A SEA URCHIN SPERM FLAGELLAR ADENYLATE KINASE WITH TRIPLED CATALYTIC DOMAINS*

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The mitochondrion of sea urchin sperm is located at the base of the sperm head and the flagellum extends from the mitochondrion for ~40 μm. These sperm have two known flagellar, non-mitochondrial, enzymatic systems to rephosphorylate ADP. The first involves the phosphocreatine shuttle, where flagellar creatine kinase (Sp-CK) uses phosphocreatine to rephosphorylate ADP. The second system, studied in this paper, is adenylate kinase (Sp-AK) that uses 2 ADP to make ATP + AMP. Cloning of Sp-AK shows that, like Sp-CK, Sp-AK has three catalytic domains. Sp-AK localizes along the entire flagellum and most of it is tightly bound to the axoneme. Sp-AK activity and flagellar motility were studied using demembranated sperm. The specific Sp-AK inhibitor Ap5A, blocks enzyme activity with an IC50 of 0.41 μM. In 1 mM ADP, flagella reactivate motility in 5 min; 1 μM Ap5A completely inhibits this reactivation. No inhibition of motility occurs in Ap5A when 1 mM ATP is added to the reactivation buffer. The pH optimum for Sp-AK is 7.7, an internal pH at which sperm are fully motile. The pH optimum for Sp-CK is 6.7, an internal pH at which sperm are immotile. In isolated, detergent permeabilized, flagella, assayed at pH 7.6, the Km for Sp-AK is 0.32 mM and the Vmax is 2.80 μmole ATP formed per min per mg protein. When assayed at pH 7.6, the Sp-CK Km is 0.25 mM and the Vmax 5.25. At the measured in vivo concentrations of ADP of 114 μM, at pH 7.6, the axonemal Sp-AK could contribute approximately 31%, and Sp-CK 69%, of the total non-mitochondrial ATP synthesis associated with the demembranated axoneme. Thus, Sp-AK could contribute substantially to ATP synthesis utilized for motility. Alternatively, Sp-AK could function in the removal of ADP, which is a potent inhibitor of dynein ATPase.

Sea urchin sperm are excellent model cells for the continuing investigation of the molecular mechanism of flagellar motility (1,2). Their flagellum is approximately 0.1-0.2 μm wide and 40-50 μm long and consists of the 9+2 arrangement of microtubule doublets of the axonemal complex covered by a plasma membrane. Methods for reactivating bending waves in nonionic detergent-demembranated flagella are well established and the effects of ions, nucleotides and inhibitors on the frequency and shape of the flagellar beat can be faithfully replicated in the demembranated flagella (3). Completion of the genome of one sea urchin species, Strongylocentrotus purpuratus (Sp), allows a proteomics approach in the continuing discovery and functional analysis of axonemal proteins of which there are more than 200 (2,4).

Sperm motility is generated by flagellar bending waves, produced by microtubule sliding, the energy being supplied by the hydrolysis of ATP by axonemal dynein ATPase (2). The microtubule sliding velocity (5) and beat frequency are both dependent on Mg2+ ATP concentrations (6). It is critical for sperm to have efficient mechanisms to synthesize and deliver ATP down the entire flagellum. Studies of the maintenance of ATP concentrations in flagella are relevant to sperm physiology and also of general importance to all flagellated and ciliated eukaryotic cells.

The mitochondrion of sea urchin sperm is located at the base of the sperm head, 40-50 μm
from the distal tip of the flagellum. The mitochondrion is active in ATP synthesis, but how do concentrations of ATP remain high enough to support the dynein ATPase driven bending waves along the entire flagellum? Two enzymatic mechanisms have been established: the phosphocreatine shuttle, and, as detailed in this report, adenylate kinase (Sp-AK). The phosphocreatine shuttle (7,8) depends on an unusual 145 kDa creatine kinase (Sp-CK) found in the flagellar plasma membrane and also bound to the axoneme (9). The Sp-CK uses high levels of phosphocreatine to rephosphorylate ADP to form ATP and creatine. Inhibition of Sp-CK by FDNB results in flagellar beating waves traveling only one-third of the way down the flagellum, roughly the distance that ATP could diffuse from the mitochondrion before being hydrolyzed by dynein (10). Sp-CK is one of the major proteins of the flagellar plasma membrane; it exists in the plasma membrane in a myristoylated form, while a non-myristoylated form associates with the axoneme (11,12).

Adenylate kinase (AK; aka myokinase) is a ubiquitous enzyme that catalyzes the reaction: 2ADP $\leftrightarrow$ ATP + AMP. The reaction constant being $\sim1$ means that the reaction proceeds equally well in either direction and depends only on the balance among the three reactants (13). AK activity has been detected in flagella of *polytoma uvella* (14), bovine sperm (13), *Chlamydomonas* (15-18), and cilia of *Tetrahymena* (19) and *Trypanosoma brucei* (20,21). To date there is only one experimental study of sea urchin sperm flagellar AK (22).

A recent study identified several flagellar proteins that become phosphorylated by cAMP dependent protein kinase (PKA) when sea urchin sperm are treated with egg jelly. Tandem mass spectrometry identified one phosphoprotein as adenylate kinase (23). Here we present the primary structure of the sea urchin flagellar Sp-AK. Sp-AK could play roles in ATP regeneration for motility, creatine rephosphorylation, and in removing ADP, which is a potent inhibitor of dynein ATPase (6).

**Experimental Procedures**

**Gametes** - Sea urchin (*Strongylocentrotus purpuratus*) gametes were spawned by injection of 0.5 M KCl into adults. Sperm was collected as undiluted semen and stored in ice.

**Cloning** - The full-length sea urchin Sp-AK cDNA was cloned by PCR amplification using a *Strongylocentrotus purpuratus* testis cDNA library in Lambda Zap II (Stratagene) as template. Oligonucleotide primers were designed from exact match sequences taken from the Sea Urchin Genome Project (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/). The nucleotide sequences were found using the eight published peptide sequences of the ~130 kDa phosphorylated Sp-AK (23). PCR reactions, cloning of PCR products and DNA sequencing were performed using standard methods.

**Sequence and Structural Analysis** - Alignments of the Sp-AK were done with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Conserved domain structures were predicted using CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and InterProScan (http://www.ebi.ac.uk/InterProScan/). PKA phosphorylation sites were predicted using ScanSite (http://scansite.mit.edu/motifscan_seq.phtml).

Topology prediction was done using SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). Helical wheel diagrams and Kyte-Doolittle hydropathy plots were performed on BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

**Phylogenetic Analysis** - Amino acid sequences of only the Sp-AK catalytic domains (Cat) were used to construct a neighbor-joining phylogenetic tree (2,000 replications) using MEGA3.1 software. GenBank accession numbers for the AK sequences used are: *Homo sapiens* AK1 (Human AK1), NP_000467; Human AK2, AAH09405; Human AK3, BAA87913; Human AK4, P27144; Human AK5, Q9Y6K8; Human AK6 Cat 1 and 2, AAO16520; Human AK7, AAH35256; *Rattus norvegicus* AK1, AAH89979; *Xenopus tropicalis*, AAH76704; *Cyprinus carpio* (Carp), P12115; *Caenorhabditis elegans*, AAG50236; *Drosophila melanogaster*, AAV36938; *Chlamydomonas reinhardtii* Cat 1, 2, and 3, AAS10182; *Escherichia coli*, AAN79072; *Dictyostelium discoideum*, XP_638872; and *Trypanosoma brucei* ADKA, XP_951719, *Trypanosoma brucei* ADKB, XP_822341; *Trypanosoma brucei* ADKE, XP_825026. The GenBank accession number of Sp-AK is DQ447969.
Production of Recombinant Sp-AK Protein and Antibodies – N-terminal His-tags were attached to the full-length Sp-AK sequence and to the COOH-terminal sequence of residues 813-920 that were then expressed in E. coli Rosetta (DE3) cells (Novagen, Madison, WI) using the pET-15b vector (Novagen). E. coli were grown at 37°C in LB broth supplemented with 100 μg/ml ampicillin and 37 μg/ml chloramphenicol and expression induced with 0.5 mM IPTG. Cells were harvested in 3 h by centrifugation at 6,000 g for 10 min at 4°C and the pellets dissolved in 8 M urea, an equal volume of sample buffer added, and the tubes boiled 3 min (24). Expressed protein, representing the COOH-terminus of Sp-AK, was purified on Ni-NTA-agarose (Qiagen, Valencia, CA) and used for commercial polyclonal antibody production (Orbigen, San Diego, CA).

Immunoblotting - Isolation of sperm heads and flagella was done as described (25). Whole sperm, isolated heads and isolated flagella were dissolved in 10% SDS, an equal volume of 2X sample buffer added, and the tubes boiled 3 min. The recombinant full-length Sp-AK protein and sperm proteins were separated on 7.5% SDS/PAGE gels and the gels stained with Coomassie brilliant blue, or transferred to PVDF membranes by standard methods. The membranes were blocked with 1 mg/ml BSA in TBST (25 mM Tris, pH 8.0, 150 mM NaCl and 0.05% v/v Tween 20) for 1 h. The blots were then incubated for 1 h at room temperature, or over night at 4°C, with anti-Sp-AK antisera diluted in blocker. After washing in TBST, rabbit antibodies were detected with an HRP-conjugated goat anti-rabbit secondary antibody. After washing, blots were developed with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) following the manufacturer’s protocol.

Immunofluorescence - Sperm were electrostatically bound to protamine sulfate-coated coverslips and fixed in 5% paraformaldehyde/0.5% glutaraldehyde in seawater. After washing in TBST, coverslips were blocked in TBST containing 5% normal goat serum and 5 mg/ml BSA. To permeabilize the cells, the TBST contained 0.1% Tween-20. Coverslips were then incubated in various dilutions of anti-Sp-AK antisera or preimmune sera diluted in blocker. After five washes, coverslips were incubated 1 h in a 1:400 dilution of Alexa-Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), washed twice in TBST and mounted in 75% glycerol.

Sp-AK and Sp-CK Activity Assays - Assays of Sp-AK enzymatic activity were performed as follows: the reaction was started by adding 6 µl of the pooled sucrose gradient fractions, or 60 µl of demembranated sperm suspension, to 900 µl of the motility reactivation solution containing 150 mM potassium acetate, 2 mM MgSO4, 2 mM EGTA, 1 mM DTT, 2% (w/v) polyethylene glycol (MW 20,000), 20 mM HEPES-NaOH, pH 7.8, and 2 mM ADP (26). To determine the effect of pH on Sp-AK and Sp-CK, enzyme activity was measured in the reactivating solution plus 1 mM phosphocreatine, 2 mM ADP and 2 mM EHNA adjusted to various pH values. For measuring Km and Vmax values of Sp-AK and Sp-CK activity, the reaction mixtures containing 12 µl of demembranated isolated flagella suspension (11 µg protein) and 882 µl of the reaction solution, plus 2 µM ADP and 2 mM EHNA adjusted to pH 7.6, were preincubated for 10 min at 16°C. For Sp-CK activity, additionally the Sp-AK assay solution plus 15 mM phosphocreatine and 20 µM Ap5A. The reaction was started by adding 18 µl of 100 mM ADP. After incubating the mixture at 16°C for 5 min, reactions were terminated by adding 10 µl 70% (w/v) perchloric acid, and the precipitated proteins removed by centrifugation for 2 min at 15,000 g at 4°C. The supernatants were neutralized by adding 15 µl of 5 M K2CO3 and the tubes centrifuged 2 min at 15,000 g at 4°C. ATP in this supernatant was measured using a coupled enzymatic assay with some modifications (7). Briefly, 400 µl of sample was added to 500 µl of reaction mixture (38 mM triethanolamine, pH 7.6, 0.33 mM NADP, 6.66 mM MgCl2, 50 mM glucose, 462 µM/ml G6PDH and 1.8 U/ml hexokinase). After incubating reactions at 23°C, the change in absorbance at 340 nm that accompanied the production of NADPH was monitored. Sigma ATP was used as standard.

ATPase Activity Assay - Fifty µl of sample was added to 950 µl of reaction buffer (150 mM NaCl, 5 mM MgCl2, 1 mM EGTA, 20 mM HEPES-NaOH (pH 7.4), 1 mM benzamidine, 1 mM PMSF and 0.5 mM DTT). The ATPase reaction was started by adding 20 µl of 50 mM ATP and the reaction stopped at 10 min by the
addition of 50 µl of 50% trichloroacetic acid. Phosphate liberated from ATP was measured by a colorimetric method (27).

**Extraction of Sp-AK from Sperm Flagella** - Isolated sperm flagella were pelleted by centrifugation at 20,000 g for 10 min. The pellet was suspended in Buffer A [0.1% w/v Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 20 mM HEPES-NaOH (pH 7.4), 1 mM benzamidine, 1 mM PMSF and 0.5 mM DTT] and extracted on ice for 30 min. This flagellar suspension was then centrifuged at 20,000 g for 10 min to obtain the first supernatant (S1) and pellet (P1). After removing S1, the extraction procedure was repeated on the P1 pellet to obtain the second supernatant and pellet (S2 and P2), and the P2 pellet extracted and centrifuged a third time to yield the S3 and P3 fractions. The S1 was further centrifuged at 100,000 g for 1 h to obtain the S100 supernatant and P100 pellet. The Sp-AK activities in each fraction were determined by enzymatic assay and immunoblotting.

The P3 pellet of axonemes was further extracted to free the outer arm dynein from the axoneme. After washing the axonemes three times in Buffer B [150 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 20 mM HEPES-NaOH (pH 7.4), 1 mM benzamidine, 1 mM PMSF and 0.5 mM DTT], each wash being separated by a 10 min centrifugation at 20,000 g, axonemes were resuspended in Buffer C [600 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 20 mM HEPES-NaOH (pH 7.4), 1 mM benzamidine, 1 mM PMSF and 0.5 mM DTT] and extracted 30 min on ice to solubilize the outer arm dynein (28). The axonemes, now without the outer arm dynein, were recovered by centrifugation at 20,000 g for 10 min. These axonemes were then washed with TED Buffer [1 mM Tris-HCl (pH 8.0), 0.5 mM EDTA and 0.5 mM DTT] to remove outer arm dynein remnants. The 20,000 g washed pellet was resuspended in TED Buffer and dialyzed against TED Buffer overnight at 4°C. The resultant suspension was centrifuged at 100,000 g for 1 h to obtain the supernatant (S-TED) and pellet (P-TED) fractions.

**Sucrose Density Gradient Centrifugation** - One ml of S-TED (2 mg protein) was layered on top of 10 ml of a 5-20% sucrose gradient in TED Buffer and centrifuged 200,000 g for 16 h in a Beckman SW41 rotor at 4°C. Fractions of 0.5 ml were collected from the tube bottom. Protein concentration, Sp-AK and ATPase activities were assayed on each fraction. Sedimentation coefficients were determined by parallel centrifugation of bovine thyroglobulin (19S) and bovine catalase (11S). Sp-AK active fractions were pooled and dialyzed against Buffer B and aliquots stored at −80°C.

**Immunodepletion** - Two hundred µl of the sucrose density gradient (SG) pooled fractions (~50 µg protein/ml) was incubated overnight with anti-Sp-AK antisera or preimmune sera at a 1:100 dilution. Twenty microliters of 50% Protein-A Sepharose CL-4B beads was then added to the mixture that was incubated for 2 h. The bead-free supernatants were then used for enzyme assays and immunoblotting. The precipitated immunocomplexes, which were bound to the Protein-A beads, were washed five times in Buffer B and the bound proteins solubilized by boiling in Laemmli sample buffer.

**Reactivation of Demembranated Sperm** - Demembranation and reactivation of sperm was performed using a slight modification of published methods (26). Undiluted sea urchin semen was suspended in ASW (486 mM NaCl, 10 mM CaCl₂, 10 mM KCl, 27 mM MgCl₂, 29 mM MgSO₄, 2.5 mM NaHCO₃ and 10 mM HEPES, adjusted to pH 8.0 with 1 N NaOH) and demembranated by addition of 15 vol of demembranating solution [0.04% (w/v) Triton X-100, 150 mM potassium acetate, 2 mM MgSO₄, 2 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM Tris-HCl, pH 8.0] for 45 sec at 16 °C. The demembranated sperm suspension was diluted with 16 vol of reactivating solution [150 mM potassium acetate, 2 mM MgSO₄, 2 mM EGTA, 1 mM DTT, 2% (w/v) polyethylene glycol (MW 20,000) and 20 mM HEPES-NaOH, pH 7.8]. For observation of sperm motility, 1 mM ATP, ADP, or AMP, (final concentration) were added to the reactivating solution.

**Microscopic Observation of sperm** - After the reactivated spermatozoa were incubated at 16°C, 10 µl of each suspension was placed on a glass slide and covered with a 22 x 22 mm coverslip. As soon as the sample was prepared, movies were taken at 30 frames per sec for 10 sec with a digital camera mounted on a phase-contrast microscope. Sperm cells were scored as either motile or immotile.
RESULTS

Cloning and Sequence Analysis of Sp-AK - Full-length Sp-AK protein has four methionines as potential translation start sites; none are in agreement with the eukaryotic translation start consensus motif (29; asterisks, Fig. 1A). In frame stop codons occur 5' to Met1. If this is the start of translation, the open reading frame is 2760-bp encoding a protein of 920 amino acids with a molecular mass of 99.1-kDa. Eight peptide sequences previously obtained from the 130-kDa protein are labeled P1-P8 (23). Sp-AK has three catalytic domains, occupying residues Ile\(^{115}\)-Val\(^{295}\), Ile\(^{626}\)-Val\(^{598}\) and Phe\(^{736}\)-Phe\(^{898}\) (Fig. 1A, defined underlines). Three potential PKA sites are Ser\(^{390}\), Thr\(^{246}\) and Ser\(^{899}\) (Fig. 1A, arrowheads). Four P-loop motifs for binding ATP or GTP (30) are at residues 120-128, 381-388, 431-439 and 740-748 (Fig. 1A, gray shade). Each catalytic domain has one P-loop and the fourth P-loop is between catalytic domains 1 and 2. An exon–intron map, constructed by comparison of the Sp-AK cDNA to the sea urchin genome, shows 19 exons, the gene occupying 18,825-bp of genomic DNA (Fig. 1B). All exons are found in one scaffold (GenBank accession number NW_678855). The Sp-AK cDNA structure with each exon numbered is shown in Figure 1C. Exons 4-6, 13-15 and 17-18 encode the three catalytic domains. Residues 3-48, in exons 1-2, consist of a dimerization-anchoring domain for cAMP-dependent type II protein kinase regulatory subunit (31). A helical wheel projection of residues 28-46 yields an amphipathic \(\alpha\)-helix that could be involved in binding calmodulin or other proteins (32). Both the Kyte-Doolittle hydropathy profile algorithm and the SOSUI algorithm predict no transmembrane segments.

Phylogenetic Analysis of AK Catalytic Domains of Various Species - The phylogenetic relationships of 24 catalytic domains of AK proteins were determined by constructing a neighbor-joining tree (Fig. 2). The tree shows that the three catalytic domains of Sp-AK are considerably divergent from each other. This is in contrast to the three catalytic domains of Chlamydomonas flagellar AK that are 98-99% identical to each other. Chlamydomonas and this sea urchin are the only flagellated cells known to date having three catalytic (Cat) domains in an AK protein. The tree suggests that Sp-AK Cat 3 is basal to Cats 1 and 2. Homo sapiens AK6 has a duplicated catalytic domain. Human AK6 Cat 1 is most similar to Sp-AK Cat 1, whereas Sp-AK Cat 2 is closest to C. elegans AK1. The percentages of amino acid sequence identity/similarity among the three Sp-AK Cat domains is: Cat 1 vs Cat 2, 38/55; Cat 1 vs Cat 3, 28/48; and Cat 2 vs Cat 3, 27/48. The three trypanosome flagellar AKs (21) separate into their own clade. However, some low bootstrap values of <50% provide low support to this topology.

Localization of Sp-AK - Rabbit antibody raised to the COOH-terminal end of Sp-AK reacted with a single protein of ~130 kDa representing the bacterial expressed full-length Sp-AK (Fig. 3, lane FL). The bacterial expressed Sp-AK has the same relative mobility as the Sp-AK reacting band in whole sperm (W), isolated sperm heads (H) and isolated flagella (F). At equal protein loads, strong signals from flagella (F) and weak signals from sperm heads (Fig. 3, lane H) were detected, showing that Sp-AK is most abundant in flagella. Immunofluorescence with either antisera shows that the Sp-AK localizes almost exclusively along the entire length of the flagellum (Fig. 4).

Extraction and Fractionation of Sp-AK from Flagella - Isolated flagella were first extracted in Buffer A containing 0.1% Triton X-100. After three extractions of the pellets, separated by 20,000 g centrifugations for 10 min, most of the Sp-AK remained in the pellet (Fig. 5A). The amount of Sp-AK enzymatic activity was measured in each fraction. Thirty-two percent of Sp-AK activity was in the S1 fraction and 68% in the P1 pellet (Fig. 5B). Slight decreases of Sp-AK in the pellet occurred with each extraction. These results show that much of the Sp-AK is tightly associated with the axoneme.

The P1 pellet was extracted by dialysis against TED buffer and the dialysate centrifuged 1 h at 100,000 g. The supernatant was then fractionated by sucrose density gradient centrifugation. After fractionation of the gradient, immunoblotting showed that Sp-AK was concentrated in fractions 13-23, with the peak fractions being 19 and 20 (apparent sedimentation coefficient ~5S; Fig. 6A). The Sp-AK enzyme activity was also highest in these fractions (Fig. 6B). Most of the ATPase activity, which is
axonemal dynein ATPase, was detected in fractions 10-16. To exclude the ATPase activity from the Sp-AK, fractions 18-23 were pooled as the partially purified Sp-AK.

To confirm that the ATP synthase activity in the pooled peak fractions was Sp-AK, the AK activity was immunodepleted by adding anti-Sp-AK, or preimmune sera as a control. After incubation, immune complexes were sedimented using Protein-A Sepharose CL-4B beads. After the beads settled, the supernatant Sp-AK enzyme activity was measured. Eighty-five percent of total activity was depleted in the immune sera, while activity in the preimmune control was unchanged (Fig. 6C).

**Inhibition of Sp-AK by Ap5A** - The effects of the AK specific inhibitor Ap5A (33) were determined on the pooled gradient fractions. Less than 10 µM Ap5A does not inhibit pyruvate kinase, hexokinase, fructose 6-phosphate kinase or creatine kinase (33). Activity of Sp-AK is inhibited by Ap5A in a dose dependent manner with 89% inhibition at 10 µM (Fig. 7A). The IC<sub>50</sub> is 0.41 µM Ap5A, a value close to previous studies (33). The effect of Ap5A on ADP-dependent ATP production was also examined in demembranated sperm. In the absence of Ap5A, demembranated sperm produce ATP with a specific activity of 0.21 µmol formed/mg protein/min (Fig. 7B). This is exactly the same specific activity found for the sea urchin Colobocentrotus atratus (22). ATP synthesis is inhibited by increasing concentrations of Ap5A with 81% inhibition at 10 µM (Fig. 7B).

**Sp-AK and the Reactivation of Sperm Motility** - To preclude the reactivation of motility by Sp-CK, assays were carried out with and without the Sp-CK specific inhibitor FDNB (20 µM; Tombes and Shapiro, 1985). Demembranated whole sperm were treated with reactivation solution plus 1 mM ATP, ADP, or AMP. Sperm treated with AMP did not show motility reactivation. The sperm treated with ATP or ADP showed excellent motility reactivation. After 30 sec in ATP, 58% of the sperm showed motility (Fig. 8A). However, after 30 sec in ADP, only 27% of the sperm were motile, with an abnormal jerky swimming pattern (Fig. 8A; see movie in the on line supplement). After 5 min, 60% of sperm in ATP, and 59% in ADP, were motile. After incubation for 10 min, the swimming patterns of demembranated sperm in ATP and ADP were comparable to intact sperm. Thus, in ADP, there is a distinct time lag before control levels of motility are achieved. This suggests that time is required for the synthesis of enough ATP to reactivate motility, which in other sea urchin species is about 5-12 µM (22,34). To confirm this possibility, 1 mM ADPβS which is a non-hydrolyzable ADP analogue, was added to the reactivation solution in place of 1 mM ADP. The complete lack of motility was observed in these sperm during 10 min of observation. This confirms that ATP synthesized from ADP is responsible for the reactivation of motility.

Sp-CK is present in sea urchin sperm flagella synthesizing ATP (7,35). Although its substrate, creatine phosphate, was not added to our media, we wanted to exclude any possibility that this other ADP rephosphorylating enzyme could be active. This was accomplished by including Sp-CK’s specific inhibitor FDNB in our assays. In the presence or absence of 20 µM FDNB, there were no differences in sperm reactivation kinetics in either ATP or ADP (Fig. 8A). These results indicate that the sperm reactivation we measure here under in vitro conditions is mediated by Sp-AK and not Sp-CK.

This hypothesis was strengthened by adding Ap5A to the motility reactivation solution containing demembranated sperm. The cells were perfectly motile 1 mM ATP in 100 µM Ap5A (Fig. 8B), showing that this inhibitor has no effect on dynein. However in ADP, the reactivation of sperm motility was completely inhibited by 1 µM Ap5A (observed at 5 min; Fig. 8B). These in vitro results show that Sp-AK activity generates enough ATP to reactivate motility.

**Sp-CK and Sp-AK Activities in Isolated, Demembranated Flagella** - Before sea urchin sperm activate motility their internal pH is 6.6 and the ADP concentration is 9 µM. When they activate motility their pH increases to 7.6, the ADP concentration rises to 114 µM, and the phosphocreatine concentration is 15.1 mM (8). Previous work on Sp-CK had determined the Km to be 0.13 mM at pH 6.7, an internal pH at which the sperm are immotile. In an attempt to determine the potential relative contributions of axonemal-bound Sp-AK and Sp-CK to ATP regeneration, we measured the pH dependency of
both enzymes in demembranated, isolated flagella between pHs 6.0-9.0. Sp-CK has a pH optimum at approximately 6.7, whereas Sp-AK’s pH optimum is at 7.7, close to the internal pH of the swimming cells (Fig. 9). For Sp-CK, the Km for ATP synthesis from ADP was 0.25 mM and the Vmax 5.25 μmole ATP formed per min per mg protein. For Sp-AK, the Km was 0.32 mM and the Vmax 2.80. Thus, at an intracellular pH of 7.6 and 114 μM ADP (8), Sp-AK might contribute 31%, and Sp-CK 69%, of total axonemal-associated ADP rephosphorylation to ATP.

DISCUSSION

Sp-AK as a Phosphoprotein - Sp-AK is phosphorylated by PKA when sea urchin sperm are treated with egg jelly (23) and cloning shows the presence of three PKA sites in this protein. We found that phosphorylation or dephosphorylation, by treatment with or without egg jelly, did not change the Sp-AK enzyme kinetics. Also, in vitro phosphorylation of demembranated sperm, by bovine PKA catalytic subunit, or dephosphorylation with calf alkaline phosphatase, did not change Sp-AK specific activity. We did not explore the possibility that phosphorylation affects the solubility of Sp-AK from flagella.

Triplication of Catalytic Domains - Molecular cloning of Sp-AK revealed all eight peptides determined by tandem mass spectrometry (23). On gels, the relative molecular masses of both full-length bacterial expressed and natural Sp-AK are 130 kDa (Fig. 3), compared to the calculated size of 99.1 kDa, therefore, this size discrepancy does not result from glycosylation. Sp-AK has three AK catalytic domains with percent amino acid identities among the three ranging from 27-38% (Fig. 1). Chlamydomonas flagellar AK is the only other AK known to date with three catalytic domains, which are 98-99% identical (Fig. 2; 17). Chlamydomonas nucleotide diphosphate kinase (NDK) is another flagellar enzyme with triplicated domains, but the triplication is not of catalytic sites (36). Two other sea urchin sperm flagellar enzymes involved in nucleotide metabolism: NDK (37) and creatine kinase (Sp-CK; 38), also possess triplications of catalytic domains. The percent amino acid identities among the three NDK domains range from 39-44%, while those for Sp-CK range from 69-72%. The greater divergence among the three Sp-AK catalytic domains could reflect more ancient gene duplication events, or less functional constraints on the Sp-AK protein as compared to Sp-CK. The fact that flagella contain several proteins with triplicated catalytic domains could be one way to increase efficiency and hence the maintenance of high levels of nucleotide triphosphates (GTP and ATP) needed for swimming. We assume, without proof, that all three catalytic domains are active. An alternative way to increase AK activity in flagella could be the reason for the presence of three different AK proteins in the flagella of trypanosomes (21). It should also be noted that in Chlamydomonas flagella, glycolytic enzymes, such as pyruvate kinase, can synthesize ATP (39).

Sperm specific isozymes of glycolytic enzymes are present in mammalian sperm and there is an extensive literature that glycolysis makes ATP needed for sperm motility. For example, gene deletion of glyceraldehyde-3-phosphate dehydrogenase-S in mice causes severe loss of sperm motility and infertility (40). Sea urchin sperm are spawned into seawater, which lacks glycolytic substrates. The great majority of ATP production in sea urchin sperm is from mitochondrial respiration (41). Although mammalian sperm contain AK activity, they have essentially no flagellar CK activity (42).

Relationship of Sp-AK to the axoneme - Western immunoblots and indirect immunofluorescence show Sp-AK to be concentrated in flagella (Figs. 3 and 4). Attempts to localize Sp-AK in axonemes by transmission electron microscopy were unsuccessful, the 6 nm gold particle aggregates being associated with all axonemal components, however preimmune controls showed no gold particle labeling. In Chlamydomonas, the triplicated AK is tightly associated with the A-tubule complex of outer dynein arm proteins 5, 8 and 10 (17).

The first extraction with 0.1% Triton X-100 solubilized 32% of the total Sp-AK enzyme activity, while sequential extractions of pellets removed less activity (Fig. 5). We do not know if the 32% detergent solubility of total activity represents a post-translational modification such as myristoylation, allowing the Sp-AK to be lipid associated. Regarding Sp-CK,
both myristoylated membrane-associated (11,12), and unmodified axonemal-associated isoforms are present in these cells (9). Approximately 68% of the total Sp-AK activity remains associated with axonemes in the P1 fraction after the first 0.1% Triton X-100 extraction. Earlier work with another sea urchin species showed that extraction of whole sea urchin sperm with 0.2% Triton X-100, followed by low force centrifugation to sediment the sperm, resulted in 80% of the AK activity being in the pellet (22).

The AKs of many cilia and flagella are also tightly associated with axonemes. For example, in *Tetrahymena*, about 95% of AK activity isolates with axonemes after extraction with 0.2% nonidet P-40 (19). In *Trypanosoma brucei*, three isoforms of AK have different 55 residue N-terminal extensions that target three AKs to the flagellum (20). The N-terminal 3-48 residues of Sp-AK share homology with the dimerization-anchoring domain of PKA regulatory subunit II. This region contains an amphipathic α-helix that could be similarly involved in binding the enzyme to the axoneme.

*What is the function of Sea Urchin Sperm flagellar AK?* - Sp-AK enzyme activity in both gradient-isolated fractions and demembranated sperm exhibits the same Ap5A inhibition curves (Fig. 7). Demembranated sperm with ADP as the substrate, show a time lag before maximum percent reactivated sperm is reached (Fig. 8A). This figure shows that, with ADP as the substrate, essentially all ATP synthesis comes from Sp-AK activity. Motility reactivation is completely blocked by Ap5A if ADP is the substrate, but not if ATP is the substrate (Fig. 8B). A time lag of 1.5 min was observed when ADP was used to reactivate demembranated trout sperm (43), and a lag of 10 min was seen in a similar experiment using sea urchin sperm (34).

At pH 7.6 and at saturating ADP and phosphocreatine concentrations, about 31% of the ATP synthesis in isolated, demembranated flagellar axonemes could be from Sp-AK and 69% from Sp-CK. These numbers are based on the *in vivo* concentrations of 114 µM ADP and 15.1 mM phosphocreatine in swimming sperm of another sea urchin species, *Psammechinus miliaris* (8). Thus, the *in vivo* contribution of Sp-AK to axonemal ATP generation might be substantial. However, in the case of flagellar AKs of *Trypanosoma brucei*, RNAi knock downs of two of the three flagellar AKs results in no change in motility, questioning whether the role of flagellar AKs is for ATP generation for use by dynein (21).

Because AKs are found tightly associated with the axonemes of essentially all eukaryotic flagella, the question arises as to what functional roles they play. AKs could be involved in ATP generation for utilization by dynein and motility. In sea urchin sperm, they could be involved in ATP generation for the rephosphorylation of creatine to phosphocreatine, to be used by the Sp-CK in the phosphocreatine shuttle (7,35). Finally, AK activity could be a way to remove ADP as a potent inhibitor of dynein ATPase (6). Assuming that they have a crucial function in sperm physiology, the presence of AKs in both invertebrate and mammalian sperm makes them potential targets for drug-mediated contraception.

REFERENCES


1 AK, adenylate kinase; CK, creatine kinase; Sp-AK, sea urchin sperm AK; Sp-Cr, sea urchin creatine kinase; PKA, cAMP-dependent protein kinase; Cat, catalytic domain; EHNA; erythro-9-[3-(2-hydroxyynonyl)] adenine; DTT, dithiothreitol; PMSF, phenylmethanesulfonylfluoride; ASW, artificial sea water; FDNB, 1-fluoro-2,4-dinitrobenzene; Ap5A, P1P5-di(adenosine-5')pentaphosphate; ATPβS, adenosine 5'-(2-O-thio) diphosphate; Sp, Strongylocentrotus purpuratus.
FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Sequence analysis. A, Amino acid sequence of Sp-AK. Three catalytic domains are marked with dashed underlines. Peptides obtained by tandem mass spectrometry are overlined P1-P8. The P-loops are shaded gray. Four Met residues marked with asterisks are possible translation initiation sites. Three residues marked with arrowheads are potential PKA phosphorylation sites. B, Intron-exon structure of the Sp-AK gene. Vertical lines indicate the exons, the relative size denoted by line width. Solid horizontal lines indicate the non-coding regions. The scaffold is from Scaffold 103869 based on the Sea Urchin Genome Project (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/). C, Diagrammatic schemes of the domain structure of Sp-AK predicted by InterProScan. Cyclic AMP-dependent protein kinase regulatory subunit II dimerization-anchoring domain is marked gray and the three catalytic domains are marked black.

Fig. 2. Phylogenetic analysis of AK catalytic domains in selected organisms. A neighbor-joining tree was constructed with 24 AK catalytic domains. Dual (human AK6) and triple (sea urchin and Chlamydomonas) catalytic domains were analyzed separately. The Sp-AK catalytic domain is shaded gray. Numbers at nodes indicate bootstrap values (2,000 replicas). A scale of branch lengths is shown at the bottom. The scale bar represents a genetic distance of 0.2 amino acid substitutions per site.

Fig. 3. Immunoblotting of Sp-AK. Left panel is immune serum, middle panel preimmune serum, and right panel parallel a Coomassie stained duplicate gel. Lane FL is, recombinant full-length His-tagged bacterial expressed Sp-AK protein; Lane W, whole sperm; lane H, sperm heads; lane F, sperm flagella. 2.5 µg protein was loaded per lane. Molecular mass standards are on right in kDa. Due to the His-tagged vector, the bacterial expressed full-length Sp-AK migrates slightly slower than the natural Sp-AK at 130 kDa.

Fig. 4. Immunofluorescent localization of Sp-AK. (A & C) are phase contrast microscopic images and (B & D) are fluorescence images. (A & B) show that pre-immune serum does not react with sperm. (C & D) show that the anti-Sp-AK reacts with flagella. The scale bar at the bottom right of C indicates 1 µm.

Fig. 5. Extraction series of Sp-AK. A, Immunoblotting of the Sp-AK in flagella sequentially extracted with Buffer A (total flagella), pellets (P1-P3), and supernatants (S1-S3). Molecular mass standards are on the right in kDa. B, Sp-AK enzymatic activity in sequential supernatants and pellets. The experiment was conducted three times. The values are expressed as the mean ± standard error.

Fig. 6. Sedimentation of the Sp-AK activity by sucrose density gradient centrifugation. A, Immunoblotting analysis of AK from the gradient fractions. Ten µl of each fraction was separated by SDS-PAGE and probed with anti-Sp-AK antisera. Fraction numbers are shown on top. Numbers on right indicate positions of molecular weight markers in kDa. B, Two mg of the TED supernatant was loaded on the gradient. Fractions were collected from the bottom to top at 0.5 ml/fraction. Protein concentrations (closed circles, axis on left), Sp-AK activity (open circles, axis on right) were determined for each fraction. Sedimentation coefficient was determined by parallel centrifugation of thyroglobulin (19S) and catalase (11.3S) (arrowheads). Sp-AK active fractions (horizontal bar) were pooled and used for further analysis. Fraction numbers are shown on bottom. C, Immunodepletion analysis using anti-Sp-AK and preimmune sera. The Sp-AK activity in the pooled fractions after the depletion of the immunocomplexes by Protein-A Sepharose CL-4B beads. The control was without either serum.
Fig. 7. Effects of Ap5A on Sp-AK activity. A, Effects of various concentrations of Ap5A on Sp-AK activity in the pooled gradient fractions. B, Effects of various concentrations of Ap5A on Sp-AK activity in demembranated sperm. The experiment was conducted three times. The values are expressed as the mean ± standard error.

Fig. 8. Sp-AK and sperm motility. Demembranated sperm were reactivated with adenine nucleotides. A, Time course of the reactivation of sperm motility in 1 mM ATP with (filled triangles) or without (filled circles) 20 µM FDNB, or 1 mM ADP with (open triangles) or without (open circles) 20 µM FDNB. Reactivated sperm, showing any motility, were scored as motile. With ADP there is a 5 min lag before motility equals that seen in ATP. B, Effects of the Sp-AK inhibitor (Ap5A) on reactivated sperm motility. The demembranated sperm were reactivated in various concentrations of Ap5A. Percentages of reactivated sperm in ATP (filled circles), or ADP (open circles), were determined. The experiment was conducted three times. The values are expressed as the mean ± standard error.

Fig. 9. Effects of pH on the Sp-AK and Sp-CK activity in isolated, demembranated flagella. The ATP synthesis activity of demembranated flagella was measured with 2 mM ADP and 1 mM phosphocreatine in the presence of 20 µM FDNB (filled circles) or 20 µM Ap5A (open circles) in various pHs. The experiment was conducted three times. The values are expressed as the mean ± standard error.
Figure 2

[Diagram of phylogenetic tree showing relationships between different species including human AK1, rat AK1, Xenopus, carp, sea urchin CAT2, C. elegans AK1, human AK5, human AK6 CAT2, Drosophila, Chlamydomonas CAT1, Chlamydomonas CAT2, Chlamydomonas CAT3, sea urchin CAT1, human AK6 CAT1, human AK3, human AK4, E. coli, human AK2, Dictyostelium, Trypanosoma ADKA, Trypanosoma ADKB, Trypanosoma ADKE, sea urchin CAT3, and human AK7.]
Figure 3

[Image of Western blots showing anti-Sp-AK, preimmune, and CBB stain lanes with molecular weight markers (kDa)].
Figure 5

A

B

ATP (µ mole/mg/min)

0 10 20

total flagella P1 P2 P3 S1 S2 S3

(kDa)

100 150
Figure 7

A

AK activity (umole ATP/ min/mg protein)

0 0.01 0.1 1 10 100

Ap5A (µM)

B

AK activity (umole ATP/ min/mg protein)

0 0.01 0.1 1 10 100

Ap5A (µM)
Figure 9

[Graph showing ATP synthase activity (μ mole ATP/min/mg protein) vs pH, with two curves indicating different treatments or conditions.]

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A sea urchin sperm flagellar adenylate kinase with triplicated catalytic domains
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