Prior to fertilization, mammalian sperm must first bind to the zona pellucida (ZP), a glycoprotein matrix surrounding the egg. Sperm specifically bind to ZP3, an 83-kDa glycoprotein which functions as both an adhesion molecule and as a secretagogue for acrosomal exocytosis (Litscher, E. S., and Wassarman, P. M. (1993) Trends Glycosci. Glycotechnol. 5, 369–388). We used acid solubilized, 125I-labeled ZPs to quantify the initial binding event on mouse spermatozoa. Live sperm could not be used since solubilized ZPs rapidly initiated exocytosis. Instead, acrosome intact mouse sperm were briefly fixed in 1% glutaraldehyde for binding studies using a standard filtration assay. The fixed sperm are suitable for sperm-zona binding assays based on two experiments: 1) incubating either live or fixed sperm in low concentrations of 125I-ZPs not sufficient to induce acrosomal exocytosis revealed no differences in binding up to 15 min and 2) solubilized, unlabeled ZPs competed for 125I-ZPs with an $K_I$ of approximately 3.78 nM. Sperm-125I-ZP binding reached equilibrium with a $t\frac{1}{2}$ of ~22 min at 37 °C. Affinity parameters were calculated using the well substantiated assumption that only ZP3 binds intact mouse sperm. The on-rate constant for association of 125I-ZP binding to the mouse sperm surface was calculated to be $3.2 \times 10^{6}$ M$^{-1}$ min$^{-1}$. The saturation binding isotherm revealed that there are approximately 30,000 binding sites, ascribed to ZP3, with an $EC_{50}$ of 1.29 nM. Further analysis indicated that this binding is complex (Hill coefficient = 1.72), suggesting involvement of multiple receptors on the sperm surface and/or multiple ligand moieties. High and low affinity ZP binding sites on the sperm surface were confirmed by dissociation experiments. 125I-ZP dissociation was clearly biphasic, and kinetic off-rate constants of 0.161 min$^{-1}$ and 0.0023 min$^{-1}$ were calculated for the low and high affinity sites, respectively. Apparent affinities ($K_I$ values) of 50 nM for the low affinity and 0.72 nM for the high affinity interaction were calculated from the rate constants. These data demonstrate that the initial adhesion event between mouse sperm and the zona pellucida is a high affinity event which is sufficient to tether a sperm to the extracellular matrix prior to the induction of acrosomal exocytosis.

When mammalian sperm first encounter an egg, they bind to the zona pellucida (ZP), an extracellular glycoprotein matrix secreted by the egg (Inoue and Wolf, 1975; Gwatkin and Williams, 1977). This binding event is a receptor-ligand-mediated interaction that serves not only to physically anchor the sperm to the egg, but also to initiate a signal transduction pathway culminating in acrosomal exocytosis (Bleil and Wassarman, 1980a, 1980b, 1983; Florman and Storey, 1982). In the mouse, acrosomal exocytosis occurs after sperm are bound to the surface of the zona pellucida (Salig et al., 1979; Florman and Storey, 1981, 1982) and is thought to be required for the penetration of this egg vestment. Thus, the initial steps in fertilization use a receptor mediated stimulus secretion coupling system to control exocytosis of the acrosomal vesicle.

Following the demonstration that sperm were acrosome intact during initial binding to the zona pellucida (Salig et al., 1979), it was soon convincingly demonstrated that the 83-kDa glycoprotein component of the zona pellucida, ZP3, is the ligand for sperm binding (Bleil and Wassarman, 1980a, 1980b, 1983; Florman and Wassarman, 1985). Surprisingly, the sperm surface receptor for ZP3 remains elusive, although numerous candidates have appeared in the literature (Bleil and Wassarman, 1990; Cheng et al., 1994; Leyton and Saling, 1989b; Leyton et al., 1992; Kalab et al., 1994; Miller et al., 1992). In addition, there has been little biochemical characterization of mammalian sperm receptor-ZP3 interactions. Sperm-zona binding is mediated by one or more of the O-linked carbohydrate moieties on ZP3 (Florman and Wassarman, 1985); however, receptor aggregation may be required to initiate signaling since glycopeptides of ZP3 will bind to sperm, but not initiate the acrosome reaction (Florman and Wassarman, 1985; Leyton and Saling, 1989a).

Unlike other receptor ligand systems, direct assessment of receptor ligand affinities, number of binding sites, cooperativity of binding, or other characteristics of sperm-ZP3 binding, has not been undertaken. This appears unusual since the initial event of sperm-egg binding is prerequisite to all subsequent steps completing fertilization. In mammalian systems, however, two intrinsic factors have presented obstacles to obtaining direct biochemical characterization of the receptor-ligand interactions. First, ligand availability has been limited by the difficulties inherent in obtaining large quantities of purified mammalian oocyte components, in this case, the zona pellucida. Second, the loss, or modification, of receptor sites on live sperm following acrosomal exocytosis creates complex interactions between the receptor and ligand populations, making experimental design and interpretation untenable.

The development of relatively straightforward mass isolation techniques (Tanphaichitr et al., 1993), which we have further modified for increased efficiency and yield, has ameliorated the problem of ligand availability. The loss of receptor sites upon exposure to the ligand appeared to be a more intractable problem since the effects that addition of inhibitors of acrosomal exocytosis might have on the receptor-ligand interactions are unknown. Following the lead of researchers char-

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* This work was supported in part by Grant HD27244 from the National Institutes of Health (to R. A. C.) and a grant from the Lalar Foundation (to C. D. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ZP, zona pellucida; HWB, Hepes supplemented Whittingham’s buffer; BSA, bovine serum albumin.
acterizing receptor-ligand binding in other systems (Hulme and Birdsal, 1992; Schroder et al., 1990; van Maurik et al., 1985; von Zastrow and Kobilka, 1992; Zidovetzki et al., 1991), we have turned to the use of fixed cells for binding studies. We have developed a sensitive, quantitative binding assay to evaluate sperm-ZP binding. This report presents the first direct quantitation of sperm-ZP binding, calculates affinity parameters for ZP3 binding based on the assumption that only ZP3 binds to acrosome intact sperm (Bleil and Wassarman 1980a, 1980b, 1983; Florman et al., 1984; Florman and Wassarman, 1985; Mortillo and Wassarman, 1991), and demonstrates the presence of both high and low affinity binding sites on acrosome-intact sperm.

EXPERIMENTAL PROCEDURES

Materials
Mice (ICR strain) were purchased from Harlan Sprague-Dawley (San Diego, CA). Na125I was purchased from DuPont NEN. Resins for gel filtration were purchased from Bio-Rad. Protease inhibitors, DNase, hyaluronidase, BSA, casein, lactalbumin, and Percoll were purchased from Sigma. Glutaraldehyde was purchased from either Electron Microscopics Sciences (Fort Washington, PA) or from Sigma. Chloramine-T was purchased from Aldrich. Whatman GF/C glass fiber filters for binding assays and all other chemicals were purchased from Fisher.

Isolation of Mouse Zonae Pellucidae
Zonae pellucidae were collected from ovarian homogenates of 3-week-old female mice according to Tanphaichitr et al. (1993) with the modifications outlined below. Ovaries were homogenized, as described, with the addition of 20 μg/ml leupeptin and aprotinin, and 200 μg/ml benzamidine. The homogenate was layered onto a 3-step Percoll gradient (3 ml 25%, 4 ml 10%, 2 ml 3%) and centrifuged at 2000 g at 4°C. The 10% step, which contained virtually all the ZPs, was collected, diluted with buffer (1:12), and centrifuged at high speed (16,000 × g, 10 min) in a standard microcentrifuge (Brinkmann Instruments) to pellet the ZPs. ZP pellets were pooled and centrifuged to obtain a single pellet. Isolated ZPs in a final volume of 100–200 μl were solubilized by adding ~2 μl of 1 N HCl to lower the pH to 2.5 and incubating at 37°C for 15 min. Insoluble material was removed by centrifugation (16,000 × g, 2 min). Solubilized ZPs were drop frozen and stored in liquid N2 until use. To minimize protein loss caused by adsorption to surfaces, handling of ZPs, during isolation and in all subsequent experimental protocols, was performed using siliconized Pasteur pipettes, pipette tips, and microcentrifuge tubes.

125I-Labeling of ZPs
Solubilized ZPs (approximately 80 μg per labeling) were concentrated to 200 μl in a vacuum concentrator (Labconco, St. Louis, MO), and the pH was adjusted to 7.0. ZPs were radioiodinated using Chloramine-T (Florman and Wassarman, 1985; Bleil and Wassarman, 1986). The free 125I was removed, and the sample was desalted by size exclusion chromatography using an 8-cm Bio-Gel P6 Pasteur pipette column. Fractions containing 125I-ZPs were pooled. The protein concentration of 125I-labeled solubilized ZPs was determined by comparison to a protein standard curve (BSA) using the Quantigold protein assay (Diversified Biotech, Piscataway, NJ). Dilutions of 125I-ZPs were quantified in a gamma counter to determine the counts/min per volume. The specific activity of the 125I-ZP preparations ranged from 4.5 × 106 cpm/ng to 1.8 × 107 cpm/ng. Free 125I contamination was estimated as a fraction of the total by densitometry of gel autoradiographs of ZPs and was less than 1% in all cases. Densitometry of the gel autoradiographs was also used to determine the contribution of 125I-ZPS to total radioactivity present in the labeled ZPs. Several exposures were made for each 125I-ZP preparation, and only exposures with the linear range of the film (Kodak XRF-5) were used to calculate the fractional contribution of 125I-ZPS. 125I-ZPS ranged from 16.3 to 22.4% of the total, and the precise contribution was calculated for every preparation of 125I-ZPs used.

Sperm Collection
 Epididymal sperm were collected from 12–15-week-old mice (Harlan Sprague-Dawley, San Diego, CA) by removing the epididymes, mincing the tissue in Hepes supplemented (20 mM, pH 7.2) (Florman and Storey, 1982; Whittingham’s Buffer (HWB) (Whittingham, 1971), and gently agitating the suspension for 10 min to release sperm. Epididymal tissue was removed by centrifugation (200 × g, 1 min). Sperm were concentrated by centrifugation (15 min, 200 × g), resuspended in HBW containing 30 mg/ml BSA and incubated at 37°C in a humidified atmosphere containing 5% CO2, for 60 min to capacitate sperm. Sperm were collected after capacitation by pelleting (200 × g, 10 min). Capacitated sperm were washed once by centrifugation in HBW without BSA, resuspended, and used in binding assays immediately or fixed. Sperm were fixed in 1% glutaraldehyde in HBW for 5 min and pelleted (250 × g, 10 min). Fixed sperm were washed by pelleting and resuspension (three times) in fresh HBW, and finally resuspended in 500 μl of HBW. Percent transmittance (%T) at 500 nm was determined for duplicate dilutions of the fixed sperm suspension. Average %T was used to calculate the sperm concentration from a standard curve of % T versus cells/ml (determined by hemacytometer counts of fixed sperm). Washed, fixed sperm were held at 4°C until use.

Binding Assays
Sperm and 125I-ZPs were equilibrated to 37°C prior to experimental manipulations. Washed, fixed sperm were added to 125I-ZPs and incubated in a water bath at 37°C. Sperm with bound 125I-ZPs were separated from free 125I-ZPs by vacuum filtration (Millipore, Bedford, MA) through Whatman GF/C glass fiber filters (nominal pore size, 1 μm) that had been incubated in BSA (50 mg/ml HBW, 1 h) to block nonspecific binding sites. Filters were washed with approximately 200 volumes of 1× HBW, with the exception of the off-rate experiments, as described below of ice-cold HBW immediately. Filters (bound 125I-ZPs) and samples of the filtrate (free 125I-ZPs) were quantified using a Beckman 5000 gamma counter (Beckman Instruments). For all assays, parallel samples without sperm were processed identically, and the nonspecific 125I-ZP binding to filters was quantified and subtracted from sperm-containing samples. All experiments were performed with duplicate samples. Following initial studies, 2 × 106 sperm/sample was assigned to be the standard sperm aliquot for binding assays, since this number of cells reproducibly gives stable readings above background. Exceptions were experiments shown in Fig. 1, as noted below. Assay volumes varied from 50 to 200 μl, but were identical for any given set of assays with the exception of the saturation assays, as noted below. Specific procedural details for each of the binding studies are outlined below.

Receptor Concentration Dependence—Increasing concentrations of live or fixed sperm were incubated for 15 min with a constant, low concentration of 125I-ZPs.

IC50—Increasing amounts of unlabeled ZPs were added to saturating concentrations of 125I-ZPs and incubated with sperm for 60 min. Samples to determine background binding for each concentration of unlabeled ZPs were processed in parallel.

Equilibrium Binding—Fixed sperm were incubated with 125I-ZPs for various times ranging from 1–120 min.

On Rate—Aliquots of fixed sperm and 125I-ZPs were incubated for increasing amounts of time from 1–10 min.

Off Rate—Fixed sperm were incubated with 125I-ZPs for 60 min to attain equilibrium. Samples were pipetted into 10 ml of HBW in siliconized 15 ml plastic culture tubes and agitated on a Lab Line three-dimensional Rotator for 10–450 min at 37°C. The samples were vacuum filtered and washed with 5 ml of ice-cold HBW. Control samples (no dilution) were processed at the end of the equilibrium binding.

Saturation—Sperm were incubated with increasing amounts of 125I-ZPs for 60 min. Assay volumes ranged from 60 to 300 μl since the stock 125I-ZP concentration was not great enough to maintain all the assay volumes at 60 μl for the higher ZP concentrations.

Ca2+ Dependence—Fixed sperm were aliquoted for equilibrium binding assays, pelleted in a standard microcentrifuge, and resuspended in HBW containing 1.7 mM Ca2+ or 2 mM EGTA with no added Ca2+ ((Ca2+) < 10−5 M using a Ca2+ electrode (Orion, Inc., Boston, MA). Aliquots of 125I-ZPs were added for 60 min and samples separated by vacuum filtration and quantified. The composition of the wash buffers were identical to the incubation buffer for each sample (i.e. 1.7 mM Ca2+ or 2 mM EGTA). Alternatively, sperm were collected and handled through incubating HBW, Ca2+ containing HBW, with no added Ca2+ and 2 mM EGTA. Since no significant differences (Student’s t test, p > 0.05) in 125I-ZP binding were detected.

Non specific binding
GF/C filters were preincubated in various protein solutions to block nonspecific binding to filters and compared by vacuum filtration of 125I-ZPs. The fraction (bound/free) of 125I-ZPs retained following different protein blocks was determined (Table I) and, subsequently, all filters were preincubated with 50 mg/ml BSA for 1 h to block nonspecific
binding sites on the filters. Nonspecific binding to BSA blocked GF/C filters by 125I-ZP or free 125I-ZPs was determined by vacuum filtration of 125I-ZPs in the absence of sperm. This background binding was subtracted from experimental samples prior to data analysis. The contribution of free 125I contamination to nonspecific binding to fixed sperm was assessed. 125I-ZPs were separated from free 125I using a Bio-Gel P6 column, as described above. Fractions from the 125I-ZP peak and fractions from the free 125I peak were incubated with 10^7 sperm for 1 h at 37 °C and bound radioactivity determined as above. Less than 0.5% of the total radioactivity was associated with sperm under these conditions (data not shown).

Data Analysis

Experiments were replicated a minimum of three times and all replicates were performed in duplicate. Exceptions were the saturation experiment for which four independent data sets are pooled and the data points appearing on the plot are binned data averages presented with error bars (± S.E.) for both x and y components. Curve fitting was performed using TableCurve2D (Jandel Scientific, San Rafael, CA) and plotted with SigmaPlot (Jandel Scientific). Goodness of fit for all curve fitting, as indicated by the r^2 values, is stated in the figure legends. All calculated affinity parameters (B_m, EC_50, k_a, and consequently K_a, and K_D), used the assumption that ZP3 is the only component binding to the intact mouse sperm, as indicated by previous studies (Bleil and Wassarman 1980a, 1980b; Florman and Wassarman, 1985). The concentration of free ZP3 was derived using the percent binding and saturation data were transformed to log((B/B max - B))/(B max - B) and plotted (free(ZP3)) and plotted for determination of the Hill coefficient. The data were fit by a double exponential decay equation: B/B max = B_e(-k_1t) + B_e(-k_2t), where k_1 and k_2 are the off rate constants for each. The saturation binding isotherm (Fig. 6) was fit to B = (B max)(L) /((EC_50)^n + [L]^n) where n is the Hill coefficient (Huile and Birdsall, 1992). The raw binding data was transformed to units of "molecules of ZP3 bound" using the published molecular weight of 83 kDa for ZP3 and assuming that all bound radioactivity was due to ZP3, since extensive studies have indicated that only ZP3 binds to intact sperm (Bleil and Wassarman, 1980a, 1980b; Florman and Storey, 1982; Florman et al., 1984; Florman and Wassarman, 1985, Mortillo and Wassarman, 1991). The concentration of free ZP3 was derived using the percent contribution of 125I-ZP3 to the total radioactivity of the sample, determined by gel autoradiography as discussed above. Additionally, saturation data was transformed to log((B/B max - B))/(log(1 - ((B)/B max))) and plotted to determine the Hill coefficient. The data were fit by linear regression for each phase and the slopes are the Hill coefficients. Significant differences in binding in the presence or absence of Ca^2+ were determined by an unpaired Student's t test with a significance level of p < 0.05.

RESULTS

Quantitative characterization of sperm-zona pellucida binding has been lacking although the zona ligand, ZP3, was identified and initially characterized over a decade ago (Bleil and Wassarman, 1980a, 1980b; Florman and Wassarman, 1985). The paucity of quantitative information concerning receptor-ligand interactions during fertilization stems from two intrinsic difficulties. First, although methods have been developed for isolating zonae pellucidae, only minute amounts are available for detailed binding studies. A mouse will typically yield less than two hundred zonae containing approximately 400 ng of ZP glycoproteins. Second, in addition to its role as an adhesion molecule, ZP3 also functions as an agonist (secretagogue) for acrosomal exocytosis. The exocytic event may lead to a loss (or modification) of receptor binding sites following exocytosis. Since this event occurs over the first 10–30 min, finding conditions which are optimal for binding studies (e.g. equilibration and saturation) is nearly impossible. Because of these inherent problems, we have developed an assay using glutaraldehyde-fixed acrosome-intact sperm to quantitatively establish the number of ZP binding sites on sperm and the affinity of the ligand for its complementary receptor.

Before carrying out binding assays, conditions for minimizing nonspecific binding of ZPs to the glass-fiber filters needed to be established. Filters were incubated in protein solutions at different concentrations in order to block potential nonspecific binding interactions with solubilized ZPs. Blocking with BSA or casein resulted in low background binding to the filters (Table I). In contrast, α-lactalbumin and ovalbumin-blocked filters retained significantly more 125I-ZPs compared to 50 mg/ml BSA (p < 0.01) and did not appear to be suitable blocking agents. Although casein blocking resulted in slightly lower background binding, the retention of insoluble aggregates of this protein by the filter suggested that it might not be a suitable choice. We therefore used 50 mg/ml BSA for blocking GF/C filters in all experiments.

To demonstrate that glutaraldehyde fixation of sperm did not affect ZP binding, different concentrations of both fixed and unfixed sperm were incubated with solubilized, 125I-labeled ZPs for 15 min. The low concentration of ZPs along with relatively short incubation times were chosen so that acrosomal exocytosis would not occur during this experiment. Indeed, using higher concentrations of ZPs, the temporal binding pattern was highly complex and did not reach equilibrium, most
likely reflecting changes in sites as sperm initiated acrosomal exocytosis (Thaler and Cardullo, 1994). However, at lower ZP concentrations and shorter times, both fixed and unfixed sperm displayed a linear dependence of ZP binding on sperm concentration, i.e., receptor number (Fig. 1). In addition, unlabeled ZPs could compete with the 125I-ZPs for binding sites on fixed sperm (Fig. 2), indicating that binding specificity is retained by the fixed sperm-zona interaction prior to acrosomal exocytosis. Further, a Kd for unlabeled ZPs was calculated from the IC50, the concentration of unlabeled ZPs at which 50% of the 125I-ZPs are displaced from their binding sites under equilibrium conditions. The Kd for ZP binding was found to be 3.78 nM by the method of Cheng and Prusoff (1973), was found to be 3.78 nM. Residual binding of 0.17 could not be displaced and was considered nonspecific binding. These data are consistent with the assumption that both the 125I-ZPs and the unlabeled ZPs recognize the same binding site and further demonstrate that fixed sperm retain ZP binding sites.

The forward rate constant ($k_{on}$) for ZP binding was determined by incubating sperm with 125I-ZPs for various lengths of time, measuring the 125I-ZPs bound and calculating the initial rate of binding (Fig. 4). Assuming that all specifically bound radioactivity is due only to ZP3, that the rate of binding is a first-order reaction ($v_{on} = k_{on}[ZP3][ZP3R]$), and that the reverse reaction is negligible at early time points, the calculated on-rate constant was $3.20 \times 10^{6} \text{ M}^{-1} \text{ min}^{-1}$ at 37°C. Dissociation experiments were used to determine the reverse rate constant, $k_{off}$. Fixed sperm were incubated with 125I-ZPs for 60 min to reach equilibrium binding, at which point sperm were diluted into a large volume of buffer and the decrease in 125I-ZPs bound over time was quantified (Fig. 5). Loss of radiolabeled ligand was clearly biphasic and followed an exponential decay function with high fidelity. Rate constants for the low and high affinity components of ZP binding were calculated to be $k_{off1} = 0.161 \text{ min}^{-1}$ and $k_{off2} = 0.0023 \text{ min}^{-1}$, respectively. These rates correspond to $\tau_{1/2}$ values for dissociation of 4.3 min and 300.8 min for the low and high affinity components.

Steady-state saturation experiments using increasing concentrations of 125I-ZPs incubated with a fixed number of sperm (2.0 $\times$ $10^{9}$ sperm) were performed under equilibrium binding conditions to determine the total number of binding sites (Bmax), the presence or absence of cooperative binding effects, and the overall affinity of ZP binding (EC50) for its complementary receptor on the mouse sperm surface. The amount of

### TABLE I

| Blocking nonspecific binding of 125I-ZPs to GF/C filters | BSA, 100 mg/ml | BSA, 50 mg/ml | $\alpha$-Lactalbumin, 100 mg/ml | Ovalbumin, 100 mg/ml | Casein, 100 mg/ml |
|---------------------------------------------------------|----------------|--------------|---------------------------|-------------------|-----------------|
| Mean (bound cpm/total cpm)                              | 0.0828         | 0.0658       | 0.1253                    | 0.2380            | 0.0493          |
| S.D.                                                    | 0.016          | 0.018        | 0.017                     | 0.008             | 0.109           |
| n                                                       | 6              | 6            | 3                         | 3                 | 4               |

**FIG. 2.** Unlabeled ZPs compete for binding sites on fixed mouse sperm. Sperm incubated with saturating concentrations of 125I-ZPs in the presence of increasing concentrations of unlabeled ZP glycoproteins showed an exponential decrease in normalized fractional binding ($B - B_{min}$)/($B_{max} - B_{min}$), of the radioactive ligand ($r^2 = 0.93$). The data are presented as mean ± S.E. The Kd for displacement of 125I-ZPs by unlabeled ligand, calculated using the correction of Cheng and Prusoff (1973), was found to be 3.78 nM. Residual binding of 0.17 could not be displaced and was considered nonspecific binding. These data are consistent with the assumption that both the 125I-ZPs and the unlabeled ZPs recognize the same binding site and further demonstrate that fixed sperm retain ZP binding sites.
were fitted to a nonlinear isotherm equation of the form thancan be suitably fit to a single-site binding model. The data from the kinetic studies in revealing that binding is more complex sites on the mouse sperm surface, and, in addition, concur with increasing free ZP concentration until available binding sites calculated to be 3.20 ± 0.06 M⁻¹ min⁻¹.

125I-ZP bound to the fixed sperm surface increased with increasing free ZP concentration until available binding sites were saturated (Fig. 6A). These studies represent the first direct quantitative determination of the number of ZP binding sites on the mouse sperm surface and, in addition, concur with the kinetic studies in revealing that binding is more complex than can be suitably fit to a single-site binding model. The data were fitted to a nonlinear isotherm equation of the form $B = B_{\text{max}} [L]^n / [K_d]^n + [L]^n$ (Hulme and Birdsell, 1992), where $n$ is the Hill coefficient, $B$ is assumed to be ZP3 bound, and $[L]$ is the concentration of free ZP3 present in the assays. The data fit well ($r^2 = 0.92$) to this multisite model with $n = 1.72$, a $B_{\text{max}}$ of 30,000, and an $K_d$ of 1.29 nm.

Sigmoidal binding curves, such as the one shown in Fig. 6A, are indicative of complex binding interactions between receptors and ligands. Accordingly, other measures of binding complexity were analyzed. Fractional occupancy rates, Scatchard plots, and Hill plots are traditionally viewed as indicators of cooperativity, although they can also reflect other complex mechanisms that yield similar plots and are usually lumped into the alternative category of “apparent cooperativity.” Fractional occupancy of the sperm-ZP3 binding sites increased from 10 to 90% within less than 1.9 log units of free ligand (1.5 log units for sperm-ZP3), suggesting cooperative, or multisite, interactions (Wells, 1992). Scatchard plots displayed a concave upward shape (data not shown), an indicator of multiple site interactions as a result of the presence of multivalent ligand and/or receptor (Lauffenburger and Linderman, 1993). Hill plots of the equilibrium saturation data produced a biphasic linear curve (Fig. 6B), also supporting multivalent interactions (Matthews, 1993). Together with the kinetic data, these steady state saturation experiments support a complex binding interaction between ZP3 and its complementary receptor on the sperm surface.

Other researchers have demonstrated that the sperm binding bioactivity resides solely with ZP3 (Bleil and Wassarman, 1980a, 1980b, 1983; Florman et al., 1984; Florman and Wassarman, 1985; Mortillo and Wassarman, 1991), and we have confirmed this using a chemical cross-linker. When the total 125I signal is attributed to ZP3, we calculated a $B_{\text{max}}$ of 30,000 ZP3 binding sites per mouse sperm (Fig. 6). In each experiment, the fractional radioactivity assigned to each of the three glycoproteins was determined. On average, ZP3 accounted for approximately 19% of the total radioactivity (range = 16.3 to 22.4%) and the ZP3 contribution was used to determine the relative concentration of free ZP3 in the assay samples. The half-maximal concentration of ZP3 needed to achieve saturation, $K_d$, was found to be 1.29 nm. The $K_d$ values for high and low affinity binding calculated from kinetic data are 0.72 nm and 50 nm, respectively, demonstrating the existence of multiple binding sites (Table II). Taken together, results from both the kinetic and steady state experiments demonstrate that the initial adhesion event between the sperm and the zona pellucida is a complex, high affinity binding interaction.

Finally, equilibrium binding studies characterizing the Ca²⁺ dependence of sperm 125I-ZP binding were conducted and no changes in binding were detected in the presence or absence of Ca²⁺ (data not shown). Previous work has suggested that Ca²⁺ is required for binding of live sperm to intact zonae (Saling et al., 1978) but these earlier qualitative experiments could not distinguish between primary adhesion events involving acrosome-intact sperm and secondary binding events following acrosomal exocytosis. We were not able to detect a Ca²⁺ requirement for binding of intact sperm to zona pellucida glycoproteins using the fixed cell models, but the applicability of this result to live cells and any requirements for Ca²⁺ at subsequent steps during sperm-zona interactions remains to be determined.

These studies demonstrate that fixed sperm retain specific ZP binding sites and have enabled us to characterize the initial sperm-ZP binding interactions. Our data indicate that the interaction has both high and low affinity components and appears to be complex in nature. In addition, this binding assay establishes a standard quantitative method to evaluate putative sperm ZP3 receptors.

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**Fig. 5.** Kinetic determination of $k_{\text{off}}$ for 125I-ZP binding. Ligand dissociation experiments were performed to determine the off rate constants(s) for 125I-ZP binding. Following equilibrium binding of fixed sperm and 125I-ZPs, samples were diluted into a large volume of buffer and the loss of bound radiolabeled ligand ($B/B_0$) over time was quantified. Ligand dissociation fit a double exponential decay function from which off rate constants, $k_{\text{off}}$, and $k_{\text{data}}$, for low and high affinity binding sites were determined to be 0.161 min⁻¹ and 0.0023 min⁻¹, respectively ($r^2 = 0.98$). These rates correspond to half-times of dissociation of 4.3 min for the low affinity and 300.8 min for the high affinity components of sperm ZP binding. The data points are averages of three independent experiments, each performed in duplicate, ± S.E.

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* C. D. Thaler and R. A. Cardullo, unpublished data.
DISCUSSION

Previous descriptions of sperm-zona adhesion have relied on microscopic binding assays using living sperm and intact zonae pellucidae. In these assays, sperm are typically incubated with eggs for some predetermined amount of time (e.g. 5–60 min), and attached sperm are subsequently counted under the light microscope. This assay successfully identified mZP3, an 83-kDa glycoprotein from the mouse zona pellucida, as both the initial adhesion ligand and as a secretagogue for acrosomal exocytosis in mice (Bleil and Wassarman, 1980b, 1983, 1986; Florman et al., 1984, Florman and Wassarman, 1985). Using solubilized ZPs, a number of researchers have shown that ZP3 is the only component that binds to acrosome intact sperm (Bleil and Wassarman, 1986; Bleil et al., 1988; Mortillo and Wassarman, 1991) and that concentrations of ZP3 exceeding 1 ng/μl (equivalent to ~1 zonae/μl) are sufficient to block sperm-
 zona binding as well as initiate the signal transduction pathway leading to acrosomal exocytosis (Bleil and Wassarman 1980a, 1980b, 1983, 1986; Florman and Wassarman, 1985; Leyton and Saling, 1989a).

The microscopic binding assay has also been used in conjunction with a variety of compounds to identify putative receptors for mZP3 including the sperm surface enzymes β-1,4-galactosyltransferase (Shur and Hall 1982a, 1982b; Lopez et al., 1985; Shur and Neely, 1988; Miller et al., 1992; Cardullo and Wolf, 1995), and mannosidase (Tulsiani, et al., 1989; Cornwall et al., 1991), as well as a trypsin inhibitor-sensitive site (Salting, 1981; Benau and Storey, 1987). Additionally, it has been demonstrated that the sperm surface galactosyltransferase could enzymatically transfer a galactose (from UDP-galactose) to ZP3 suggesting that it may act as a receptor (Miller et al., 1992). Other putative receptors have been characterized by affinity methods: sp56, by ZP3 affinity chromatography and cross-linking (Bleil and Wassarman, 1990; Cheng et al., 1994), and p95/116, by affinity blotting with 125I-ZP3 (Leyton and Saling, 1989b; Leyton et al., 1992). Subsequently, p95/116 has been shown to share significant sequence homology to hexokinase (Kalab et al., 1994).

Although useful for studying the native interactions between mZP3 and its complementary receptor on the sperm surface, using intact zonae pellucidae may preclude accurate determination of binding characteristics between ligand and receptor. The quantitative, standardized binding assay presented here, using solubilized ZP glycoproteins, has revealed molecular details about the specific interactions between mammalian sperm and egg and will aid in the identification of bona fide ZP3 receptors on acrosome-intact sperm.

Initial attempts to characterize solubilized ZP binding to living mouse sperm proved difficult because equilibrium was never achieved (Thaler and Cardullo, 1994). This was presumably due to loss of binding sites following the acrosome reaction (Bleil and Wassarman, 1986; Bleil et al., 1988; Mortillo and Wassarman, 1991). Removal of binding sites is common in living cells and, in such cases, binding events can be distinguished from downstream physiological processes by blocking the removal of binding sites through use of low temperatures, pharmacological inhibitors, or fixation. In our studies, ZP binding experiments below 15 °C were precluded since solubilized ZPs precipitated out of solution under these conditions (data not shown). Pharmacological inhibitors, including pertussis toxin (Endo et al., 1987, 1988) and 3-quinuclidinyl benzilate (Florman and Storey, 1981) block ZP3 induced acrosomal exocytosis, but the rate of spontaneous acrosomal exocytosis is unaffected and is sufficient to significantly decrease the fraction of acrosome-intact sperm over reasonable binding times (Florman and Storey, 1982). In contrast, fixed cell models have been widely used to characterize ligand receptor interactions including those of growth factor (van Maurik et al., 1985; Zidovetzki et al., 1991), cytokine (Smith et al., 1979), adrenergic (von Zastrow and Kobilla, 1992), and acetylcholine (Schroder et al., 1990) receptors. In this study, we have shown that 125I-labeled ZPs bind to glutaraldehyde-fixed sperm in a concentration dependent manner, that unlabeled ZPs compete for binding sites of 125I-ZPs on mouse sperm, and that binding of 125I-ZPs at low concentrations and short times (conditions that do not favor ligand-induced acrosomal exocytosis) are virtually identical for live and fixed sperm. These experiments demonstrate that glutaraldehyde-fixed mouse spermatozoa are a good model system for characterizing the initial binding events between sperm and zona pellucida and provide a stable population of acrosome intact sperm (typically greater than 80%) for binding experiments.

Both kinetic and equilibrium binding studies undertaken to quantify the sperm-ZP binding interactions were performed using whole solubilized 125I-labeled ZPs containing all three ZP glycoproteins. The binding parameters calculated from these studies have assumed that all specifically bound radioactivity is solely due to the binding of ZP3 as suggested by previous work (Bleil and Wassarman 1980a, 1980b, 1983; Florman et al., 1984; Florman and Wassarman, 1985; Mortillo and Wassarman, 1991). Studies quantitatively determining the binding parameters of individual ZP glycoproteins are planned pending availability of cloned ZPs since such studies require great quantities of ZF glycoproteins and are not feasible with biochemically isolated material.

Kinetic studies to determine the forward and reverse rate constants for sperm-ZP binding suggest a complex interaction for this receptor-ligand pair. The slow association kinetics indicated by the $k_{on}$ of 3.2 × 10^3 M^{-1} s^{-1} could indicate a complex mechanism requiring the interaction of multiple ligands and/or receptor subunits, or changes in conformational state during binding, as has been suggested for other ligand-receptor interactions, such as transferrin (Ciechanover et al., 1983), insulin (Lipkin et al., 1986), and IgE (Pruzansky and Patterson, 1986), in which the forward rate constant is significantly below the diffusion limited rate for surface bound receptors and soluble ligands (Lauffenburger and Linderman, 1993). Dissociation experiments delineating the off rate constants demonstrate the presence of high and low affinity components of sperm-ZP binding, again pointing to the complexity of this interaction. Analysis of equilibrium saturation studies suggests a multisite mechanism for binding, and Scatchard and Hill analysis suggest possible cooperative interactions. It is not possible, however, to differentiate between true cooperativity (i.e. a change in affinity dependent upon the occupancy state of the receptor population) or apparent cooperativity (due to other complex mechanisms including multivalent receptors or multivalent ligands) from these data alone. However, other research has strongly indicated that ZP3 is a multivalent ligand, since proteolytically derived, monovalent glycopeptides of ZP3 bind to sperm but are not able to induce acrosomal exocytosis (Florman and Wassarman, 1985), but subsequent antibody cross-linking of ZP3 glycopeptides results in acrosomal exocytosis (Leyton and Saling, 1989a). Additionally, monovalent oligosaccharides do not inhibit sperm-ZP binding, but synthetic multivalent oligosaccharides show an enhanced ability to block sperm binding to intact ZPs as the number and length of branches is increased (Litscher et al., 1995). These data may suggest that the binding behavior observed here in both kinetic and steady state experiments is due to an apparent cooperativity resulting from the multivalent nature of the ligand for this system.

Evidence which may suggest the presence of multiple receptors in addition to a multivalent ligand comes from the observations that sperm-ZP adhesion and, consequently, fertilization...
sperm-ZP binding remain controversial. Some evidence suggests that the terminal monosaccharide directly responsible for adhesion is a α-galactose (Bleil and Wassarman, 1988; Shalgi et al., 1991; Litscher et al., 1995), but transgenic mice lacking all 1,3α-galactose epitopes were fertile (Thall et al., 1995), suggesting that α-galactose may not be solely responsible for the sperm-ZP interactions required for fertilization. Other evidence suggests that the terminal monosaccharide is a β-GlcNAc which acts as an acceptor for the sperm β-1,4-galactosyltransferase (Shur and Hall, 1982a, 1982b; Shur and Neely, 1988; mannosidase (Cornwall et al., 1991; Tulsiani et al., 1989), fucosyltransferase (Cardullo et al., 1989), and the tryptic inhibitor-sensitive site (Benau and Storey, 1987). While it is widely recognized that the binding activity of ZP3 resides within O-linked oligosaccharides of ZP3 (Bleil and Wassarman, 1980b; Florman and Wassarman, 1985; Litscher and Wassarman, 1993) and not with the highly conserved polypeptide chain (Chamberlin and Dean, 1990; Litscher and Wassarman, 1996), the specific saccharide residues responsible for sperm-ZP binding remain controversial. Some evidence suggests that the terminal monosaccharide directly responsible for adhesion is an α-galactose (Bleil and Wassarman, 1988; Shalgi et al., 1991; Litscher et al., 1995), but transgenic mice lacking all 1,3α-galactose epitopes were fertile (Thall et al., 1995), suggesting that α-galactose may not be solely responsible for the sperm-ZP interactions required for fertilization. Other evidence suggests that the terminal monosaccharide is a β-GlcNAc which acts as an acceptor for the sperm β-1,4-galactosyltransferase (Shur and Hall, 1982; Shur and Neely, 1988; Miller et al., 1992), but synthetic oligosaccharides containing GlcNAc in a β-linkage at the nonreducing terminus had no effect on sperm-ZP binding (Litscher et al., 1995). Despite the obvious contradictions among these studies, it is clear that none of these oligosaccharide blockers effectively inhibit sperm binding to intact ZPs, as the IC50 values reported are in the micromolar to millimolar range. One hypothesis arising from such studies is that multiple adhesion molecules are involved in sperm-ZP binding, and the high IC50 values reflect a number of low affinity interactions. The complex nature of the sperm-ZP binding isotherm presented here may thus reflect the involvement of one or more of these receptors leading to the formation of a fertilization complex that is required for adhesion and/or acrosomal exocytosis.

Alternatively, the high IC50 values derived from these assays may be due to limitations intrinsic to quantifying a competitive interaction between a ligand embedded within a matrix (the intact ZP) and a soluble competitor. Further, live, acrosome-intact, sperm and zonae are never in equilibrium above a critical ZP3 concentration because acrosome reactions are initiated rapidly and determining an IC50 or Kf for agents interfering with binding is therefore impossible. Consequently, an assay using fixed sperm, or some method which prevents the acrosome reaction but not ZP binding in live sperm, is absolutely required to identify and characterize putative receptors. The Kf determined for unlabeled ZP displacement of 125I-ZPs is in close agreement with the EC50 from saturation binding studies and this suggests that the soluble binding assay is also a suitable approach to determine the relative affinities of ZP3 and its putative competitors for binding sites on the sperm surface. The conditions and binding assay presented in this study should provide guidelines for testing models of sperm-ZP binding in a quantitative fashion.

While the results presented here are essential for characterizing sperm-zona interactions at a molecular level, they may only partially address the interactions that occur on the intact zona pellucida. Theoretical arguments based on biophysical measurements of sperm adhesion have suggested that only a few sperm-zona bonds are needed to tether a sperm to the egg (Baltz et al., 1988). The rate-limiting step for sperm adhesion on the intact zona pellucida is related to the surface density of ZP3 on the intact zona, the concentration of complementary ZP3 receptors on the sperm surface, the contact area between sperm and zona and the diffusion coefficient of the membrane bound receptor on the sperm surface (Baltz and Cardullo, 1989). The ZP3 density has been calculated to be as high as 300 molecules/μm2 and the contact area has been calculated to be from 0.1 to 5 μm2 (Baltz and Cardullo, 1989). However, no number has been determined for either the ZP3 receptor density on the sperm surface or its diffusion coefficient. The data presented here demonstrate that the total number of ZP3 binding sites is approximately 30,000 per sperm, and we are now using a recently developed fluorescent conjugate of ZP3 (Chen and Cardullo, 1994) to determine both the density of ZP3 binding sites on the sperm surface along with the mobility of these molecules in order to more precisely examine the initial interactions between sperm and egg. At present, the high concentration of ZP3 presented by the intact ZP combined with the relatively high number of ZP3 binding sites on sperm predicts that adhesion between sperm and egg is inevitable once physical contact is made. In concert with receptor localization, continued studies in our laboratory are underway to evaluate the mechanism of sperm-ZP interactions and to identify the mouse sperm receptor for ZP3 using the soluble binding assays described here.

In summary, the binding data presented here show, by several analyses, that the nature of sperm binding to the zona pellucida is complex, resulting in the apparent cooperativity observed in sperm-ZP binding. The complex nature of the sperm-ZP binding isotherm may thus reflect the interaction of multiple sperm proteins with a multivalent ZP3, leading to the formation of a fertilization complex that is required for adhesion and/or acrosomal exocytosis.

Acknowledgment—We thank Dr. David Johnson for advice and helpful discussions regarding experimental design.

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J. Biol. Chem. 1996, 271:23289-23297.
doi: 10.1074/jbc.271.38.23289

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