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A novel pyruvate kinase (PK-S) from boar spermatozoa is localized at the fibrous sheath and the acrosome

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Abstract

Boar spermatozoa contain a novel pyruvate kinase (PK-S) that is tightly bound at the acrosome of the sperm head and at the fibrous sheath in the principal piece of the flagellum, while the midpiece contains a soluble pyruvate kinase (PK). PK-S could not be solubilized by detergents, but by trypsin with no loss of activity. Purified PK-S as well as PK-S still bound to cell structures and soluble sperm PK have all kinetics similar to those of rabbit muscle PK-M1. The PK-S subunit had a relative molecular mass of $64 \pm 1 \times 10^3$ ($n = 3$), i.e. slightly higher than that of PK-M1, and carried an N-terminal extension (NH_2-TSEAM-COOH) that is lacking in native PK-M1. Evidence is provided that PK-S is encoded by the *PKM* gene. Antibodies produced against the N-terminus of purified PK-S (NH_2-TSEAMPKAHMDAG-COOH) were specific for PK-S as they did not react with somatic PKs or soluble sperm PK, while anti-PK-M1 recognized both sperm PKs. Immunofluorescence microscopy showed anti-PK-S to label the acrosome and the flagellar principal piece, whereas the midpiece containing the mitochondria was labelled only by anti-PK-M1. Immunogold labelling confirmed the localization of PK-S at the acrosome. In the principal piece, both polyclonal anti-PK-M1 and anti-PK-S were found at the fibrous sheath. Our results suggest that PK-S is a major component in the structural organization of glycolysis in boar spermatozoa.

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Introduction

Mammalian spermatozoa are elongated and polarized motile cells with functionally different compartments. The head contains the nucleus with condensed chromatin which has to be transferred to an egg for fertilization. At the front part, the nucleus is covered by the acrosome which releases hydrolytic enzymes to facilitate penetration of the zona pellucida of an egg. The flagellum harbours the central axoneme with the machinery for swimming. Its dynein-ATPases hydrolyse ATP to ADP and inorganic phosphate thus providing free energy for flagellar beating. The axoneme is surrounded by dense fibres which probably improve stability and elasticity of the flagellum. The sperm head is connected to the relatively short midpiece of the flagellum where all sperm mitochondria are concentrated surrounding the dense fibres. The principal piece lacks mitochondria but contains the fibrous sheath, a structure typical for mammalian (Fouquet & Kann 1994) and some reptilian (Jamieson *et al.* 1996) spermatozoa. It appears segmented by semi-circular ribs connecting two longitudinal columns thus providing flagellar flexibility.

Mammalian spermatozoa use extracellular sugars as the main substrate for ATP production (Mann & Lutwak-Mann 1981, Kamp *et al.* 1996, Marin *et al.* 2003), but species-specific differences have been reported (Rodriguez-Gil 2006). Glycolysis seems to be essential for fertilization in sperm of mouse, rat, hamster and man but not in bovine sperm (Miki *et al.* 2004, Galantino-Homer *et al.* 2004). The mechanisms by which glycolysis affects fertility of spermatozoa remain still not clear. Glycolysis is involved in capacitation by stimulation of protein tyrosine phosphorylation (Urner & Sakkas 2003) and/or in providing the principal piece of the flagellum with ATP particularly for the vigorous whiplash motility (hyperactivity) that produces the thrust to penetrate the zona pellucida (Yanagimachi 1994, Bedford 1998, Bone *et al.* 2000, Williams & Ford 2001). Whether local glycolytic ATP-production is essential for sperm motility or can be substituted by other means has comprehensively been discussed by Ford (2006). Several glycolytic enzymes show unusual properties, suggesting that glycolysis in the principal

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piece is structurally organized along the fibrous sheath of the flagellum (Visconti *et al.* 1996, Westhoff & Kamp 1997, Bunch *et al.* 1998, Travis *et al.* 1998, Krisfalusi *et al.* 2006). Sperm specific isozymes are known for hexokinase (HK1-S; Travis *et al.* 1998) and glyceraldehyde 3-phosphate dehydrogenase in mice (GAPDH-S; Bunch *et al.* 1998). HK1-S and GAPDH-S possess unique N-terminal amino acid sequences which probably anchor the enzymes to sperm structures (see Eddy *et al.* 2003). In the flagellum of boar sperm, GAPDH is located along the fibrous sheath, whereas it could not be detected in the midpiece (Westhoff & Kamp 1997) probably because it had been removed by maturating sperm in cytoplasmic droplets together with the endoplasmic reticulum. Release of GAPDH from sedimented sperm fragments required tryptic digestion. In contrast, HK1-S was located not only at the fibrous sheath, but also at mitochondria and at membranes of the head, and unlike GAPDH it was solubilized by detergents like Triton X-100 (Travis *et al.* 1998).

Association with sperm structures had also been reported of pyruvate kinase (PK, EC 2.7.1.40) the last enzyme of glycolysis. PK of rabbit spermatozoa could not be washed out from hypotonically treated sperm, although its catalytical properties were similar to those of muscle PK (PK-M1), which is a soluble enzyme (Storey & Kayne 1980). Recently Krisfalusi *et al.* (2006) have demonstrated that PK is bound to the purified fibrous sheath of mouse spermatozoa. However, it is not known whether spermatozoa contain a somatic and/or a sperm-specific PK form and whether PK is located exclusively at the fibrous sheath. Moreover, it is still an open question by which type of binding PK is attached to sperm structures. The physiological functions of the PK-binding are also not clear. Does it serve the spatial organization of glycolysis in sperm or does it affect the kinetics of the enzyme or both?

In order to answer some of these questions we have purified the bound PK from ejaculated boar sperm and produced specific antibodies against its N-terminal amino acid sequence. Using these antibodies and polyclonal anti-PK-M1, we have studied whether boar spermatozoa contain different PKs and where these are localized. Furthermore, we have tested whether the kinetics of PK from boar spermatozoa differ from those of somatic PKs.

Materials and Methods

Samples and chemicals

Fresh ejaculates from fertile boars diluted tenfold with Beltsville thawing solution (BTS) were provided by a breeder association (59387 Ascheberg, Germany) and swine tissues as well as blood from a local butcher. Goat antibodies against PK from rabbit muscle (anti-PK-M1) were obtained from DPC Biermann (61231 Bad Nauheim, Germany), peroxidase-conjugated secondary antibodies from Sigma–Aldrich Inc., fluorescence-labelled secondary antibodies from Molecular Probes (2333 AA Leiden, The Netherlands) and gold-labelled secondary antibodies from Aurion (6702 AA Wageningen, The Netherlands).

Biochemicals and enzymes were obtained from Roche Diagnostics GmbH (68305 Mannheim, Germany) and Sigma–Aldrich Inc. Fractogel EMD SO_3^- (S) and Fractogel EMD Propyl (S) as well as other chemicals were purchased from Merck KGaA (64271 Darmstadt, Germany), Roti-Load 1, Roti-Block and glycerol from Carl Roth GmbH and Co. (76185 Karlsruhe, Germany) and the nitrocellulose membrane (Protran BA 85 Cellulosenitrate) from Schleicher and Schuell (37582 Dassel, Germany). Amersham Biosciences Europe GmbH (79111 Freiburg, Germany) provided standard proteins low molecular weight (LMW), Bio-Rad Laboratories GmbH (80939 München, Germany) the Bio-Rad Protein Assay (dye reagent concentrate). Ovalbumin and Tween 20 were purchased from AppliChem GmbH (64291 Darmstadt, Germany) and phenylmethylsulfonyl fluoride (PMSF), cold-water fish gelatine as well as 4′-6-diamidino-2-phenylindol and LR White acrylic resins from Sigma–Aldrich Inc. Other chemicals were obtained from Sigma–Aldrich Inc. and Serva Electrophoresis GmbH (69115 Heidelberg, Germany). Mounting media Mowiol was obtained from Hoechst (65926 Frankfurt, Germany).

Enzyme activity assays

Activities of PK (EC 2.7.1.40) and GAPDH (EC 1.2.1.12) were measured using coupled assays with $NADH_2$ as indicator (Bergmeyer 1983) at 25 °C and pH 7.6. The assay for PK comprised 50 mmol/l triethanolamine (TRA), 50 mmol/l KCl, 4 mmol/l $MgCl_2$, 0.2 mmol/l $NADH_2$, 1 mmol/l phosphoenolpyruvate (PEP), 1 mmol/l ADP, lactate dehydrogenase (4 U/ml); for GAPDH 50 mmol/l TRA, 1 mmol/l EDTA, 4 mmol/l $MgSO_4$, 2.4 mmol/l glutathione (reduced), 0.2 mmol/l $NADH_2$, 1 mmol/l ATP, 7 mmol/l 3-phosphoglycerate, phosphoglycerate kinase (10 U/ml). If PK was measured in samples (e.g. extracts) containing adenylate kinase, its specific inhibitor diadenosine pentaphosphate (AP_5A) was added (10 µmol/l final concentration). The assays of enzyme activity were verified by testing whether the amount of substrate transformed was linear with the amount of enzyme. Enzyme activities are given as U (µmol of substrate transformed per minute, 25 °C) together with S.D. ($\pm$S.D.) and the number n of independent measurements.

Extraction of PK from spermatozoa

Boar spermatozoa were separated from seminal plasma and BTS by centrifugation (3000 ***g***; 17 °C,

15 min) and stored at −20 °C. Thawed sperm were resuspended in 10 ml/g buffer (pH 7.3) comprising 25 mmol/l sodium phosphate, 1 mmol/l EDTA and 1 mmol/l dithiothreitol (DTT). Sperm were disrupted by sonication (Branson Sonifier, 6×6 s with intermittent cooling). The crude homogenate was centrifuged (10 000 ***g***; 4 °C; 4 min) and the supernatant discarded. The sediment was washed twice in the same buffer, sonicated and centrifuged as before. Finally the sediment was resuspended in sodium phosphate buffer (10 mmol/l; 1 mmol/l EDTA, 1 mmol/l DTT; pH 7.3) and incubated with trypsin (3 mg/g sperm wet weight) at room temperature for 5 min. Trypsin was then inhibited by adding 20 µl of 0.1 mol/l PMSF per gram of sperm. The sample was immediately centrifuged at 40 000 ***g*** at 4 °C for 20 min. The supernatant was used for purification of the proteolytically solubilized PK (PK-S).

Purification of PK-S

The solubilized PK was purified by ion exchange and hydrophobic interaction chromatography. The supernatant containing PK was adjusted to pH 7.15 and applied to a column of Fractogel EMD SO_3^- (S) (bed volume 10 ml, flow rate 2 ml/min) that had been equilibrated with sodium phosphate buffer (10 mmol/l; pH 7.15) containing 1 mmol/l DTT and 10% (v/v) glycerol. The column was extensively rinsed overnight with the same buffer. Proteins were eluted by increasing the phosphate concentration stepwise first to 15 mmol/l (flow rate 3 ml/min), then to 38 mmol/l (flow rate 5 ml/min). The activities of PK and GAPDH were determined in fractions of 5 ml (in the 15 mmol/l eluate) and 3 ml (in the 38 mmol/l eluate). The fractions of the main PK peak were combined and adjusted to pH 7.3 and 30% (w/v); $(NH_4)_2SO_4$. The sample was then applied to a column of Fractogel EMD Propyl (S) (bed volume 10 ml, flow rate 2 ml/min) that had been equilibrated with sodium phosphate buffer (25 mmol/l; pH 7.3) containing 1 mmol/l EDTA and 1 mmol/l DTT as well as 30% (w/v) $(NH_4)_2SO_4$ and 10% (v/v) glycerol. The column was extensively rinsed overnight with the same buffer. Proteins were eluted by reducing the $(NH_4)_2SO_4$ concentration stepwise to 20.5, 19.5, 14, 12.5 and 11% (flow rate 2 ml/min). Activities of PK and GAPDH were measured in fractions of 5 ml (in the 20.5% eluates) and 2 ml (in the 19.5, 14, 12.5 and 11% eluates). Protein was determined according to Bradford (1976). The final purification was assessed with respect to specific activity, purification factor and PK yield as well as by SDS–PAGE.

Extraction of PK from boar tissues and erythrocytes

Fresh tissues were generally homogenized in 10 volumes (v/w) but in case of kidney in 2.5 volumes of sodium phosphate buffer (50 mmol/l; 1 mmol/l EDTA, 1 mmol/l DTT; pH 7.3) using an Ultra Turrax (6×6 s, in case of muscle 20×6 s). After centrifugation (40 000 ***g***; 4 °C, 20 min), the supernatant as well as the washed (sodium phosphate buffer, see above) and resuspended sediment were assayed for PK activity. Blood (∼750 ml) was immediately mixed with 50 ml sodium phosphate buffer (5 mmol/l; 154 mmol/l NaCl, 64.5 mmol/l EDTA; pH 8.0) to prevent agglutination. Erythrocytes were collected by centrifugation, first at 600 ***g*** (7 °C, 10 min), after which the sediment was washed with sodium phosphate buffer (5 mmol/l; 154 mmol/l NaCl, 4 mmol/l EDTA; pH 8.0) and again centrifuged at 750 ***g*** (7 °C, 10 min). The collected cells were homogenized in 2.5 volumes (v/w) of sodium phosphate buffer (5 mmol/l; 1 mmol/l EDTA, 1 mmol/l DTT; pH 7.3) by sonication (6×6 s) and centrifuged (40 000 ***g***, 4 °C, 20 min). Both supernatant and resuspended sediment were assayed for PK activity. For Western blot analysis, PK extracted from erythrocytes was concentrated by adding ammonium sulphate (0.26 g/ml; 0 °C), centrifugation of the precipitated PK (30 000 ***g***, 4 °C; 15 min) and then dissolved in H_2O.

Electrophoresis and immunoblotting

SDS–PAGE was performed according to Laemmli (1970) in a PHERO-minivert electrophoresis chamber (Biotec-Fischer GmbH, 35447 Reiskirchen, Germany). Proteins were precipitated by mixing the sample with 10% (v/v) of each 0.15% (w/v) sodium deoxycholic acid and 72% (w/v) trichloroacetic acid. The samples were centrifuged at 10 000 ***g*** for 10 min and the sediments were dissolved in Roti-Load 1 (diluted fourfold with 0.5 mol/l Tris/HCl; pH 6.8) and incubated in closed tubes at 95 °C for 6 min. Proteins were separated in SDS gels consisting of 4% stacking gel and 10% running gel at 4 °C and 70 V (stacking gel) and at 130 V (running gel). Proteins were then stained with Coomassie blue or transferred to a nitrocellulose membrane by Western blotting using a Trans-Blot Semi-Dry (Bio-Rad Laboratories; 15 V, $\leq$5.5 mA/cm^2, 20 min). LMW standard proteins for the determination of the relative molecular mass (M_r) were phosphorylase, 97×10^3; albumin, 66×10^3; ovalbumin, 45×10^3; carbonic anhydrase, 30×10^3 and trypsin inhibitor 20.1×10^3.

Before the immunochemical test, protein transfer from the SDS gel to the membrane was assessed by reversible protein staining with Ponceau S (0.2% (w/v) in 3% (v/v) acetic acid) and compared with the corresponding SDS gel. After washing the membrane four times with PBS (139 mmol/l NaCl, 12 mmol/l Na_2HPO_4, 3.6 mmol/l KH_2PO_4; pH 7.2), non-specific protein-binding sites were blocked with Roti-Block (diluted 1:10 with H_2O) at room temperature for 1 h. The membrane was washed again four times in PBS and was then incubated overnight at room temperature with the primary antibody

(goat anti-PK-M1 1:12 500 or rabbit anti-PK-S 1:500) in Roti-Block (1:10 diluted with H_2O) which was further diluted in the ratio of 1:3 with PBS. The membrane was then washed with PBS and incubated with the appropriate peroxidase-conjugated secondary antibody (anti-goat IgG or anti-rabbit IgG (each 1:10 000 diluted in the same solution as the primary antibody)) for 1 h at room temperature, and washed again four times in PBS. The immune complexes were visualized by incubation with 3,3′-diaminobenzidine for 1–3 min. The incubation was stopped by washing the membrane with PBS.

Sequencing of the N-terminus of purified boar sperm PK-S

For partial sequencing of the structure-bound PK-S from boar spermatozoa, the purified enzyme was run on SDS–PAGE (10% gel, 0.75 mm thick) and transferred by Western blotting (semi-dry phase) to a PVDF-membrane. Proteins on the membrane were stained with Coomassie blue. The PK band at 64.5×10^3 was cut out for partial sequencing which was performed with an amino acid sequenator ABI 473A at Würzburg University (Theodor-Boveri-Institut, Physiologische Chemie II, Biozentrum, 97074 Würzburg, Germany).

Production of antiserum against PK-S

A polyclonal antiserum against boar sperm PK-S was produced by CovalAb (69007 Lyon, France). Before immunization the pre-immune sera of two rabbits were tested for unspecific immune reactions. An appropriate rabbit (New Zealand White, Norm SPF (specific pathogen free)) was then immunized following a standardized protocol (CovalAb). Purified PK-S (4×100 μg) was injected on multiple spots on day 0 (dissolved in complete Freund's adjuvant) and, for the booster injections, on days 21, 42 and 63 (in incomplete Freund's adjuvant). Terminal blood collection was on day 88.

Purification of antibodies

From the above antiserum, antibodies (anti-PK-S) were immunopurified using a peptide-coupled sepharose column (peptide synthesis and peptide immobilization on sepharose beads were also performed by CovalAb). To verify the specificity of the partial N-terminal amino acid sequence of PK-S, protein databases (Swiss-Prot and TrEMBL) were searched. A peptide (NH_2-TSEAMP-KAHMDAG-COOH) corresponding to the N-terminus of purified PK-S (see Fig. 3) was synthesized by CovalAb and coupled to sepharose in order to purify antibodies against this peptide by immunoaffinity chromatography of the final antiserum generated against PK-S.

The antiserum (10 ml) was diluted in the ratio of 1:1 with PBS and mixed with the peptide-sepharose (1 ml in 5 ml PBS containing 0.02% (w/v) sodium azide). The sepharose-serum-suspension was carefully shaken at 37 °C for 1 h and then incubated overnight at 4 °C. The suspension was filled in a column, washed with 15 ml PBS containing 0.1% (v/v) Tween 20 and then with 30 ml PBS, while release of protein was followed at 280 nm. Antibodies were eluted by stepwise increasing the percentage (10, 20, 30, 40, 60, 80 and 100% (v/v)) of a glycin solution (100 mmol/l; pH 2) in PBS. Fractions of 1 ml each were collected in vessels containing 200 μl Tris (1 mol/l; pH 8). Various fractions were used in immunoblotting to identify fractions that reacted specifically with PK-S.

Immunofluorescence labelling of sperm PK

Boar ejaculate was diluted in the ratio of 1:10 in BTS and 5 μl of this sperm suspension were each smeared on a poly-L-lysine-precoated coverslip and left to dry. Sperm were then permeabilized with 0.1% (v/v) Triton X-100 in PBS at room temperature for 5 min. Thus, pretreated sperm were incubated, first with 0.01% (v/v) Tween 20 in PBS, then with 50 mmol/l NH_4Cl in PBS for 10 min each and washed again with PBS before being treated with blocking solution (0.5% (w/v) cold-water fish gelatine plus 0.1% (w/v) ovalbumin in PBS) for 2 h at room temperature to occupy non-specific-binding sites. The spermatozoa were then incubated with primary antibodies (either goat anti-PK-M1 1:10 000 or rabbit anti-PK-S 1:5) in blocking solution overnight at 4 °C. On the following day, after washing with PBS, sperm were incubated, for 2 h at room temperature in the dark, with secondary antibodies that were fluorescence-labelled (anti-goat IgG, Alexa Fluor 546 or anti-rabbit IgG, Alexa Fluor 568; dilution 1:400 in PBS). Finally sperm were washed in PBS and in bidistilled water and mounted in Mowiol 4.88. Fluorescence labelling was analysed with a DM RP microscope (Leica, 64625 Bensheim, Germany). Images were taken with a Hamamatsu ORCA ER CCD camera (Hamamatsu, 82211 Herrsching, Germany) and processed using Photoshop (Adobe Systems Inc., Version 7.0).

Immunogold labelling

Fresh boar sperm were fixed and embedded in LR White as previously described (Westhoff & Kamp 1997). Ultrathin sections were collected on polyvinyl formal (Formvar)-coated nickel grids, etched for 2 min with saturated sodium periodate and further processed for immunogold labelling as described by Wolfrum & Schmitt (2000). The antibodies were diluted as follows: goat anti-PK-M1 1:10 000; rabbit anti-PK-S 1:5; anti-goat

IgG 1:15 and anti-rabbit IgG 1:28. Sections were counterstained for 10–20 min with 2% (w/v) aqueous uranyl acetate and subsequently for 2 min with lead citrate according to Hanaichi *et al.* (1986). Immunogold labelling was analysed by electron microscope (FEI Tecnai 12 Biotwin; 5600 KA Eindhoven, The Netherlands).

As an additional control for antibody specificity, primary antibodies were pre-incubated with their respective antigens at 37 °C under gentle agitation for 1 h and subsequently at 4 °C for 2 h. The immune complexes were then sedimented (10 000 ***g***; 4 °C, 15 min) and the supernatant was used as in immunogold labelling. One hundred micrograms of the sperm-specific PK-peptide per 6 µl immunopurified antibodies and 160 µg rabbit muscle PK per microgram of anti-rabbit muscle PK were used for pre-absorption of primary antibodies.

In case of immunogold localization with anti-PK-S, the labelling density of the fibrous sheath was determined. The area of the fibrous sheath in an electron micrograph was calculated by pixel counting based on scale bars (μm^2 per pixel). All gold particles in this area of fibrous sheath were counted. Labelling density is the number of gold particles per micrometer square of fibrous sheath area and given as mean ± S.D., based on counting four separate electron micrographs of slices treated with anti-PK-S (total fibrous sheath area 0.98 μm^2) as well as four controls treated with pre-incubated anti-PK-S (total fibrous sheath area 1.14 μm^2). Counting was done without knowing how the specimens had been treated. Statistical significance was analysed with a Student's *t*-test. Results were considered to be significant if $P<0.05$.

Results

Extraction of PK from boar sperm

When boar sperm were sonicated in sodium phosphate buffer (25 mmol/l; pH 7.3) and centrifuged (100 000 ***g***, 1 h) more than 60% of the total PK activity, which was 0.41 ± 0.14 U per 10^8 spermatozoa (at 25 °C, $n=8$), was sedimented. This PK could not be washed out from the sediment by phosphate buffer, indicating that soluble and sedimented PKs are not in equilibrium. For better differentiation we designate the tightly bound sperm PK as PK-S. Triton X-100, which solubilized sperm hexokinase (Travis *et al.* 1998), did not solubilize PK-S in the range of 0.5–3% (v/v in 25 mmol/l NaP_i, 1 mmol/l EDTA and 1 mmol/l DTT; pH 7.3), but the bound PK-S was considerably activated by Triton X-100 (>50%), whereas the activity of soluble PK was not affected. A brief tryptic digestion, however, as had been used for solubilizing GAPDH (Westhoff & Kamp 1997), did release catalytically active PK-S from boar sperm structures. Trypsin (at 3 mg/g sperm wet weight) solubilized about 80% of PK-S in 5 min at room temperature with no loss of catalytic activity. Consequently, trypsin treatment was efficient for purification of PK-S (Table 1). However, GAPDH was also prominent in extracts after trypsin treatment of sperm sediment and had to be separated from PK-S by chromatography.

Purification of boar sperm PK-S

The trypsin extract containing solubilized PK-S was applied to a column of Fractogel EMD SO_3^- (S) and eluted with 38 mmol/l phosphate buffer. Although this ion exchange chromatography increased sevenfold the specific activity of PK-S (Table 1), it was not efficient in separating PK-S from GAPDH (Fig. 1A). Separation was achieved by hydrophobic interaction chromatography on Fractogel EMD Propyl (S) to which both enzymes were bound at 30% ammonium sulphate (Fig. 1B). In presence of 20.5% ammonium sulphate PK-S remained bound to the column, whereas most GAPDH was released. PK-S was eluted at 14% ammonium sulphate. The combined PK-S fractions had a specific activity of 116.7 U/mg protein. Thus, the purification factor for PK was almost 1000, but the preparation still contained traces of GAPDH (M_r 42×10^3) in SDS–PAGE (Table 1 and Fig. 2A). The prominent protein at 64.5×10^3 was identified as PK in a Western blot using anti-rabbit

Table 1 Purification of boar sperm PK-S.

	Volume (ml)	Protein (mg)	Pyruvate kinase activity (U) Total	Per milligram of protein	Yield (%) of PK-S	Purification factor	PK:GAPDH ratio
Sperm homogenate[a]	720	9415	1085	0.12			
Supernatant after trypsin incubation	390	243	720	2.96	100	24.7	
PK fractions after ion exchange chromatography[b]	198	22.7	461	20.3	64	169.2	0.2
PK fractions after hydrophobic interaction chromatography[b]	12	2.1	245	116.7	34	972.5	32.1

[a]Sperm homogenate of 88 g frozen sperm was obtained by sonication and the resulting sperm fragments were centrifuged and washed before bound PK (PK-S) was solubilized by trypsin (see Materials and Methods). [b]After centrifugation solubilized PK-S was subjected to ion exchange chromatography (see Fig. 1A) and hydrophobic interaction chromatography (see Fig. 1B).

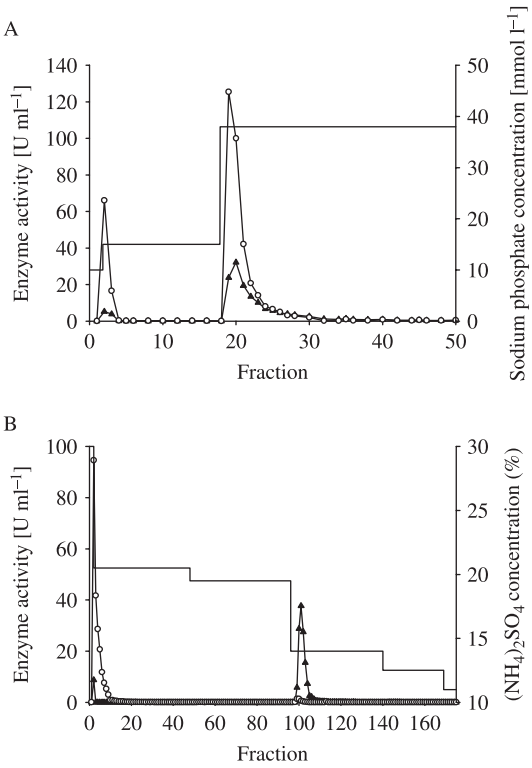

Figure 1 Elution profiles of PK-S and GAPDH activities extracted from boar spermatozoa by trypsin treatment and subjected to an ion exchange (A) and subsequently to a hydrophobic interaction chromatography (B). (A) Extract of trypsin-treated sperm fragments containing solubilized PK-S and GAPDH were chromatographed on Fractogel EMD SO_3^- (S). Both enzymes were eluted by stepwise increasing the sodium phosphate concentration. Most PK-S (–▲–) but also much GAPDH (–○–) activity was eluted with 38 mmol/l sodium phosphate. (B) The fractions of PK-S and GAPDH eluted at 38 mmol/l sodium phosphate from chromatography (A) were pooled and adjusted to 30% $(NH_4)_2SO_4$ and pH 7.3. Applied on Fractogel EMD Propyl (S) nearly all GAPDH (–○–) was eluted by reducing $(NH_4)_2SO_4$ to 20.5%, whereas most PK-S activity (–▲–) appeared in a narrow peak at 14% $(NH_4)_2SO_4$.

muscle PK (anti-PK-M1; Fig. 2B). This PK-S preparation was used for producing an antiserum in rabbit.

Kinetics of somatic and sperm PKs

PK isozymes from mammalian tissues differ in their kinetic properties. Muscle (PK-M1) is characterized by high affinity for the substrate PEP. It lacks activation by fructose 1,6-bisphosphate (F1,6P_2) and inhibition by L-alanine, two properties that are typical for the liver isozyme (PK-L) as well as for the isozymes PK-M2 and PK-R (see Table 2). PK-S bound to boar sperm structures showed kinetic behaviour similar to that of PK-M1. Solubilization of PK-S by trypsin did not significantly change the kinetics suggesting that binding to cell structure did not influence the catalytic properties of boar sperm PK-S. Soluble sperm PK also showed similar kinetics as PK-S and PK-M1 (not shown). In control

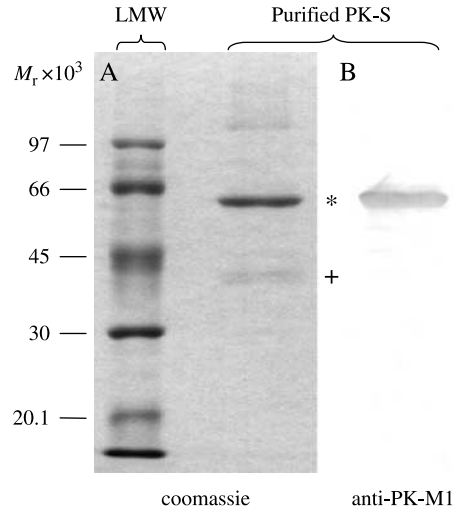

Figure 2 SDS–PAGE (A) and Western blot (B) of PK-S purified from boar spermatozoa. (A) The purified boar sperm PK-S gave rise to a prominent protein band at 64.5×10^3 (*). LMW standard proteins (lane 1) are listed in Materials and Methods. The minor band at M_r 42×10^3 (+) is probably due to GAPDH (see text). (B) Boar sperm PK-S was recognized by anti-rabbit PK-M1. This PK-S preparation was used for antibody production. PK-S was also cut out from a blot and used for N-terminal sequencing.

experiments, we extracted PK-L from mouse liver and showed it to be activated by F1,6P_2 and inhibited by L-alanine (data not shown), properties that we did not observe with rabbit muscle and boar sperm PKs assayed under identical conditions.

Sequencing of boar sperm PK-S

The purified boar sperm PK-S was run on SDS–PAGE (Fig. 2) and blotted onto PVDF, and the prominent PK band was cut out for sequencing the N-terminus. Eighteen of the first 19 amino acids were identified: NH_2-TSEAMPKAHM-DAGXAFIQT-COOH (X = unidentified). Comparison with sequences from protein databases (Swiss-Prot, TrEMBL) revealed that 7–11 of 14 amino acids were identical with the N-termini of PK-M1 or PK-M2 if the first five amino acids of the solubilized PK-S were neglected (Fig. 3). When compared with the corresponding sequences of PK-L or PK-R, only 4 out of 14 amino acids were identical. The sequences of PK-M1 which were derived from protein sequencing indicate that PK-M1 lacks the five N-terminal amino acids that were found in the solubilized PK-S. Consequently, the designation of the structurally bound boar sperm PK as PK-S is justified.

Most PK sequences in protein databases have been derived from the analysis of cDNA. One of the PK-M2 (Homo) thus deduced would have an N-terminal extension including the sequence NH_2-TSAA-COOH where PK-S has NH_2-TSEA-COOH. Another sequence, most probably encoding a PK, derived from a cDNA library of bovine ileum, showed the sequence NH_2-TSEA-COOH in an N-terminal extension at the same

Table 2 Comparison of boar sperm PK-S (structure bound and solubilized by trypsin) and somatic pyruvate kinases with respect to kinetics and molecular mass.

	PK-S					
Isozyme	Bound	Solubilized	**PK-M1**[a]	**PK-L**[a]	**PK-R**[a]	**PK-M2** (−A)[a]
Source	Boar sperm		Skeletal muscle, cardiac muscle, and brain	Liver (parenchymal cells)	Erythrocytes	Kidney, lung, leukocytes, adipose tissue, liver (stromal cells), and fetal tissues
Relative molecular mass/molecular weight per subunit ($\times 10^{-3}$)		64±1	52.5–62.5	50—60	50–60	47.5–62.5
K_m (PEP; mM)	0.02–0.05	0.02–0.03	0.04–0.09	0.3–1.0	0.5–0.6	0.2–0.4
K_m (ADP; mM)	0.52–0.56	0.46	0.3–0.4	0.1–0.4	0.4–0.6	0.2–0.4
Activation by $F1,6P_2$	No[b]	No[b]	No[b]	Yes[b]	Yes	Yes
Inhibition by L-alanine	No[b]	No[b]	No[b]	Yes[b]	Yes	Yes

[a]Data from Hall & Cottam (1978). [b]The effect of fructose 1,6-bisphosphate ($F1,6P_2$, 0.2 mmol/l) was assayed at 0.1 mmol/l PEP, phosphoenolpyruvate, the effect of L-alanine (1 mmol/l) at 1 mmol/l PEP. ADP was given at 1 mmol/l in all assays.

position as in PK-S (see Fig. 3). Interestingly, in the bovine ileum PK this sequence is preceded by a lysine thus defining a trypsin cleavage site. A lysine at the same position in boar sperm PK-S would explain the tryptic cleavage of this enzyme from boar sperm structures and suggest that the N-terminus of native PK-S is longer than that obtained by tryptic cleavage.

Specificity of anti-PK-S

The N-terminal peptide (NH_2-TSEAMPKAHMDAG-COOH) of purified boar sperm PK-S was synthesized and immobilized on sepharose for purification of PK-S antibodies from the rabbit antiserum as described in Materials and Methods. The purified anti-PK-S was tested for specificity in Western blots after SDS–PAGE (Figs 4–6). One fraction of anti-PK-S eluted from the peptide-sepharose column specifically labelled PK-S. In contrast, rabbit muscle as well as boar sperm GAPDH were not or only faintly labelled even if the protein concentrations of these enzymes were high (Fig. 4). PK-S was detected in extracts of trypsin-treated boar sperm fragments (Fig. 4, TSP) and in an enriched PK-S preparation which was still contaminated with GAPDH (Fig. 4, PK/GAPDH). Boar sperm extract not treated with trypsin was not labelled although it contained soluble PK (Fig. 4, SP). Even when enriched by ion exchange and hydrophobic interaction chromatography soluble sperm PK (sPK) was not detected by anti-PK-S (Fig. 5). Hence, the anti-PK-S discriminates between PK-S and soluble PK from boar spermatozoa and can be regarded as specific for PK-S, which is a prerequisite for identification and localization of PK-S by fluorescence or immunogold labelling. In contrast to anti-PK-S, anti-rabbit PK-M1 detected both the soluble boar sperm PK and PK-S (data not shown). Specificity of anti-PK-S for sperm PK-S was also indicated by the observation that it did not recognize PKs from boar organs and cells such as muscle, liver, kidney or erythrocytes, whereas anti-PK-M1 did (Fig. 6). Unlike sperm PK-S, all PKs of these somatic cells did not require tryptic digestion for solubilization. More than 90% of their activities were extracted in sodium phosphate buffer.

Localization of PK-S in boar sperm

Ejaculated boar spermatozoa were permeabilized (with Triton X-100) on poly-L-lysine-precoated coverslips and incubated with anti-PK-S (Fig. 7A and C) and anti-PK-M1 (Fig. 7B and D) for immunofluorescence microscopy. Figure 7A and B shows differential interference contrast (DIC) images merged with antibody fluorescence, whereas in Fig. 7C and D only the red fluorescence is seen. Incubating sperm with anti-PK-S resulted in compartmentation of the fluorescence label, which is conspicuous in the principal piece of the flagellum and in the acrosomal area of the sperm head (Fig. 7A and C) but hardly noticeable in the midpiece. A significant fluorescence was also detected at the head–midpiece junction. Under otherwise identical conditions, controls without the primary antibody showed no fluorescence (data not shown).

With anti-PK-M1, the whole flagellum and the acrosomal area were stained (Fig. 7B and D). Controls without the primary antibody showed only unspecific fluorescence at the acrosome and midpiece which was much weaker than the reaction to anti-PK-M1 (data not shown).

In summary, labelling of sperm with both antibody preparations indicates that PK-S is mainly confined to the

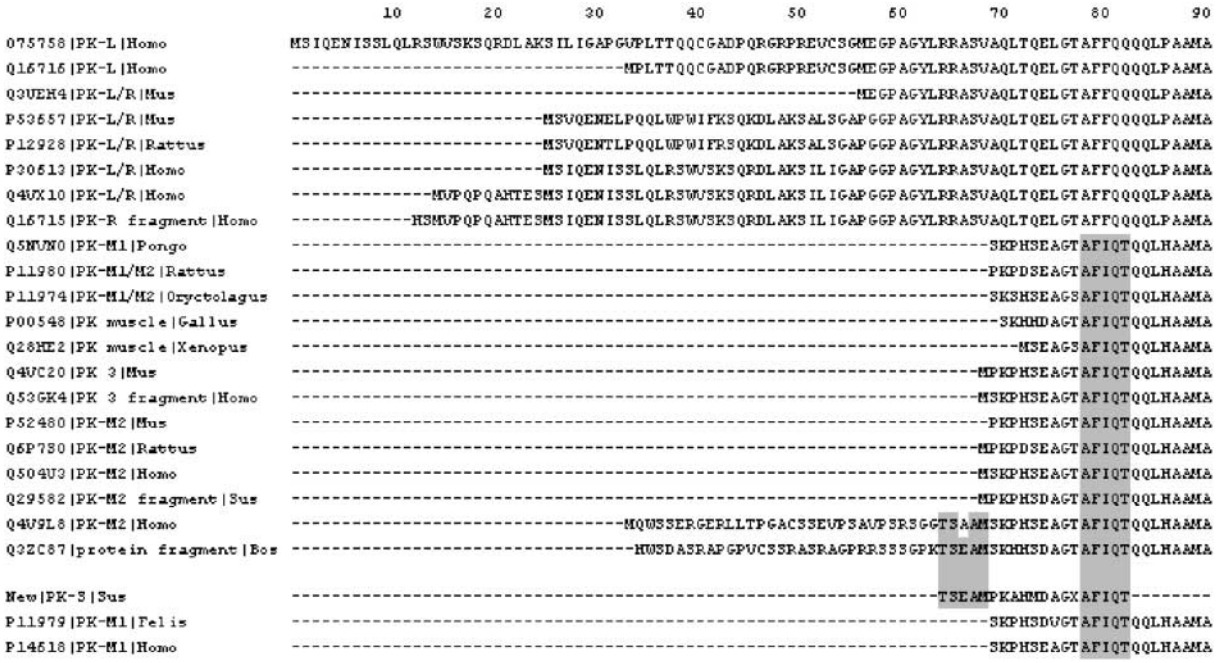

Figure 3 Comparison of the N-terminal amino acid sequences of the tryptically solubilized sperm PK-S and of somatic pyruvate kinases of various sources. Sequences of somatic pyruvate kinases obtained from Swiss-Prot and TrEMBL are compared with the N-terminal sequence of purified PK-S (New |PK-S| Sus; X = unidentified amino acid). The 21 sequences of somatic pyruvate kinases that are listed above PK-S were derived from cDNA and/or genomic DNA analyses, the sequence of PK-S and the two last sequences from protein sequencing. Except for the first five amino acids (NH_2-TSEAM-COOH), the sperm PK-S sequence shows a high degree of similarity to the respective sequences of PK-M1 and PK-M2 (50–78%). The sequence NH_2-AFIQT-COOH is present in all PK-Ms and also in PK-S (under laid), but not in PK-L and PK-R. Two PK-M sequences (Homo and Bos) deduced from cDNAs show identical or nearly the same amino acids (under laid) at corresponding positions as the N-terminal extension of PK-S.

principal piece of the flagellum and to the acrosomal area, whereas labelling of the midpiece with anti-PK-M1 only, suggests that soluble PK is present in this area.

For ultrastructural localization of PK, immunogold electron microscopy was used (Figs 8 and 9). With anti-PK-M1 relatively few gold particles were found in the midpiece, but the principal piece was markedly labelled, mainly at the fibrous sheath (Fig. 8A and B). In sperm head, the acrosomal area was labelled, but also the nucleus and the postacrosomal substance showed some gold particles (Fig. 8D and E). Controls with anti-PK-M1 pre-incubated with rabbit muscle PK were virtually free from label (Fig. 8C and F).

With anti-PK-S, labelling at the fibrous sheath (Fig. 9A and B) occurred to a lesser extent than with anti-PK-M1. Yet anti-PK-S significantly labelled the fibrous sheath when compared with controls with pre-incubated antibodies (Fig. 9C). Labelling density was 66 ± 27 vs 26 ± 5 in controls (see Materials and Methods). The acrosomal area was conspicuously labelled (Fig. 9D, E and H), while the postacrosomal substance was virtually free from labels (Fig. 9D and G). Controls with anti-PK-S

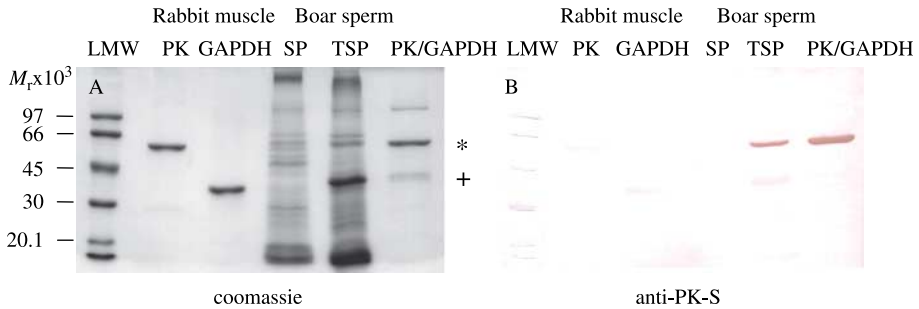

Figure 4 Specificity of antibodies purified by immune affinity from a serum raised against boar PK-S. (A) Purified rabbit muscle PK and GAPDH as well as proteins extracted from boar sperm without (SP, soluble proteins) and with trypsin treatment (TSP, tryptically solubilized proteins) as well as a sample enriched in PK-S containing traces of GAPDH (PK/GAPDH) were run by SDS–PAGE. LMW standard proteins are listed in Materials and Methods. (B) The Western blot of (A) indicates that PK-S (*) in sperm extract after trypsin treatment and in the PK/GAPDH preparation is recognized by anti-PK-S. Although their protein concentrations were high, muscle PK and GAPDH as well as boar sperm GAPDH ($^+$) were hardly stained. Other proteins extracted from boar spermatozoa were not detected. Standard proteins were faintly marked with a pencil.

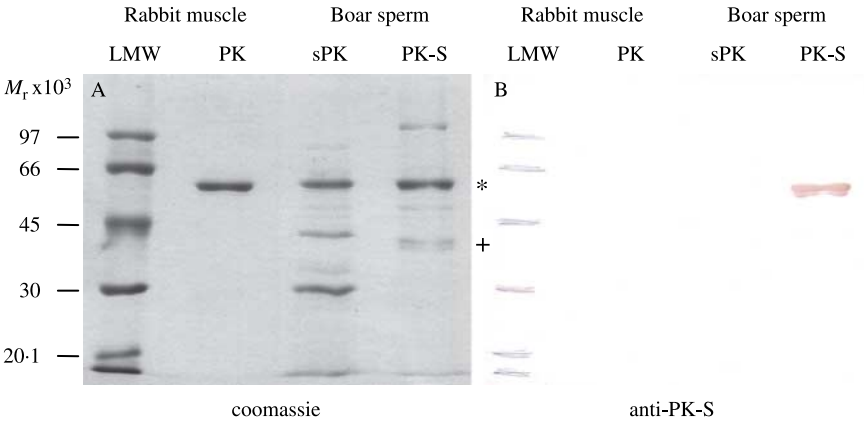

Figure 5 The soluble PK (sPK) from boar sperm is not recognized by anti-boar PK-S. To test whether or not sPK was labelled by anti-PK-S, sPK was partly purified by a procedure similar to that shown in Fig. 1 for PK-S and run on a gel (A) together with rabbit muscle PK and boar sperm PK-S. (A) sPK and PK-S produced significant protein bands (*) and weak GAPDH band ($^+$). LMW standard proteins are listed in Materials and Methods. (B) The western blot of (A) shows that anti-PK-S did recognize PK-S but neither the soluble sperm PK (sPK) nor the rabbit muscle PK. Hence, anti-PK-S is regarded specific for sperm PK-S. LMW standard proteins were marked with a pencil after reversible protein staining.

that had been pre-incubated with the PK-S-specific peptide showed only sporadic labels (Fig. 9F).

Discussion

Two PKs in boar spermatozoa

Novel forms of glycolytic enzymes in sperm were identified from nucleotide sequences of GAPDH (GAPDH-S; Welch *et al.* 1992, 2000, 2006) and HK (HK1-S; Mori *et al.* 1993, Travis *et al.* 1998). The corresponding amino acid sequences of both enzymes have unusual N-termini. While GAPDH-S is N-terminally extended, HK1-S lacks the N-terminal porin-binding domain of HK1, which is replaced by a spermatogenic cell-specific region. During spermatogenesis both proteins appear first in spermatids. The PK-S of boar spermatozoa is the third glycolytic enzyme containing an unusual N-terminus. PK-S is similar to the native muscle isozyme PK-M1, but like GAPDH-S N-terminally extended (see Fig. 3: P11979|PK-M1|Felis, Muirhead *et al.* 1986; P14618|PK-M1|Homo, Gevaert *et al.* 2003), so that its relative molecular mass slightly exceeds that of rabbit PK-M1.

Two genes, *PKM* and *PKL*, are known for mammalian PK isozymes. PK-M1 and PK-M2 are encoded by the *PKM* gene in rat and man and differ due to alternative splicing of the same primary transcript (Takenaka *et al.* 1989, 1991). In case of the *PKL* gene, alternative tissue-specific promoters lead to the production of *PK-L* and *PK-R* transcripts in rat (Noguchi *et al.* 1987). The N-termini of PK-L and PK-R are considerably extended when compared with PK-M1/M2 and might be responsible for differences in kinetic properties (cf. Muirhead *et al.* 1986). The sequenced part of the novel isoform PK-S is more similar (7–11 of 14 amino acids identical) to PK-M1 and PK-M2, than to PK-L and PK-R (4 out of 14 amino acids identical) if the first five amino acids of the N-terminus of PK-S were neglected. Especially the highly conserved sequence NH_2-AFIQT-COOH is present in PK-M1/M2 and in PK-S, but not in PK-L/R (see Fig. 3, positions 79–83). Therefore, we assume that PK-S is encoded by the *PKM* gene and that the different N-terminus arises from alternative initiation or posttranscriptional modification.

The N-terminus of the native PK-M1 begins at position 70 (see Fig. 3) which is preceded by a methionine in the N-terminal extension of PK-S (NH_2-TSEAM-COOH). This methionine is also present in some sequences deduced from cDNA/mRNA or genomic DNA for PK-M1 and PK-M2 (see Fig. 3). From human cDNA (source: chondrosarcoma lung metastasis), a sequence of mRNA for PK-M2 has been derived that contains an AUG start codon upstream of an extension sequence so that the deduced protein would have an N-terminal extension with a methionine at position 35 and a sequence similar to PK-S at positions 65–69 (TrEMBL accession number Q4V9L8; Fig. 3). In addition a hypothetical protein from bovine ileum (TrEMBL accession number Q3ZC87) seems to be an N-terminally extended PK-M1 or PK-M2 with the same

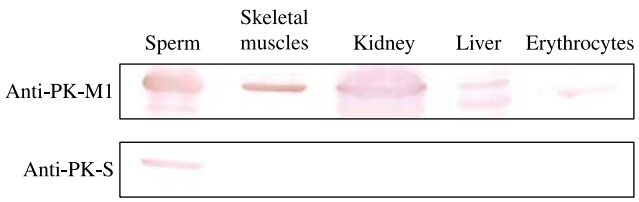

Figure 6 Western blots of extracts from boar organs and cells incubated with antibodies directed against PK-M1 and PK-S. Unlike sperm PK-S, pyruvate kinases were readily extracted from somatic organs and cells by homogenization in phosphate buffer (see Materials and Methods) and were recognized by anti-PK-M1 but not by anti-PK-S. Anti-PK-S stained only PK-S which is further evidence that PK-S is a sperm specific enzyme.

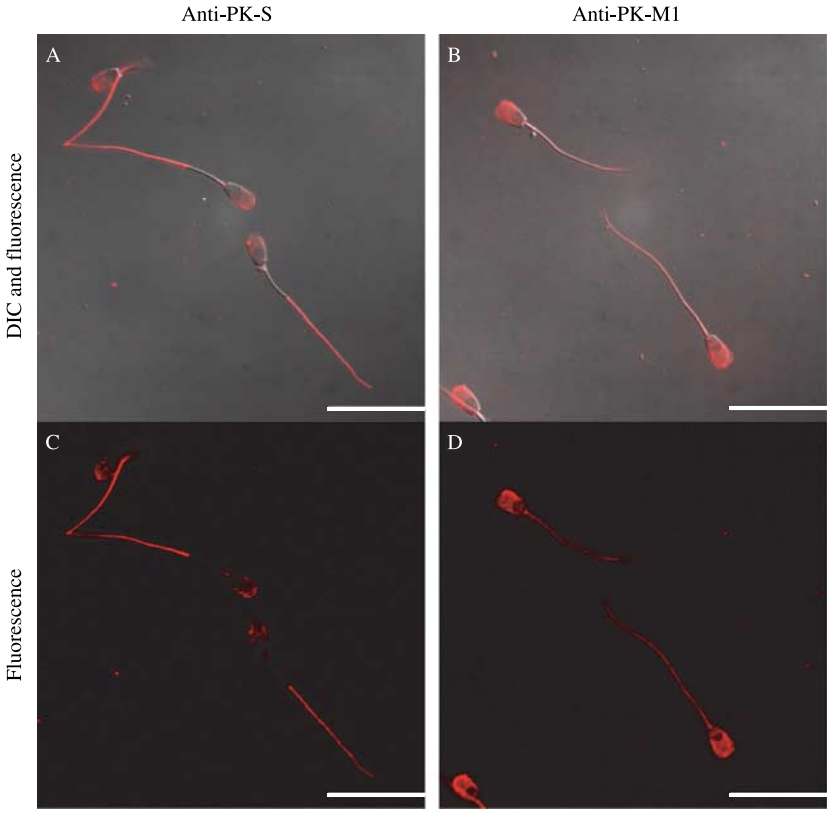

Figure 7 Localization of PK in boar spermatozoa by immunofluorescence microscopy. Immunofluorescence of boar sperm merged with differential interference contrast (DIC) images is shown in (A and B), fluorescence only in (C and D). Rabbit anti-PK-S (A and C) and goat anti-PK-M1 (B and D) were used for labelling PK in boar spermatozoa. With anti-PK-S the principal piece of the flagellum and the acrosome as well as the head–midpiece junction are specifically labelled. In contrast to anti-PK-S, anti-PK-M1 labelled the midpiece as well. The control without anti-PK-M1 had a weak unspecific fluorescence at the acrosome and the midpiece (not shown). Scale bars = 16.5 μm.

amino acid sequence (NH_2-TSEA(M)-COOH) as PK-S. Nucleotide sequence databases (GenBank, EMBL, DDBJ and PDB) contain *PKM* mRNAs that have sequences preceding the 5′-site of the predicted start codon AUG that encode amino acids similar to those of the extension of PK-S (Fig. 10). This is a further indication that PK-S is encoded by the *PKM* gene. Since N-terminal sequences of native PK-M2 are not available it cannot be excluded that PK-M2 may show a similar extension as PK-S. Kinetic properties, however, indicate that PK-S resembles PK-M1 rather than PK-M2. Therefore, we assume that PK-S is an N-terminally extended PK-M1.

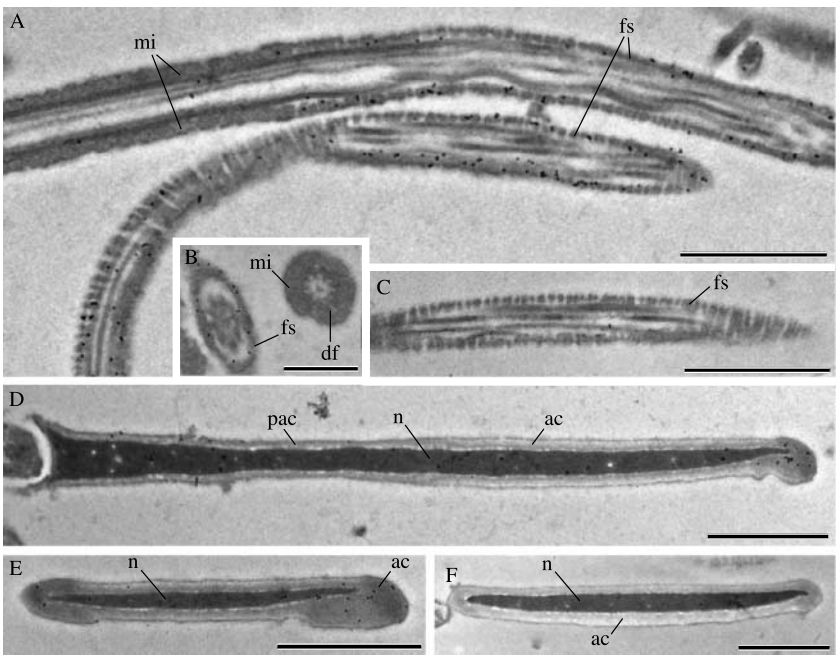

Figure 8 Ultrastructural localization of PK in boar sperm by immunogold electron microscopy using anti-PK-M1. Anti-PK-M1 significantly labelled the fibrous sheath, while only few gold particles were found on other structures of the flagellum (A and B). The area of the acrosome was also labelled (D and E). Few gold particles were found in the nucleus and the postacrosomal substance. When anti-PK-M1 was pre-incubated with rabbit muscle PK-M1, slices were not labelled (controls; C and F). Scale bars = 1 μm, in *B* = 0.5 μm. ac, acrosome; df, dense fibres; fs, fibrous sheath; mi, mitochondria; n, nucleus; pac, postacrosomal substance.

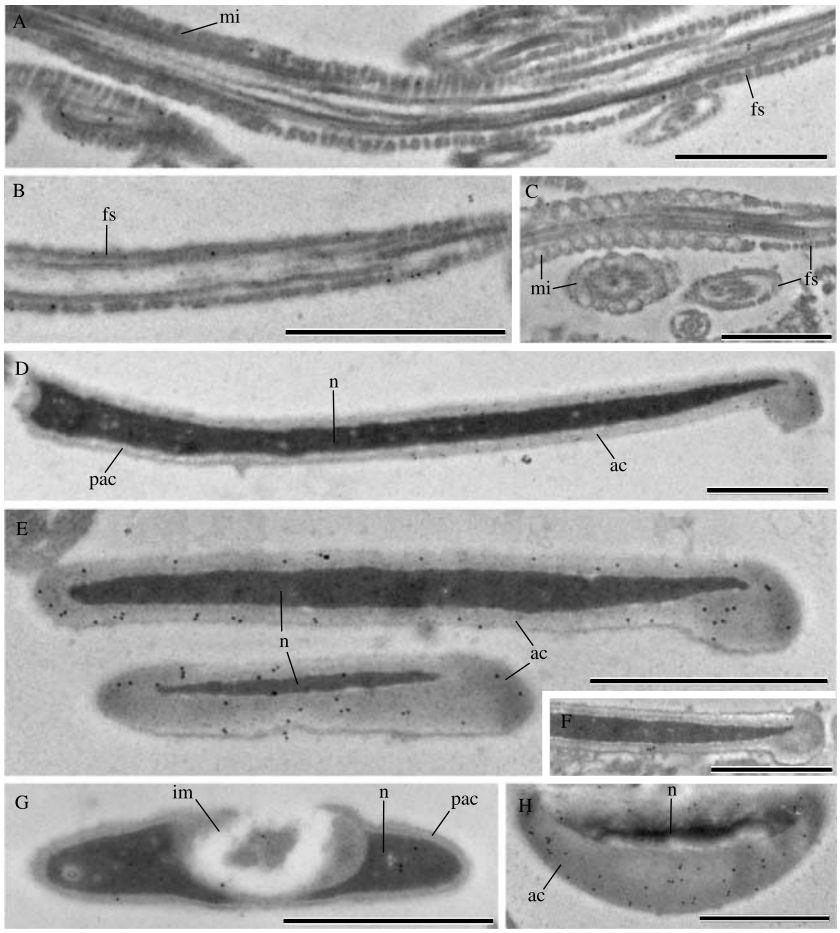

Figure 9 Ultrastructural localization of PK-S in boar sperm by immunogold electron microscopy using anti-PK-S. Although the flagellum of sperm was only slightly labelled, significantly more gold particles were found at the fibrous sheath of sperm slices exposed to anti-PK-S (A and B; see text) than in control slices treated with anti-PK-S that had been pre-incubated with the synthetic peptide of the N-terminal sequence of PK-S (C). Anti-PK-S efficiently labelled the acrosomal area (D, E and H), while the nucleus and the postacrosomal substance (D and G) were nearly free of labels. The control with pre-incubated antibodies (F) was almost not labelled. Scale bars = 1 μm. ac, acrosome; df, dense fibres; fs, fibrous sheath; im, implantation fossa; mi, mitochondria; n, nucleus; pac, post-acrosomal substance.

The N-terminal sequence (NH_2-TSEA(M)-COOH) of purified boar PK-S is certainly part of a longer extension that has been split by trypsin. This proposed sequence could resemble the N-terminal extension of the hypothetical PK corresponding to a cDNA from bovine ileum but also the N-terminal extensions encoded by nucleotide sequences for PK-M1 and -M2 which are, with only one exception (accession numbers BC096823 and Q4V9L8 respectively), predicted as non-translated (Fig. 10). The hypothetical bovine PK contains a lysine, and many of the predicted non-translated extensions contain an arginine directly before sequences that are similar to the PK-S extension (NH_2-TSEA-COOH). If either lysine or arginine were also present in native PK-S this would define a trypsin cleavage site and explain the tryptically generated PK-S.

Beside PK-S there exists a soluble form of PK in boar spermatozoa with kinetics similar to those of PK-M1. This soluble PK must have another N-terminus than PK-S since it was not recognized by anti-PK-S. We assume that the soluble PK is a PK-M1 which is not N-terminally extended and might be present in the midpiece of the flagellum where anti-PK-M1, but not anti-PK-S brought about immunofluorescence labelling. Thus, our results indicate the existence of two different forms of PK in boar spermatozoa, a soluble PK and the bound PK-S.

Localization of PK and other glycolytic enzymes in mammalian spermatozoa

Immunofluorescence using anti-PK-M1 and anti-PK-S (Fig. 7) has shown that PK-S is localized in the principal piece of the flagellum where label was associated with the fibrous sheath as shown by immunogold labelling using anti-PK-M1 (Fig. 8) and anti-PK-S (Fig. 9). This is in line with the recent report that PK is tightly bound to purified fibrous sheath from mouse spermatozoa (Krisfalusi *et al.* 2006). With anti-PK-S relatively few gold labels were found at the fibrous sheath, yet their number was significantly larger than in the control. The comparatively poor gold labelling could, at least in part, be due to paraformaldehyde (PFA) fixation of the LR White-embedded spermatozoa. PFA is known to cross-link proteins and may reduce the accessibility

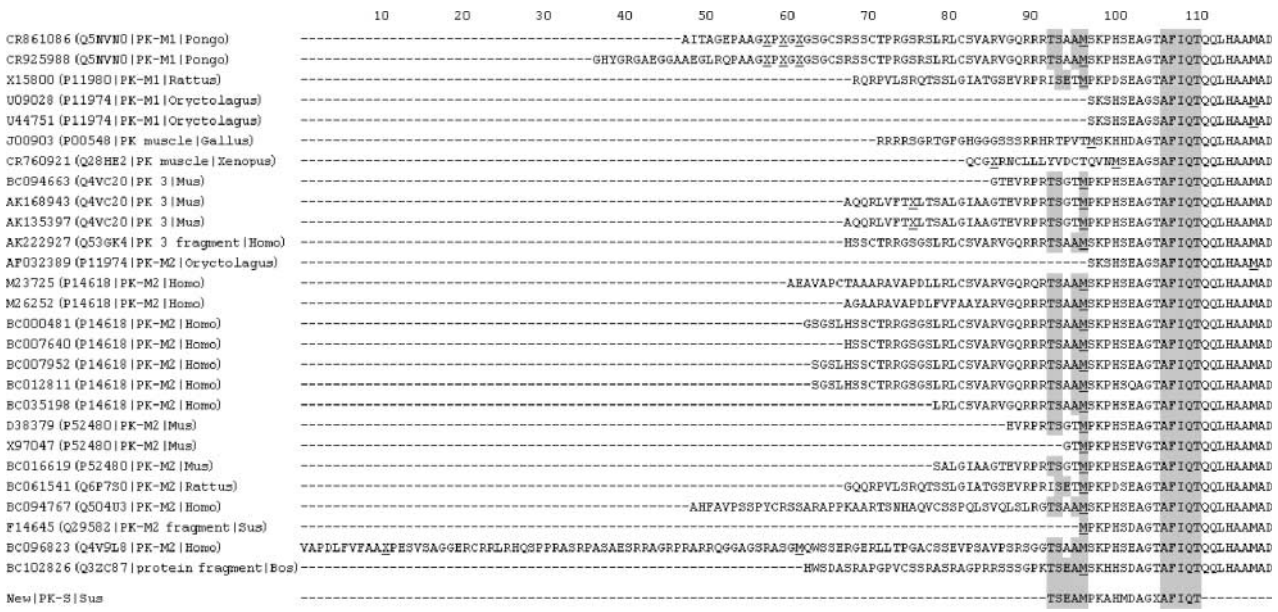

Figure 10 The N-terminus of the tryptically solubilized sperm PK-S when compared with corresponding amino acid sequences encoded in *PKM* mRNAs. For this comparison, *PKM* mRNAs were completely translated into amino acid sequences. Accession numbers of nucleotide sequences (GenBank) are given together with those of the deduced proteins (in parentheses). The N-terminus of purified PK-S (New |PK-S| Sus; X = unidentified amino acid) is shown in the last line. The first 5′-AUG start codon is marked as M (methionine), stop codons are represented as X. *PKM* mRNAs often have nucleotide sequences at the 5′-end before the predicted start codon that encode amino acid sequences (positions 93–96; under laid) similar to the extension of PK-S. In case of PK-M1, translation is probably initiated at the usually predicted start codon AUG for methionine at position 97 (see native PK-M1 in Fig. 3; P11979 and P14618), whereas in PK-S another upstream start codon would be used. The sequence NH_2-AFIQT-COOH (positions 107–111; under laid) is found in all PK-Ms and also in PK-S.

of antigens. It is not unusual that the access and conservation of antigens for their antibodies differ between various techniques (e.g. Wallimann *et al.* 1986, Kaldis *et al.* 1996). Even more important could be the fact that the antigenic epitope of anti-PK-S is only a 13 amino acid stretch of PK-S close to the part by which the enzyme is firmly attached to the fibrous sheath. This spatial arrangement could further reduce antigen accessibility for anti-PK-S when compared with anti-PK-M1 which is expected to bind to various epitopes that are more exposed to antibodies than the antigenic structure recognized by anti-PK-S.

The fact that some glycolytic enzymes are tightly bound to the fibrous sheath strongly suggests that glycolysis is structurally organized along the flagellum as already proposed by Storey & Kayne (1975). A structural organization of glycolysis has also been proposed for somatic tissues (Arnold & Pette 1968, Lynch & Paul 1983, Masters *et al.* 1987, Srere 1987, Knull & Walsh 1992) but binding to the cell structures show much lesser degrees of stability in these instances.

Unlike the principal piece which was labelled by both anti-PK-M1 and anti-PK-S, the midpiece of boar spermatozoa was only labelled by anti-PK-M1 but not by anti-PK-S in immunofluorescence analysis. This suggests that only the soluble sperm PK, which is not recognized by anti-PK-S, is present in the midpiece. Whether or not glycolysis is active in the midpiece of mature mammalian spermatozoa is still not clear because GAPDH has not yet been demonstrated in this part of sperm (Westhoff & Kamp 1997, Welch *et al.* 2006). Interestingly, the head–midpiece junction showed PK-S staining (Fig. 7) suggesting that PK-S is bound to structures at this site. It is tempting to speculate that PK-S at this site is functionally linked to secretory pathway Ca^{2+}-ATPase that was localized at the head–midpiece junction and might be implicated in the control of sperm Ca^{2+}-homeostasis (for review, see Jimenez-Gonzalez *et al.* 2006).

Anti-PK-S substantially labelled the acrosomal area. This is evident from both immunofluorescence (Fig. 7) and immunogold labelling (Fig. 9). Immunogold particles were not found in the nucleus and the postacrosomal substance. The strong gold labelling at the acrosome is in contrast to the weak labelling of the fibrous sheath and suggests that the micro-environment of the PK-S at the acrosome differs from that at the fibrous sheath with the effect that anti-PK-S can easily reach its antigen, the proposed anchor peptide, at the acrosome but less so at the fibrous sheath. Where exactly PK-S is located at the acrosome requires further study.

PK-S is the third glycolytic enzyme located in the acrosomal area. Travis *et al.* (1998) found HK1-S in the membranes around the acrosome and the whole sperm head. We have recently demonstrated 6-phosphofructokinase in the acrosomal area of boar spermatozoa (Kamp *et al.* 2007). Interestingly, the glucose and fructose

transporting GLUT8 was found also in this region in mouse and human sperm (Schürmann *et al.* 2002) and GLUT3 was reported in membranes of boar sperm head (Medrano *et al.* 2006). Taken together these data would suggest glycolytic activity around the acrosome. On the other hand, GAPDH has not yet been found at the acrosome (Bunch *et al.* 1998, Welch *et al.* 2006), and there is no information as to whether the before-mentioned enzymes are catalytically active or inactive remnants of protein synthesis during spermatogenesis (cf. Ramalho-Santos *et al.* 2002, Kierszenbaum & Tres 2004) or have other than glycolytic functions as proposed for HK (Travis *et al.* 1998).

Kinetics and possible functions of PKs in boar sperm

Isozymes of somatic PKs differ in their kinetic properties and this reflects tissue-specific control of glycolysis (Newsholme & Leech 1983). Both forms of boar sperm (soluble PK and PK-S) lack activation by F1,6P_2 and inhibition by L-alanine, hence resembling muscle PK (PK-M1; Hall & Cottam 1978) and suggesting similar functions and control of glycolysis in muscle and sperm. Our results back a report on the kinetics of structure-bound PK from rabbit epididymal spermatozoa (Storey & Kayne 1980).

Kinetic properties of enzymes can be modulated by binding of enzymes to cell structures (Masters *et al.* 1987, Low *et al.* 1993, Gutowicz & Terlecki 2003). If binding is reversible and dependent on specific effectors, enzyme activity can thus be regulated. Boar sperm PK-S, however, is irreversibly bound to the fibrous sheath and the acrosomal area and the kinetics with respect to the substrates PEP and ADP and the potential effectors F1,6P_2 and L-alanine were independent of whether the enzyme was bound or solubilized. This and the presence of a soluble PK with similar kinetics indicate that the novel PK-S serves the spatial arrangement of glycolytic enzymes in specific compartments rather than regulatory adaptations to sperm metabolism.

Rationale for glycolysis in the principal piece and at the acrosome

There is evidence that the fibrous sheath functions as a scaffold for spatial organization of glycolytic enzymes thus enabling ATP-production by sugar fermentation to lactate in the principal piece of the flagellum. We have proposed (Westhoff & Kamp 1997) that glycolysis in the principal piece is important to supply ATP locally for dynein-ATPases since the mitochondria are confined to the midpiece and the phosphocreatine/creatine kinase shuttle for transport of mitochondrial energy-rich phosphate is poorly developed in mammalian spermatozoa (Kamp *et al.* 1996). Support for this hypothesis comes from the observations that lactate production by boar spermatozoa occurs even under normoxic conditions (Kamp *et al.* 2003) and is increased if sperm are stimulated to hyperactivity (unpublished results) which is vital for fertility (Yanagimachi 1994, Stauss *et al.* 1995, Mortimer 1997, Bedford 1998).

The role of glycolysis at the acrosome is puzzling because we do not know yet whether all glycolytic enzymes are present and active at the acrosome (see above). The presence of the transporters GLUT8 and GLUT3 together with hexokinase, 6-phosphofructokinase and PK led us speculate about possible functions of glycolysis at the acrosome. One function could be local ATP-supply for Ca^{2+}-ATPases that keep the intracellular Ca^{2+} concentration low thus preventing premature acrosome reaction (cf. Feng *et al.* 2006). Other ATPases are also located in the acrosomal region and are important for sperm function (Na^+, K^+-ATPase: ATP1A4, Thundathil *et al.* 2006; H^+-ATPase: V-ATPase E1-subunit isoform, Sun-Wada *et al.* 2002).

In summary, our results and reports from others suggest that mammalian spermatozoa need local ATP-production for flagellar movement and control of the acrosomal reaction. If glycolytic ATP in the principal piece powered motility, especially hyperactivity, and in the head secured proper acrosomal functioning, glycolysis will prove essential for sperm fertility *in vivo*.

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