 114–156 (Peterson et al. 1987). The lysine residue at position 125 would seem to be especially crucial as shown by site-directed mutagenesis. Tertiary folding of antithrombin III is also important as reduction and alkylation abolishes its capacity to bind to heparin-Sepharose (Ferguson and Findlay, 1983). In total, these results emphasise the importance of complementarity between the orientation of sulphate groups on ZPGPs on the one hand and their docking site on proacrosin on the other. Variations in the stereochemistry of either reactant could conceivably impart species specificity on the system.

Lastly, it should not be construed from the foregoing that we regard proacrosin/acrosin as the only zona ligand in mammalian spermatozoa. Evidence from several laboratories has indicated that there are multiple egg-binding proteins on spermatozoa, e.g. in the mouse, galactosyltransferase (Shur, 1986), tyrosine kinase (Leyton and Saling, 1989), a trypsin-like enzyme (Benau and Storey, 1987) and an $M_r$ 56×10$^3$ antigen (Biel and Wassarman, 1990) are all contenders as zona ligands. Such ligands must be present on the surface membrane overlying the sperm head (to account for those species in which acrosome intact spermatozoa bind to the zona, e.g. mouse) and also within the acrosome (to account for those species in which acrosome reacted spermatozoa bind to the zona, e.g. guinea-pig, rabbit). These two levels of recognition are not mutually exclusive and one may override the next depending on the species. A model incorporating these concepts has recently been discussed in detail (Jones, 1990). In this scheme, proacrosin is considered to be a secondary ligand operative after the acrosome reaction. Further work is necessary to establish the behaviour of this molecule on the surface of the zona at the exact point of sperm attachment.

The author records with pleasure numerous stimulating conversations with Dr Bert Urch, UC, Davis, in the course of this work. The assistance of Mr P. Barker (protein sequencing), and Mrs L. Notton and Mrs D. Styles (manuscript preparation) is acknowledged.

References


(Accepted 28 January 1991)