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Assembly of the fluorescent acrosomal matrix and its fate in fertilization in the water strider, *Aquarius remigis*

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Abstract

Animal sperm show remarkable diversity in both morphology and molecular composition. Here we provide the first report of intense intrinsic fluorescence in an animal sperm. The sperm from a semi-aquatic insect, the water strider, *Aquarius remigis*, contains an intrinsically fluorescent molecule with properties consistent with those of Flavin Adenine Dinucleotide (FAD) which appears first in the acrosomal vesicle of round spermatids and persists in the acrosome throughout spermiogenesis. Fluorescence recovery after photobleaching reveals that the fluorescent molecule exhibits unrestricted mobility in the acrosomal vesicle of round spermatids, but is completely immobile in the acrosome of mature sperm. Fluorescence polarization microscopy shows a net alignment of the fluorescent molecules in the acrosome of the mature sperm but not in the acrosomal vesicle of round spermatids. These results suggest that acrosomal molecules are rearranged in the elongating acrosome and FAD is incorporated into the acrosomal matrix during its formation. Further, we followed the fate of the acrosomal matrix in fertilization utilizing the intrinsic fluorescence. The fluorescent acrosomal matrix was observed inside the fertilized egg and remained structurally intact even after gastrulation started. This observation suggests that FAD is not released from the acrosomal matrix during the fertilization process or early development and supports an idea that FAD is involved in the formation of the acrosomal matrix. The intrinsic fluorescence of the *A. remigis* acrosome will be a useful marker for following spermatogenesis and fertilization.

Keywords

Insect sperm; Acrosomal matrix; Intrinsic fluorescence; Flavin adenine dinucleotide

INTRODUCTION

Most animal sperm are highly differentiated cells that possess a flagellated motility apparatus (the tail) that is separated from a head region containing a haploid nucleus and a specialized organelle known as the acrosome. In the most studied systems, the acrosome is known as a secretory vesicle that undergoes a requisite exocytotic event (the acrosome reaction) prior to sperm-egg fusion. During the acrosome reaction, the acrosome releases

hydrolytic enzymes and other components that facilitate sperm binding and penetration through the outer vestments of the egg (Yanagimachi, 1994; Neill and Vacquier, 2004).

In contrast to well-studied systems such as mice and sea urchins, little is known about the role of acrosomes in insect sperm. Insect eggs are encased in a shell that permits sperm entry only through a specialized structure known as the micropyle (Margaritis, 1985, Triplehorn and Johnson, 2005). It is unclear whether the acrosome plays a role in enabling sperm passage through the micropyle. However, Wilson et al. (2006) suggested a different function for the acrosome in the fruit fly, *Drosophila melanogaster* (Order Diptera). Rather than undergoing an exocytotic event, the acrosome in these sperm remains intact after entry through the micropyle and may facilitate sperm plasma membrane breakdown inside the egg. The acrosome is released into the egg cytoplasm after this event and persists in the cytoplasm at least as late as prometaphase of the first embryonic cycle (Wilson et al., 2006). Although the function of the released acrosome in the egg cytoplasm is still unknown, this study suggests that the functions of the acrosome are not the same across all species.

Sperm from the semi-aquatic insect, the water strider, *Aquarius remigis* (Hemiptera; Gerridae) have a markedly different morphology than most sperm. These giant sperm (≈ 5 mm long) (Pollister, 1930) are substantially longer than the eggs they fertilize (egg lengths range from ≈ 1.3 to 1.5 mm, and widths from ≈ 0.5 to 0.6 mm; Fairbairn unpublished data). Unlike most sperm, including those of *D. melanogaster*, where the length of the tail far exceeds the head length, *A. remigis* sperm heads and tails are nearly equal in length. A $5\text{ }\mu\text{m}$ -long nucleus is located proximal to the tail and the remainder of the head, which is more than $2500\text{ }\mu\text{m}$ in length, contains an acrosome (Tandler and Moriber, 1966). This unusually long structure of the *A. remigis* acrosome would be a good system to study its structure and function both microscopically and biochemically. A previous ultrastructural study using transmission electron microscopy revealed that the acrosome is filled with a tubular structure that runs parallel to the long axis of the acrosome and is likely responsible for its rigidity (Tandler and Moriber, 1966). However, the process and the molecular components that lead to the assembly of this long tubular acrosomal matrix are largely unknown. Further, the role of this unusually long acrosome in the fertilization process is enigmatic, particularly as its length exceeds that of the egg by at least $1000\text{ }\mu\text{m}$.

In the present study, we report that the acrosome of *A. remigis* sperm possesses an intrinsically fluorescent molecule with its properties consistent with those of Flavin Adenine Dinucleotide (FAD). Fluorescence recovery after photobleaching (FRAP) showed that the fluorescent molecule became immobilized during spermiogenesis. We also demonstrate that the fluorescent molecule exhibits a static, partial alignment in the acrosome of mature sperm using fluorescence polarization microscopy (FPM). These results suggest that FAD assembles into the acrosomal matrix during spermiogenesis in a fashion that results in a net alignment of FAD. The function of FAD in the acrosomal matrix will be discussed. Further, the intrinsic fluorescence of the *A. remigis* acrosome would serve as a useful biomarker for elucidating reproductive strategies in water striders. Utilizing this intrinsic fluorescence we followed the fate of the acrosomal matrix during the fertilization process and early development.

MATERIALS AND METHODS

Sample preparations

A. remigis is a common and abundant semi-aquatic water strider found throughout North America (Preziosi and Fairbairn, 1992). Sexually mature *A. remigis* males were obtained from a laboratory culture where they had been mating ad libitum. Females mate with many males and are able to store sperm for at least 3 weeks in the spermathecal tubes (Rubenstein,

1989, Campbell and Fairbairn, 2001). Males were anaesthetized with chloroform and testes and seminal vesicles were dissected under a dissecting microscope and transferred into phosphate-buffered saline (PBS: 7.7 mM Na₂HPO₄, 2.7 mM NaH₂PO₄ and 150 mM NaCl, pH 7.2). Mature sperm were obtained by teasing the seminal vesicles in PBS. To observe sperm bundles and spermatids, the testes were transferred to a drop of PBS on a glass slide and squashed with a cover slip. Laid eggs were collected from a culture tank.

Microscopy and image processing

Cells were observed under differential interference contrast (DIC) or epifluorescence optics using an Olympus BX-71 microscope or a Zeiss Axiovert 10 microscope. A GFP filter set (model 41012, Chroma Technology) was used for intrinsic fluorescence. Three-dimensional images were generated using a deconvolution microscope system (DeltaVision, Applied Precision) that consisted of an Olympus IX-71 microscope with a 250 W Xenon lamp and a GFP filter set. To determine an intrinsic fluorescence emission spectrum, a Leica TCS-SP2 confocal microscope was used in lambda scan mode with an excitation wavelength of 351 nm or 458 nm and a detection bandwidth of 10 nm. DIC and epifluorescence micrographs were processed for contrast enhancement using Image J (National Institutes of Health) and Adobe Photoshop 6.0.

Fluorescence Recovery After Photobleaching (FRAP)

FRAP analysis was performed on a Leica-SP2 confocal microscope equipped with a 63x/1.20 water immersion objective lens. Photobleaching of the intrinsic fluorescence was carried out using the 351 nm, 364 nm, 442 nm, 458 nm, 476 nm and 488 nm laser lines at full power for 20 iterations. Images were obtained with an excitation wavelength of 476 nm at 10% laser power, a detection bandwidth of 500 nm to 550 nm and a temporal resolution between frames of 823 msec. The collected images were analyzed with Leica Confocal Software (LCS, 2.61.1537) in combination with Microsoft Excel 2004 with double normalization performed as described by Phair et al. (2004). For the normalization of mature sperm bundles, the acrosome region in the image was used instead of the entire acrosome. The mobile fraction M_f was calculated using the equation: $M_f = (F_{\infty} - F_0)/(F_i - F_0)$ where F_{∞} is the fluorescence in the bleached region after full recovery, F_i is the average fluorescence before bleaching and F_0 is the fluorescence immediately following the bleach. To obtain F_{∞} for round acrosomes, fluorescence recovery curves were fitted to a single exponential function using nonlinear regression (Prism 5, GraphPad).

Fluorescence Polarization Microscopy (FPM)

We analyzed the polarization of the acrosomal fluorescence using a liquid crystal universal compensator that is usually employed to measure the birefringent fine structure in living cells and other transparent objects (LC-PolScope, CRi, Woburn MA, [Oldenbourg and Mei, 1995; Shribak and Oldenbourg, 2003]). For measuring the fluorescence anisotropy, the LC compensator was placed into the analyzer slot of a Zeiss Axiovert 200M microscope stand and operated as a fast switching polarization analyzer. Using epi-illumination, acrosomes were illuminated by unpolarized light (450 nm – 490 nm) and the polarization of the emitted fluorescence (510 nm – 550 nm) was analyzed sequentially using four LC compensator settings representing linear polarization states with azimuthal angles of 0°, 45°, 90° and 135°. In addition, a fifth LC compensator setting returned to the angle of 0° for assessing the effect of photobleaching. For each setting a fluorescence image was automatically recorded (exposure time 1 sec) in quick succession (total recording time 5.1 sec) using custom software. The first four intensity images were used to calculate the fluorescence anisotropy in each pixel after correcting for photobleaching based on the intensity reduction between the first and the fifth image. The fluorescence anisotropy coefficient was calculated using image arithmetic and the following formula: Anisotropy Coefficient (AC) = $((I_0 - I_{90})^2 +$

$(I_{45} - I_{135})^2)^{1/2}/(I_0 + I_{90} + I_{45} + I_{135})$. The anisotropy coefficient is related to the polarization ratio $= I_{\max}/I_{\min}$, in which $I_{\max}$ is the maximum fluorescence intensity observed for a specific azimuth angle $\Theta_{\max}$. The minimum intensity $I_{\min}$ is necessarily observed for an azimuth of $\Theta_{\max} + 90^\circ$. The following relationship holds: polarization ratio $= I_{\max}/I_{\min} = (1+2\cdot AC)/(1-2\cdot AC)$. The polarization ratio is 1 for randomly oriented fluorophores and increases with increasing mutual alignment. The azimuth angle associated with the fluorescence maximum is computed using: $\Theta_{\max} = \text{atan2}((I_0 - I_{90}), (I_{45} - I_{135}))/2$. The $\text{atan2}(x, y)$ function is the same as $\arctan(y/x)$, except that the signs of the numerator and denominator are used to determine the quadrant of the angle. More details on the fluorescence polarization measurements will be presented in a forthcoming publication.

Tricine-SDS-PAGE and fluorescence spectrophotometry

Tricine-SDS-PAGE was performed as described by Schagger and von Jagow (1987) using 16.5 % polyacrylamide gel. Fluorescent signals on the gel were detected with ImageMaster VDS-CL (Amersham Bioscience) using a 321-nm ultraviolet transilluminator and a 520-nm bandpass filter (full width at half maximum: 50 nm). Excitation and emission spectra of a liquid sample were obtained using a Hitachi F-2000 fluorescence spectrophotometer.

RESULTS

Morphology of mature *A. remigis* sperm

A schematic drawing of *A. remigis* sperm is shown in Figure 1A based on our observations using both DIC and epifluorescence optics. As reported previously (Pollister, 1930), these sperm are extremely long cells with a well-defined head, nucleus, and tail that were readily observed in DIC. A periodic, wavelike, structure was found proximal to the nucleus that extends for approximately 300 μm and then tapers to a thinner, linear structure for the remainder of the sperm head (Fig. 1A). Using a GFP filter set we observed an intense fluorescent signal that was restricted to the periodic and linear regions of the sperm head anterior to the nucleus with no signal observed in the tail (Fig. 1B). When filter sets for DAPI (model 31000, Chroma Technology) or TRITC (model 31002, Chroma Technology) were used, no fluorescent signals were detected in the periodic and linear regions of the sperm head suggesting that the intrinsic fluorescence has a defined excitation and emission spectrum.

The intrinsic fluorescent signal enabled us to investigate the three-dimensional structure of the head. To create a three-dimensional image, sperm were observed using a deconvolution microscope system with a GFP filter set. The periodic wavelike fluorescent structure was detected in both the X-Z projected plane and the Y-Z projected plane, indicating that this region of the head is helical (Fig. 2A and 2B). This helical structure was confirmed when sperm were observed in Z-sections down the shaft (Fig. 2C). The fluorescent signal rotated around a central axis with a pitch of approximately 7 μm in the Z-axis, consistent with the images in the X-Z and Y-Z projected planes.

The intrinsically fluorescent signal in *A. remigis* sperm first appears during spermatogenesis in the developing acrosome

Localization of the intrinsic fluorescence anterior to the nucleus in *A. remigis* sperm suggested that the fluorescence is in the acrosome. To see when the fluorescent signal in *A. remigis* sperm first appears, and to see if its appearance coincides with the formation of the acrosome, we used cells from the testis and observed cells in various stages of spermatogenesis using both DIC and epifluorescence optics. As with other spermatogenic cells, the acrosome of *A. remigis* sperm first appears following meiosis in round spermatids. In *A. remigis* spermatids, the nascent acrosome appears as a spherical organelle that consists

of two distinct regions (Fig. 3A). This observation is consistent with a previous ultrastructural study that identified two distinct acrosomal regions with different electron densities: a “core” that is proximal to the nucleus and is less electron-dense than a more distal region described as the “sheath” (Tandler and Moriber, 1966). When we observed the intrinsic fluorescence that was restricted to the acrosome of spermatids, the fluorescence intensity in the core was higher than the sheath (Fig. 3A).

The fluorescent signal was also detected in the developing acrosome of condensing spermatids (Fig. 3B). In this stage, the acrosome begins to elongate and is bounded by two basal bodies that surround an acrosome that is proximal to the nucleus (Fig. 3B) (Pollister, 1930; Tandler and Moriber, 1966). The intrinsic fluorescent signal, however, was not observed in these basal bodies and was only detected in the acrosome (Fig. 3B).

Mature sperm in the testis were observed in bundles with their long heads and tails precisely aligned (Fig. 3C). Consistent with mature sperm from seminal vesicles, the fluorescent signal in testicular sperm was restricted to the linear and helical regions of the head (Fig. 3C). Taken together these results suggest that the fluorescent molecule is restricted to the acrosome of *A. remigis* sperm.

The fluorescent molecule becomes immobilized during spermiogenesis

Since the acrosome of *A. remigis* is filled with a tubular acrosomal matrix (Tandler and Moriber, 1966), we hypothesized that the fluorescent molecule may be associated with this structure. This is supported by the fact that we detected no fluorescent signals in the basal bodies of the condensing spermatid (Fig. 3B) where the tubular structure is absent (Tandler and Moriber, 1966). In order to test this hypothesis, we determined the mobile fraction (M_f) of the fluorescent molecule using Fluorescence Recovery After Photobleaching (FRAP) in sperm bundles. If the fluorescent molecule is tightly associated with the acrosomal matrix, we argued that the fluorescence intensity should not recover and the mobile fraction should be low. Indeed, the mobile fraction of the fluorescent molecule associated with the acrosomes in mature sperm bundles was low ($M_f = 7.23 \pm 2.07\%$, $n = 5$, Fig. 4A). In contrast, the fluorescent molecule was found to freely move in both sheath and core regions of round spermatids (Sheath; $M_f = 96.84 \pm 5.99\%$, $n = 9$, Fig. 4A, Core; $M_f = 96.53 \pm 6.78\%$, $n = 8$). Since the tubular acrosomal matrix first appears when the round acrosome begins to elongate (Tandler and Moriber, 1966), these results suggest a model in which the fluorescent molecule is incorporated into the acrosomal matrix during its formation.

To test this model further, we isolated the acrosomal matrix as a heat/detergent resistant fraction by boiling the mature sperm in PBS containing 2% SDS. There were no tails and nuclei observed after this treatment under DIC optics. However, the acrosomal matrix was insoluble under this harsh condition and retained an intense intrinsic fluorescence (Fig. 4B). In contrast, when 100 mM DTT or 1% β -mercaptoethanol was added under this condition, the acrosomal matrix dissolved completely. This result supports a model in which the acrosomal matrix includes the fluorescent moiety and suggests that the matrix is stabilized by disulfide bonds.

The fluorescent molecules are aligned in the acrosome of mature sperm

In order to investigate further the assembly of the fluorescent molecule we observed both the round acrosomes of spermatids and the extended acrosome of mature sperm using Fluorescence Polarization Microscopy (FPM) that can analyze the orientation distribution of fluorophores (Axelrod, 1979; Inoue et al., 2002; Vrabioiu and Mitchinson, 2006). We applied a liquid crystal compensator for rapidly and sensitively measuring the static fluorescence anisotropy in every resolved image point. The LC-compensator was used as a

tunable linear polarization analyzer to determine the azimuth angle and the ratio of highest to lowest polarized fluorescence (see Materials and Methods). The fluorescence signal throughout the acrosomes of mature sperm exhibited a high polarization ratio of 3.6 with an azimuth angle nearly perpendicular to the long axis of the acrosome (i.e. the maximum intensity was observed when the linear analyzer was oriented nearly perpendicular to the extended acrosome, Fig. 5B and 5C). Our measurements gave nearly identical results using live sperm ($I_{\max}/I_{\min} = 3.64 \pm 0.43$, mean $\pm$ SD, $n = 7$) and sperm that were fixed with 4% formaldehyde in PBS for 10 minutes ($I_{\max}/I_{\min} = 3.63 \pm 0.57$, mean $\pm$ SD, $n = 8$). In contrast, the fluorescence signal of round acrosomes of either live or fixed spermatids exhibited no anisotropy. These results indicate that the fluorescent molecules are incorporated into the acrosomal matrix during spermiogenesis in a fashion that results in a net alignment of the emission transition dipole moment of the fluorescent molecules.

Properties of the fluorescent molecule are similar to those of Flavin Adenine Dinucleotide (FAD)

The FRAP and FPM analyses suggest that the fluorescent molecule is incorporated into a rigid acrosomal structure. In order to determine the fluorescence characteristics of the matrix, we obtained the emission spectrum of the fluorescent signal from *A. remigis* sperm *in vivo* using a confocal microscope. Sperm bundles from the testes were used instead of a single sperm in order to maximize the overall fluorescent signal. A maximum emission wavelength of 515 nm was detected using an excitation wavelength of 351 nm (Fig. 6A). Although keratin, collagen, and elastin have all been reported to form tubular matrices in cells or connective tissues that display intrinsic fluorescence (Fujimoto, 1977; Wu et al., 2004; Pena et al., 2005; Wu and Qu, 2006), the emission spectrum of these molecules is not similar to that of the fluorescence in *A. remigis* sperm. Rather, the spectrum of *A. remigis* sperm is similar to that of flavin or a flavin-containing molecule (Weber, 1950; Kleiner et al., 1999). The flavin molecule has two absorption maxima at 375 nm and 455 nm (Weber, 1950). When an excitation wavelength of 458 nm was used instead of 351 nm, a similar spectrum was obtained. To test if the emission spectrum is similar to that of flavin or a flavin-containing molecule, we determined the emission spectrum of flavin adenine dinucleotide (FAD), a common flavin-containing molecule, using an excitation wavelength of 351 nm. As reported previously, a maximum emission wavelength of about 530 nm was detected for FAD (Fig. 6A). This emission spectrum is similar to that of the fluorescent molecule in the matrix although the spectrum of FAD is red-shifted about 15 nm. This difference could be due to differences in microenvironments of flavin in the matrix.

In cells, flavin is a component of FAD, riboflavin and flavin mononucleotide (FMN). To further investigate the fluorescent molecule in the matrix, the acrosomal matrix that was isolated from the *A. remigis* sperm as a SDS-insoluble fraction was subjected to tricine-SDS-PAGE and fluorescent signals were examined on the gel. The mass-to-charge ratio of the fluorescent molecule from the matrix was similar to that of FAD but not riboflavin or FMN (Fig. 6B). After the gel was stained with coomassie blue, there was a major protein band with a molecular weight of approximately 75 kDa, but there was no protein band observed in the same region as the fluorescent signal (Fig. 6B).

We also investigated the excitation and emission spectra of the isolated acrosomal matrix that was solubilized by the addition of β -mercaptoethanol. Consistent with the excitation spectrum *in vivo* (Fig. 6A), a maximum emission wavelength of 520 nm was detected using an excitation wavelength of 370 nm (Fig. 6C). There were 2 excitation peaks detected at 370 nm and 440 nm (Fig. 6D). These emission and excitation spectra were matched with those of FAD. Further, the fluorescent intensity of FAD but not riboflavin or FMN increases dramatically when pH is low (Bessey et al., 1949). When the spectra were investigated at pH 7.1, the fluorescence intensities decreased about 10-fold as compared to those of pH 2.5.

Taken together, these results strongly suggest that the fluorescent molecule in the acrosomal matrix is FAD.

The fate of FAD that is associated with the acrosomal matrix in the fertilization process and early development

In well-studied systems such as mice and sea urchins, the acrosomal contents are released prior to sperm-egg fusion and facilitate sperm binding and penetration through the outer vestments of the egg (Yanagimachi, 1994; Neill and Vacquier, 2004). In order to examine if it is also the case for FAD from the *A. remigis* sperm acrosome, we followed its fate during the fertilization process and early development utilizing its intrinsic fluorescence. Both helical and thinner linear regions of the fluorescent matrix were observed inside the fertilized egg (Fig. 7A), indicating that the long acrosomal matrix enters the egg during the fertilization process. The single fluorescent acrosomal matrix was often found near the micropyle of the fertilized egg. Further, the fluorescent matrix remained structurally intact even gastrulation started (Fig. 7B). The fertilized egg that contained the acrosomal matrix inside developed normally into a nymph. These results suggest that FAD is not released from the acrosomal matrix during the fertilization process or early development and is likely an important component of the acrosomal matrix.

DISCUSSION

Our characterization of the *A. remigis* acrosome reveals a unique fluorescent molecule that becomes immobilized and oriented during spermiogenesis. To our knowledge, a bright fluorescence in the acrosome has not been previously reported in any other animal species. Acrosomes that are filled with tubular structures are also found in other Heteropteran species including *Corixa sp.*, *Hydrometra sp.*, and *Notonecta glauca* although their lengths vary substantially (Afzelius et al., 1976; Dallai and Afzelius, 1980). Comparison among the sperm of these species may shed light on the evolution of the intrinsic fluorescence in the acrosome.

The apparent continuous filamentous structure of *A. remigis* sperm previously made it difficult to distinguish head and tail regions using brightfield microscopic methods. However, previous ultrastructural work using transmission electron microscopy revealed that the tail contains both two mitochondrial derivatives and a “9+9+2” microtubule-containing axoneme (Tandler and Moriber, 1966), which are commonly observed in insect sperm (Jamieson et al., 1999) and a head that contains an unusually long acrosome. Using the intrinsic fluorescence of the acrosome we confirmed that the acrosome is unusually long, is formed subsequent to meiosis, and is derived from a spherical organelle adjacent to the nucleus of spermatids.

Our results of FRAP and FPM suggest that the fluorescent molecule is incorporated into a tubular acrosomal matrix, and thus may play functional role to form the tubular structure in the acrosome. The phenomenon of cellular intrinsic fluorescence has been reported previously in other cell types (Aubin, 1979; Benson et al., 1979; Anderson et al., 1998) with three major sources of intrinsic fluorescence in animal cells routinely identified: NADH, lipofuscin, and flavin. These are obvious candidates for the intrinsic fluorescence in the acrosome of *A. remigis*. Although the emission spectrum can be influenced by intracellular and environmental conditions to some degree, only flavin appears to be a viable candidate. The intrinsic fluorescence in *A. remigis* acrosome has an emission peak at 515 nm *in vivo*, while NADH has an emission peak at approximately 440 nm (Avi-Dor et al., 1962). Lipofuscin, a pigment that is composed of a complex mixture of lipids, metals, and organic molecules that result from oxidative degradation in lysosomes or mitochondria, has a broad emission spectrum with a peak at approximately 630 nm (Delori et al., 1995; Brunk and

Terman, 2002; Haralampus-Grynaviski et al., 2003). The most likely candidate here is flavin with an emission peak of approximately 520 nm that is excited by both ultraviolet and blue light (Weber, 1950; Kleiner et al., 1999). This idea is further supported by the results of tricine-SDS-PAGE and *in vitro* fluorescence spectrophotometry. These results also suggest that the flavin-containing molecule is FAD but not riboflavin or FMN.

FAD exists in cells in isolation or as a prosthetic group of flavoproteins that are capable of catalyzing various oxidation-reduction reactions (Massey, 2000; Joosten and Berkel, 2007). Tricine-SDS-PAGE of the acrosomal matrix indicates that there are several proteins that form the acrosomal matrix. It is likely that FAD is associated with one of these proteins. If the fluorescence in the acrosome is due to FAD, it is unclear why the acrosome possesses a significant amount of FAD that is associated with the acrosomal matrix, which renders the matrix strongly fluorescent. Considering the redox ability of FAD, it is possible that this molecule may facilitate the formation of the acrosomal matrix through oxidation-reduction reactions. Since the acrosomal matrix is stabilized by disulfide bonds, there should be a molecule that accepts electrons caused by the disulfide bond formation in the acrosome. In other organelles such as endoplasmic reticulum and mitochondria, FAD is known to accept electrons caused by the disulfide bond formation (Riemer et al., 2009).

Acrosomal proteins of mammalian species are also stabilized by disulfide bonds (Mate et al., 1994; Westbrook-Case et al., 1995; Kim et al., 2001). Further, protein disulfide isomerase, which catalyzes the rearrangement of disulfide bonds, is expressed in the developing acrosome of rats (Ohtani et al., 1993). It is interesting to investigate if the acrosome of these species also contains FAD. Although it is not reported that the acrosome of these species exhibits a flavin-like intense intrinsic fluorescence, they may contain less amount of FAD than the *A. remigis* acrosome considering its much smaller size.

The functions of FAD in the acrosomal matrix remains to be determined, but this intrinsic fluorescence can be used as a marker for the *A. remigis* acrosome to follow its fate subsequent to fertilization. Using this intrinsic fluorescence, we observed that the unusually long acrosomal matrix remained associated with the sperm during the fertilization process. This observation suggests that this long acrosome may play a structural role during the passage of the sperm through the female reproductive tract. A part of the *A. remigis* female reproductive tract is narrow ($\approx 12 \mu\text{m}$ in diameter) and tightly wound (Campbell and Fairbairn, 2001). It seems difficult for the long, relatively rigid acrosome to pass through this tightly winding region of the female reproductive tract. This structure may be stretched and straightened by females to ensure that the sperm with the long acrosomal matrix can move thorough this region. Our observation also suggests that it is unlikely that the long acrosomal matrix contributes to physiological processes within the fertilized egg during the early development because the acrosomal matrix remained structurally intact even after gastrulation started. One explanation for sperm gigantism is that sperm bring a large amount of molecules that facilitate embryonic development (Simmons, 2001). In the case of the *A. remigis* long acrosome, this idea may not be the case although the long acrosomal matrix enters the egg in fertilization.

In summary, our study describes a novel phenomenon in which the *A. remigis* acrosome possesses an intrinsically fluorescent molecule with properties similar to those of FAD that becomes immobilized and aligned during the acrosome formation. Further, utilizing the intrinsic fluorescence, we observed that the fluorescent acrosomal matrix remained structurally intact in the embryo during the early development. Further detailed investigations on the fluorescent molecule of the *A. remigis* sperm will provide an insight into the mechanisms of the acrosomal matrix formation.

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Fig. 1. Morphology of mature *A. remigis* sperm. (A) A schematic drawing showing the major features of water strider sperm. The sperm contains a 2500 μm linear tail structure and a complex head region separated by a 5 μm nucleus. The head region consists of a 300 μm periodic structure followed by a 2200 μm thinner, linear apical region. Both of these structures in the head region contain a fluorescent molecule. (B) Panels on the left are DIC images with corresponding fluorescence images in the right panels using a GFP filter set. Top. The region of the head proximal to the nucleus contains a regularly periodic fluorescent structure that extends for 300 μm . Bottom. The head region consists of both the 300 μm periodic structure and a thinner linear region. The contrast of the fluorescence image is enhanced to show the presence of fluorescence in the thinner linear region. h_p , periodic region of the head; h_l , linear region of the head; n , nucleus; t , tail. Scale bars = 10 μm .

Fig. 2.

The periodic structure of the water strider sperm head is a helix. (A) A schematic drawing showing the water strider acrosome. The periodic structure is helical with a pitch of approximately 7 μm. (B) Images of the periodic structure from a sperm head in the X-Z projected plane (left panel) and Y-Z projected plane (right panel). Scale bar = 5 μm. (C) Z-sections down the shaft of the periodic structure reveal that it rotates about a central axis to form a helix. The panels from left to right are successive images moving toward the nucleus. Scale bar = 1 μm.

Fig. 3.

The fluorescent signal in the sperm head appears during spermatogenesis and is localized to the acrosome in spermatids. Panels on the left are DIC images with corresponding fluorescence images (a GFP filter set) on the right. (A) A mid-stage spermatid shows that the fluorescent signal originates in the spherical acrosome. (B) A condensing spermatid with associated fluorescence in the elongating acrosome. (C) Top. The periodic structure is bundled together with adjacent sperm when they are stored in the testis. Bottom. Bundled sperm showing both the periodic and linear regions of the sperm head. a_c , core region of the acrosomal vesicle; a_s , sheath region of the acrosomal vesicle; ba , basal body of the acrosome; h_p , periodic region of the head; h_l , linear region of the head; n , nucleus; t , tail. Scale bars = 10 μ m.

Fig. 4.

The fluorescent molecule becomes immobilized during spermiogenesis. (A) FRAP analysis was performed on the acrosome of a round spermatid (RS) and a mature sperm bundle (MS). Panels on the left are images before photobleaching with corresponding images just after photobleaching in the middle and plots of fluorescence recovery on the right. Red rectangles in the pre-bleach images show the areas of photobleaching. Scale bars = 10 μm. (B) An isolated acrosomal matrix still exhibits a bright fluorescence. A panel on the left is a DIC image with a corresponding fluorescence image (a GFP filter set) on the right. Scale bar = 5 μm.

Fig. 5.

The intrinsic fluorescence of mature sperm exhibits strong anisotropy. Fluorescence polarization microscopy was performed on the acrosome of fixed mature sperm. (A) A DIC image of the acrosome. Scale bar = 5 μm . (B) An image showing fluorescence polarization ratios for each pixel as indicated in the grayscale bar. High polarization ratios were observed in the acrosome. A white square shows the area magnified for C. (C) A magnified image showing azimuth angles with red lines. The azimuth angle is nearly perpendicular to the long axis of the acrosome.

Fig. 6.

Properties of the fluorescent molecule in the matrix is similar to those of FAD. (A) A sperm bundle from the testis (white circle) and 1 mM FAD (black circle) were imaged with a confocal microscope using an excitation wavelength of 351 nm. An emission spectrum was collected from 410 nm to 650 nm with a maximum wavelength of 515 nm for the fluorescent signal of the acrosome and 530 nm for FAD. (B) An isolated acrosomal matrix, riboflavin, FMN and FAD were subjected to tricine-SDS-PAGE and the fluorescent signals were detected. The gel was also stained for proteins. Mr, molecular weight. (C, D) Emission (C) and Excitation (D) spectra of the acrosomal matrix that was solubilized by the addition of β -mercaptoethanol were determined at pH 2.5 (red line) and pH 7.1 (blue line) using a fluorescence spectrophotometer. The spectra were matched with those of FAD at pH 2.5 (green line).

Fig. 7.

The fate of the fluorescent molecule associated with the acrosomal matrix during the fertilization process and early development. Helical regions of the acrosome were observed in a fertilized egg (A) and an embryo that underwent gastrulation (B). Panels on the left are bright field images with fluorescence images (a GFP filter set) on the right. Black squares show the area magnified for the fluorescence images. An arrowhead indicates the region where invagination occurred. Scale bars for bright field images = 100 μm and fluorescence images = 10 μm .