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Recombinant Sea Urchin Flagellar Adenylate Kinase

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Adenylate kinase (AK) is localized in sea urchin sperm flagella and embryonic cilia. To investigate sea urchin *Strongylocentrotus purpuratus* AK (SpAK) enzymatic characteristics, the full-length recombinant protein of 130 kDa (SpAKr) and each of its three catalytic domains were expressed in *Escherichia coli*. Although the full-length SpAK had high enzymatic activity, each of the three catalytic domains had no activity. The K_m for ATP synthesis from ADP was 0.23 mM and the V_{max} was 4.51 μmol ATP formed per minute per milligram of protein. The specific AK inhibitor, Ap5A, blocks SpAKr enzymatic activity with an IC_{50} of 0.53 μM. The pH optimum for SpAKr is 8.1, as compared to 7.7 for the natural SpAK. Calcium inhibits SpAKr activity in a dose-dependent manner. Although SpAKr has three cAMP-dependent protein kinase phosphorylation sites, and can be phosphorylated *in vitro*, the enzymatic kinetics after phosphorylation are not significantly altered. SpAK and *Chlamydomonas* flagellar AKs are the only AKs with three catalytic sites. Further study of the SpAKr will aid in understanding the active site of this interesting and important ATP synthase.

Key words: adenylate kinase, protein expression, ATP synthesis, cell motility, axoneme, protein phosphorylation.

Abbreviations: AK, adenylate kinase; SpAK, sea urchin *Strongylocentrotus purpuratus* AK; 130 kDa SpAK, *S. purpuratus* AK of 130 kDa relative molecular mass; SpAKr, the recombinant full-length 130 kDa SpAK protein expressed in *Escherichia coli*; PKA, cyclic AMP-dependent protein kinase; ALP, calf intestinal alkaline phosphatase; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; Ap5A, P1,P5-di(adenosine-5′)-pentaphosphate.

Adenylate kinase (AK; myokinase) is a ubiquitous enzyme that catalyses the reaction $2ADP \leftrightarrow ATP + AMP$. AK is important to the maintenance of ATP concentrations in most cells. AK activity exists in flagella of sea urchin sperm (*1*, *2*), bovine sperm (*3*), *Chlamydomonas* (*4–6*), embryonic sea urchin cilia (*7*), cilia of *Tetrahymena* (*8*) and *Trypanosoma brucei* (*9*). These AKs probably function in ATP regeneration from ADP to support the high demand for ATP in flagellar and ciliary motility.

The kinetics of several AKs have been studied using recombinant AK expressed in *Escherichia coli*. For example, human AK1 (*10–12*), chicken AK (*13*), porcine AK (*14*), rice AK (*15*) and *Chlamydia pneumoniae* (*16*) have been expressed and kinetically analysed. We previously discovered a 130 kDa sea urchin *Strongylocentrotus purpuratus* AK (SpAK) in sperm flagella (*2*) and embryonic cilia (*7*). The flagellar SpAK becomes phosphorylated when sperm are treated with egg jelly to induce the acrosome reaction (*17*). In this study, we constructed and expressed the full-length 130 kDa SpAK in *E. coli*. This is the first study of the kinetics of the recombinant SpAK, which is unusual in possessing three catalytic domains (*2*).

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MATERIALS AND METHODS

Recombinant SpAK protein—The cDNA encoding full-length SpAK (GenBank accession no. DQ447969) was amplified by PCR using an *S. purpuratus* testis cDNA library in Lambda Zap II (Stratagene, La Jolla, CA, USA) as template. The forward primer for the full-length SpAK (F) was 5′-AGGAATTCCATATGACATCAAGAGAA GACACCAAATCGTA-3′ and the reverse primer was 5′-TCGGATCCGTTATTATTCTCCACTTAAGGCTGACAC AGCATCGAG-3′. The cDNA encoding each of the three SpAK catalytic domains (C) were also amplified. The forward primer for C1 (Ile^{116}- Asp^{304}) was 5′-AGGAATTC CATATGATCTTGGTCATTGGCGGGCCTGGCAGTGG-3′ and the reverse primer was 5′-TCGGATCCGTTATTAG TCAATCATGTTGGCAACATCAAAGAACA-3′. The forward primer for C2 (Lys^{425}- Ile^{605}) was 5′-AGGAATTC CATATGAAGATCATTTTTGTTGTAGGGGGACCAGG-3′ and the reverse primer was 5′-TCGGATCCGTTATTAG ATCTCCTTCACCAGGACGAAGACCTCCTC-3′. The forward primer for C3 (Phe^{736}- Asp^{912}) was 5′-AGGAATTC CATATGTTCTTTGTCGTTGGTGCCCCCGGTACTGG-3′ and the reverse primer was 5′-TCGGATCCGTTATTAA TCGAGGACGGTTGAAAGATCGTTAGCAAC-3′. PCR products were cloned into pET-15b (Novagen, Madison, WI, USA) with an NH_2-terminal His tag. The vector was transformed into *E. coli* Rosetta-gami (DE3) competent cells. Expression was induced with 0.5 mM IPTG at 16°C overnight in LB broth with 100 μg/ml ampicillin and 37 μg/ml chloramphenicol. Cells were harvested by

centrifugation at 6,000*g* for 10 min (4°C). The pellet was suspended in ice-cold lysis buffer containing 500 mM NaCl, 50 mM imidazole, 50 mM HEPES-NaOH (pH 7.6), 5 mM benzamidine, 1 mM PMSF and 5 mM 2-mercaptoethanol and lysed by sonication for 3 min. One milligram/millilitre of crude cell lysate supernatant was obtained by centrifugation at 20,000*g* for 30 min and incubated 1 h with 50% (v/v) lysis buffer suspension of 500 µl of Ni-NTA (Qiagen, Valencia, CA, USA). The beads were then washed thrice with 10-vol lysis buffer and used for the SpAK activity assay. The expressed protein coupled to Ni-NTA was further purified by elution with lysis buffer containing 300 mM imidazole.

Electrophoresis and Immunoblotting—Samples were added to Laemmli sample buffer (*18*) and boiled for 3 min. The proteins were separated on 7.5% SDS/PAGE gels for the full-length SpAK and on 17.5% SDS/PAGE gels for each of the three catalytic domains. The bands visualized by silver staining (*19*) or Coomassie brilliant blue staining. For immunoblotting, proteins were transferred to PVDF membrane by standard methods. The membranes were blocked with 0.1% 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 incubated overnight with the phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling Technology, Danvers, MA, USA; Catalog number 9621) or anti-130 kDa SpAK antisera (*2*) diluted in blocker (4°C). 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, USA) following the manufacturer's protocol. For HisProbe-HRP antibody (Pierce; Catalog number 15165) reactions were done following the manufacturer's protocol.

SpAK Activity Assays—Assays of SpAK enzymatic activity were performed as described previously (*2*). Reactions were started by adding 20 µl of the Ni-NTA suspension (2 µg protein), or bead eluate, to 450 µl of the assay solution containing 150 mM potassium acetate, 2 mM $MgSO_4$, 2 mM EGTA, 1 mM DTT, 2% (w/v) polyethyleneglycol (MW 20,000) and 20 mM HEPES-NaOH, pH 7.6 (*20*). The dynein ATPase inhibitor, 10 µM of sodium orthovanadate (*21*, *22*), was also added. For determining the K_m and V_{max}, the reactions were started by adding 0.1–5 mM ADP. For determining the effects of Ap5A, the reactions were started by adding 2 mM ADP and 0.01–100 µM Ap5A. For determining the pH or Ca^{2+} effects, the reactions were started by adding 4 mM ADP at various pHs (*6–9*) or Ca^{2+} concentrations (10^{-3}–10^{-9} and 0 M). Free Ca^{2+} concentration was calculated using CALCON (http://user.ecc.u-tokyo.ac.jp/~ckam/calcon.html), which is based on Goldstein's algorithm (*23*). After incubating the reaction at 16°C for 5 min, reactions were terminated by adding 5 µl of 70% (w/v) perchloric acid. The precipitated proteins were removed by centrifugation for 2 min at 15,000*g* at 4°C. The supernatants were neutralized by adding 7.5 µl of 5 M K_2CO_3 and the tubes centrifuged for 2 min at 15,000*g* at 4°C. ATP in the supernatant was measured using a coupled enzymatic assay with some modifications (*24*). Briefly, 400 µl of sample was added to 500 µl of reaction mixture (31.2 mM triethanolamine, pH 7.6, 0.33 mM NADP, 6.66 mM $MgCl_2$, 50 mM glucose, 462 mU/ml G6PDH and 1.8 U/ml hexokinase). After incubating the reactions for 5 min at 23°C, the change in absorbance at 340 nm that accompanied the production of NADPH was monitored. ATP (Sigma, St. Louis, MO, USA) was used as a standard.

In Vitro Phosphorylation and Dephosphorylation—Twenty microlitres of the Ni-NTA bead suspension was treated with 50 µl of reaction buffer containing 150 mM NaCl, 20 mM HEPES-NaOH (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT. For *in vitro* phosphorylation, 2 U catalytic subunit of cAMP-dependent protein kinase (PKA) from human recombinant protein (Sigma; Catalog number C-8482), 1 mM ATP and 200 µM sodium orthovanadate were added to the reaction buffer. For dephosphorylation, 2 U calf intestinal alkaline phosphatase (ALP; Boehringer Mannheim, Indianapolis, IN, USA) was added. Reactions were performed for 30 min at room temperature. The samples were washed thrice with reaction buffer and enzyme assays and immunoblotting performed.

RESULTS

The Recombinant SpAK Protein—The SpAK (SpAKr) was expressed in *E. coli* and the bacterial lysate showed that the major band at 130 kDa was only in cells treated with IPTG. The lysate proteins coupled to Ni-NTA showed that the dense band at 130 kDa was specifically detected after IPTG induction, while numerous bacterial proteins were detected in both the induced and non-induced cells (Fig. 1A). The 130 kDa band reacted with the 130 kDa SpAK-specific antisera (*2*) and also with His-Probe antibody for detection of recombinant poly-His-tagged fusion proteins (data not shown). To measure the SpAKr activity, the activity assay was performed using the bacterial lysate (prepared with or without IPTG induction) coupled to Ni-NTA. The expressed protein had a 36-fold higher AK activity than the non-induced control (Fig. 1B). The SpAKr coupled to Ni-NTA was further purified by elution with 300 mM imidazole (Figs 1C and D). The SpAKr was collected in fractions 1–5 as shown by SDS/PAGE and silver staining. The 70 kDa band is bacterial (Fig. 1C). Each of the three SpAK catalytic domains were also expressed in *E. coli* and the bacterial protein showed a major band at 22 kDa for catalytic domain 1, 21 kDa for catalytic domain 2 and 22 kDa for catalytic domain 3 (Fig. 1E). To measure the SpAKr activity, the activity assay was performed using the bacterial lysate (prepared from induced and non-induced cells) coupled to Ni-NTA. Although the full-length SpAK had high AK activity, each of the three catalytic domains, when expressed singly had no AK activity (Fig. 1F).

Characteristics of SpAKr—To compare the kinetics of SpAKr, to those of SpAK in sperm flagella and embryonic cilia, the K_m and V_{max} values were determined. For SpAKr, the K_m for ATP synthesis from ADP was 0.23 mM and the V_{max} was 4.51 µmol ATP formed per minute per milligram of protein. In flagella, the K_m

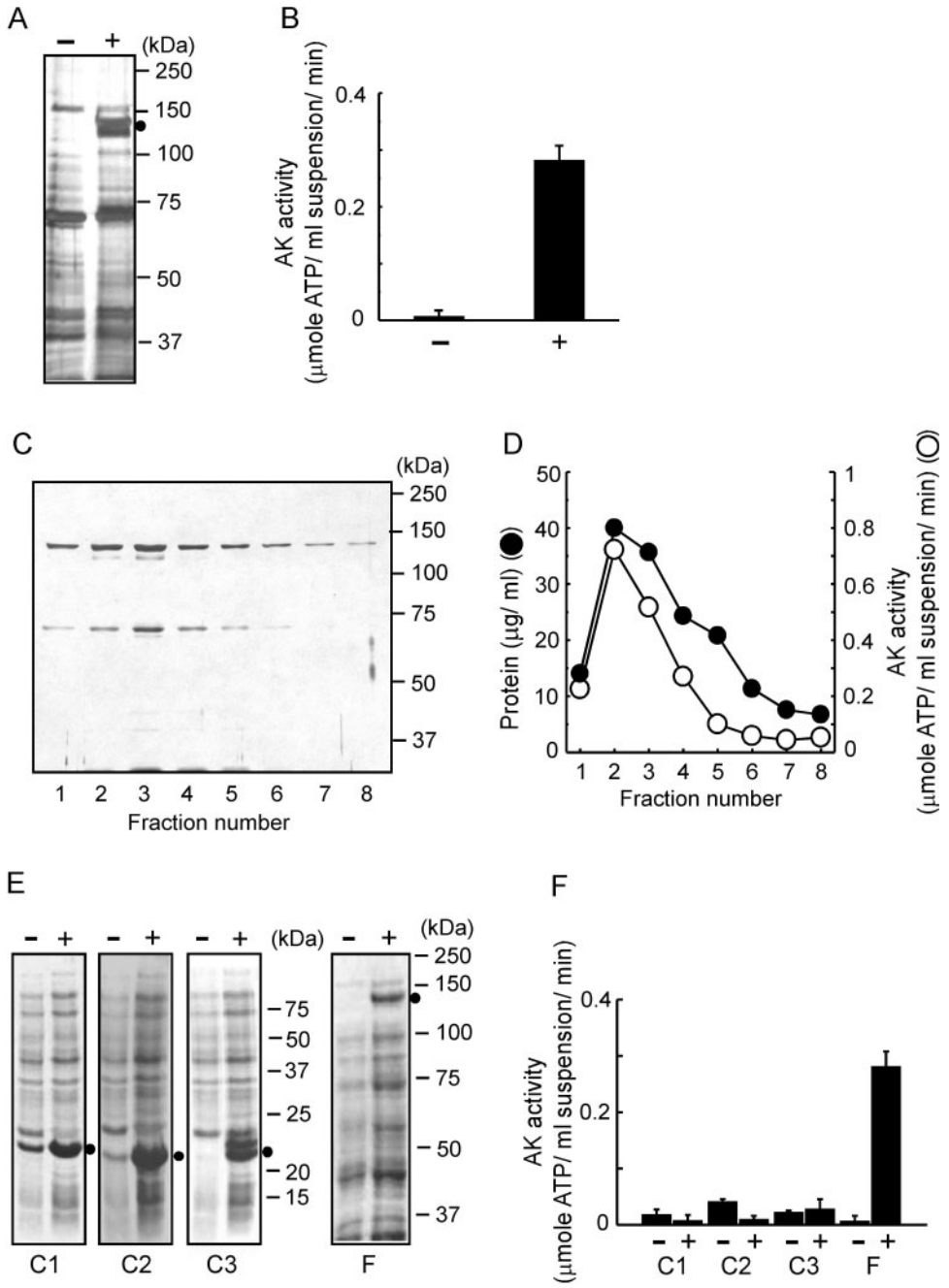

Fig. 1. **Expression and purification of the recombinant 130 kDa SpAK.** (A) Silver-stained gel of the *E. coli* cell lysate proteins that bind to Ni-NTA without (–) or with (+) IPTG induction. The recombinant 130 kDa SpAK migrates at ~130 kDa (black dot). Molecular mass standards are on the right in kilodaltons. (B) SpAKr enzymatic activity of Ni-NTA without (–) or with (+) IPTG induction. The experiment was conducted three times. The values are expressed as the mean ±SE. (C) Ten milligrams of the *E. coli* lysate after induction by IPTG was passed over the Ni-NTA column. The recombinant protein coupled to the Ni-NTA was eluted with lysis buffer containing 300 mM imidazole, 1 ml fractions were collected. Ten microlitres of each fraction was separated on SDS/PAGE and the gel stained with silver. Molecular mass standards are on the right in kilodaltons. (D) Protein concentrations (filled circle, axis on left) and SpAKr activity (open circle, axis on right) were determined for each fraction. (E) Coomassie brilliant blue stained gel of the *E. coli* cell proteins without (–) or with (+) IPTG induction (~10 μg protein per lane). The recombinant SpAK catalytic domains (C1, C2 and C3) migrate at ~20 to 25 kDa (black dot). The recombinant SpAK full-length (F) migrates at ~130 kDa (black dot). Molecular mass standards are on the right in kilodaltons. (F) Each of the three catalytic domains and the full-length SpAKr enzymatic activity of Ni-NTA without (–) or with (+) IPTG induction. The experiment was conducted three times. The values are expressed as the mean ±SE.

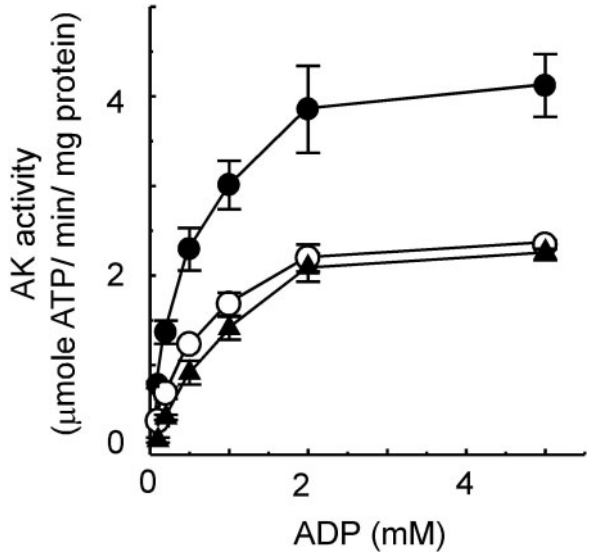

Fig. 2. **Plot of substrate dependency of SpAKr, and SpAK in sperm flagella [taken from (2)] and embryonic cilia [taken from (7)].** The assay was determined by adding ADP to a final concentration of 0.1–5 mM. *E. coli* expressed SpAKr (filled circle), SpAK in isolated flagella (open circle) and cilia (filled triangle) is shown. The experiment was conducted three times. The values are expressed as the mean ±SE.

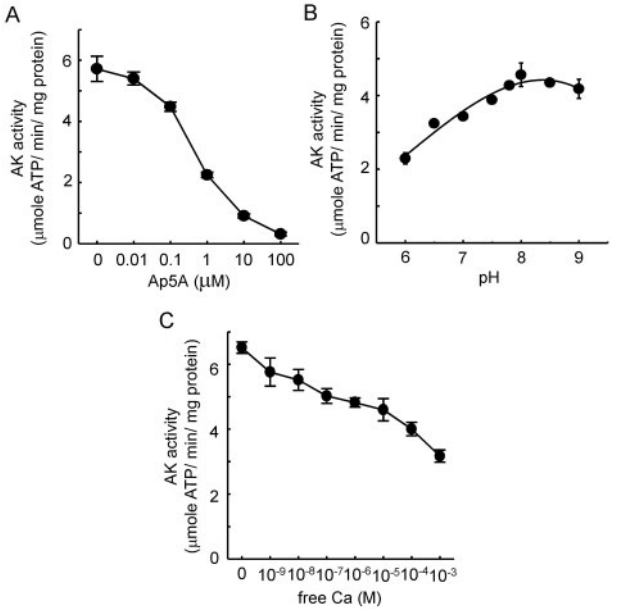

Fig. 3. **Characterization of SpAKr.** (A) Effects of various concentrations of Ap5A on SpAKr activity. (B) Effects of pH on SpAKr activity. The pH optimum for SpAKr was ~8.1. (C) Effects of various free Ca^{2+}concentrations on SpAKr activity. The activity was decreased as free Ca^{2+} increased. The experiment was conducted three times. The values are expressed as the mean ±SE.

was 0.32 mM and V_{max} 2.8 (*2*), and in cilia, the K_m was 0.69 mM and V_{max} 3.34 (*7*). The K_m value of ciliary AK was higher than flagellar SpAK and SpAKr, indicating that at least one or more other AK isozymes with higher K_m value is in sea urchin embryonic cilia (*7*). The V_{max} of SpAKr was higher than those of flagella and cilia, indicating the purity of SpAKr was greater than the other two AK activities (Fig. 2).

The effects of the AK-specific inhibitor, Ap5A (*25*), were determined on SpAKr coupled to Ni-NTA. Activity of SpAKr was inhibited by Ap5A in a dose-dependent manner with 84% inhibition at 10 μM (Fig. 3A). The IC_{50} was 0.53 μM Ap5A, which was close to the 0.41 μM in partially purified sperm flagellar SpAK (*2*).

The pH dependency of SpAKr between pH 6.0 and 9.0 was determined. Although the pH optimum of SpAK in sea urchin sperm flagella is 7.7 (*2*), that of SpAKr is approximately 8.1 (Fig. 3B). Thus, a slight shift of pH optimum of SpAKr was observed.

The effect of free Ca^{2+} on AK activity was determined in the range of between 10^{-9} and 10^{-3} M and when Ca^{2+} is totally chelated by EGTA. Ca^{2+} inhibited the SpAKr activity in a concentration-dependent manner with 51% inhibition at 10^{-3} M Ca^{2+} compared to no free Ca^{2+} in EGTA (Fig. 3C).

Phosphorylation and Dephosphorylation of SpAKr—SpAK is phosphorylated by PKA in sea urchin sperm treated with egg jelly (*17*). To confirm that SpAKr can be phosphorylated, *in vitro* reactions with human recombinant PKA catalytic subunit were performed. In SpAKr without PKA, or with ALP treatment, phosphorylation was not detected by immunoblotting with the anti-PKA substrate antibody, indicating that *E. coli* expressed SpAKr did not get phosphorylated on PKA sites. In SpAKr phosphorylated by PKA, the anti-PKA substrate antibody specifically reacted with a 130 kDa band (Fig. 4A left panel). The ~45 kDa phosphorylated band is most probably auto-phosphorylated PKA catalytic His-tagged human recombinant protein, whose molecular weight is 43.5 kDa (Fig. 4A, left panel). After PKA treatment, SpAKr coupled to Ni-NTA reacted with the anti-130 kDa SpAK antisera, confirming that the recombinant protein was expressed only after induction of IPTG (Fig. 4A, right panel). In the *in vitro* phosphorylated SpAKr, the K_m for ATP synthesis from ADP was 0.33 mM and the V_{max} was 5.43 μmol ATP formed per minute per milligram of protein. In SpAKr treated with alkaline phosphatase, the K_m was 0.32 mM and V_{max} 4.58. Thus, the K_m value is not changed by phosphorylation, but a 19% increase of V_{max} was observed after PKA treatment (Fig. 4B).

DISCUSSION

Based on the amino acid sequence, the predicted molecular mass of SpAK is 99.1 kDa. However, the expressed protein migrates at 130 kDa (*2*) (Fig. 1), showing that glycosylation is not responsible for this discrepancy. Natural flagellar and ciliary SpAK also migrate at 130 kDa (*2*, *7*). Both the natural and the recombinant proteins migrate on SDS-PAGE at 130 kDa indicating that the full-length protein is faithfully expressed. SpAKr has high enzymatic activity suggesting that all three catalytic domains are active. However, each of the three catalytic domains (C1, Ile^{116}–Asp^{304}; C2, Lys^{425}–Ile^{605}; C3, Phe^{736}–Asp^{912}), when expressed

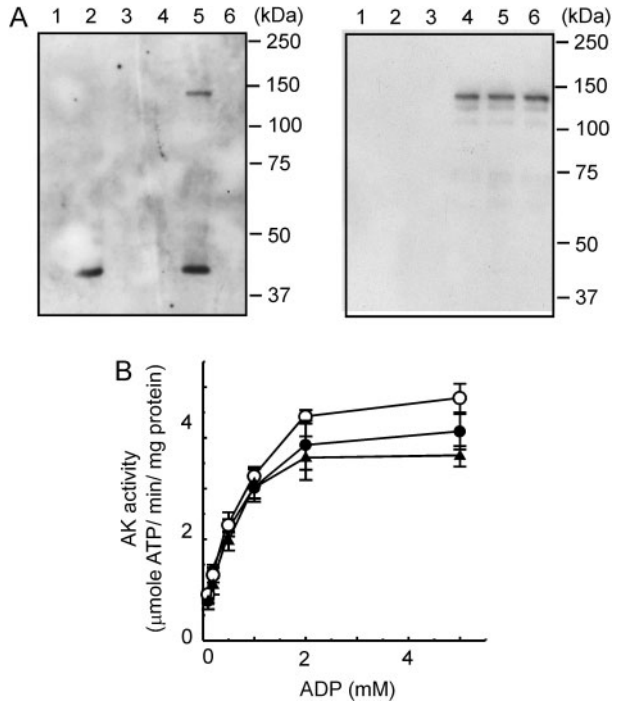

Fig. 4. **Phosphorylation and dephosphorylation of SpAKr *in vitro*.** (A) Immunoblotting of SpAKr after phosphorylation or dephosphorylation. Left panel is treated with the phospho-(Ser/Thr) PKA substrate antibody and right panel with 130 kDa SpAK antisera. Lanes 1–3 are *E. coli* lysate coupled to Ni-NTA without IPTG induction, and lanes 4–6 are with IPTG induction. Lanes 1 and 4 are no-treatment, lanes 2 and 5 are PKA treatment, lanes 3 and 6 are ALP treatment. Molecular mass standards are on the right in kilodaltons. (B) Substrate dependency plot of SpAKr fraction. The assay was determined by adding ADP to a final concentration of 0.1–5 mM. Bacteria expressed recombinant SpAK protein (filled circle), SpAKr treated with PKA catalytic (open circle) and SpAKr treated with ALP (filled triangle) was shown. The experiment was conducted three times. The values are expressed as the mean ±SE.

singly in bacteria, had no AK activity (Fig. 1F). In the case of SpAK, although all three catalytic domains are not active when expressed alone, a neighbouring AK catalytic domain might exert a cooperative effect on folding/stabilization of active neighbouring AK catalytic activity. Alternatively, one of the catalytic domains of SpAK might be inactive. Comparisons of the percent identity of amino acids in the three catalytic domains show them to be highly divergent from each other. The arginine kinase from the razor clam *Ensis directus* consists of two tandem catalytic domains and kinetic analysis suggests that only the second domain is active (*26*). As we found with SpAK, expression of each *Ensis* catalytic domain by itself produces inactive protein (*26*).

In other recombinant AKs, important amino acid positions have been found by site-directed mutagenesis. In human AK1, several Arg residues (Arg^{44}, Arg^{97}, Arg^{132}, Arg^{138}) are essential for catalytic activity (*11*). When comparing human AK1 to SpAK catalytic domains, C1 (Arg^{150}, Arg^{208}, Arg^{243}, Arg^{249}), C2 (Arg^{460}, Arg^{513}, Arg^{548}, Arg^{554}) and C3 (Thr^{769}, Lys^{821}, Lys^{857}, Arg^{863}), sequence alignments show that Arg residues in C1 and C2 are completely conserved, but not in C3.

The K_m value for ATP synthesis from ADP in SpAKr is 0.23 mM, which is closer to that of natural SpAK in flagella with a K_m of 0.32 mM, and not that of cilia with a K_m of 0.69 mM. This suggests that the only AK in flagella is the 130 kDa SpAK. In cilia, K_m and solubility data suggest the presence of more than one AK isozyme. In isolated, Triton-X100 extracted flagella, the V_{max} of the native SpAK was 2.8, and in the His-tagged SpAKr, purified on the Ni-NTA column, the Vmax is 4.5. Thus, the V_{max} value of the recombinant protein was 1.6-fold higher. One possible explanation for this difference in V_{max} is that it could either be due to a fraction of the recombinant SpAKr molecules being misfolded, or the His tag might interfere with the native conformation/stability of the SpAKr. Alternatively, the native SpAK in the isolated flagella tightly binds to axonemal proteins and an activating factor may increase the AK V_{max} value. Analysis of the sea urchin *S. purpuratus* genome predicts five other AK isozymes, all having single catalytic domains. The pH dependencies of the AK activities compared between SpAKr and natural SpAK show similar curves, but the pH optimum is slightly higher in SpAKr (8.1) as compared to the natural protein (7.7) (*2*).

Ca^{2+} inhibited the SpAKr activity in a concentration-dependent manner (Fig. 3C). Concentrations of 1 mM are not found in living cells, the normal range of free Ca^{2+} being 10^{-7}–10^{-6} molar. What is interesting in Fig. 3C is the decrease of about 27% in the SpAKr activity at zero-free Ca^{2+} compared to 10^{-7} M. At higher concentration, for example, in rod outer segments of bovine retina, 10^{-3} Ca^{2+} inhibits the AK activity (*27*). The motility of flagella and cilia is also regulated by Ca^{2+} concentrations (*28*, *29*). In sea urchin sperm, demembranated and then ATP reactivated, motility ceased in 10^{-4} M free Ca^{2+} (*30*, *31*). Concentrations of 10^{-6}–10^{-5} M Ca^{2+} increase the asymmetry of the flagellar waveform and the microtubule sliding pattern (*32*). This study shows that SpAK could be one candidate enzyme negatively regulated by Ca^{2+}.

Egg jelly increases sperm PKA activity by the increase in adenylate cyclase activity (*33*) and the subsequent elevation of cAMP (*34*, *35*). When sea urchin sperm are treated with egg jelly, 130 kDa SpAK is phosphorylated (*17*). The sequence of 130 kDa SpAK shows three PKA phosphorylation sites (*2*). In this study, we found that SpAKr can be phosphorylated *in vitro* by treatment with PKA (Fig. 4A). According to the specificity for the PKA substrate antibody used in this study, at least Thr^{246} could be the target of PKA phosphorylation. After SpAKr phosphorylation, the K_m value for ATP synthesis from ADP does not change. To support this result, phosphorylation or dephosphorylation by treatment of sperm with egg jelly did not change SpAK enzymatic kinetics. Also, *in vitro* phosphorylation or dephosphorylation by treatment with bovine heart PKA or ALP did not change the natural SpAK activity (*2*). In this study, a 19% increase of V_{max} in SpAKr was observed with phosphorylation by PKA (Fig. 4B). The phosphorylation of SpAK might alter its affinity for axonemal proteins without changing its kinetics. SpAK and *Chlamydomonas* AK are the only AKs known with

three catalytic domains. Now that SpAK can be expressed in active form, site-directed mutagenesis could be used to determine the importance of each residue to its activity.

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