Protein thiols in spermatozoa and epididymal fluid of rats

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Summary. Thiol (SH) oxidation to disulphides (SS) is thought to be involved in sperm chromatin condensation and tail structure stabilization, which occur during maturation of spermatozoa. Previously developed procedures, using the fluorescent labelling agent monobromobimane (mBBR), enabled us to study the thiol-disulphide status of spermatozoa. Electrophoretic separation of labelled sperm proteins from the caput and cauda regions showed that during maturation thiol oxidation occurs in many protein fractions from the tail and that the magnitude of oxidation differs between proteins. Among the protein bands, one major band (MPB), probably a dense fibre constituent, is quantitatively prominent. *N*-Ethylmaleimide (NEM) or mBBR alkylation (of intact spermatozoa) changes the mobility of the caput MPB, but not that of the cauda MPB. The results indicated that the altered mobility of MPB is mainly due to a change in its shape, possibly resulting from the alkylation of a few critical SH groups. Epididymal fluid proteins contain both SH and SS. The thiol and disulphide content of the various epididymal proteins appears similar, although some diminution in fluorescence is seen in epididymal fluid proteins from the cauda region as compared with those from the caput region. The prominent changes in thiol status occur in the spermatozoa.

Keywords: thiols; epididymis; sperm maturation; rat

Introduction

Motility and fertilizing capacity of spermatozoa develop as they mature during passage through the epididymis (Yanagimachi, 1988). During maturation, most sperm structures, such as the chromatin, midpiece and tail components, gradually become stabilized (Calvin & Bedford, 1971; Bedford *et al.*, 1973; Bedford & Calvin, 1974a; Pellicciari *et al.*, 1983). The involvement of disulphide formation in stabilizing sperm structures during sperm maturation, mainly in the sperm chromatin, has been studied by several biochemical methods (Marushige & Marushige, 1975; Mercado *et al.*, 1976; Saowaros & Panyim, 1979; Miller & Masui, 1982; Pellicciari *et al.*, 1983), but there has been little information on the thiol status of proteins from the tail.

Attempts to follow thiols in sperm protein fractions have been based on the use of [¹⁴C]iodoacetamide (Calvin *et al.*, 1975; Sattayasai & Panyim, 1982). We have developed procedures for the determination of sperm thiols (SH) and disulphides (SS) (Shalgi *et al.*, 1989) based on the use of the fluorescent thiol labelling agent monobromobimane (mBBR) (Kosower & Kosower, 1987). Labelling by mBBR can be carried out in the intact spermatozoa prior to fractionation, thus avoiding possible thiol modification and loss during subsequent cell fractionation and analysis. These procedures allow (i) the morphological evaluation and quantitative determination of SH and SS in whole spermatozoa and subcellular fractions and (ii) the analysis of changes in thiol status by electrophoretic separation of labelled sperm proteins. During maturation in rat epididymis, oxidation of sperm thiols occurs both in sperm heads and in tails between the caput and

cauda regions (Shalgi *et al.*, 1989). In this study, the dynamics of sperm protein thiols during maturation was further investigated and the thiol disulphide status in epididymal tissue and fluid surrounding the spermatozoa was studied.

Materials and Methods

Materials and media. Dithiothreitol (DTT), sodium dodecyl sulphate (SDS), *N*-ethylmaleimide (NEM), ethylenediamine-tetraacetic acid (EDTA) and phenyl methyl sulphonyl fluoride (PMSF) were purchased from Sigma Chemical Co. Monobromobimane (mBBR, Calbiochem-Behring) was dissolved in acetonitrile and kept as a 50mM stock solution in the dark (Kosower *et al.*, 1979). Buffers used were: (a) phosphate-buffered saline (PBS) containing 135mM NaCl and 10mM sodium phosphate buffer pH = 7.4; (b) Krebs-Ringer-phosphate (Ringer), was prepared as described by Brooks & Higgins (1980) with a final composition of 150mM Na⁺, 4.8mM Ca²⁺, 1.2mM Mg²⁺, 129mM Cl⁻, 15.5mM PO₄³⁻, 1.2mM SO₄²⁻ and 10mM glucose, and (c) 10mM Tris-HCl, pH = 7.6.

Isolation of epididymal luminal fluid and spermatozoa. Spermatozoa and epididymal luminal fluid were obtained from sexually mature Wistar-derived male rats. The isolation of epididymal luminal fluid and spermatozoa was carried out as described by Brooks & Higgins (1980). Epididymal tissue was cleared of blood and the caput, corpus and cauda regions were obtained by cutting the tube. Pieces of tissue were shaken in Ringer at 37°C for 15 min to allow the release of the spermatozoa and the fluid originally contained within the lumen of the epididymal tubule. The tissue was collected with a Pasteur pipette and discarded and the suspension was centrifuged at 600 g for 10 min to sediment the spermatozoa. The supernatant containing the luminal fluid was retained. The spermatozoa were washed twice more by resuspension and centrifugation in fresh Ringer.

Labelling of thiol groups with monobromobimane. Sperm thiol groups were labelled with mBBR as described by Shalgi *et al.* (1989). Briefly, aliquots of washed spermatozoa were incubated with or without 1mM DTT at 37°C for 10 min. Samples were then washed twice by centrifugation for 10 min at 600 g, resuspended in PBS to 4–8 × 10⁶ spermatozoa/ml and labelled with mBBR. To block reactive thiols, samples were treated with 10mM NEM at 37°C for 30 min, washed and then labelled with mBBR. In some experiments, spermatozoa were first solubilized in 5% SDS and 1mM DTT and the labelling of the solubilized spermatozoa carried out at a final mBBR concentration of 2.5mM for 20 min.

Electrophoretic analysis of sperm proteins. After labelling of intact spermatozoa, the cells were solubilized by incubation in 5% SDS and 1mM DTT in 37°C for 30 min in the presence of 1mM EDTA and 0.5mM PMSF. For electrophoretic analysis, 3% 2-mercaptoethanol (ME) was added to the solubilized spermatozoa, followed by sonication for 10 s to liquefy the DNA. The samples were loaded on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). After the electrophoretic separation, the gels were observed and photographed under ultraviolet illumination to detect the fluorescent protein bands (Kosower & Kosower, 1987). The gels were then stained with Coomassie Brilliant Blue and photographed again. Densitometric profiles of the fluorescent and the stained gels were obtained by using the films of the gels.

Labelling and analysis of epididymal luminal fluid. The luminal fluid was diluted with Ringer buffer and incubated for 10 min without or with 1mM DTT at 37°C for 10 min. Labelling was then carried out in the dark for 20 min at a final mBBR concentration of 2.5 mmol/l in the presence or absence of DTT. The labelled solution was centrifuged at 50 000 g for 90 min at 4°C and the supernatant was dialysed for 24 h at 4°C against distilled water, freeze-dried and redissolved in 10mM Tris-HCl, pH 7.6. The samples were then electrophoresed on SDS-PAGE and the gels were analysed as described for sperm proteins.

Histochemical detection of thiols and disulphides in the epididymal tissue. Epididymal tissues were fixed in a solution of paraformaldehyde and picric acid. Serial sections of 6 µm were prepared and mounted on slides. One set was stained with haematoxylin and eosin and the others were retained for histochemical labelling. The procedure for labelling with mBBR was carried out according to the methods of Gainer & Kosower (1980) with slight modifications. The sections were washed in xylene for 40 min to remove the embedding material, followed by washes in 100, 96, 70 and 50% ethanol for 15 min each and then washed with PBS + 1mM EDTA. The sections were incubated with or without 10mM DTT in PBS + EDTA at 37°C for 10 min, washed twice with PBS + EDTA and labelled with mBBR. Labelling was carried out in the dark for 10 min, at a final mBBR concentration of 1.5 mmol/l and was followed by 2 washes.

Results

Spermatozoa, isolated from the caput and cauda of the rat epididymis, were solubilized in SDS + DTT, and proteins were analysed by SDS-PAGE electrophoresis. When stained with Coomassie blue, electrophoretic profiles displayed a series of protein bands ranging in molecular mass from ~95 kDa to 16 kDa with one major protein band (MPB) at ~27 kDa (Fig. 1). The protein profiles

Fig. 1. Polyacrylamide–sodium dodecyl sulphate gel electrophoresis of sperm proteins from caput (lanes 1 and 3) and cauda (2 and 4) epididymides; *N*-ethylmaleimide alkylated (lanes 3 and 4), without alkylation (lanes 1 and 2). Approximate molecular masses are as indicated from migration of known protein standards.

of spermatozoa from the caput and cauda regions appeared similar for most bands, with the exception of a single band at 14 kDa (Fig. 1).

To characterize the differences in protein thiols during maturation, fluorescence and protein staining following electrophoretic separation of sperm proteins from the caput and cauda regions were compared. Caput spermatozoa presented a high number of fluorescent protein bands, including the MPB and some minor proteins of low molecular mass ranging from ~30 kDa to 16 kDa (Fig. 2b). Pretreatment of caput spermatozoa with DTT had only a slight effect on the focusing of one fluorescent band (23 kDa) but did not alter the intensity of the various bands. These results indicate that in the caput spermatozoa, the thiols in most or all of the proteins are present as SH. Less fluorescence was observed in cauda sperm samples in most regions of the gel than in that of caput sperm samples, especially in the region of proteins of low molecular mass. Pretreatment of cauda spermatozoa with DTT prior to labelling with mBBBr resulted in a significant increase in fluorescence of these bands, indicating that most of the thiols in these proteins are oxidized to SS in the cauda. The cauda MPB contained both SH and SS as indicated by the moderate decrease in fluorescence from caput to cauda and by the enhancement in fluorescence following DTT treatment of cauda spermatozoa (Fig. 2b).

Under our experimental conditions (1mM DTT for 10 min at 37°C), spermatozoa remained intact and morphology was not altered. In order to expose any possible inaccessible thiols and disulphides, spermatozoa were solubilized in SDS and DTT and the proteins in the solubilized spermatozoa labelled with mBBBr. Solubilization of caput spermatozoa, prior to mBBBr labelling, did not significantly alter the extent of fluorescence of the various bands (Fig. 2b, lanes 3, 5).

Fig. 2. Polyacrylamide-sodium dodecyl sulphate gel electrophoresis of monobromobimane-labelled sperm proteins from caput (lanes 1, 3 and 5) and cauda (2, 4 and 6) epididymides. (a) Coomassie Brilliant Blue stain, (b) fluorescence illumination. Without dithiothreitol (DTT) pretreatment (lanes 1 and 2), after DTT treatment (3 and 4) and after solubilization with SDS + DTT (5, 6).

Fig. 3. Densitometry illustration of the polyacrylamide gel electrophoresis profile of sperm proteins labelled with monobromobimane from caput (—) and cauda (---) epididymides; (a) fluorescence, (b) Coomassie Brilliant Blue stain (for origin of the gel on the left, see Fig. 2a lanes 1 and 2, and Fig. 2b lanes 1 and 2).

Solubilization of cauda spermatozoa prior to labelling led to a slight increase in fluorescence of most bands, compared to that of DTT-treated intact spermatozoa (Fig. 2b lanes 4, 6). It was therefore concluded that most of the disulphides in the intact cauda spermatozoa are reactive to 1mM DTT. A small fraction of disulphides in the cauda spermatozoa is not reduced by DTT and becomes exposed upon sperm solubilization.

Fig. 4. Densitometry illustration of the polyacrylamide gel electrophoresis profile of sperm proteins labelled with monobromobimane from (a) caput and (b) cauda epididymides. After treatment with dithiothreitol (—); untreated (---); (for origin of the gel on the left, see Fig. 2a lanes 1 and 3 for caput and lanes 2 and 4 for cauda epididymides).

The fluorescence of protein bands compared with protein content is illustrated by densitometry bands of proteins labelled with mBBBr and stained with Coomassie blue. In the absence of DTT, there are some highly fluorescent proteins in caput spermatozoa (Fig. 3). One group is that of low molecular weight proteins. These bands stain weakly in Coomassie blue but show high fluorescent labelling. A marked decrease in the fluorescence of these proteins in cauda spermatozoa is not related to protein content which remained unchanged. The amount of MPB also remained unchanged with a moderate decrease in its fluorescence. Densitometry of cauda spermatozoa after DTT treatment showed an increase in labelling in most protein bands. Under these conditions, the fluorescence profiles of caput and cauda proteins appeared similar (Fig. 4), demonstrating that the total amount of thiols and disulphides was not altered during maturation. Densitometric scans of cauda sperm proteins labelled after solubilization revealed that an additional small amount of SS is reduced, as compared with the DTT-treated intact spermatozoa (Fig. 5).

Blocking reactive thiols in spermatozoa with NEM changed the mobility of caput MPB (Fig. 1, lanes 1 and 3). The NEM-alkylated MPB from caput spermatozoa moved slower in the gel and was concentrated in a sharp band. In contrast, the mean mobility of cauda MPB was shifted slightly as the apparent molecular mass of the altered, diffuse species ranged between 27 and 30 kDa (Fig. 1, lanes 2 and 4). Corpus MPB behaved like caput MPB (not shown).

As in NEM alkylation, blocking reactive thiols with mBBBr in spermatozoa changed the mobility of caput MPB with no apparent effect on the mobility of cauda MPB (Fig. 2a, lanes 1–4). Densitometry also clearly revealed the change of MPB mobility after alkylation (Fig. 3). Solubilization of caput spermatozoa prior to labelling did alter migration of MPB; yet, under the same conditions, MPB from cauda spermatozoa changed its mobility and migrated similarly to caput MPB (Fig. 2a, lanes 5 and 6).

Electrophoresis of epididymal fluid samples collected from different regions of the duct gave similar banding patterns (Fig. 6), with 6–8 major bands and over 30 total bands in all regions. The degree of expression of some bands differed. Analysis of fluorescence patterns of epididymal fluid proteins revealed that they contained SS and SH in both the caput and cauda epididymides; the fluorescence being stronger for the caput epididymal fluid proteins.

Histochemical evaluation of thiol status of epithelial cells in caput and cauda epididymides are presented in Fig. 7. Moderate fluorescence was seen in the different elements of the tube in both caput and cauda segments. Following DTT treatment, the fluorescence of all the elements was

Fig. 5. Densitometry illustration of the polyacrylamide gel electrophoresis profile of sperm proteins labelled with monobromobimane from cauda epididymis; (a) fluorescence, (b) Coomassie Brilliant Blue stain. After DTT treatment with dithiothreitol (---); after solubilization (—); (for origin of the gel on the left, see Fig. 2a lanes 4 and 6).

Fig. 6. Polyacrylamide-sodium dodecyl sulphate gel electrophoresis of monobromobimane-labelled epididymal fluid from caput, corpus and cauda epididymides. Fluorescence (middle lanes) and Coomassie Brilliant Blue stain; with (+) and without (−) treatment with dithiothreitol (DTT).

stronger, indicating the presence of SS and SH groups in the epithelial cells and in the connective tissue, with no significant difference between caput and cauda regions. In contrast, the spermatozoa in the lumen showed a difference in the fluorescent intensity between caput and cauda, with significant enhancement of the fluorescence in cauda spermatozoa after DTT treatment (Fig. 7). These results are consistent with those obtained by fluorescent microscopy of the labelled sperm cells isolated from the caput and cauda (Shalgi *et al.*, 1989).

Fig. 7. Cross-section of caput (a and c) and cauda (b and d) epididymal tissue. Epifluorescence; sections labelled with monobromobimane without (a and b) and with (c and d) treatment with dithiothreitol ($\times 250$).

Discussion

A shift in the status of protein thiols occurs in rat spermatozoa as they undergo maturation during their passage through the epididymis (Shalgi *et al.*, 1989). We previously found that the total content of thiols and disulphides in spermatozoa did not change during passage from the caput to the cauda region, but that thiols were oxidized to disulphides in the heads and tails of spermatozoa. Electrophoretic analysis of tail proteins from mBBBr-labelled cauda spermatozoa showed the presence of disulphides in many fractions (Shalgi *et al.*, 1989). In the present work, we found that, during the passage of spermatozoa from caput to cauda epididymides, thiol oxidation occurred in many tail protein fractions and the magnitude of oxidation differed among proteins. Densitometric scans indicated that the total amount of thiols in caput and cauda proteins is similar. These results are thus consistent with those obtained by quantitative analysis (Shalgi *et al.*, 1989).

The rat sperm tail is very large and contains 4 times as much protein as the head (Shalgi *et al.*, 1989). Most of the protein bands revealed in this study by SDS-PAGE were those of the tail structures, since, under our experimental conditions, the protamine, which is the major head protein, does not migrate. Little is known about highly organized flagella, and the distribution, organization and function of specific sperm tail proteins have not been elucidated (Eddy, 1988).

The dense fibres are very pronounced in the rat sperm tail. Dense fibres from rat spermatozoa have been isolated (Price, 1973; Calvin, 1979; Olson & Sammons, 1980; Vera *et al.*, 1984; Oko, 1988). Various protein fractions have been described, ranging in molecular mass from 11 to 87 kDa, but no consensus has been reached on the number of protein fractions present or their molecular size. Fibrous sheath and perforatorium polypeptides have been described also (Oko, 1988; Oko & Clermont, 1988), ranging in molecular mass from 11 to 90 kDa. In the present work, the sperm protein profiles included proteins in these molecular mass ranges, which include mostly thiols in the caput region and both thiols and disulphide groups in the cauda region.

Using SDS and DTT, Bedford & Calvin (1974a) demonstrated the presence of thiols and disulphides in dense fibres, connecting piece, fibrous sheath and outer mitochondrial membrane. Other studies reported high cysteine content in proteins from the outer mitochondrial membrane (Pallini *et al.*, 1979), accessory fibres (Baccetti *et al.*, 1976) and perforatorium (Olson *et al.*, 1976). Several studies have confirmed that the low molecular mass polypeptide bands of the dense fibres have a high cysteine content (Calvin, 1979; Baccetti *et al.*, 1976; Olson & Sammons, 1980; Vera *et al.*, 1984). A major band/s in the range of 26–32 kDa corresponds probably to the MPB observed in the present study. Using [^{14}C]iodoacetamide, Sattayasai & Panyim (1982) determined SH concentrations in some sperm protein fractions in the range of 13–32 kDa. Although the total amount of thiols and disulphides was not evaluated in their work, they showed that these proteins included a major band of 32 kDa which contained high concentrations of thiols in caput, but not in cauda, spermatozoa.

In the present work we found that, in caput spermatozoa MPB contains mostly SH, as shown by the few additional thiols labelled following reduction with DTT in either the intact or solubilized cells. In cauda spermatozoa, some MPB thiols remain reduced while a significant proportion is present as disulphides, as shown by the extent of fluorescence of labelled MPB in the DTT-treated compared with the untreated intact cells. Some additional SS groups are reduced only upon sperm solubilization in DTT–SDS. These results indicate the presence of inter- or intrachain nonreactive ‘buried’ disulphides in the cauda MPB.

Alkylation with mBBr of DTT–SDS solubilized spermatozoa changed the mobility of caput and cauda MPB. Under these conditions, both caput and cauda MPB moved in the gel more slowly than cells solubilized in SDS–DTT and not alkylated (compare Fig. 1 lanes 1 and 2 with Fig. 2 lanes 5 and 6). Changes in the mobility of dense fibre proteins occur also after carboxymethylation (Calvin, 1979; Olson & Sammons, 1980). NEM or mBBr alkylation of intact spermatozoa changed the mobility of caput MPB, but not that of cauda MPB. DTT treatment of intact cauda cells reduced most of the disulphides (Fig. 5), only a small additional fraction of SS being reduced upon solubilization. However, alkylation of this fraction of SH was sufficient to change MPB mobility significantly. A change in protein migration induced by reduction of disulphide bonds has been reported by Knudsen *et al.* (1985) and Ruoslahti & Pierschbacher (1987). It has been proposed that internal disulphide bonds are more critical to the conformation of those protein bands (Knudsen *et al.*, 1985). The change in MPB mobility observed here could be due to a change in the size and/or shape of the molecule upon thiol alkylation. Our results indicate that the altered mobility is mainly due to a change in the shape of the protein and possibly results from the alkylation of a few critical SH groups.

A direct involvement of epididymal secretory proteins as mediators in the process of sperm maturation has been reported. Association of antibodies directed against epididymal proteins inhibits fertilization (Moore & Hartman, 1986; Cuasnicu *et al.*, 1984). Special attention has been focused on some acidic glycoproteins in the range of 27–37 kDa. These glycoproteins have been given several names (Cameo & Blaquier, 1976; Lea *et al.*, 1978; Brooks & Higgins, 1980; Jones *et al.*, 1980; Faye *et al.*, 1980; Wong & Tsang, 1982). In the present study, electrophoretic separation of epididymal fluid showed proteins in these molecular mass ranges which contain both SS and SH. The protein profiles from the various epididymal regions appeared similar with only a few differences between caput and cauda. These results are consistent with those of Hamilton (1981). In the present work we showed that the proteins in the various epididymal regions contain both SS and SH.

Attempts to clarify the involvement of disulphide formation in sperm maturation have focused mainly on sperm structures; there is no information on epididymal fluid thiols. Brooks & Tiver (1983) reported on some rat epididymal glycoproteins of 26 kDa, which bind by disulphide linkages to an integral sperm membrane protein of ~200 kDa. These glycoproteins contain free thiol groups (Brooks, 1985) and a high content of cysteine residues (14 out of 93) in the C-terminal half of the molecule (Brooks *et al.*, 1986). One or several of the proteins shown here to contain both

thiol and disulphide groups may correspond to these polypeptides, but further study is necessary to identify them.

The epididymal epithelial cells and the intercellular connective tissue contain both SH and SS with no significant differences between the caput and cauda regions, as indicated by the fluorescence of mBBR-labelled histological preparations. Although some differences in thiols and disulphides were observed between caput and cauda in epididymal fluid and tissue, the major change in thiol status involved the sperm cells.

The mechanisms responsible for the selective oxidation of sperm protein thiols to disulphides during sperm maturation are unknown. Thiol oxidase activity in the male reproductive tract has been reported (Chang & Zirkin, 1978) and it was proposed that this enzyme might play a role in the formation and maintenance of sperm disulphide bonds. The enzyme has been characterized with respect to its reaction with thiols of low molecular mass, but whether it can oxidize thiol groups in proteins is not known. However, it is unlikely that the enzyme would penetrate the sperm cell. Small, nonprotein, epididymal thiols, may be able to penetrate the spermatozoa and could play a role in the oxidation of thiols within the sperm cell. The participation of such thiols in these processes remains to be studied.

Previous studies have suggested that thiol oxidation may play a role in the development of sperm motility (Calvin & Bedford, 1971; Bedford & Calvin, 1974b; Bedford, 1975). A more recent study (Cornwall *et al.*, 1988) provides direct evidence for the relationship between thiol oxidation and acquisition of motility by epididymal spermatozoa, demonstrating that caput epididymal spermatozoa require oxidation of thiols to become progressively motile.

The maintenance of thiols in the correct degree of oxidation appears to be important for the events preceding fertilization, since either blockage of free thiols (Reyes & Rosado, 1975), or reduction of disulphides with dithiothreitol inhibits capacitation, acrosome reaction and egg attachment (Miller & Masui, 1982; Fleming *et al.*, 1982; Yanagimachi *et al.*, 1983). The possible interaction between thiol proteins (SS and SH) in epididymal fluid and sperm surface proteins during epididymal transit and maturation requires further study.

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