

## Identification of two distinctly localized mitochondrial creatine kinase isoenzymes in spermatozoa

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### SUMMARY

The creatine kinase (CK) isoenzyme system is essential for motility in rooster and sea urchin sperm. In the present study, biochemical characterization as well as immunofluorescence and confocal laser microscopy with highly specific antibodies against various chicken CK isoenzymes revealed that cytosolic brain-type CK isoenzyme (B-CK) is the only CK isoenzyme in rooster seminal plasma, while three isoenzymes, cytosolic B-CK, sarcomeric mitochondrial CK (Mi<sub>b</sub>-CK), and a variant of ubiquitous Mi-CK ('Mi<sub>a</sub>-CK variant'), are found in rooster spermatozoa. These three isoenzymes are localized in different regions of the sperm cell. B-CK and Mi<sub>b</sub>-CK were localized along the entire sperm tail and in the mitochondria-rich midpiece, respectively. The 'Mi<sub>a</sub>-CK variant', on the other hand, was found predominantly at the head-midpiece boundary, in a

non-uniform manner in the midpiece itself and, surprisingly, at the distal end of the sperm tail as well as at the acrosome. Several lines of evidence show that the 'Mi<sub>a</sub>-CK variant' shares some characteristics with purified Mi<sub>a</sub>-CK from chicken brain, but also displays distinctive features. This is the first evidence for two different Mi-CK isoenzymes occurring in one cell and, additionally, for the co-expression of Mi<sub>b</sub>-CK and cytosolic brain-type B-CK in the same cell. The relevance of these findings for sperm physiology and energetics is discussed.

Key words: Creatine kinase compartmentation, Sperm motility, Fertility, Immunofluorescence, Confocal microscopy, Anti-peptide antibody, Chicken, Rooster, Seminal plasma

### INTRODUCTION

Sperm motility, as one of the crucial determinants for male fertility, depends on the availability of chemical energy in the form of ATP. The creatine kinase (CK, EC 2.7.3.2) isoenzyme system participates in the energy metabolism of sperm by catalyzing the reversible transfer of the *N*-phosphoryl group from phosphorylcreatine (PCr) to ADP in order to regenerate ATP (for a review see Wallimann and Hemmer, 1994). In a series of elegant experiments with sea urchin sperm, selective inhibition of the CK isoenzymes by 2,4-dinitrofluorobenzene (DNFB) was shown to result in loss of flagellar motility in the distal two thirds of the sperm tail, indicating that the tail cannot be supplied with adequate amounts of 'high-energy phosphates' by ATP and ADP diffusion alone (Tombes and Shapiro, 1985). Qualitatively similar results were also obtained with rooster sperm (Wallimann et al., 1986).

In general, CK isoenzymes are expressed in tissues with high and fluctuating energy demands, e.g. in skeletal and cardiac muscle, brain, retina, and spermatozoa (Wallimann et al., 1992). In most tissues, cytosolic as well as mitochondrial CK isoenzymes are co-expressed. The three cytosolic CK isoenzymes form exclusively dimeric molecules composed of

two types of subunits (MM-CK, MB-CK and BB-CK; M standing for the 'muscle' isoform; B standing for the 'brain' isoform). They are either freely soluble components of the cytosol or are associated with subcellular structures, e.g. the myofibrillar M-line or the sarcoplasmic reticulum, where they are functionally coupled to the acto-myosin ATPase (Krause and Jacobus, 1992; Wallimann et al., 1984) and the Ca<sup>2+</sup>-ATPase (Korge et al., 1993; Korge and Campbell, 1994; Rossi et al., 1990), respectively. In contrast, the mitochondrial CK (Mi-CK) isoenzymes are associated with the outer side of the inner mitochondrial membrane (Jacobus and Lehninger, 1973) and form both dimeric and octameric molecules that are readily interconvertible. Two different Mi-CK isoenzymes were found in all vertebrates examined so far: a sarcomeric (Mi<sub>b</sub>-CK) and a ubiquitous isoform (Mi<sub>a</sub>-CK), which are expressed predominantly in striated muscle and brain, respectively (Payne et al., 1991; Schlegel et al., 1988a; Wyss et al., 1990). Studies on isolated mitochondria have shown that mitochondrial oxidative phosphorylation and the Mi-CK reaction are functionally coupled, that is, Mi-CK preferentially utilizes the ATP synthesized through oxidative phosphorylation for PCr synthesis (for reviews see Wallimann et al., 1992; Wyss et al., 1992).

In sea urchin sperm, two different CK isoenzymes were

found: a Mi-CK isoenzyme with a subunit  $M_r$  of 47,000 and a so-called tail CK (T-CK) with a  $M_r$  of 145,000 (Tombes and Shapiro, 1985). T-CK is myristoylated at the N terminus and most likely originates from triplication of an ancestral CK gene (Quest et al., 1992; Wothe et al., 1990). T-CK was localized exclusively along the sea urchin sperm tail while Mi-CK is restricted to the midpiece containing the mitochondrion (Tombes and Shapiro, 1987). It is interesting to note that invertebrate species such as echinoderms and polychaeta which otherwise express arginine kinase or other guanidino kinases, specifically accumulate CK in their spermatozoa (Moreland et al., 1967; Thoai and Robin, 1964). This fact may be explained by the differences in thermodynamic properties between PCr and the other naturally occurring phosphagens (Ellington, 1989).

Up to now, only a few studies have focused on CK in vertebrate spermatozoa. In rooster and human sperm, B-CK and an undefined Mi-CK isoform were identified and preliminarily assigned to the tail and the midpiece, respectively (Huszar et al., 1992; Wallimann et al., 1986). Furthermore, the relative proportion of Mi-CK was shown to be of diagnostic value for predicting the fertilizing potential of human spermatozoa (Huszar et al., 1992).

Using a set of specific antibodies against all chicken CK isoenzymes, the CK isoenzymes of rooster sperm were biochemically characterized and localized by immunocytochemistry. We demonstrate for the first time the simultaneous presence of two different mitochondrial CK isoenzymes within the same cell and, additionally, the co-expression of cytosolic brain-type B-CK with sarcomeric Mi<sub>b</sub>-CK, a mitochondrial CK isoenzyme found so far only in sarcomeric muscle. Possible explanations for these surprising findings in terms of the very specialized physiology and energy requirements of spermatozoa are discussed.

## MATERIALS AND METHODS

### Collection of rooster sperm and seminal plasma

Fresh rooster sperm were obtained by massage of the back of a rooster. The ejaculate (300 µl) was collected in a tube containing protease inhibitors (8.5 mM phenylmethylsulfonyl fluoride (PMSF), 8.5 mM EDTA, 1.5 mM leupeptin, 14 µg Trasylol (Bayer, Leverkusen) and 1.5 mM ADP; all final concentration). The sperm were diluted in 10 ml of buffer A (20 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES), 150 mM NaCl, 10% glycerol, pH 7.4) and centrifuged for 5 minutes at 12,000 *g*. The pellet was either dissolved in SDS-PAGE sample buffer or extracted under native conditions. For extraction, the sperm were dissolved in 300 µl of Milli-Q water (Millipore) and swollen for 30 minutes on ice. Subsequently, 300 µl of extraction buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 2% Triton X-100, 5 mM 2-mercaptoethanol) was added and incubation continued for 2 hours on ice. These extracts were analyzed by cellulose polyacrylate electrophoresis or by SDS-PAGE. In some experiments a mixture of up to ten protease inhibitors was added to the ejaculate (final concentrations: 74 µM antipain-dihydrochloride, 0.3 µM aprotinin, 0.17 µM chymostatin, 28 µM E-64, 1.34 µM EDTA, 10 µM leupeptin, 1.6 µM pefabloc SC, 0.375 µM phosphoramidon, 7.2 µM pepstatin, and 325 µM bestatin; Boehringer Mannheim). As an alternative, washed sperm pellets were dissolved in 200 mM HCl, which resulted in complete lysis of sperm, as confirmed by phase contrast microscopy.

For the analysis of seminal plasma, the ejaculate was first cen-

trifuged in a microcentrifuge for 10 minutes at 10,000 *g*, and the supernatant was then clarified by ultracentrifugation in an airfuge (Beckman) for 30 minutes at 30 psi (178,000 *g*). Inspection by phase contrast microscopy revealed that the seminal plasma samples thus prepared were free of spermatozoa. For analysis with two-dimensional (2-D) gel electrophoresis, seminal plasma was concentrated approximately 3-fold with Centricon-30 concentrators (Amicon).

### Antibodies

The following primary antibodies against CK isoenzymes, generated and characterized previously in this laboratory, were used for immunolocalization and staining of western blots: rabbit anti-B-CK serum (Hemmer et al., 1993), rabbit anti-Mi<sub>a</sub>-CK serum (Wyss et al., 1990), rabbit anti-Mi<sub>b</sub>-CK serum (Schlegel et al., 1988a), monoclonal mouse anti-Mi<sub>b</sub>-CK 30a supernatant (Schlegel et al., 1988b), and rabbit anti-M-CK serum (Caravatti et al., 1979). In control experiments, preimmune sera were used at the same dilution as the corresponding primary antibodies.

Since the available antibody against Mi<sub>a</sub>-CK was not sufficiently discriminative, the specificity of this antibody was increased by cross-absorption against Mi<sub>b</sub>-CK bound to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia). In addition, highly specific anti-peptide antibodies were raised against the Mi-CK isoenzymes. The N terminus was selected as antigenic determinant, since it is among the most variable regions within the CK sequences (Mühlebach et al., 1994). Peptide synthesis was performed on a Milligen-9050 continuous-flow synthesizer using an adapted software package for TPTU activation (O-(1,2-dihydro-2-oxopyrid-1-yl) *N,N,N',N'*-tetramethyluronium tetrafluoroborate) (Knorr et al., 1989) and Fmoc/tBu strategy. The carboxamide peptides corresponding to Mi<sub>b</sub>-CK amino acids 1-15, Mi<sub>a</sub>-CK amino acids 1-8, 1-15, and 175-183 have been synthesized using Fmoc protection for the N $\alpha$  function on tentagel resin (Rapp Polymere, Tübingen, Germany) modified with *p*-[R,S- $\alpha$ -1(9H-fluoren-9-yl) methoxyformamido 2,4-dimethoxybenzyl] phenoxycetic acid (Fmoc-TMBHA, Novabiochem, Läfelfingen, Switzerland). Side-chain protection was afforded by the following: N $^{\omega}$ -(2,2,5,7,8-pentamethylchroman-6-sulfonyl) (Pmc) for arginine, trityl (Trt) for glutamine and histidine, and tert-butyl (tBu) for serine, threonine, tyrosine, aspartic acid, and glutamic acid. The cleavage reaction was performed for 105 minutes in a mixture of 1.6 ml water, 1.6 ml thioanisole, 0.8 ml ethanedithiol, 2 g phenol, and 32 ml trifluoroacetic acid (TFA). Peptides were purified on a Delta-Prep-3000 HPLC column (Waters) and characterized by analytical HPLC (Merck-Hitachi L-6200A) and ion spray mass spectrometry, performed on an API III (Sciex). The peptides were coupled to keyhole limpet hemocyanin at room temperature as follows (Schneider et al., 1983): hemocyanin (Pierce) was dissolved in 50% glycerol (10 mg protein in 80 µl solution) and mixed with 420 µl of buffer B (140 mM NaCl, 1.6 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4). Subsequently, 20 µl of freshly prepared 100 mM disuccinimidyl suberate in dimethylsulfoxide was added and the mixture stirred for 10 minutes. Then, 6.3 mg of solid peptide was added and stirring continued for 90 minutes. Finally, the mixture was diluted with 500 µl of buffer B. After stirring for another 30 minutes, 160 µl aliquots were frozen and stored at -20°C. Rabbits were injected intracutaneously with a mixture of 160 µl of hemocyanin-coupled peptide solution, 340 µl of buffer B and 500 µl of either Freund's complete adjuvant (day 0) or incomplete adjuvant (days 14, 28, 42, 67 and 91). Rabbits were bled by ear puncture on days 35, 42, 55, 67, 84, 91, 98 and 105. Sera were analyzed for cross-reaction with other chicken CK isoenzymes by enzyme-linked immunosorbent assay (ELISA) (data not shown) and western blotting and were stored in aliquots at -20°C.

As far as terminology is concerned, the term 'polyclonal antibody against a particular CK isoenzyme' stands for antibodies raised against the whole CK molecule, i.e. amino acids 1-380. On the other hand, polyclonal antibodies raised against peptides of Mi-CK isoen-

zymes are always explicitly and properly named ('anti-peptide antibodies').

### Electrophoretic techniques and immunoblotting

Cellulose polyacetate electrophoresis was performed for 90 minutes at 100 V and room temperature as described (Wyss et al., 1990). Fresh, washed sperm were always applied after preincubation in extraction buffer. SDS-PAGE was performed according to the method of Laemmli (1970). After electrophoresis, proteins were semi-dry-blotted at a constant current of 1 mA/cm<sup>2</sup> for 90 minutes onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). These blots were 'stained' either with Coomassie Brilliant Blue R-250 or with specific antibodies against the different CK isoenzymes. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell et al. (1977), using nonequilibrium pH gradient electrophoresis (NEPHGE; pH range 3-10, Pharmalyte, Pharmacia) for 6 hours at 400 V in the first dimension and standard SDS-PAGE in the second dimension. Alternatively, 2-D gel electrophoresis was performed according to the method of O'Farrell (1975) using isoelectric focusing (IEF; pH range 5-8, Pharmalyte, Pharmacia) for 10,000 to 16,000 Vh in the first dimension and standard SDS-PAGE in the second dimension. Proteins separated by 2-D gel electrophoresis were either silver-stained or semi-dry-blotted onto PVDF membranes and stained as described above.

### Immunofluorescence and confocal laser microscopy

Rooster ejaculate was centrifuged for 1 minute at 2,500 *g*. From the supernatant, a thin layer of sperm was smeared on a microscope slide. The sperm were air-dried for 30 minutes at room temperature followed by permeabilization and fixation with a mixture of 0.2% Triton X-100 and 4% paraformaldehyde in TBS. Thereafter, excess paraformaldehyde was quenched with 100 mM glycine in TBS for 10 minutes. Nonspecific binding sites were blocked with 2% horse serum in TBS for 30 minutes. Samples were incubated with the primary antibody (in the dilution indicated in the individual experiments) for 2 hours in a moist chamber and then washed with three changes of TBS for 10 minutes each. Rhodamine-conjugated goat anti-rabbit IgG (Pierce, 1:500 diluted), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Nordic, 1:100 diluted) or Texas red-conjugated donkey anti-mouse IgG (Jackson Immuno Research Laboratories, 1:300 diluted) served as secondary antibodies (1 hour incubation) and were all diluted in TBS containing 2% horse serum. After three washes with TBS, the specimens were mounted either in buffered polyvinyl alcohol medium (Lenette) in the presence of the anti-fading agent *p*-phenylene diamine (1 mg/ml) or in *n*-propyl gallate medium.

The confocal system consisted of a Zeiss Axioplan fluorescence microscope, a Bio-Rad MRC-600 confocal scanner and a Silicon Graphics workstation. The fluorochromes were excited at 488 nm (FITC) and 568 nm (Texas red) using an argon-krypton mixed gas laser. A background value corresponding to the first relative minimum in the grey level histogram has been subtracted from all data; after expanding the remaining data to maximal contrast, they were reconstructed. The data sets were processed using the program Imaris (Bitplane AG, Zürich, Switzerland).

## RESULTS

### Production of specific antibodies against Mi-CK isoenzymes

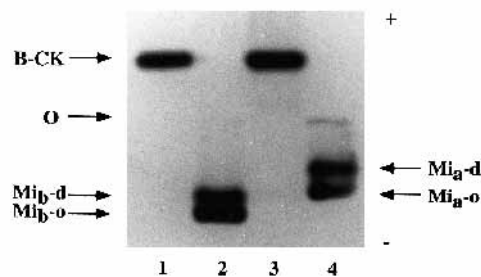
The CK isoenzymes in rooster sperm were identified by using specific antibodies against all chicken CK isoenzymes. Monoclonal antibodies against Mi<sub>b</sub>-CK (Schlegel et al., 1988b) and several polyclonal antibodies against all chicken CK isoenzymes were available in our laboratory. Since the polyclonal

antibody against Mi<sub>a</sub>-CK also stained Mi<sub>b</sub>-CK on western blots (see Fig. 4D, lane 4) and cross-reacted with several CK isoenzymes in ELISA (Kaldis, 1994), it was cross-absorbed against Mi<sub>b</sub>-CK in order to improve its specificity. In ELISA experiments, this cross-absorbed antibody did not cross-react with Mi<sub>b</sub>-CK or other CK isoenzymes (Kaldis, 1994). On western blots, a very faint cross-reactivity with Mi<sub>b</sub>-CK remained (not shown). In an attempt to get highly specific antibodies against Mi-CK isoenzymes, polyclonal antibodies were raised in rabbits against N-terminal or internal peptides of chicken Mi<sub>a</sub>- and Mi<sub>b</sub>-CK. Peptides corresponding to Mi<sub>b</sub>-CK amino acids 1-15 and Mi<sub>a</sub>-CK amino acids 1-8, 1-15, and 175-183 served as antigenic determinants. Control experiments revealed that the anti-peptide antibody against chicken Mi<sub>b</sub>-CK 1-15 is highly specific for chicken Mi<sub>b</sub>-CK and does not cross-react with other chicken CK isoenzymes in ELISA and immunoblotting experiments (Kaldis, 1994). The anti-peptide antibodies against chicken Mi<sub>a</sub>-CK 1-15 (Kaldis, 1994) and 175-183 cross-reacted slightly with Mi<sub>b</sub>-CK in ELISA experiments and were therefore cross-absorbed against Mi<sub>b</sub>-CK. The anti-peptide antiserum against chicken Mi<sub>a</sub>-CK 1-8 was highly specific, but had a rather low titer.

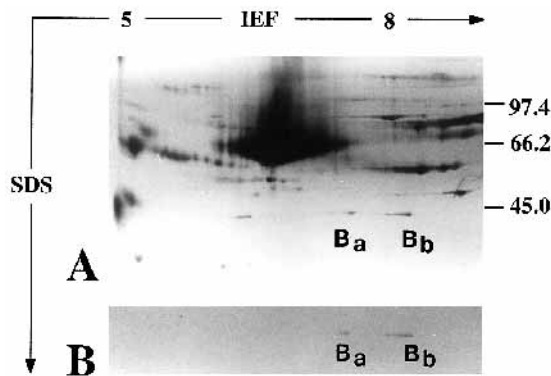
### Biochemical characterization of the CK isoenzyme system of seminal plasma

For the analysis of rooster seminal plasma and sperm, several electrophoretic methods were used, i.e. cellulose polyacetate electrophoresis, SDS-PAGE, and 2-D gel electrophoresis (using NEPHGE or IEF in the first dimension). Cellulose polyacetate electrophoresis allows reproducible separation of the chicken CK isoenzymes under native conditions.

Cellulose polyacetate electrophoresis (Fig. 1), immunoblotting of SDS-polyacrylamide gels (with several antibodies against Mi<sub>a</sub>-CK, Mi<sub>b</sub>-CK, M-CK, and B-CK; not shown), and 2-D gel electrophoresis (Fig. 2) demonstrated that rooster seminal plasma contains exclusively the cytosolic brain-type B-CK isoenzyme. Fig. 2 shows additionally that seminal plasma contains the Ba-CK and Bb-CK subtypes in a 1:1 ratio as found previously for many other tissues (Hemmer et al., 1993; Quest et al., 1990). Comparison of total ejaculate and washed sperms on 2-D gels revealed an increased B-CK signal



**Fig. 1.** Identification of B-CK in rooster seminal plasma. Seminal plasma was analyzed for the presence of CK isoenzymes by cellulose polyacetate electrophoresis (see Materials and Methods). Seminal plasma (lane 3) was run together with purified B-CK (lane 1), Mi<sub>b</sub>-CK (lane 2), and Mi<sub>a</sub>-CK (lane 4). CK isoenzymes were visualized by coupling the CK enzyme activity to a colour reaction. Note that seminal plasma contains exclusively B-CK. Mi<sub>a</sub>-d and Mi<sub>a</sub>-o, dimeric and octameric Mi<sub>a</sub>-CK; Mi<sub>b</sub>-d and Mi<sub>b</sub>-o, dimeric and octameric Mi<sub>b</sub>-CK. O, origin (point of sample application).



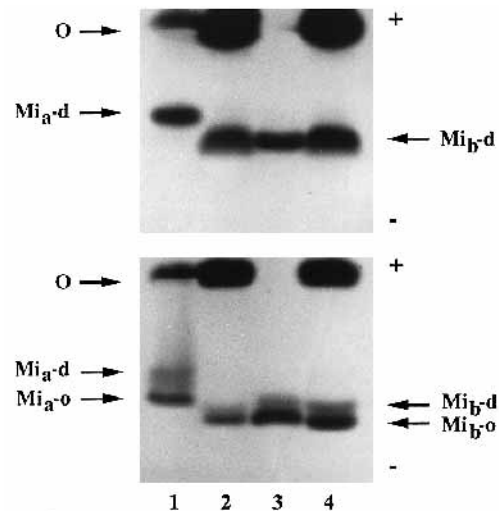
**Fig. 2.** Two-dimensional gel electrophoresis of rooster seminal plasma. Concentrated seminal plasma was subjected to two-dimensional gel electrophoresis, using isoelectric focusing in the pH range 5-8 in the first dimension and standard SDS-PAGE in the second dimension. One gel was silver stained (A) while the other was blotted onto PVDF membrane and stained with a polyclonal antibody against B-CK (B). Molecular mass markers are indicated at the right side of the silver stained gel (A). The section shown in B corresponds to the 45 kDa region of A. Note that rooster seminal plasma contains B<sub>a</sub>-CK, B<sub>b</sub>-CK (denoted as B<sub>a</sub> and B<sub>b</sub>, respectively), as well as some minor subtypes (see spots between B<sub>a</sub> and B<sub>b</sub>), thus showing the well-known microheterogeneity of B-CK (Quest et al., 1990; Soldati et al., 1990).

for ejaculate (data not shown), corroborating the fact that there is B-CK in the seminal plasma.

### Biochemical characterization of the CK isoenzymes in rooster sperm

For cellulose polyacetate electrophoresis, washed sperm were hypotonically swollen, permeabilized, extracted and applied to cellulose polyacetate strips. Extracts pre-incubated with transition state-analogue complex (TSAC) substrates (4 mM ADP, 5 mM MgCl<sub>2</sub>, 20 mM creatine, 50 mM nitrate), which induce dissociation of Mi-CK octamers into dimers, showed only a single dimeric Mi-CK band (Fig. 3, upper panel). 'Untreated' sperm extracts (Fig. 3, lower panel) gave rise to two bands for Mi-CK, corresponding to octameric and dimeric molecules (Wyss et al., 1990). Under both conditions, two CK isoenzymes were observed for rooster sperm, with electrophoretic mobilities that were identical to purified B-CK and Mi<sub>b</sub>-CK. Analysis of a mixture of sperm extract and purified Mi<sub>b</sub>-CK did not reveal additional CK bands (Fig. 3, lane 4), thus corroborating the occurrence of sarcomeric Mi<sub>b</sub>-CK in rooster sperm. A very faint band in the region of Mi<sub>a</sub>-CK was occasionally observed (not shown), indicating that there might be a second mitochondrial CK isoenzyme which constitutes a very small proportion of the total CK activity.

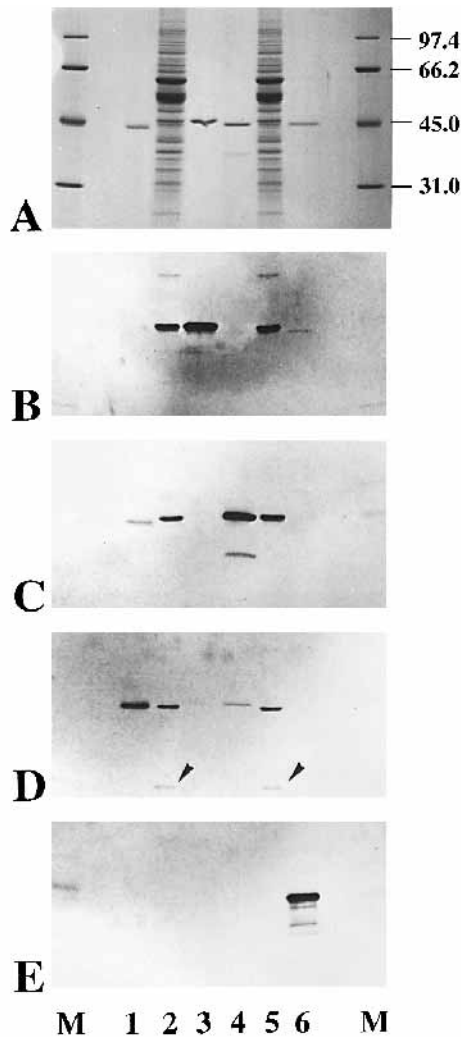
For SDS-PAGE and 2-D gel electrophoresis, washed sperm were dissolved in SDS-PAGE sample buffer and in 9 M urea, respectively. Staining of western blots with specific antibodies confirmed that B-CK, but not M-CK, is present in rooster sperm (Fig. 4B and E). Furthermore, a polyclonal antibody against Mi<sub>b</sub>-CK stained a protein band of rooster sperm with the same electrophoretic mobility as purified Mi<sub>b</sub>-CK (Fig. 4C). This band had a clearly lower mobility than purified Mi<sub>a</sub>-CK, as can be judged from the cross-reactivity of the anti-Mi<sub>b</sub>-CK antibody with purified Mi<sub>a</sub>-CK (Fig. 4C, lane 1). Staining



**Fig. 3.** Cellulose polyacetate electrophoresis of rooster sperm extracts. 'Untreated' samples (containing Mi-CK octamers and dimers) were separated in the lower panel, whereas samples run in the upper panel were incubated with TSAC substrates (inducing dissociation of Mi-CK octamers into dimers) before electrophoresis. A chicken brain extract (containing B-CK and Mi<sub>a</sub>-CK, lane 1), rooster sperm extract (lane 2), purified Mi<sub>b</sub>-CK (lane 3) and a mixture of purified Mi<sub>b</sub>-CK and rooster sperm extract (lane 4) were electrophoresed on cellulose polyacetate membranes. For staining and abbreviations see Fig. 1. Note that the band near the anode, above the origin, represents B-CK which is abundant in rooster sperm.

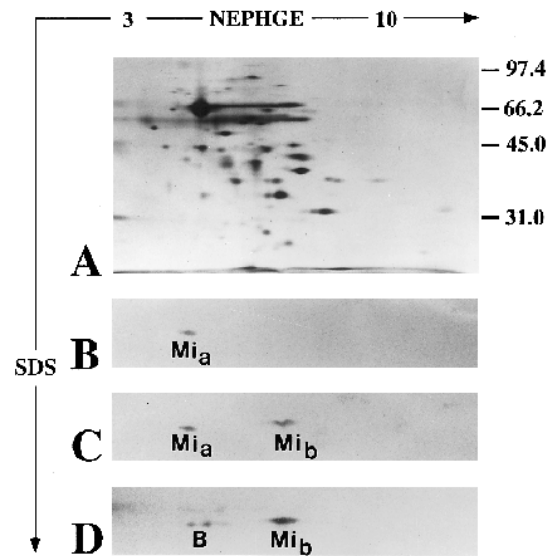
with the specific anti-peptide antibody against Mi<sub>b</sub>-CK 1-15 gave rise to the same bands, except that no cross-reaction with purified Mi<sub>a</sub>-CK was observed (see Fig. 5C,D; data not shown). The polyclonal antibody against Mi<sub>a</sub>-CK, on the other hand, recognized a sperm protein on western blots with the same mobility as purified Mi<sub>a</sub>-CK (Fig. 4D), but with a slightly higher mobility than purified Mi<sub>b</sub>-CK which was also stained due to cross-reactivity (Fig. 4D, lane 4). Again, the polyclonal antibody against Mi<sub>a</sub>-CK that was cross-absorbed against Mi<sub>b</sub>-CK resulted in staining of the same bands except that no cross-reaction with Mi<sub>b</sub>-CK was observed (see Figs 5B and 6B; not shown).

In order to get more detailed information about the different Mi-CK isoenzymes in rooster sperm, an analysis by 2-D gel electrophoresis was undertaken, using IEF or NEPHGE in the first and SDS-PAGE in the second dimension. Fig. 5A shows a silver stained gel, while two others were blotted onto PVDF membranes and stained subsequently with antibodies against different CK isoenzymes (Fig. 5B-D). The polyclonal, cross-absorbed antibody against Mi<sub>a</sub>-CK stained one spot in the acidic region (Fig. 5B). Subsequent staining of the same blot with the specific anti-peptide antibody against Mi<sub>b</sub>-CK 1-15 gave rise to an additional spot in the basic region which co-migrated with purified Mi<sub>b</sub>-CK (Fig. 5C; spot in the middle). The polyclonal antibody against B-CK recognized two spots corresponding to B<sub>a</sub>- and B<sub>b</sub>-CK (Fig. 5D; two spots on the left side of the blot; see also Fig. 6 and Discussion). Note that this same blot was also stained with the anti-peptide antibody against Mi<sub>b</sub>-CK 1-15 resulting in a spot in the middle. The spot stained with the antibody against Mi<sub>a</sub>-CK was much more

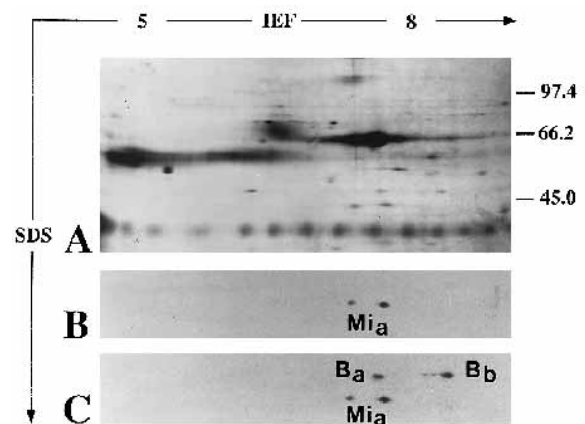


**Fig. 4.** SDS-PAGE of rooster sperm proteins and immunodetection of the CK isoenzymes. Rooster sperm proteins (lanes 2 and 5) and purified CK isoenzymes, i.e.  $Mi_a$ -CK (lane 1), B-CK (lane 3),  $Mi_b$ -CK (lane 4), and M-CK (lane 6), were analyzed by SDS-PAGE (A) and immunoblotting (B-E). An SDS-polyacrylamide gel was stained with Coomassie blue (A). Corresponding western blots were 'stained' with polyclonal antibodies against B-CK (B),  $Mi_b$ -CK (C),  $Mi_a$ -CK (D), and M-CK (E). M, low molecular mass standards (Bio-Rad) comprising phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa) and bovine carbonic anhydrase (31 kDa). Possible proteolytic degradation products of  $Mi_a$ -CK are marked by arrowheads (lanes 2 and 5 in D).

acidic than expected for chicken  $Mi$ -CK isoenzymes (Schlegel et al., 1988b) and was even slightly more acidic than B-CK. A mixture of sperm proteins and purified  $Mi_a$ -CK from chicken brain, analyzed by 2-D gel electrophoresis and staining with the polyclonal antibody against  $Mi_a$ -CK yielded two different spots, a basic one corresponding to purified  $Mi_a$ -CK and a more acidic one corresponding to the sperm protein stained in Fig. 5B (data not shown). To further elucidate the differences between B-CK and the spot stained by the polyclonal antibody against  $Mi_a$ -CK, we improved resolution by performing IEF in the pH range 5-8. One gel was silver stained (Fig. 6A) while another was blotted onto a PVDF membrane and stained with



**Fig. 5.** Two-dimensional gel electrophoresis (NEPHGE) of rooster sperm proteins. Rooster sperm proteins were separated by nonequilibrium pH gradient electrophoresis (NEPHGE, pH 3-10) in the first dimension, followed by conventional SDS-PAGE in the second dimension. One gel was silver stained (A); others were blotted onto PVDF membranes followed by visualization of the CK isoenzymes by staining with polyclonal antibodies. The first blot was stained with the cross-absorbed antibody against  $Mi_a$ -CK (B) and subsequently with the anti-peptide antibody against  $Mi_b$ -CK 1-15 (C). The other blot was stained with both the polyclonal antibody against B-CK and the anti-peptide antibody against  $Mi_b$ -CK 1-15 (D). Corresponding CK spots are indicated as  $Mi_a$  ( $Mi_a$ -CK),  $Mi_b$  ( $Mi_b$ -CK), and B (B-CK). The B-CK comprises two spots, most probably corresponding to  $B_a$ -CK and  $B_b$ -CK (see Fig. 6). For molecular mass standards see Fig. 4.



**Fig. 6.** Two-dimensional gel electrophoresis (IEF) of rooster sperm proteins. Rooster sperm proteins were separated by isoelectric focusing (IEF, pH 5-8) in the first dimension, followed by conventional SDS-PAGE in the second dimension. One gel was silver stained (A), another was blotted onto PVDF membrane. CK isoenzymes were visualized by staining consecutively with the cross-absorbed antibody against  $Mi_a$ -CK (B) and the polyclonal antibody against B-CK (C). Note the well-known microheterogeneity of B-CK including not only  $B_a$ - (denoted as  $B_a$ ) and  $B_b$ -CK (indicated as  $B_b$ ) but also some minor spots (between  $B_a$  and  $B_b$ ; as also shown for seminal plasma, see Fig. 2). The cross-absorbed antibody against  $Mi_a$ -CK stained two different spots that displayed a higher mobility and more acidic migration as compared to B-CK (C). For molecular mass standards see Fig. 4.

antibodies against  $Mi_a$ -CK and B-CK. The polyclonal antibody against  $Mi_a$ -CK stained two major spots in the 43 kDa region (Fig. 6B) as well as some minor low molecular mass spots most likely representing degradation products (not shown). The polyclonal antibody against B-CK stained two major spots corresponding to  $B_a$ -CK and  $B_b$ -CK as well as some minor spots on the acidic side close to the main spots, thereby reflecting the well-known microheterogeneity of B-CK (Hemmer et al., 1993; Quest et al., 1990). These results confirm that the various spots originate from different proteins and correspond in fact to B-CK and either a modified  $Mi_a$ -CK or a novel sperm protein with high homology to  $Mi_a$ -CK.

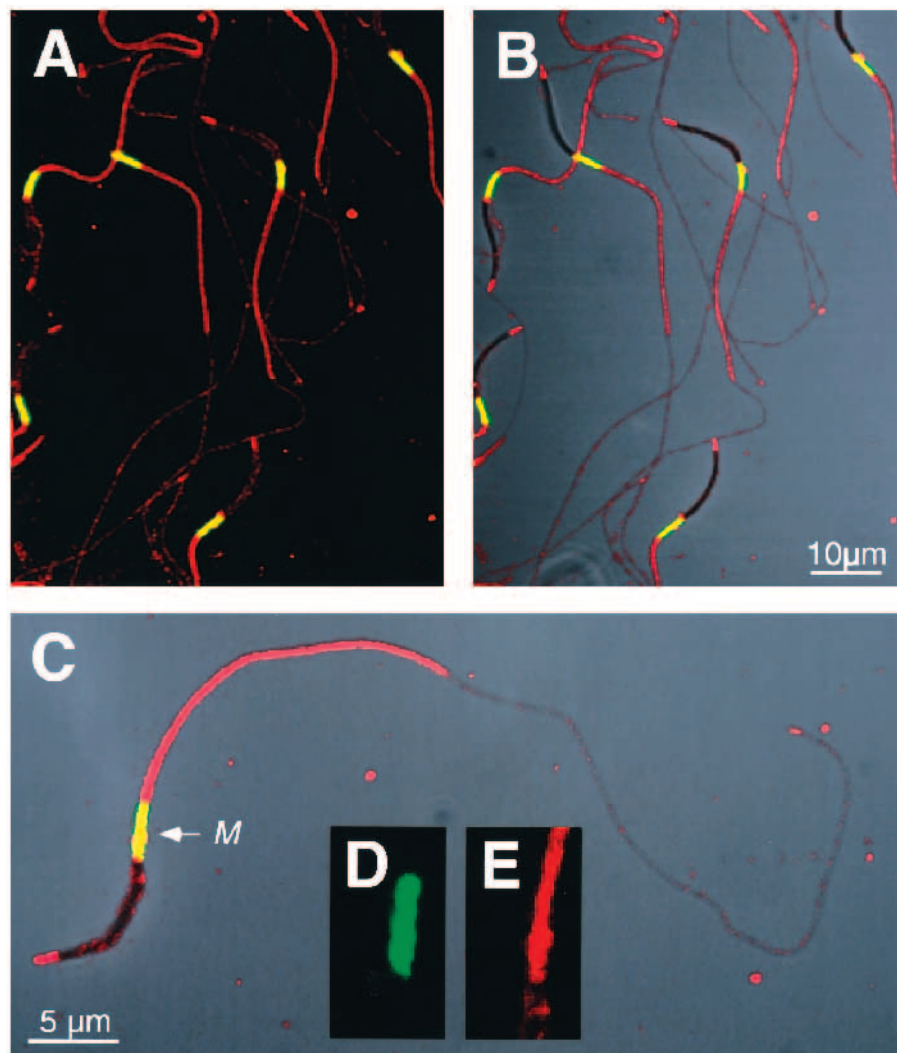
Surprisingly, none of the anti-peptide antibodies against  $Mi_a$ -CK recognized sperm proteins on western blots after SDS-PAGE or 2-D gel electrophoresis (not shown, see also Discussion). In addition, extraction of sperm under native conditions caused total loss of the original  $Mi_a$ -CK band, while  $Mi_b$ -CK and B-CK were recovered quantitatively. Even extraction with 10% Nonidet P-40, 100 mM phosphate, 300 mM KCl and 20% glycerol and the use of a cocktail with up to ten protease inhibitors did not increase the yield of  $Mi_a$ -CK. Undegraded  $Mi_a$ -CK could be extracted only if the sperm were dissolved in 200 mM HCl. These results favour the notion that  $Mi_a$ -CK is degraded specifically and rapidly in sperm extracts.

In conclusion, the  $Mi_a$ -CK-like protein found in rooster sperm displays some distinctive features from that purified from chicken brain. Therefore, this CK species will be denoted as ' $Mi_a$ -CK variant'.

#### Localization of the CK isoenzymes in rooster sperm by confocal laser microscopy

Fresh, intact sperm were smeared on microscope slides, air-dried for a short period of time and fixed with paraformaldehyde in the presence of detergent. The immunolabelling was carried out according to the procedure described in Materials and Methods.

With confocal laser microscopy, high resolution can be achieved and phase contrast pictures can easily be superimposed with two different fluorescence pictures. This method allowed us to localize the CK isoenzymes relative to each other and relative to the sperm cell. B-CK is found predominantly in the tail of the sperm, at the acrosome, and in low concentration where cytoplasm is found in sperms (Fig. 7A-C). The intensity of the staining decreased from the proximal to the distal end of the tail (Fig. 7C), most probably due to leakage during fixation or staining of the sperms. The antibody against B-CK stained also the midpiece (Fig. 7E) but with a lower intensity than the antibody against  $Mi_b$ -CK. The B-CK staining



**Fig. 7.** Localization of B-CK and  $Mi_b$ -CK in rooster spermatozoa. Confocal laser microscopy was used to visualize the distinct localization of B-CK and  $Mi_b$ -CK in rooster spermatozoa. (A) Superimposition of both fluorescence channels; (B,C) superimposition of both fluorescence channels with the phase contrast image; (D and E) only one fluorescence channel is shown. (A-E) Polyclonal antibody against B-CK (red fluorescence) at a dilution of 1:200; (A-C and D) monoclonal antibody against  $Mi_b$ -CK 30a (green fluorescence) at a dilution of 1:2 served as primary antibodies. (D and E) Higher magnifications from the region of the midpiece that is indicated in C with an arrow (M, midpiece).



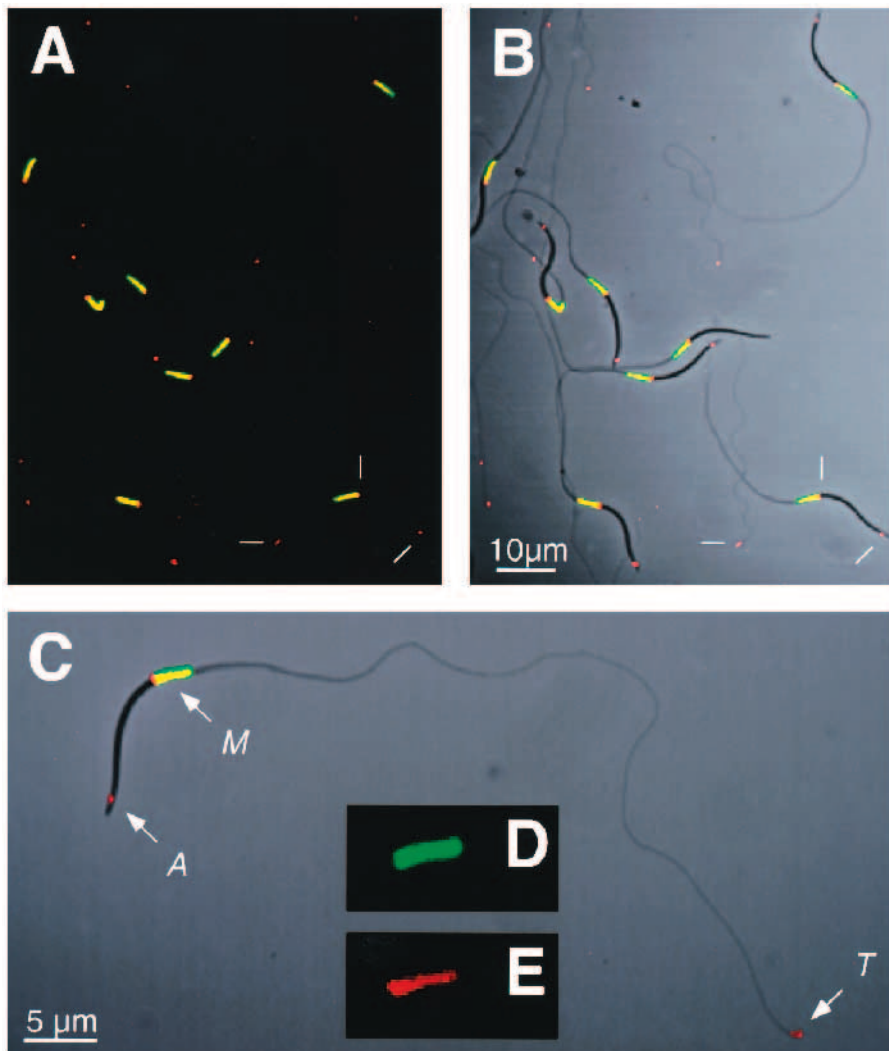
in the midpiece is likely to detect antigen in the cytoplasm around the mitochondria. Antibodies against  $Mi_b$ -CK (monoclonal antibody 30a as well as anti-peptide antibody  $Mi_b$ -CK 1-15) stained the mitochondria-rich midpiece strongly and with very high specificity (Fig. 8D, see also A-C). The staining was uniform throughout the whole midpiece (Figs 7D, 8D), a finding that was corroborated by optical sectioning with confocal laser microscopy. The polyclonal antibody against  $Mi_a$ -CK which was cross-absorbed against  $Mi_b$ -CK strongly stained the midpiece at the boundary to the head. Additionally, the proximal part of the mitochondria-rich midpiece was stained in a non-uniform manner, with the intensity gradually decreasing away from the head-midpiece junction (Fig. 8). This non-uniform staining of the midpiece was also observed when the midpiece had previously been saturated with the monoclonal antibody against  $Mi_b$ -CK. Double staining experiments with the polyclonal antibody against  $Mi_a$ -CK and the monoclonal antibody against  $Mi_b$ -CK revealed distinct localizations of  $Mi_b$ -CK and of the ' $Mi_a$ -CK variant' (Fig. 8D,  $Mi_b$ -CK, and E,  $Mi_a$ -CK). The red anti- $Mi_a$ -CK staining overlapped in part with the green fluorescence of the anti- $Mi_b$ -CK staining, giving rise to a yellow signal in the midpiece (Fig. 8C, see arrow M). Surprisingly, the distal end of the sperm tail and the tip of the head were also stained by the antibody against  $Mi_a$ -

CK (Fig. 8C, arrows T and A). All these results were reproduced using conventional immunofluorescence microscopy (not shown).

## DISCUSSION

In accordance with previous studies on human seminal plasma (Asseo et al., 1981; Soufir, 1979), B-CK was identified in our experiments as the sole CK isoenzyme present in seminal plasma of the rooster. The occurrence of CK in seminal plasma is not surprising since large amounts of PCr and Cr are present in seminal vesicle epithelium cells and seminal vesicle fluid (Lee et al., 1991), with the latter making up 50-70% of the seminal plasma. Considering the fact that addition of PCr to human ejaculate or isolated sperm enhances sperm motility and velocity (Fakih et al., 1986), it seems plausible that B-CK in seminal plasma contributes to the activation of, or supplies external 'energy' to rooster sperm.

In previous experiments on human and rooster sperm (Huszar et al., 1992; Wallimann et al., 1986), B-CK and an unspecified  $Mi$ -CK isoenzyme had been identified and tentatively assigned to the sperm tail and to the mitochondria in the midpiece, respectively. Both characterization and localization



**Fig. 8.** Localization of the ' $Mi_a$ -CK variant' and of  $Mi_b$ -CK in rooster spermatozoa. Confocal microscopy pictures manifest the distinct localization of the ' $Mi_a$ -CK variant' and of  $Mi_b$ -CK. (A) Superimposition of both fluorescence channels; (B,C) superimposition of both fluorescence channels with the phase contrast image; (D and E) only one fluorescence channel is shown. The monoclonal antibody against  $Mi_b$ -CK 30a (green fluorescence) was used at a dilution of 1:2 in A-D, and the polyclonal antibody against  $Mi_a$ -CK (which was cross-absorbed against  $Mi_b$ -CK; red fluorescence) at a dilution of 1:300 in A-C and E. In A and B the different localizations of the ' $Mi_a$ -CK variant' are indicated with white lines, corresponding to the arrows in C. Note that the polyclonal antibody against  $Mi_a$ -CK stained mainly the mitochondria (C, arrow; M, midpiece), but also the distal end of the sperm tail and the tip of the head (C, arrows; T, tail and A, acrosome).  $Mi_a$ -CK and  $Mi_b$ -CK overlap in the midpiece, as reflected by the yellow fluorescence in A-C.

of the CK isoenzymes in rooster sperm were worked out in more detail in the present experiments, since CK isoenzyme expression patterns proved to be more complex than previously assumed, and since a complete set of highly specific antibodies against the chicken CK isoenzymes was available only recently.

Biochemical characterization revealed the presence of three CK isoenzymes in rooster sperm, namely B-CK, Mi<sub>b</sub>-CK and a 'Mi<sub>a</sub>-CK variant', with Mi<sub>b</sub>-CK representing the major and the 'Mi<sub>a</sub>-CK variant' the minor Mi-CK isoform. These findings are surprising since Mi<sub>b</sub>-CK had never been found in a cell in combination with B-CK or Mi<sub>a</sub>-CK. Furthermore, these results question a previous study (Payne et al., 1991) which concluded that sarcomeric Mi-CK (Mi<sub>b</sub>-CK) is exclusively expressed in heart and skeletal muscle. However, species differences have to be taken into account, since recent investigation revealed Mi<sub>a</sub>-CK to be the sole mitochondrial CK isoform in mouse sperm (Steehgs et al., 1995).

Several lines of evidence point to the presence of a Mi-CK isoform different from Mi<sub>b</sub>-CK in rooster sperm. On SDS-polyacrylamide gels this isoform and genuine Mi<sub>a</sub>-CK displayed the same  $M_r$  which is slightly, but distinctly lower than that of Mi<sub>b</sub>-CK (Fig. 4). Relative intensities of the two Mi-CK bands in rooster sperm on immunoblots using polyclonal antibodies against Mi<sub>a</sub>-CK and Mi<sub>b</sub>-CK, with each of them cross-reacting slightly with the other Mi-CK isoenzyme (Fig. 4C,D), also suggests that the additional Mi-CK band more resembles genuine Mi<sub>a</sub>-CK than Mi<sub>b</sub>-CK. Furthermore, the additional Mi-CK band was selectively stained by the specific, cross-absorbed antibody against Mi<sub>a</sub>-CK, but not by a specific anti-peptide antibody against Mi<sub>b</sub>-CK (not shown). The assignment of the additional CK species to the Mi-CK isoforms is supported by the fact that it was mainly localized in the midpiece of the sperm where the mitochondria are located using confocal laser (Fig. 8) and immunofluorescence microscopy (not shown). Finally, northern blot analysis indicated that besides B-CK and Mi<sub>b</sub>-CK mRNA, genuine Mi<sub>a</sub>-CK mRNA is present in rooster testis (M. Stolz, unpublished observation). The testis Mi<sub>a</sub>-CK protein co-migrated on 2-D gels with genuine Mi<sub>a</sub>-CK purified from chicken brain. The latter finding suggests that within rooster testis, Mi<sub>a</sub>-CK might be present in sperm precursor cells (M. Stolz, unpublished).

On the other hand, the additional Mi-CK isoform found in rooster sperm also displayed distinctive features when compared to genuine Mi<sub>a</sub>-CK from chicken brain. The pI of the additional Mi-CK isoform was more acidic than expected for chicken Mi<sub>a</sub>- or Mi<sub>b</sub>-CK. On immunoblots, the additional Mi-CK band was not stained by anti-peptide antibodies against Mi<sub>a</sub>-CK and specific antibodies against other CK isoenzymes. These latter findings are surprising since these anti-peptide antibodies specifically recognize Mi<sub>a</sub>-CK on blots of chicken brain extracts as well as on brain sections (Kaldis et al., 1996). The apparent discrepancy may be due to buried epitopes or to a different protein sequence.

The additional Mi-CK isoform might represent a genuine, new sperm-specific CK isoenzyme. In previous northern blot experiments, a cDNA probe against the coding, but not against the non-coding region of rat ubiquitous Mi-CK hybridized with rat testis mRNA, thus suggesting alternative splicing of ubiquitous Mi-CK or the presence of a novel Mi-CK gene (Payne et al., 1991). However, these results should be interpreted with

caution since the hybridization signal was very weak even when a fair amount of testis poly(A<sup>+</sup>) RNA was used.

In order to unambiguously resolve the question of whether the additional Mi-CK isoform in sperm is simply a posttranslationally modified Mi<sub>a</sub>-CK species or a novel sperm-specific CK isoenzyme, protein sequencing will be required. However, many attempts to N-terminally sequence the additional Mi-CK spot obtained by 2-D gel electrophoresis of rooster sperm proteins have failed so far. This is probably due to a blocked N terminus as previously found for sea urchin sperm T-CK (which is N-terminally myristoylated; Quest et al., 1992) and for one of the sea urchin sperm Mi-CK bands (Wyss et al., 1995). Since the additional Mi-CK isoform in rooster sperm is closely related to Mi<sub>a</sub>-CK, but also displays some distinctive features, it is termed 'Mi<sub>a</sub>-CK variant'. If it turns out to be a modified Mi<sub>a</sub>-CK, the more acidic pI might be explained by multiple phosphorylation of the polypeptide chain, since most other modifications, e.g. myristoylation (Quest and Shapiro, 1991), have only a minor influence on the pI of a protein. In fact, several CK isoenzymes (chicken Mi<sub>b</sub>-CK, chicken and rabbit B-CK and M-CK) were shown to be autophosphorylated (Hemmer et al., 1991). In addition, B-CK represents a substrate for protein kinases, in particular for protein kinase C (Chida et al., 1990a,b; Quest et al., 1990).

Similar to the situation in rooster sperm, two Mi-CK bands with slightly different pI (on IEF) were observed in sea urchin (*Psammechinus*) sperm (Wyss et al., 1995). One of these Mi-CK bands was not amenable to amino acid sequencing, most probably due to N-terminal blockage, while for the other band, a stretch of 22 amino acids could be sequenced. In sperm of the sea urchin *Strongylocentrotus purpuratus*, specific radiolabelling of the CK isoenzymes by the CK inhibitor DNFB revealed three Mi-CK bands with apparent  $M_r$  values of 44,000, 47,000, and 50,000 on SDS-polyacrylamide gels (Tombes and Shapiro, 1985; Tombes and Shapiro, 1987). Additional evidence for the co-existence of two mitochondrial isoenzymes within an organ comes from characterization of the CK isoenzymes in chicken cerebellum. Besides B-CK (Quest et al., 1989), M-CK (Hemmer et al., 1994), and Mi<sub>a</sub>-CK (Schlegel et al., 1988a), we detected an unexpected, slightly more acidic 'Mi<sub>b</sub>-CK variant' in cerebellum (Kaldis et al., 1996).

In both confocal laser and immunofluorescence experiments, the 'Mi<sub>a</sub>-CK variant' was localized mainly at the head-midpiece boundary, with the intensity of the staining gradually decreasing away from the head-midpiece junction (Fig. 8E). Most surprisingly, some minor immunostaining of the 'Mi<sub>a</sub>-CK variant' was also found at the distal end of the sperm tail and at the acrosome (Fig. 8C). The localization of the 'Mi<sub>a</sub>-CK variant' at the head-midpiece junction is clearly distinct from the localization of Mi<sub>b</sub>-CK in the midpiece and corresponds exactly to the position of the proximal centriole (see Lake et al., 1968; Tingari, 1973). The distal centriole is found in the center of the midpiece and is surrounded by mitochondria. If the distal centriole is also stained by the antibody against Mi<sub>a</sub>-CK, the non-uniform staining may be due to limited access of the antibody to the centriole.

B-CK staining was found mainly in the proximal half of the rooster sperm tail (Fig. 7A-C), where the axoneme is surrounded by the fibrous sheets and thus the flagellum is significantly thicker. The distal half of the sperm tail is very thin and accordingly less stained by the antibody against B-CK,



however, B-CK staining can be found along the entire sperm flagellum. Accounting for the use of detergent during fixation and staining of the sperms (see Material and Methods) it seems plausible that B-CK was lost due to diffusion. B-CK was also found at the acrosome and in the midpiece (Fig. 7), therefore we conclude that B-CK can be found wherever cytoplasm is located in rooster sperm.

Experiments on sea urchin sperm strongly favour a transport function for 'high-energy phosphates' for the CK system. Inhibition of the CK isoenzymes in sperm by DNFB led to an inhibition of flagellar motility in the distal two thirds of the tail (Tombes and Shapiro, 1985). This problem may be overcome by Cr and PCr which are present in much higher concentrations and, due to the smaller size and to the lower negative charge, diffuse faster than ADP and ATP (for reviews see Wallimann et al., 1992; Wyss et al., 1992). Accordingly, the fertilizing potential of sperm may depend critically on the proper function of the CK system.

When compared to sperm from sea urchins and fowl (e.g. rooster and turkey) which show very high CK activity, sperm from stallions and bulls seem to have much lower or negligible amounts of CK (G. Kamp, University Münster, Germany, personal communication). In addition, a transgenic mouse mutant lacking Mi<sub>a</sub>-CK, which is present as the main Mi-CK isoenzyme in sperm of normal mice, was shown to produce motile sperm and to be fertile (Steehgs et al., 1995). In contrast to sea urchin sperm which thrive exclusively on mitochondrial fatty acid oxidation (Tombes and Shapiro, 1985), mammalian sperm must remain motile also under the low-oxygen conditions prevalent in the female tract, deriving chemical energy from glycolytic pathways (Hammerstedt and Lardy, 1983) or from external sources, in the form of glucose, fructose, mannose or even PCr, substances all shown to be present in seminal plasma (Hammerstedt and Lardy, 1983). Therefore, Mi-CK may not be absolutely required for sperm motility and fertilization in mammals. However, the fact that rooster sperm, like sea urchin sperm, responded to the CK inhibitor DNFB (Wallimann et al., 1986) indicates that the CK/PCr system may also be physiologically important in this species. Therefore, depending on the animal species and the specific energy requirements (e.g. whether spermatozoa are released into sea water without exogenous support, whether they are contained in a closed system in the female tract, and/or whether sperm can count on an external energy supply, etc.) the importance of the CK system for proper sperm function and fertility may range from essential (sea urchin) to marginal (some mammals).

In this study, we found a sperm-specific Mi<sub>a</sub>-CK variant together with genuine sarcomeric Mi<sub>b</sub>-CK, but both with differential localisation within a single sperm cell. Future studies, will attempt to localize the CK isoenzymes, in particular, the 'Mi<sub>a</sub>-CK variant', at higher resolution e.g. by immuno-electron microscopy, to evaluate possible interactions of CK isoenzymes with subcellular structures and with each other. This will hopefully help to elucidate more precisely the functions of the different CK isoenzymes in sperm.

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