# PHOSPHOPROTEIN PHOSPHATASE OF BOVINE EPIDIDYMAL SPERMATOZOA

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SUMMARY A protein phosphatase which dephosphorylates proteins phosphorylated by cAMP-dependent protein kinase from sperm has been identified in sonic extracts of bovine epididymal spermatozoa (BES). Phosphate-labeling experiments indicate that the protein phosphatase regulates the rates of phosphorylation and dephosphorylation of sperm proteins.

The BES protein phosphatase has been purified 8-fold by salt fractionation and DEAE cellulose chromatography. The apparent $K_m$ of the purified phosphatase is 9.6 μM [$^{32}$P]-mixed histone. $ZnCl_2$ (5 mM) inhibits the purified protein phosphatase by 83%. The distribution of protein phosphatase activity in sperm heads, midpieces, tail fragments, and cytosol is 11, 9, 23, and 57 percent, respectively.

The sperm of several mammalian species contain active adenylate cyclase, cAMP-dependent protein kinase (cAMP-PrK), and cAMP phosphodiesterase activities as well as significant levels of endogenous cyclic AMP (1-4). The function of these components of the cAMP second messenger system in spermatozoa is largely unknown except that cyclic nucleotides or inhibitors of cAMP degradation are known to stimulate spermatozoan respiration, fructolysis, and motility (4, 5). Hoskins et al. (6) suggested that the cAMP-dependent protein kinases from sperm phosphorylate specific proteins associated with cellular motility, basing their suggestion on the fact that motility is a major energy-consuming function of spermatozoa, that the protein kinases are major constituents (10%) of the sperm cytosol (6, 7), and that many of the physiological functions of cAMP in other tissues are expressed through cAMP-dependent protein kinases (8, 9). Concurrent studies are being carried out in this laboratory to substantiate this theory and to determine what role, if any, phosphoprotein phosphatases play in regulating the steady-state levels of these hypothetical phosphorylated motility proteins.

This communication reports that sonic extracts of both rhesus ejaculated and bovine epididymal spermatozoa contain a highly active phosphoprotein phosphatase which

dephosphorylates proteins phosphorylated by the cAMP-dependent protein kinase derived from sperm. The activity of this enzyme in extracts in dephosphorylating histones is comparable to that reported previously for histone phosphorylation by cAMP-dependent protein kinases (6, 7). In addition, we present data from phosphate-labeling experiments which indicate that phosphoprotein phosphatases are involved in regulating the rate of protein phosphorylations and dephosphorylations in sonic extracts of bovine sperm. We also describe the partial purification of the phosphatase from bovine epididymal sperm through $(NH_4)_2SO_4$ fractionation and DEAE cellulose chromatography and the properties of the partially purified phosphatase.

## METHODS AND MATERIALS

Bovine spermatozoa from the distal caudal epididymis were collected by the method of Henle and Zittle (10) and those from the caput epididymis by the procedure described by Hoskins et al. (1). Spermatozoa were collected within 24 hours after slaughter, diluted in a buffer solution containing 130 mM KCl, 5 mM $MgSO_4$, and 10 mM TRIS-HCl, pH 7.2, and washed three times in this same diluent (4). Washed sperm, at a concentration of $10^9$ cells/ml, were disrupted by sonic oscillation (three 1-min periods, maximum intensity) at $0^\circ$ with a Bronwill Biosonik III sonifier and centrifuged at 100,000 x g for 60 min, and the supernatant was stored in liquid nitrogen until used for phosphatase purification.

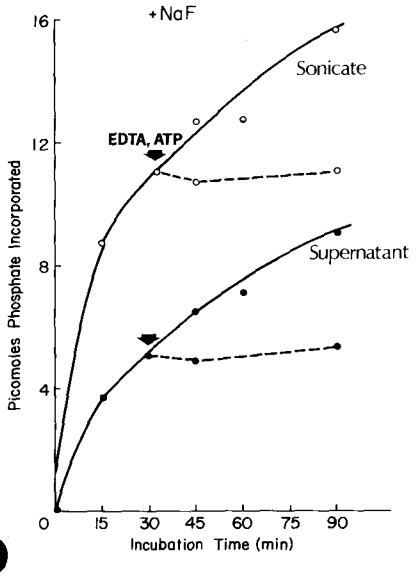
Phosphatase assays were performed by the method of Maeno and Greengard (12) in a final volume of 200 µl. [$^{32}$P]-labeled histones (Sigma) were prepared as described by these same authors except that phosphorylation with [$\gamma$-$^{32}$P]ATP (New England Nuclear, SA 22.7 µCi/50 nmoles) was carried out with bovine sperm cAMP-dependent protein kinase (46 units) purified through the DEAE cellulose chromatography step (6). One unit of protein phosphatase activity is defined as the release of one pmole [$^{32}$P] from [$^{32}$P]-labeled substrates during a 5-min assay period. Phosphorylated mixed histones were assumed to have an MW of approximately 10,000. Protein was determined by the method

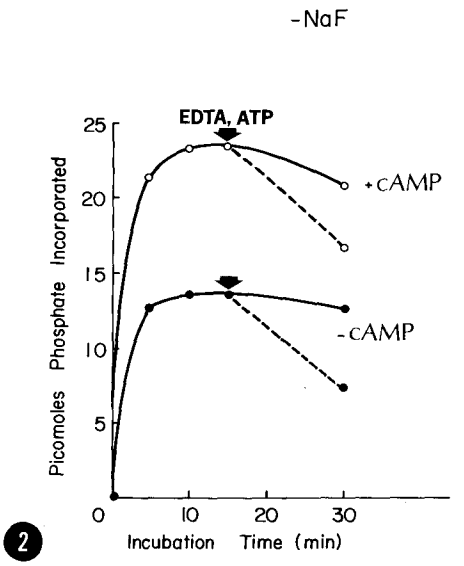
of Lowry et al. (11). Subcellular fractionation was performed by an adaptation of the method of Stambaugh and Buckley (13) for use with bovine spermatozoa. Five hundred µl ($10^9$ cells/ml) of a sperm sonicate were centrifuged at 4080 x g for 21 min through 10.5 ml of a 5-65% sucrose gradient. Twenty-five drop portions were collected and 50 µl of each were used for phosphatase assay.

## RESULTS AND DISCUSSION

Since bovine epididymal sperm contain high levels of cAMP-dependent protein kinase (6, 7), we sought preliminary evidence to determine whether the proteins phosphorylated by this enzyme in sonic extracts were dephosphorylated by a corresponding protein phosphatase. We took advantage of the known ability of NaF (12) and EDTA (12) to inhibit protein phosphatases and protein kinases, respectively. Figure 1 shows that the incorporation of label from $AT^{32}P$ into endogenous proteins in unfractionated sonicates and in sonicate supernate (100,000 x g, 1 hr) preparations proceeds in a nearly linear fashion for up to 90 min in the presence of NaF. The addition of 7.40 mM EDTA and 0.78 mM ATP to these preparations after initiation of the reaction prevented further phosphorylation. The deletion of NaF from the sonicate supernatant preparation (Fig. 2) facilitates dephosphorylation of endogenous proteins, presumably by allowing full expression of phosphatase activity. The absence of NaF also allows the attainment of maximal rates of phosphorylation within 5 min. The addition of 1 µM cAMP is seen to stimulate incorporation of label into protein by some 70%. These data suggest that bovine sperm cytosol contains a phosphoprotein phosphatase capable of dephosphorylating proteins phosphorylated by cAMP-dependent protein kinase and that the phosphatase is involved in regulating the rates of endogenous protein phosphorylations in sperm cytosol preparations. After three 1-min intervals of sonic irradiation and separation of subcellular particles, 57% of the phosphatase activity is found in a 100,000 x g (60 min) soluble fraction. Sperm heads, midpieces, and tail fragments contain 11, 9, and 23% of the total activity, respectively.

The partial purification of this enzyme from the 100,000 x g sonicate supernatant




Figures 1 and 2. Time course of phosphorylation of proteins from bovine caudal spermatozoa with and without NaF.

Incubation conditions were as described for the assay of cAMP-PrK (6) except that endogenous sperm proteins were labeled with [$\gamma$-$^{32}$P]ATP. At the time of incubation indicated by the arrows, 0.2 ml of 0.78 mM nonradioactive ATP and 7.40 mM EDTA (12) were added to the incubations to inhibit PrK activity. In Fig. 1 (no cAMP, 10 mM NaF) incubations were made with both sperm sonicates and sperm cytosols derived from $5.0 \times 10^7$ spermatozoa. In Fig. 2 ($\pm$ 1 µM cAMP, no NaF), incubations were made with sperm cytosol derived from $4.3 \times 10^7$ spermatozoa. The incubations were terminated by precipitation with 3 ml 5% TCA-0.25% sodium tungstate and separation of the protein pellet by centrifugation (27,700 x g, 15 min, 4°C). The pellets were washed three times by suspension in 100 µl 1 N NaOH and reprecipitation with 2 ml 5% TCA-0.25% sodium tungstate. The final pellet was resuspended in 100 µl 1 N NaOH, transferred with 1 ml $H_2O$ to 10 ml scintillation fluid (16), and assayed for radioactivity with a Packard Tricarb (2425) liquid scintillation spectrometer. Solid lines (without EDTA, ATP); dashed lines (with EDTA, ATP).

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fraction is shown in Table 1. Note that the specific activity of this enzyme in extracts is some 8000 units/mg proteins, a value similar to the 9700 units/mg protein previously reported (6) for cAMP-dependent protein kinase. The phosphatase was partially purified in the following manner: Pooled supernates from $1.9 \times 10^{11}$ sperm were precipitated with solid $(NH_4)_2SO_4$ between 0 and 50% saturation at 4°; the precipitate obtained after centrifugation at 30,000 x g for 15 min was dissolved in 25 ml of 10 mM TRIS-HCl, pH 7.2. This solution was dialyzed for 24 hrs against 3 x 2 liter volumes of this same buffer and clarified

Table 1

Partial purification of bovine sperm phosphatase

| Purification step | Total protein (mg) | Total units $\times 10^{-6}$ | Specific activity $\times 10^{-6}$ | Purification | % Recovery (activity) |
|---|---|---|---|---|---|
| 100,000 x g supernatant | 252.0 | 4.3 | 17.4 | 1.0 | 100 |
| 0-50%$(NH_4)_2SO_4$ | 77.0 | 1.9 | 22.8 | 1.3 | 44 |
| DEAE cellulose (tubes 71-95) | 40.4 | 1.6 | 136.9* | 7.9 | 37 |

*Peak tube (83) only.

by centrifugation at 30,000 x g for 15 min. The resultant supernate (12 ml) was applied to a 2.5 x 10.0 cm DEAE cellulose column that had been previously equilibrated with 10 mM TRIS-HCl, pH 7.2. Sperm phosphatase was eluted with a linear NaCl gradient (0.0 to 0.7 M, 300 ml containing 1 mM dithiothreitol) and collected in 2.5 ml fractions. A broad peak of activity was eluted between 0.35 and 0.55 M NaCl (Fig. 3). Peak activity fractions were dialyzed and concentrated with a ProDiCon apparatus (Biomolecular Dynamics, Hillsboro, Oregon) to 3.8 mg/ml in 10 mM TRIS buffer and stored at -10°C in the presence of 1 mM dithiothreitol. The enzyme at this stage lost 60-70% of its activity after storage for 2 months. Further attempts at purification by preparative disc gel electrophoresis and

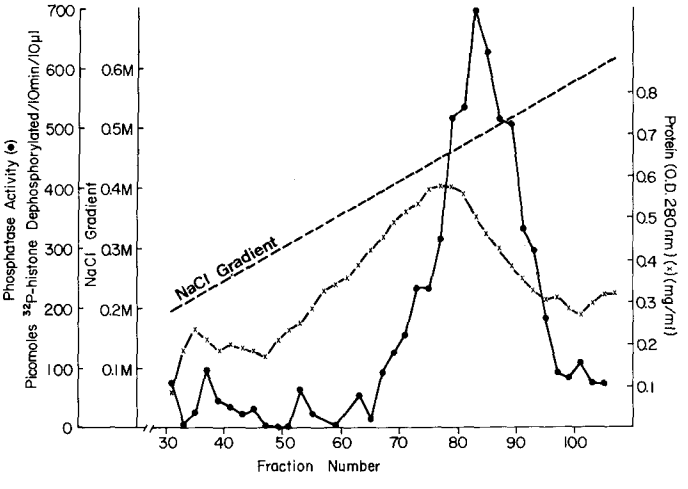


Figure 3. Elution profile of sperm phosphatase after DEAE cellulose chromatography.

at MW determination by thin-layer gel filtration were unsuccessful because of total loss of activity.

The partially purified phosphatase is devoid of cAMP-dependent protein kinase and exhibits linear kinetics with respect to time (up to 30 min) and to enzyme concentration (up to 190 μg protein). The apparent $K_m$ for phosphohistone with 36 μg enzyme protein is 9.6 μM.

Sperm phosphatase is affected by metal ions in the same way as brain phosphatase (12) (Table 2). Five mM $Zn^{++}$ inhibits the enzyme to the greatest degree (83%). The same concentration of $Zn^{++}$ was observed to completely inhibit motility in bovine caudal cells. Whether the $Zn^{++}$ inhibition of motility is related to the inhibition of phosphatase activity remains to be established. Sperm contain large quantities of $Zn^{++}$ and its partial removal from human sperm significantly stimulates motility (14, 15). Five mM $MnCl_2$ stimulates the sperm phosphatase whereas a number of other compounds including cyclic AMP and theophylline are without significant effect on the partially purified sperm enzyme (Table

Table 2

Effects of various compounds on partially purified phosphatase activity

| Reagent | Relative activity |
|---|---|
| $ZnCl_2$ | 17 |
| $CoCl_2$ | 30 |
| $KH_2PO_4$ (1 mM) | 72 |
| Mg Acetate | 73 |
| NaF (10 mM) | 79 |
| $CaCl_2$ | 87 |
| Cyclic AMP (10 μM-10 mM) | 95 |
| Theophylline (10 μM-10 mM) | 103 |
| EDTA | 120 |
| $MnCl_2$ | 148 |

Protein phosphatase was assayed in duplicate under standard conditions. All compounds were added to a final level of 5 mM unless otherwise noted. Activity is expressed as the percentage of that without any added compound.

Table 3

Alkali lability of the phosphoester bond of [$^{32}$P]histone

| | cpm | % control |
|---|---|---|
| TCA pellet (control) | 1664 | |
| 0.8 M Hydroxylamine | 1708 | 102% |
| 0.8 M NaCl | 1559 | 93% |
| 1 N NaOH | 48 | 3% |

Ten µl [$^{32}$P]histone were boiled for 10 min with 1 ml of the above compounds. The control received no treatment. Each of the above treatments and the control were subsequently precipitated with 2 ml 25% TCA, 0.1 ml 1.2 N $H_2SO_4$, and 200 µl 0.63% bovine serum albumin as carrier. The TCA pellets were washed three times by resuspension in 100 µl 1 N NaOH and precipitation in 25% TCA. The final TCA pellet was resuspended in 100 µl 1 N NaOH and counted in 10 ml of scintillation fluid:3g Omnifluor (New England Nuclear), 250 ml Triton X-114 (Sigma Chemical Corp.), 750 ml xylene (16).

2). Ten mM NaF inhibits the sperm phosphatase, but to a lesser degree than it inhibits the brain protein phosphatase (12). However, NaF appears to be a more specific inhibitor of the sperm phosphatase since it did not inhibit the cAMP-PrK in sonicate extracts of bovine sperm as did 5 mM $ZnCl_2$ (Stephens and Hoskins, data unpublished).

The enzyme shows a pH optimum in the range 6.5-7.5 in the presence of 100 µM Hepes buffer. The enzyme dephosphorylates phosphorylated arginine-rich histone and mixed histones at about equal rates. Phosphorylated lysine-rich histone is a less effective substrate for the protein phosphatase; phosphoprotamine is not dephosphorylated. The data shown in Table 3 indicate that 97% of the radioactivity of the [$^{32}$P]-labeled mixed histone substrate used in this study was associated with alkali-labile phosphoester bonds and not acyl phosphate bonds which are cleaved by hydroxylamine.

The data reported here represent the first description of phosphoprotein phosphatase activity in mammalian sperm. The high activity of the enzyme in sonic extracts (some 20,000 units per $10^9$ sperm in both BES caudal and rhesus ejaculated sperm preparations) and its specificity for proteins phosphorylated by sperm protein kinase suggest that the pro-

tein phosphatase plays a significant role in the regulation of protein dephosphorylations in sperm.

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