EPIDIDYMAL SULFHYDRYL OXIDASE: A SPERM-PROTECTIVE ENZYME FROM THE MALE REPRODUCTIVE TRACT

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SUMMARY: A new enzyme catalyzing the reaction $2R\text{-}SH + O_2 \longrightarrow R\text{-}S\text{-}S\text{-}R + H_2O_2$ has been found in the male reproductive tract. The enzyme is specific for sulfhydryl compounds and does not require cofactors for activity. Physiologically, the enzyme appears to protect sperm structure and function against damage by endogenous sulfhydryls. Consequently its inhibition may be of contraceptive significance.

Several enzymes which catalyze the oxidation of sulfhydryl compounds by atmospheric oxygen have been reported. Mandels described an enzyme from Myrothecium verrucaria which oxidized glutathione, cysteine, homocysteine and thiophenol with the formation of the corresponding disulfides and water as products (1). Thiol oxidase (EC 1.8.3.2), initially reported in Piricularia oryzae by Neufeld et al (2) and later purified by Aurbach and Jakoby (3), catalyzes the oxidation of compounds containing ethylenic-linked thiols to their disulfide forms with the production of water. Recently a sulfhydryl oxidase was isolated and characterized from bovine milk by Janolino and Swaisgood (4). This enzyme was observed to catalyze the oxidation of the low molecular weight thiols, glutathione and cysteine, as well as thiol-containing milk proteins. The products of this iron-dependent reaction were the corresponding substrate disulfides and hydrogen peroxide. Here we report the catalytic reaction, substrate specificity and cofactor requirement of a unique sulfhydryl oxidase enzyme found in the male reproductive tract. The possible role of the enzyme in the preservation of sperm structure and function in vivo is discussed.

METHODS

Crude enzyme solutions were prepared by extruding the contents of hamster caudal epididymides into 0.2M phosphate buffer, pH 7.6 in a ratio of 1 epididymis per 1 ml buffer. The spermatozoa were allowed to disperse at 37°C, then centrifuged at 30,000g for 10 minutes. The resulting supernatant fraction was filtered through Whatman No. 1 filter paper to remove insoluble lipids. The filtrate containing epididymal fluid and enzyme was stored at 0°C.

The stoichiometry of the enzymatic reaction was determined by addition of 2mM dithiothreitol (DTT) to a mixture containing 0.4ml enzyme solution, 0.6ml phosphate buffer, pH 7.6, and 12 units catalase (Sigma). To determine the ratio of oxygen consumed to sulfhydryl oxidized, the oxygen uptake was measured with a Clark electrode at 37°C according to the method of Kielley (5). When the oxygen tension in the mixture decreased approximately 50-80%, the reaction was stopped by addition of 5% TCA. The sample was then centrifuged and the supernatant fraction assayed for sulfhydryl content by the method described by Ellman (6).

To determine the ratio of oxygen consumed to disulfide produced, the rate of oxygen consumption of an enzyme solution was first determined in the absence of added catalase. 2mM azide was present to inhibit endogenous epididymal catalase activity. The rate of DTT oxidation was then monitored at 285mu in a Gilford spectrophotometer using an identical enzyme reaction mixture. Formation of disulfide was quantitated using oxidized DTT, prepared according to the method of Cleland (7), as a standard.

Production of hydrogen peroxide was detected in enzyme reaction mixtures containing 2mM azide by the horseradish peroxidase-catalyzed oxidation of o-dianisidine (8).

RESULTS

An initial observation that the addition of DTT to a suspension of hamster epididymal spermatozoa resulted in a significant increase in the rate of oxygen uptake led to the subsequent finding that 100% of the DTT-stimulated oxygen uptake activity was present in the 5,000g supernatant of the sperm suspension. Lysis of the spermatozoa in the pellet by three times freeze-thawing at CO_2-acetone temperatures did not result in the appearance of DTT-stimulated oxygen uptake in the lysate. Since freeze-thawing did not markedly affect the DTT-stimulated oxygen uptake activity in the supernatant, these results indicated that the DTT-activity was present in the epididymal fluid fraction, which was extruded along with the spermatozoa from the epididymides, and not in the cellular fraction of the sperm suspension.

The enzymatic nature of the DTT-stimulated oxygen uptake activity was established by experiments which demonstrated that the activity was non-dialy-

zable, heat-labile at 100°C for 10 minutes, acid labile in 5% TCA, and sensitive to proteolytic digestion by trypsin and bacterial protease.

Hydrogen peroxide produced by the enzymatic reaction was observed to non-enzymatically oxidize the DTT substrate. Therefore measurements of the stoichiometric amount of sulfhydryls oxidized by the enzymatic reaction alone were possible only when catalase was added to the reaction mixture to remove the peroxide product. Endogenous catalase activity coincidentally found in the epididymal fluid was not sufficient to react with all the peroxide produced by the enzyme under the conditions of our assay. The addition of excess catalase necessitated that the observed oxygen uptake be multiplied by a factor of two to obtain the actual amount of oxygen consumed in the enzymatic reaction. This procedure was justified by the observation that samples containing excess catalase exhibited one-half the reaction rate of azide-inhibited samples.

Although the production of hydrogen peroxide was not quantitated due to the rapid non-enzymatic reaction with the DTT in the azide-inhibited system, formation of hydrogen peroxide was confirmed by experiments in which the reaction was allowed to run to completion.

Table 1 indicates that over four experiments, an average of 2.06 umoles sulfhydryls were oxidized per umole oxygen consumed. Table 2 shows that for each umole oxygen consumed, 1.08 umole disulfide was produced. From the accumulated data, the stoichiometry of the enzymatic reaction was calculated to be $2R\text{-}SH + O_2 \rightarrow R\text{-}S\text{-}S\text{-}R + H_2O_2$.

The substrate specificity of the enzyme was studied by measuring the enzymatic activity at pH 7.6 in the presence of 10mM concentrations of DTT, cysteine, glutathione, 2-mercaptoethanol, thioglycolate, ethanol, resorcinol, spermine, putricine, and ascorbate. 1mM FADH and NADH were also tested as substrates. Table 3 indicates that all the sulfhydryls tested were utilized as substrates. Alcohols, amines, ascorbate, FADH and NADH were not oxidized by the enzyme. These results indicate that the enzyme has a general speci-

Table 1: Stoichiometric relationship between sulfhydryl oxidation and oxygen consumption in the epididymal sulfhydryl oxidase reaction.

Expt. No.	umoles -SH oxidized	umoles O_2 consumed	umoles -SH/ umoles O_2
1	0.98 ± 0.03	0.41 ± 0.01	2.39
2	0.59 ± 0.07	0.34 ± 0.01	1.74
3	0.57 ± 0.05	0.25 ± 0.01	2.28
4	0.69 ± 0.04	0.38 ± 0.01	1.81
		Average ratio	2.06

Data from each experiment represents an average of four determinations. See Methods for experimental conditions.

ficity for sulfhydryl compounds.

The enzyme cofactor requirement was investigated by measuring the enzyme activity in the presence of 10mM EDTA, EGTA, o-phenanthroline, and diethyldithiocarbamate using 2mM DTT as substrate. Diethyldithiocarbamate was not oxidized by the enzyme. Enzyme solutions were also dialyzed overnight at 4°C against 2mM KBr and 10mM EDTA, then re-dialyzed against phosphate buffer alone before measurement of enzyme activity. Epididymal catalase was inhibited by 2mM azide. Table 4 indicates that the enzyme activity was not significantly affected by addition of metal chelators or by extensive dialysis against KBr and EDTA. These findings suggest that the enzyme does not require losely bound cofactors for activity. Possible prosthetic group involvement in the catalytic reaction has not been determined.

DISCUSSION

This communication describes an enzyme distinct from previously reported sulfhydryl oxidizing enzymes in its catalytic properties and reactivity. Epididymal fluid sulfhydryl oxidase differs from thiol oxidase and the sulfhydryl oxidase found in *Myrothecium verrucaria* in that the product of its reac-

Table 2: Stoichiometric relationship between disulfide production and oxygen consumption in the epididymal sulfhydryl oxidase reaction.

Expt. No.	umoles DTT oxidized/min	umoles O_2 consumed/min	umoles disulfide/ umoles O_2
1	0.10 ± 0.01	0.09 ± 0.01	1.11
2	0.09 ± 0.01	0.08 ± 0.01	1.12
3	0.07 ± 0.01	0.07 ± 0.01	1.00
		Average ratio	1.08

Data from each experiment represents average of three determinations. Experimental conditions described in Methods.

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tion is hydrogen peroxide and not water as is the case for the latter two enzymes. A further distinction is that whereas the epididymal fluid enzyme is capable of utilizing mercaptoethanol, glutathione, cysteine and thioglycolate as substrates, thiol oxidase is inhibited by all these compounds except thioglycolate, and the enzyme from Myrothecium verrucaria is not capable of oxidizing thioglycolate. The sulfhydryl oxidase from epididymal fluid is different from the enzyme found in milk in two respects: it is not affected by high concentrations of glutathione (40mM), and does not require metals for activity. The milk enzyme is inhibited by concentrations of glutathione greater than 0.8mM and requires iron for catalytic activity.

In a preliminary distribution survey, the enzyme activity was found in hamster testicular and epididymal cells, hamster, guinea pig, dog and bull epididymal fluids and human seminal plasma and blood serum. The enzyme was not present in the 250,000g supernatants of rat brain, heart, kidney, liver, and lung homogenates.

In vitro studies have demonstrated that sulfhydryl compounds can damage spermatozoa by reducing structurally vital disulfide crosslinks present in the sperm nuclear and tail structures (9-11). In vivo, relatively high con-

Table 3: The ability of various compounds to serve as substrates for epididymal sulfhydryl oxidase.

Substrate	nmoles O_2 consumed/min	% Maximal Activity
DTT	185.6	100.0
Cysteine	37.7	20.3
Glutathione	25.1	13.5
2-mercaptoethanol	23.3	12.6
Thioglycolate	20.6	11.1
Ethanol	0	-
Resorcinol	0	-
Spermine	0	-
Putricine	0	-
Ascorbate	0	-
FADH	0	-
NADH	0	-

All compounds were present at 10mM except for FADH and NADH which were 1mM. Other conditions were described in text.

centrations of free sulfhydryls are present (6), yet sulfhydryl-related sperm lesions are rarely observed. The presence of the new enzyme reported here offers an explanation for this discrepancy. We have observed that under physiological conditions, the harmful effects of sulfhydryls upon spermatozoa were prevented when epididymal fluid sulfhydryl oxidase was added to the incubation medium containing spermatozoa and DTT (Chang and Morton, unpublished). These findings suggest that the enzyme functions as a protective agent in the male reproductive tract, oxidizing potentially damaging physiological sulfhydryl groups before they can harm the spermatozoa, thereby preserving sperm structure and function.

Table 4: Effect of metal chelators and KBr on epididymal sulfhydryl oxidase activity.

Treatment	% Maximal Activity
Enzyme (control)	100.0
+ EDTA	101.5
+ EGTA	95.6
+ o-phenanthroline	93.8
+ diethyldithiocarbamate	97.1
Enzyme dialyzed against 2mM KBr	100.0
" " " 10mM EDTA	104.0

The discovery of this enzyme has significant implications in the field of fertility regulation. If an inhibitor to the enzyme can be found, it would make an ideal male contraceptive, as it would permit damage to the spermatozoa in the male reproductive tract by physiological concentrations of sulfhydryl agents such as glutathione and cysteine.

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