The Catalytic Subunit of the cAMP-dependent Protein Kinase of Ovine Sperm Flagella Has a Unique Amino-terminal Sequence*

Jovenal T. San Agustin‡, John D. Leszyk§, Lydia M. Nuwaysir¶, and George B. Witman‡

From the ‡Department of Cell Biology and §Protein Chemistry Facility, University of Massachusetts Medical Center, Worcester Foundation Campus, Shrewsbury, Massachusetts 01545 and ¶Perkin Elmer Sciex, Applied Biosystems Division, Foster City, California 94404

The basis for the unusual properties of the catalytic subunit (C) of ram sperm cAMP-dependent protein kinase was investigated. Ram sperm C was purified and found by mass spectrometry (MS) to be ~890 Da smaller than Cβ, the predominant somatic isoform. Partial internal amino acid sequence from ram sperm C was an exact match to that of bovine Cα, but differed from the predicted sequences for the Cγ and Cγ isoforms. MS analysis of 2-nitro-5-thiocyanatobenzoic acid fragments showed that the mass difference originated in the amino-terminal region. A unique blocked amino-terminal fragment was isolated from sperm C and sequenced by a combination of tandem mass spectrometry and Edman degradation of a subfragment. The results revealed that the amino-terminal myristate and the first 14 amino acids of Cα are replaced by an amino-terminal acetate and six different amino acids in sperm C. The predicted mass difference due to these changes is 899 Da. The region of homology between sperm C and Cα begins at the exon 1/exon 2 boundary in Cα, suggesting that sperm C results from use of an alternate exon 1 in the Cα gene. The different amino terminus of sperm C may be related to a unique requirement for localization of the “free” C subunit within the sperm flagellum.

*This study was supported by National Institutes of Health Grant HD23858, National Science Foundation Grant 9512226, and a grant from the Campbell and Hall Charity Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The cAMP-dependent protein kinase (PKA) is a major enzyme in cellular signal transduction and is thought to mediate most of the physiological responses to cAMP in eukaryotic cells (1). Below a cAMP threshold concentration, PKA exists as an inactive tetramer of two catalytic and two regulatory subunits (Cα(R)). The two R subunits form a dimer with each protomer inactive tetramer of two catalytic and two regulatory subunits. Activation of adenylate cyclase by extracellular signals raises the intracellular concentration of cAMP, and at a certain threshold concentration cAMP binds to the R subunits of the PKA tetramer, releasing C to phosphorylate its substrates (for reviews, see Refs. 1 and 5–8).

Cyclic AMP-dependent signaling has an important role in the control of sperm movement. Mammalian sperm are nonmotile in the testis, but as they pass through the epididymis they acquire the capacity for motility. This process is known as “epididymal maturation” and is essential for the sperm to fertilize an egg (9). Several types of studies have shown that changes in sperm cAMP levels are involved in epididymal maturation (see Refs. 10–12 for reviews). The most direct evidence for a role of cAMP in the acquisition of the capacity for motility has come from studies of demembranated, reactivated sperm (13–15). When caudal epididymal or ejaculated sperm were demembranated by treatment with nonionic detergents, and then placed in an appropriate solution containing ATP, they were reactivated with a waveform very similar to that of intact ejaculated sperm. In contrast, under the same conditions demembranated testicular sperm exhibited very poor motility. However, if cAMP was added to the reactivation medium, the demembranated testicular sperm began to beat with a motility similar to that of the mature sperm models. Cyclic AMP-dependent motility also could be demonstrated in the demembranated ejaculated sperm if the sperm were metabolically inhibited to reduce their motility prior to demembranation (16, 17). The requirement for cAMP could be bypassed by addition of exogenous C to the reactivation solution, confirming that cAMP was acting via PKA (17). Therefore, cAMP-dependent phosphorylation is critical for sperm motility.

Sperm C (Cα) appears to have unusual solubility properties. It is generally accepted that, in the presence of cAMP, C is soluble in the cytoplasm (1, 18). However, in a previous study we observed that neither detergent nor cAMP alone released C activity from demembranated sperm, but that cAMP plus nonionic detergent did release the activity (17). These results suggested that Cα is bound to internal sperm structures by two types of bonds, one sensitive to detergent and one sensitive to cAMP.

There are at least three different isoforms of mammalian C, Cα, Cβ, and Cγ, which are the products of different genes. Cα occurs in a wide variety of tissues (19); Cβ also is widely distributed but is expressed in lesser amounts than Cα in most tissues except the brain (19, 20). Cγ appears to be expressed only in testis (21). Cα appears to be the predominant isoform in mammalian germ cells (22). Splice variants of both Cα and Cβ also are known (23–25).

We now have further characterized Cα and investigated its relationship to the previously known isoforms of C. We found that Cα is the major, and perhaps the only, PKA catalytic subunit in sperm. We isolated Cα from ram sperm, which could be obtained in amounts sufficient for protein biochemistry.
Purification also was aided by the solubility properties of the subunit, which made it possible to obtain a homogeneous preparation of Cα by a simple two-step protocol. Mass spectrometry (MS) revealed that the mass of Cα was ~890 Da less than that of C from ovine striated muscle. The amino terminus of Cα was blocked, but partial amino acid sequence from internal tryptic and CNBr fragments was an exact match to the sequence of bovine Cα. Cα clearly differed in sequence from the Cβ and Cγ isoforms. An amino-terminal endoproteinase lysine-C fragment was isolated from Cα and sequenced by a combination of tandem mass spectrometry (MS/MS) and Edman degradation of an endoproteinase aspartate-N subfragment. The results revealed that the amino-terminal 14 amino acids and amino-terminal myristate of Cα are replaced by six different amino acids and an amino-terminal acetate in Cα. The predicted mass difference due to this replacement is 899 Da, in excellent agreement with an amino-terminal acetate in Cα. The predicted mass difference due to this replacement is 899 Da, in excellent agreement with the MS data. The region of homology between Cα and C has been attributed to this replacement.

Materials and Methods

Materials used and their sources were: 4-aminobenzenamide, cAMP, CNBr, 3,5-dimethoxy-4-hydroxycinnamic acid, and NTGB from Aldrich; [14C]-methylated protein molecular weight markers were from Amersham; Aquapore RP-300 C8 column (0.1 × 25 cm), Aquapore OD-300 C8 column (1 × 100 mm), and C18 300 A column (0.5 × 150 mm) from Applied Biosystems; Tween 20 and SDS from Bio-Rad; endoproteinase aspartate-N from Boehringer Mannheim; bovine serum albumin (fatty acid free, 300 mg/ml) from Sigma-Aldrich; nitrocellulose BA 83, 0.2 μm, from Millipore; [32P]ATP from NEN Life Science Products Inc; CM Fast Flow, Source 15S, from Pharmacia LKB; endoproteinase lysine-C and modified trypsin from Prozyme; nitrocellulose BA 83, 0.2 μm, from Schleicher and Schuell; alkaline phosphatase labeled goat anti-rabbit IgG (A9397), Amido Black, ammonium bicarbonate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium buffered tablets, α-cyano-4-hydroxycinnamic acid, pH 6.5 (PBSI). Subsequent steps were done at 4 °C. The sperm suspension in each tube was sonicated on ice (Branson Sonifier Model 200 fitted with a 3-mm double-stepped microtip) using four 10-s continuous pulses with a power setting of 25% (37.5 watts). The above sonication conditions produced about 70% sperm decapitation.

The flagella were separated from heads and residual whole sperm by centrifugation at 4 °C in a discontinuous sucrose gradient as described earlier (28) except that the bottom 2.2 M sucrose layer was omitted. The sonicated sperm (48 ml) were stirred into 108 ml of 2.2 M sucrose and 13-ml aliquots were pipetted into 32-ml polycarbonate centrifuge tubes each containing 19 ml of 2.05 M sucrose. The tubes were centrifuged at 72,000 × g for 1.5 h (Beckman SW 28 rotor). Heads and whole sperm pelleted at the bottom of the flagella while the flagella collected at the 1.5–2.05 M sucrose boundary.

The isolated flagella were transferred to 12-ml thick-walled polypropylene tubes, diluted 1:2 with PBSI, and centrifuged at 18,000 × g for 15 min (Sorvall SS 34 rotor). The pellets were resuspended in PBSI, and the concentration of the flagellar suspension determined using a hemacytometer. Usually about 10 × 10⁶ flagella were obtained from 6 ml of ram semen.

Isolation of sperm heads was as described (28). Two passes through the sucrose gradient were done to make sure that the preparation was free of flagella and undecapitated sperm.

Preparation of Demembranated Ovine Ejaculated Sperm, Sperm Heads, and Sperm Flagella

Ovine ejaculated sperm were demembranated as described earlier (26). Isolated sperm heads and flagella were treated the same way except that flagella were separated from the demembranated medium by centrifugation through 40% Percoll, and heads were separated from the demembranated medium by centrifugation through 70% Percoll.

Renaturation of Protein Kinases Blotted on PVDF Membrane

Samples were dissolved in SDS-PAGE sample buffer (10% glycerol, 3% SDS, 0.03% bromphenol blue, 50 mM DTT, 62.5 mM Tris-HCl, pH 6.8) and then electrophoresed in a 1.5-mm thick gradient gel (5–15% acrylamide, 12 × 14 cm). Protein kinases were renatured on the blot and detected using a protocol adapted from Ferrell and Martin (29) and described earlier (30). In some experiments, 1% poly(Glu,Tyr) 4:1, a tyrosine kinase substrate, was used as blocking solution in place of 5% bovine serum albumin.

Western Blotting

Samples were dissolved in SDS-PAGE sample buffer, electrophoresed in 0.75-mm thick 10% minigels (4.5 × 8 cm), and blotted to PVDF membranes (TE 22 transfer apparatus, Hofer Scientific, 28 V for 5 min followed by 84 V for 20 min). The transfer buffer composition was 50 mM Tris base, 192 mM glycine, 20% methanol, and 0.1% SDS. After transfer, the membrane was blocked with TBS-Tween 20 (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 20 min, incubated with anti-bovine C (in TBS-Tween 20, 1:20 dilution) for 1 h, washed four times (5 min each wash) with 200 ml of TBS-Tween 20. Incubation with the secondary antibody (alkaline phosphatase labeled goat anti-rabbit IgG in TBS-Tween 20, 1:800 dilution) was for 1 h, followed by washing twice, 10 min each wash, with 200 ml of TBS-Tween 20. Final wash was 200 ml of 30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 for 20 min. The blot was then exposed to 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (1 tablet dissolved in 10 ml water) to reveal cross-reacting proteins.

Isolation of the PKA Catalytic Subunit (Cα) from Ovine Sperm Flagella

Extraction of Cα from Sperm Flagella by cAMP—Extraction was done at 4 °C. Sperm flagella (in PBSI) were centrifuged (1750 × g, 15 min) and resuspended for 30 min in a Triton X-100/NaCl buffer (5 mM potassium phosphate, pH 6.5, 0.5%, v/v, Triton X-100, 150 mM NaCl, 1 mM EDTA, 25 μM leupeptin, 1 mM DTT), at a concentration of 3 × 10⁶ flagella/ml. This treatment removed the plasma membrane and most of the soluble flagellar proteins that otherwise would coextract with Cα upon subsequent treatment with cAMP; Cα itself remained bound to the demembranated flagella. The suspension then was centrifuged (1750 × g, 30 min), the supernatant discarded, and the pellet dispersed in KPNELD wash buffer (5 mM potassium phosphate, pH 6.5, 50 mM

NaCl, 1 mM EDTA, 25 μM leupeptin, 1 mM DTT, 0.22 mM of buffer/108 flagella) and centrifuged (1750 × g, 10 min). This was repeated with 0.167 mM of buffer/108 flagella. The washed pellet was then extracted for 20 min with KPNEFLD + 10 μM CAMP, 0.167 mM/108 flagella. The CAMP extract, which contained Csm, was transferred to a polypropylene tube that was treated with Triton X-100 to minimize nonspecific binding of Csm and then centrifuged at 27,000 × g for 15 min to remove residual flagella.

Fast Protein Liquid Chromatography of CAMP Extract of Sperm Flagella—The CAMP extract was made 0.2% (w/v) in β-octylglucoside and applied at 0.3 ml/min flow rate to a CM Fast Flow column (0.5 × 8.5 cm) previously equilibrated with chilled buffer A (20 mM potassium phosphate, pH 6.5, 1 mM EDTA, 50 mM NaCl, 0.1%, w/v, β- octylglucoside, 1 mM DTT). The column was washed with buffer A until the absorbance, which rose due to cAMP in the extract, returned to baseline. The column was eluted with 7 ml of a cold linear NaCl gradient of buffer A plus buffer B (20 mM potassium phosphate, pH 6.5, 1 mM EDTA, 50 mM NaCl, 0.1%, w/v, β-octylglucoside, 1 mM DTT) at 0.2 ml/min flow rate. Csm was detected in fractions between 180 and 215 mM NaCl. Yield was typically 20–25 μg/6 ml of semen. β-Octylglucoside was included in the buffers to prevent nonspecific binding of Csm to the glass column, tubings, and tubes, as well as to stabilize the native conformation of Csm (31).

Isolation of the PKA Catalytic Subunit (Csm) from Ovine Skeletal Muscle

The procedure was adapted from Okuno and Fujisawa (31) for the isolation of bovine heart Csm. Working at 4 °C, about 600 to 800 g of skeletal muscle tissue from the hind legs and back of a ram were stripped of fat and connective tissue and then passed through a meat grinder (coarse setting). The ground tissue was mixed with 1.5 liters of a homogenization buffer (10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 0.1 mM DTT) and further processed to a smooth consistency in a Waring blender. The homogenized mass was then centrifuged at 125,000 × g for 1 h and then transferred to a Centriplus 10 concentrator (Amicon) and washed with 20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 50 mM NaCl, 0.1% (w/v) β-octylglucoside, 1 mM DTT and then incubated overnight at 37 °C followed by direct injection of the supernatant onto the HPLC. Tryptic peptides were separated on a 1-mm × 25-cm Applied Biosystems (Aqauapor RP-300) column using a linear gradient from 100% solvent A (0.1% trifluoroacetic acid) to 55% solvent B (0.08% trifluoroacetic acid in acetonitrile/water: 70/30) in 30 min, then from 55% solvent B to 85% solvent B in 10 min at a flow rate of 150 μl/min. The eluant was monitored at 210 nm and fractions were collected manually.

CNBr Cleavage of Csm and Csm at Methionyl Residues

For electrophoretic analysis of fragments, about 2 μg each of Csm and Csm in 50 μl of buffer A were lyophilized. To each lyophilized sample was added 12.5 μl of 100 mM DTT and then allowed to stand at room temperature for 1 h. To start the cleavage reaction, 37.5 μl of 47 mM CNBr (in 88% formic acid) were added. The reaction mixture was incubated overnight (about 19 h) at room temperature in the dark. To stop the reaction, 100 μl of water was added and the resulting solution was lyophilized to remove the formic acid and unreacted CNBr. The pellet was resuspended in 100 μl of water and lyophilized again, and afterward 15 μl of SDS sample buffer (50 mM Tris-HCl, pH 8.8, 15% glycerol, 5% SDS, 0.003% bromphenol blue) was added to the lyophilized sample. The sample was electrophoresed in a 0.75-mm thick 13% Tris-Tricine/SDS-polyacrylamide gel (32); the gel was silver stained to reveal the fragments.

For sequencing of fragments, the starting sample contained about 10–15 μg of C. The cleavage products were separated by electrophoresis in a 1.5-mm gel and transferred to a PVDF membrane according to the protocol of Otter et al. (33), except that the transfer time was shortened from 17 to 12 h. The blot was stained with Amido Black and the fragments excised and sequenced.

NTCB Cleavage of Csm and Csm at Cysteinyl Residues

The procedure was adapted from Jacobson et al. (34). The concentration of Csm and Csm (in fresh buffer A) was adjusted to 40 μg/ml and 50 μl of each was then lyophilized. The dried samples were redissolved in reduced denaturation buffer (45 mM potassium phosphate, pH 6.8, 0.1% DTT), and allowed to stand for 30 min at room temperature. A 15-μl aliquot of freshly prepared NTCB (66.5 mM) was added and allowed to react for 20 min. The amount of NTCB added gave about a 10-fold excess of NTCB over the combined sulphydryl groups of DTT (present in buffer A) and Csm or Csm (presumed to be two, as with bovine C). NTCB (15 mg) was first dissolved in 0.333 ml of ethanol and then made up to 1 ml with the reduced denaturation buffer. The pH of the reaction mixture after the addition of NTCB was about 8.4. The cleavage reaction was initiated by the addition of 4.8 μl of 1 N NaOH, which brought the pH up to about 11.6. The reaction was allowed to proceed for about 16 h. The mixture was then transferred to a Centricon 10 concentrator (Amicon) and washed with buffer A until the urea and excess NTCB were reduced to below 30% and the final volume was reduced to about 200 μl. The concentrate was then mixed with the same volume of 2 × Schägger sample buffer and electrophoresed in a 10% Tris-Tricine/SDS-polyacrylamide gel (32). The fragments were revealed by silver staining.

Preparation of NTCB fragments for mass spectrometry was the same, except that 30 μg each of Csm and Csm were used as starting material. The Tris-Tricine gel was transferred to nitrocellulose (33) at 20 °C for 5 min and then at 4 °C for 5 h. The bands were revealed by staining with Ponceau S and then cut out for MALDI TOF MS.

Endopeptidase Lysine-C Digestion of Csm and Csm

Csm and Csm were blotted on nitrocellulose which was then cut into 1 × 1-mm pieces and submerged under 50 μl of Digest Buffer. An aliquot of endopeptidase lysine-C (0.5 μg in 0.5 μl of 25 mM sodium phosphate, pH 7.5, 1 mM EDTA) was then added and the samples were incubated overnight at 37 °C followed by direct injection of the supernatant onto
the HPLC. Endoproteinase lysine-C peptides were separated on a 0.5 ×
150-μm Applied Biosystems column (C18, 300 A) using a linear gradient
from 100% solvent A to 46% solvent B in 35 min, then from 46% solvent
B to 60% solvent B in 10 min at a flow rate of 20 μL/min. The eluent was
monitored at 210 nm and fractions collected manually.

Endoproteinase Aspartate-N Digestion of Blocked Amino-terminal
Peptide of Cs

The blocked amino-terminal peptide isolated from the endoproteinase
lysine-C digest of Cs was dissolved in 25 μL of 100 mM ammonium
bicarbonate, and 0.12 μg of endoproteinase aspartate-N in 3 μL of 10 mM
Tris-HCl, pH 7.5, was added. Digestion proceeded overnight at 37 °C.
The digested peptide was desalted in a C18 microcartridge (0.8 mm × 5
mm, LC packing, San Francisco, CA) prior to direct application to
Edman sequence analysis.

Peptide Synthesis

Four peptides were synthesized in order to compare their MS/MS
product ion spectra to the MS/MS product ion spectrum of the amino-
terminal blocked endoproteinase lysine-C peptide derived from Cs: 1) acetyl-SANPNDVQEFLAK; 2) acetyl-ASNPNDVKEFLAK; 3) acetyl-
ASGPNGDVKEFLAK; and 4) acetyl-ASPGGDVKEFLAK. Peptides
were synthesized on a Perkin-Elmer 432A Synergy Peptide Synthesizer
using HBTU activation and the 9-fluorenylmethoxycarbonyl protecting
strategy. Crude peptide mixtures were purified by reverse phase
HPLC using an Aquapore OD-300 C18 column (1 × 100 mm) and a
water/acetonitrile/trifluoroacetic acid gradient at 40 μL/min and 37 °C.

RESULTS

Cs Has an Unusual Mobility in SDS-PAGE—Our previous
results (17) showed that demembranated ram sperm models retained >90% of the sperm PKA activity; moreover, preparations of demembranated sperm with initially poor motility in the presence of ATP became highly motile when cAMP was
added, indicating that the cAMP-dependent pathway that initi-
tates motility was still intact. To begin characterization of Cs
and other protein kinases in these cytosol-free models, the
models were analyzed using protein kinase blots. Proteins of
the demembranated sperm were separated by SDS-PAGE, blot-
ted to PVDF membranes, renatured by incubation in a buffer
containing the non-ionic detergent Tween 20, and then incubated in the presence of [γ-32P]ATP to allow the bound kinases to phosphorylate themselves or the blocking reagent. A typical autoradiogram of such a blot is shown in Fig. 1A. Six to eight
putative kinase bands were observed in the demembranated
sperm. Two prominent bands at −Mr 40,000 and 39,000 mi-
grated slightly faster than porcine C (−Mr 41,000). To deter-
mine if either of these was Cs, the incubation with [γ-32P]ATP was carried out in the presence of PKI(5–24), a potent inhibitor of
C (35), including ram Cs (17) activity. The inhibitor com-
pletely and specifically blocked the labeling of porcine C and
the −Mr 40,000 band from sperm (Fig. 1B), indicating that the latter is likely to represent Cs. Labeling of the other bands was
not affected by PKI(5–24). Interestingly, labeling of the prom-
inent band at −Mr 40,000 was enhanced when the blocking
reagent was the tyrosine kinase substrate poly(Glu,Tyr) in-
stead of bovine serum albumin (results not shown), suggesting that this band represents a tyrosine kinase. Tyrosine kinases
also have been implicated in the control of sperm motility (36, 37).

To confirm that the sperm protein kinase inhibited by

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Protein kinase blots of demembranated ram sperm. Panel A, autoradiogram of blotted and renatured protein kinases after labeling with [γ-32P]ATP. Lane S, demembranated ram sperm (3.3 × 10⁶); lane C, porcine C (400 ng). Panel B, same as panel A except labeling with [γ-32P]ATP was done in the presence of 600 nM of the PKA inhibitor PKI(5–24). Arrows indicate PKI(5–24)-sensitive protein kinase bands. Asterisks indicate a possible tyrosine kinase (see text). Numbers indicate molecular weights of 14C-methylated marker proteins.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Immunodetection of Cs and C(sm) by Western blot using polyclonal antibodies to bovine C. Panel A, ram sperm (lane S, 1 ×
10⁶ washed sperm) contain a cross-reacting protein with a slightly faster mobility than porcine C (lane C, 62 ng). This protein is released
from sperm flagella by extraction with Triton X-100 (lane Ex, extract from 2 × 10⁶ flagella). Panel B, comparison of electrophoretic mobilities of porcine C (lane C, 20 ng), ram skeletal
muscle C (lane C(sm), 20 ng), and ram sperm head C (lane H, 3 × 10⁶ heads), and ram sperm flagellar C (lane F, 1 × 10⁶ flagella). Lane F + C(sm)
shows a mixture of ram skeletal muscle C (20 ng) and ram sperm flagella (1 × 10⁶ flagella). Panel C, the C subunits from ram testicular,
epididymal, and ejaculated sperm have identical electrophoretic mobilities. Lane 1, porcine C (31 ng); lanes 2 and 7, ejaculated ram sperm
flagella (6 × 10⁵ flagella); lanes 3–5, ram sperm from cauda, corpus, and caput regions, respectively, of the epididymis (1 × 10⁶ sperm each);
lane 6, ram sperm from rete testis (1 × 10⁶ sperm); Numbers indicate molecular weight of marker proteins (not shown).
PKI(5–24) is the catalytic subunit of PKA, the proteins of demembranated ram sperm were probed in Western blots with a polyclonal antibody against bovine C (Fig. 2A). The antibody reacted with a single sperm protein that migrated slightly faster than porcine C in the SDS-polyacrylamide gels. This protein could be extracted from sperm flagella by treatment with cAMP in the presence of Triton X-100 (Fig. 2A), in agreement with our previous observation that much of the PKI(5–24)-inhibitable protein kinase activity could be released from sperm by this same treatment (17). These results provided independent evidence that the −M, 40,000 band from sperm is an isof orm of C.

C, Is a Tissue Variant—The unusual mobility of ram C, could have been due to species (porcine versus ovine) or tissue (somatic versus sperm) variation. To clarify this, ram skeletal muscle C (Csm) was partially purified and compared directly to the sperm isoform (Fig. 2B). Because the predominant isof orm of C in skeletal muscle is Cα (19), the isoform that we purified from muscle is presumed to be predominantly or entirely Cα. The skeletal muscle subunit had the same mobility as porcine C, and both migrat ed slightly slower than the sperm subunit. When purified ram Csm and ram sperm flagella were mixed together, two distinct, nonmerging bands were observed. These results clearly indicated that the unusual mobility of Cs is tissue specific.

C, Is Contained Primarily in Flagella—The relative distribution of C, in demembranated, isolated sperm heads and tails also was investigated. The vast majority of C, was located in the flaggella (Fig. 2B, lanes H and F); note that lane H was loaded with 3 × 10⁶ sperm heads versus 1 × 10⁶ flaggella in lane F; however, some C, was detectable in the sperm heads. Similar results were obtained with intact sperm heads and intact sperm tails (results not shown). These results are consistent with previous reports that PKA is located primarily in the sperm flaggella (38–43).

The Unusual Mobility of C, Is Not the Result of Epididymal Processing—Some sperm proteins undergo processing during epididymal maturation (44–46). To determine if such processing was responsible for the unusual mobility of C, sperm were isolated from the testis and regions of the epididymis, and the relative mobilities of their PKA catalytic subunits compared in Western blots. Fig. 2C shows a Western blot of C, from demembranated ram testicular sperm, demembranated epididymal sperm (cauda, corpus, and caput), and demembranated ejaculated sperm flaggella. The mobility of C, was identical in sperm from all stages, and in all cases was slightly faster than that of somatic C. These results indicate that the apparently smaller size of C, is not due to processing during sperm maturation.

Purification of C, —To understand the difference between C, and Csm, it was necessary to purify C, for structural character-

FIG. 3. Purification of C, Silver-stained SDS-polyacrylamide gel illustrating, from left to right, sequential steps in C, purification. Starting material was 8.2 × 10⁸ ram sperm flaggella. Lanes are as follows: TX-100, proteins extracted by Triton X-100/NaCl (from 5.6 × 10⁹ flaggella; washes, supernatants recovered from washes of the flaggella after extraction with Triton X-100/NaCl; aex, cAMP extract from washed flaggella (4.5 × 10⁹); FT, flow-through during introduction of cAMP extract to the CM Fast Flow column; 10, 12, 14, 15, 16, 17, 18, 19, 20, 21, fractions collected from the CM Fast Flow column during elution with the NaCl gradient; Csm, ram Csm (140 ng); M, molecular weight markers. Fractions 12–20 were pooled to yield about 25 μg C,.
dicted for bovine somatic Ca with a myristylated glycine at the amino terminus (40,858 Da) (see below).

Because Cs is smaller than Csm, the difference in mass between the two subunits cannot be due to the addition of an unusual post-translational modification to Cs. Somatic Ca, which Csm is presumed to be, has been extensively characterized. Its major post-translational modifications are myristylation of the amino-terminal glycine (47), which adds 210 Da to the mass predicted by sequence alone, and phosphorylation at Thr-197 and Ser-338 (48), each of which would add 80 Da to the predicted mass. Therefore, even the total absence of these post-translational modifications in Cs could not account for the difference in mass. By the process of elimination, the difference must arise from differences in the primary structures of the two proteins. It seemed unlikely that this difference was due simply to proteolysis during fractionation of the sperm or purification of Cs, because: 1) no band was ever observed in sperm that migrated with somatic Ca, and 2) attempts to obtain amino-terminal amino acid sequence from Cs were unsuccessful, suggesting that its amino terminus was blocked (see below).

The Partial Amino Acid Sequence of Cs Is Identical to That of Ca—Beebe et al. (21) reported the isolation and sequencing of human cDNA clones encoding an unusual tissue-specific isoform of Ca, which they termed Cy. Cy mRNA was found in detectable levels only in testis. Cy has not yet been isolated from testis, but when it was expressed and purified from transfected cells it migrated in SDS-PAGE at 39–40 kDa versus 41–42 kDa for Ca (49). Although Cy reportedly is not sensitive to PKI, its expression in testis and the similarity in its apparent mass to that of Cs raised the possibility that Cs might be Cy.

Human Cy and Ca differ at 74 amino acid residues, suggesting that the ovine homologs of these two isoforms should be distinguished readily by even partial amino acid sequences. Attempts to obtain amino-terminal sequence directly from the intact ram Cs were unsuccessful, suggesting that its amino terminus is blocked. Consequently, purified Cs was digested with trypsin to generate tryptic fragments, and the fragments purified by HPLC. Similarly, purified ram Cs or Csm was cleaved with CNBr, and the larger fragments separated by SDS-PAGE and transferred to PVDF membrane. Selected tryptic and CNBr fragments were then sequenced by automated Edman degradation. Fig. 5 shows the resulting amino acid sequences compared with similar sequences predicted for bovine Ca and Cb, and human Ca and Cy. There is no published sequence for ovine Ca or Cb. However, the ovine Cs sequence exactly matched that of bovine Ca (78 out of 78 residues). Moreover, in 17 out of 18 positions where human Ca differed from human Cy, ovine Cs was identical to human Ca. Therefore, Cs is not Cy. Similarly, ovine Cs was identical to bovine Ca at 5 out of 5 positions where bovine Cs differed from bovine Cb, indicating that Cs is not Cb. These results strongly suggested that Cs is a short variant of Ca.

**FIG. 4.** MALDI TOF mass spectrum of Cs and Csm mixture. Equimolar amounts of ram Cs and Csm were mixed together and analyzed. Two discrete peaks were observed, differing in mass by 889.9 Da.

**FIG. 5.** Partial sequence of Cs and Csm from CNBr and tryptic fragments. Sequences obtained for ram Cs and Csm fragments are aligned with the corresponding fragments from bovine Ca and Cb, and human Ca and Cy. Residues different from those of Cs and Csm are highlighted.

**FIG. 6.** NTCB fragments from Cs and Csm. Panel A, Tris-Tricine gel of products resulting from NTCB cleavage of ram Cs (Cs) and Csm (Csm). The three bands are intact C (C) and fragments 1 (1) and 2 (2). Starting material was 2 μg each of Cs and Csm. Positions of molecular weight markers (M) are indicated. Panel B, MALDI TOF mass spectra of fragments 1 and 2 of Cs and Csm. Singly, doubly, and triply charged ion peaks are evident.
A single CNBr fragment of C₈ was analyzed and found to have a sequence identical to that of bovine Cₐ (Fig. 5). Although this sequence is inadequate to distinguish between Cₐ and C₈, the results are consistent with our presumption that C₈ is the conventional Cₐ isoform.

**Localization of the Region of Mass Difference**—To delimit the regions that are different between Cₛ and C₈, the purified subunits were treated with NTCB, which cleaves at cysteinyl residues. There are only 2 cysteinyl residues (Cys-199 and Cys-343) out of a total of 350 amino acids in either bovine or human Cₐ. The three fragments resulting from a complete cleavage of Cₐ by NTCB are predicted to have masses of 0.9 kDa (residues 343–350), 16.6 kDa (residues 199–342), and 23.0 kDa (residues 1–198). When the NTCB fragments of Cₛ and C₈ were electrophoresed in a Tris-Tricine/SDS-polyacrylamide gel, three major bands were seen (Fig. 6A). The largest band corresponded to the intact polypeptide. Based on the predicted sizes of the fragments, fragment 1 must be the amino-terminal fragment, and fragment 2 must correspond to residues 199–342. Fragment 2 from Cₛ and fragment 2 from C₈ had identical mobilities, whereas fragment 1 of Cₛ migrated more rapidly than fragment 1 of C₈. The fragments were then transferred to nitrocellulose and analyzed by MALDI TOF MS (Fig. 6B). Fragment 2 of Cₛ and fragment 2 of C₈ had nearly identical masses of 17,970 and 17,967 Da, respectively. In contrast, fragment 1 of Cₛ had a mass of ~23,620 Da, whereas fragment 1 of C₈ had a mass of ~24,444 Da, a difference of ~824 Da. Because this difference is similar to the difference in masses between the intact polypeptides (~890 Da), most of the difference in mass must be due to structural differences in the amino-terminal halves of the proteins. Substantial sequence in this part of Cₛ (residues 30–45, 72–91 and 129–133; see Fig. 5) already had been found to match the sequence of Cₐ, so these regions could be ruled out as being the source of the difference.

**Identification of a Unique Endoproteinase Lysine-C Fragment from Cₛ**—There are 34 lysyl residues in bovine Cₐ of which 8 occur in the first 59 residues. It therefore seemed likely that digestion of Cₛ and C₈ with endoproteinase lysine-C would allow the detection of any dissimilar fragments. Fig. 7A shows HPLC chromatograms of endoproteinase lysine-C fragments from Cₛ and C₈. A prominent peak eluting at 26 min was observed in the Cₛ digest but not in the C₈ digest. MALDI TOF MS analysis of this peak indicated that it contained a single peptide with a mass of 1474 Da (results not shown). Similarly, a peak at 1475 Da was observed in a MALDI TOF mass spectrum of the endoproteinase lysine-C digest of Cₛ, but not in the C₈ digest (Fig. 7B). An attempt to determine the amino-terminal sequence of the 1474-Da peptide obtained by HPLC was unsuccessful, suggesting that its amino terminus was blocked. These results indicated that this fragment probably represented the amino terminus of Cₛ.

**Amino Acid Sequence of a Unique Amino-terminal Fragment from Cₛ**—The structure of the 1474-Da peptide was solved by a combination of MS/MS on a triple quadrupole mass spectrometer and Edman sequence analysis of an endoproteinase aspartate-N cleavage product.

In product ion MS/MS, precursor ions (or parent ions) of a particular m/z value are selected in the first quadrupole (Q1) of a triple quadrupole mass spectrometer and allowed to enter the second quadrupole (Q2). The second quadrupole acts as a collision cell and is filled with a neutral gas (in this case nitrogen). The parent ions undergo fragmentation through collisions with this neutral gas, a process called collisionally activated dissociation, or CAD. These product ions (or daughter ions) are then analyzed in the third quadrupole (Q3). For peptide ions, fragmentation specifically at the amide bonds results...
in a series of ions with charge retention on either the carboxyl terminus ("y-ions") or amino terminus ("b-ions"). The sequence of the peptide can be deduced from these, as well as other fragment ions in the daughter ion spectrum.

The doubly charged ion (m/z 738.3) was selected and fragmented, producing the spectrum shown in Fig. 8B. Initial interpretation of the spectrum confirmed an amino-terminal blocked residue (acetyl-AS) and provided much of the carboxyl-terminal sequence (DV(K/Q)EF(I/L)AK). Leucine and isoleucine have identical masses and lysine and glutamine have nearly identical masses and so could not be distinguished. Because only a single aspartate was detected in the sequence, the 1474-Da peptide was cleaved with endoproteinase aspartate-N, and the fragments then sequenced by Edman degradation. As predicted, a single Edman sequence (DVKEFLAK) was obtained. This indicated that the residue following valine was lysine and confirmed the rest of the carboxyl-terminal sequence. Determination of the amino-terminal sequence of the 1474-Da fragment was complicated because the data were consistent with either glycine-glycine or asparagine on either side of the proline; glycine-glycine and asparagine have identical masses. To resolve this uncertainty, a series of synthetic peptides were made containing permutations of asparagine and glycine-glycine. The tandem mass spectrum of only one of these peptides, acetyl-ASNPNDVKEFLAK, was virtually identical to that of the 1474-Da amino-terminal peptide isolated from Cs (Fig. 8A and B), indicating that this is the correct sequence.

Although the 1474-Da peptide was derived from an endoproteinase lysine-C digest, the appearance of an internal lysine in the fragment is not completely unexpected because this lysine is followed by a glutamic acid. Previous literature have suggested that this enzyme may be hindered at glutamic acid residues (50).
A comparison of this sequence to the amino-terminal portion of bovine Ca (Fig. 9) showed that residues 7–13 of Ca (VKFLAK) are identical to residues 15–21 of Cs. Residues 1–6 of Cs are completely different from residues 1–14 of Ca. The calculated mass difference between the amino-terminal sequence of bovine Ca and Ca (including an amino-terminal myristyl group) and that of Cs (including an amino-terminal acetyl group) is 899.2 Da, in excellent agreement with the difference in mass determined by MALDI TOF MS.

**DISCUSSION**

The PKA catalytic subunit of ram sperm (Cs) is unusual in its solubility properties (17) and its electrophoretic mobility in SDS-PAGE (see Figs. 1 and 2). The solubility properties were utilized as a critical first step in a simple purification protocol that permitted the isolation of ram sperm Cs in amounts sufficient for characterization by peptide mapping, protein sequencing, and mass spectrometry. The results showed that ovine Cs is identical to bovine Ca in 85/85 amino acids sequenced between Ca residues 15 and 317, but that the amino-terminal 14 amino acids of Ca have been replaced with 6 amino acids of completely different sequence in Cs. Moreover, the amino terminus of Cs is acetylated, rather than myristylated as in Ca. As a result, Cs is predicted to be 899 Da smaller than Ca, in excellent agreement with the mass difference (∼890 Da) determined by MS. This difference undoubtedly accounts for the more rapid mobility of Cs in SDS-PAGE.

A closer inspection of the amino-terminal sequences of ovine Cs and bovine Ca reveals that the homology between the two subunits begins precisely at the site (Val-15 in Ca to the first two turns of a long amphipathic α helix, the catalytic core (residues 42–297), then turns sharply inward to terminate with the myristyl group occupying a deep hydrophobic pocket (55). The myristate is tightly anchored by interactions with hydrophobic groups from four parts of the molecule that are widely separated in the linear sequence. The myristate in turn anchors one end of the A-helix, which covers a large and mostly hydrophobic portion of the catalytic core. Bacterially expressed recombinant Co lacking the myristyl group is more heat labile than the myristylated subunit (56), and a deletion mutant lacking the myristyl group and residues 1–14 also has decreased thermal stability (57). These results indicate that the myristylated motif is important for stabilizing the protein. Interestingly, deletion of the myristylated motif did not affect the catalytic properties of the recombinant Cs. Therefore, Cs might be expected to be less thermodynamically stable than Ca, but to be similar with regard to catalytic activity. In crystals of the non-myristylated recombinant Cs, the amino-terminal 14 residues were not visible, indicating that they are unstructured in the absence of the myristyl group (58). It would be of interest to determine if the short amino-terminal domain of Cs is similarly unstructured.

In Cs the amino acid residues of the myristylation motif and the rest of the A-helix shield the myristate and hydrophobic surface of the catalytic core from the aqueous environment, with the result that free Ca is soluble. It is possible that replacement of the myristylation motif with a shorter stretch of residues, coupled with replacement of the myristate with an acetate, would leave a portion of the hydrophobic core exposed.
in Cn. Additionally, because the shorter A-helix would be more loosely held to the hydrophobic core as a consequence of the absence of the myristyl anchor, even more of the hydrophobic surface beneath it might be exposed. In this case, the surface of Cn would have a hydrophobic patch that might cause it to bind to other hydrophobic surfaces in the flagellum.

Indeed, such a hydrophobic site may exist on Cn. In the course of developing our purification protocol for Cn, we observed that Cn eluted from a Source 15S column as a broad peak (results not shown). This was in contrast to Cα which eluted as a sharp peak. Source 15S is an anion exchanger with a polystyrene/divinylbenzene matrix. Two possible explanations for the different elution profiles are that either the Cn sample was heterogeneous, or there was a substantial nonspecific interaction of the matrix with Cn but not with Cα. The former is unlikely, because only one band was observed in silver-stained SDS-PAGE and only one peak was detected in mass spectra of purified Cn. The broad peak observed with Cn was more likely due to hydrophobic interaction with the column matrix. The corollary of this is that Cn has an exposed hydrophobic domain not present in Cα (Ca).

The presence of a hydrophobic patch on Cn could explain why cAMP plus Triton X-100 (this report) may elute Cn from Flagellar axonemes more readily than its free subunit to a specific compartment within the highly ordered sperm tail.

Many proteins are potential substrates for phosphorylation by C. In the sperm, phosphorylation of specific proteins by C may be achieved by controlling access of C to its potential substrates. For example, if C were tethered to a flagellar structure by its unique amino terminus or a hydrophobic patch, it might be released from sperm in the presence of cAMP. Cα also eluted as a broad peak (results not shown). This was in contrast to Cα sm, which was released as a sharp peak (results not shown). This was in contrast to Cα sm, which was released as a sharp peak (results not shown). This was in contrast to Cα sm, which was released as a sharp peak (results not shown). This was in contrast to Cα sm, which was released as a sharp peak (results not shown). This was in contrast to Cα sm, which was released as a sharp peak (results not shown). This was in contrast to Cα sm, which was released as a sharp peak (results not shown). This was in contrast to Cα sm, which was released as a sharp peak (results not shown).
The Catalytic Subunit of the cAMP-dependent Protein Kinase of Ovine Sperm Flagella Has a Unique Amino-terminal Sequence
Jovenal T. San Agustin, John D. Leszyk, Lydia M. Nuwaysir and George B. Witman

doi: 10.1074/jbc.273.38.24874

Access the most updated version of this article at http://www.jbc.org/content/273/38/24874.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 31 of which can be accessed free at http://www.jbc.org/content/273/38/24874.full.html#ref-list-1