Vol. 87, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1979 Pages 171-176

A FLAVOPROTEIN RESPONSIBLE FOR THE INTENSE SULFHYDRYL OXIDASE ACTIVITY OF RAT SEMINAL VESICLE SECRETION

M.C. Ostrowski, W.S. Kistler and H.G. Williams-Ashman*

Chemistry Department and School of Medicine, University of South Carolina, Columbia, SC 29208 and *Ben May Laboratory for Cancer Research, Department of Biochemistry, and Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637

Received January 29, 1979

Summary. A flavoprotein isolated in substantial yields from rat seminal vesicle secretion accounts for most if not all of the capacity of this fluid to catalyze the aerobic oxidation of a number of low molecular weight thiol compounds. The nature and possible physiological significance of this enzyme are discussed.

A soluble enzyme catalyzing the oxidation of a number of low molecular weight sulfhydryl compounds was reported to be present in various male reproductive organs and their secretions in rats and hamsters (1,2). The enzyme converts sulfhydryl-containing substances to the corresponding disulfides at the expense of molecular oxygen and with apparent generation of hydrogen peroxide. The activity of rat seminal vesicle secretion (SVS)[1] was especially intense. Comparable thiol oxidase activity determined in the presence of azide could not be detected in extracts of rat muscle, liver, kidney, brain, or lung (1).

In the course of purification of a major bulk protein from rat SVS (3), a yellow colored protein was obtained as a by-product. This paper describes the isolation of this protein in an apparently homogeneous form and summarizes evidence that its prosthetic group is a flavin (probably FAD). The capacity of this flavoprotein to oxidize low molecular weight thiols accounts for most if not all of the sulfhydryl oxidase activity of rat SVS.

EXPERIMENTAL PROCEDURES

Purification of thiol oxidase. Seminal vesicles from sexually mature Sprague-Dawley rats were dissected free of coagulating glands. The secretion was manually expressed from the single central lumens of the seminal vesicles into ice-cold 150 mM NaCl containing 50 mM Tris-Cl and 5 mM EDTA at pH 8.0 (25°). Insoluble material was removed by centrifugation at 2,000 x g for 10 min at 2°. The supernatant fluid was fractionated by addition of solid $(NH_4)_2SO_4$. The material precipitating as a result of addition of 17.6 g of $(NH_4)_2SO_4$ per 100 ml of solution was discarded. The precipitate formed by addition of 21.4 g of $(NH_4)_2SO_4$ to the supernatant fluid was collected and dissolved in a small volume of 100 mM sodium acetate pH 5.6, and desalted by passage through

[1]Abbreviations: SVS, seminal vesicle secretion; SDS, sodium dodecylsulfate.

0006-291X/79/050171-06$01.00/0

a column of Sephadex G-25 previously equilibrated with sodium acetate. The protein material was applied to a column (2.5 x 30 cm) of carboxymethyl Sephadex C-25 equilibrated against the same acetate buffer. After washing with two column volumes of the loading buffer, the yellow protein was eluted almost immediately by application of acetate buffer containing 125 mM NaCl, and before any other SVS proteins present in the fraction was applied to the column.

Assay of thiol oxidase activity. Aerobic oxidation of sulfhydryl compounds was determined by monitoring oxygen disappearance with a Gilson Oxygraph Model KM equipped with a Clark oxygen electrode. The standard assay mixture consisted of 100 mM 2-mercaptoethanol and 200 mM sodium phosphate pH 7.0. The total volume of the reaction was 1.6 ml with the temperature maintained at 25° with stirring. Suitable amounts of SVS protein fractions were added to ensure that the oxygen consumption measured was proportional to the amount of protein added and to the time of incubation. Oxygen uptake in the absence of enzyme preparations was negligible under these conditions. The consumption of oxygen was calculated assuming that the initial concentration of oxygen in the assay solution was 0.25 μmol/ml at 25°.

Other methods. Polyacrylamide gel electrophoresis was performed according to Laemmli (4) at acrylamide concentration of 15%. Protein was estimated by the biuret method (5) or the procedure of Lowry et al. (6) with bovine serum albumin as standard. Absorbtion spectra were determined with a Cary Model 219 spectrophotometer.

RESULTS AND DISCUSSION

In preliminary experiments we readily confirmed the observation of Chang and Zirkin (2) that crude soluble extracts of rat SVS readily oxidized 2-mercaptoethanol and some other low molecular weight thiol compounds. Under the standard assay conditions employed, the aerobic oxidation of thiols was unaffected by 10 mM sodium azide. The protein in rat SVS catalyzing 2-mercaptoethanol oxidation was purified by the procedures described above. The results of a typical study are summarized in Table I. This simple three-step procedure yielded an apparently homogeneous yellow protein, as illustrated in Fig. 1. Other saline-soluble SVS proteins eluted from the carboxymethyl-Sephadex column used in step 3 by either lower or higher salt concentrations than were employed for discrete separation of the yellow protein were devoid of sulfhydryl oxidase activity. More than 50% of the total thiol oxidase activity in the initial saline extract of SVS (step 1) was associated with the single protein isolated in step 3 of Table I. Since trailing portions of thiol oxidase activity unassociated with significant material with absorbancy at 280 nm were not included in the pooled fraction 3 of Table I, it is apparent that most and perhaps all of the thiol oxidase activity in saline extracts of rat SVS can be accounted for by the yellow protein. It is noteworthy that this material constituted less than 2% of the total soluble protein in the initial extract, and thus represents only a minor protein fraction of rat SVS (3,7). The capacity of pooled fractions from step 3 of this purification procedure (Table I) to oxidize 2-

Table 1

Purification of Rat Seminal Vesicle Secretion Thiol Oxidase

Step	Total Protein(mg)	Total thiol oxidase activity (μmoles O_2 reduced/min)	Recovery (%)	Specific Activity (units/mg protein)
1) Soluble extract of secretion	286	77.4	(100)	0.27
2) Ammonium sulfate fraction	212	62.6	81	0.30
3) Carboxymethyl-Sephadex	3.5	41.2	53	11.7

The procedures for SVS protein fractionation and assay of thiol oxidase activity are described in the text. The SVS used as starting material for this preparation was obtained from both seminal vesicles of 20 rats.

mercaptoethanol, when stored at a protein concentration of 1.5 mg per ml, was stable on storage at 4° for 3 months.

Precipitation of the enzyme with 10% trichloroacetic acid, or by boiling, left a flavin component in solution. After suitable concentration, the yellow and fluorescent substance so liberated migrated to virtually the same position as authentic FAD, but far from FMN, in chromatographic systems 5 and 6 of Kilgour et al. (8). The absorption spectrum of the unresolved enzyme in 60 mM sodium phosphate at pH 7.0 exhibited an intense peak at 275 nm (absorbancy of 1.5 at a protein concentration of 1.0 mg per ml), and two nearly symmetrical bands with maxima at 365 and 455 nm. The ratio of absorbancies at 365: 455: 275 nm were 1:1.4:14.6. The yellow color of the enzyme was bleached on addition of 100 mM 2-mercaptoethanol. From its migration during SDS-polyacrylamide gel electrophoresis, performed alongside reference proteins under conditions where plots of distance migrated were a linear function of the logarithm of molecular weights, the SVS flavoprotein appeared to be comprised of one or more subunits of apparent molecular weight of 66,000. This estimate combined with the spectral data, permits the conclusion that the purified flavoprotein contains close to 1 mole of FAD per subunit of 66,000 daltons.

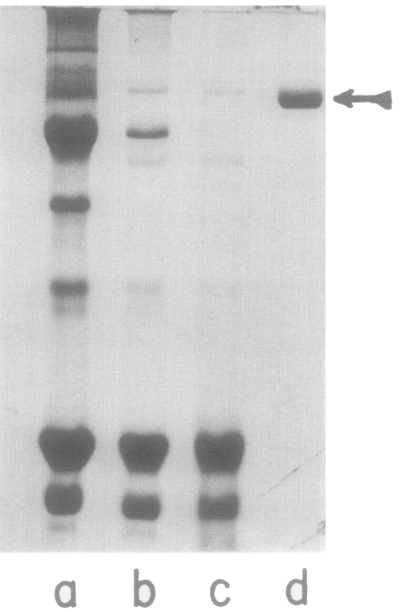

FIG. 1. SDS-Polyacrylamide gel electrophoresis of rat seminal vesicle secretion thiol oxidase at various stages of purification. The tracks from left to right illustrate the migration of proteins in the following preparations: (a) fresh, unfractionated seminal vesicle secretion; (b) proteins in SVS that remain soluble on dilution with 0.15 M NaCl containing 50 mM Tris and 5 mM EDTA at pH 8.0 (step 1 material in Table 1); (c) ammonium sulfate fraction; (d) purified thiol oxidase eluted from carboxymethyl-Sephadex. The arrow indicates the position of migration of the purified thiol oxidase.

Both crude and purified preparations of the enzyme oxidize a number of low molecular weight thiol compounds. At substrate concentrations of 10 mM and at pH 7.0, the following relative rates of oxidation were observed : dithiothreitol, 100; glutathione, 66; L-cysteine, 33; 2-mercaptoethanol, 11. Preliminary experiments with the purified enzyme indicated that the apparent K_m for dithiothreitol was less than 1 mM, whereas the value was greater than 10 mM for glutathione, and even higher for L-cysteine and 2-mercaptoethanol (inhibitions at very high thiol substrate concentrations were observed). The sulfhydryl oxidase activity of the purified flavoprotein was heat labile, with 50 percent loss in activity after heating at 60° for 3.5 min.

The purified SVS thiol oxidase did not catalyze aerobic oxidation of the following substrates at 25° and pH 7.0: xanthine, NADH, NADPH, putrescine, benzylamine, L-leucine, D-leucine and glucose. Similarly, NADPH and NADH were not oxidized by 10 μM 2-methyl-1,4-naphthoquinone in the presence of 3-(4,5-dimethyl thiazolyl-2-)-2,5-diphenyl tetrazolium bromide as terminal electron acceptor. These findings suggest that the SVS thiol oxidase is not identical with a number of well characterized flavo-

protein oxidases or dehydrogenases, but obviously do not exclude its identity with many other known flavoprotein enzymes. The spectrum of the purified SVS sulfhydryl oxidase provided no indication of either heme or non-heme iron sulfur components. On the grounds of its flavoprotein nature, thiol substrate specificity, and other properties, the SVS enzyme is clearly different from the sulfhydryl oxidase of bovine milk (9,10), the thiol oxidase of Piricularia oryzae that attacks ethylenic-linked thiols (11), and the mammalian microsomal protein disulfide exchange enzyme originally described by Goldberger et al. (12,13). We did not determine whether the rat SVS thiol-oxidizing flavoprotein can catalyze the oxidation of sulfhydryl groups in proteins.

Chang and Morton (1) provided evidence that oxidation of dithiothreitol by crude rodent epididymal extracts (which is probably catalyzed by the enzyme considered here) is accompanied by formation of hydrogen peroxide, although the eventuality that other substances such as superoxide radical might represent the immediate products of oxygen reduction (14-16) was not excluded. We did not quantitate relationships between disulfide and hydrogen peroxide formation during the oxidation of thiols by the rat SVS enzyme, but we noticed that addition of crystalline catalase to assay mixtures containing relatively low concentrations of thiol substrates reduced the oxygen consumption by exactly one-half. Neither 10 mM EDTA nor 10 mM azide influenced oxidation of 2-mercaptoethanol by the SVS flavoprotein in the absence of catalase.

The physiological significance, if any, of the rat SVS thiol oxidizing enzyme we have isolated is mysterious. It is by no means certain that any thiols represent the natural substrates for the enzyme. Without providing experimental details, Chang and Morton (1) stated that the harmful effects of sulfhydryl compounds on spermatozoa were prevented when crude preparations of epididymal sulfhydryl oxidase were added to incubation mediums containing spermatozoa and dithiothreitol. Although exogenous low molecular weight thiols can indeed evoke damage to spermatozoa as a result of reducing disulfide bridges in sperm nuclear and flagellum components, these effects have usually been demonstrated only under unnatural conditions involving high pH, or addition of detergents and/or denaturing agents such as urea (17-20). Furthermore, it is well known that sulfhydryl compounds can exert beneficial effects on the structural integrity and motility of spermatozoa under physiological circumstances (21). In addition, the status of various low molecular weight thiols in mammalian seminal plasmas has not been explored exhaustively, especially in various rodent species (21,22). Several other potential functions of rodent SVS thiol oxidase are easily imaginable but a paucity of relevant physiological data makes it unprofitable to elaborate on these speculations.

Acknowledgements. These studies were supported in part by NIH research grants HD-10793 and HD-04592; and by a Basil O'Connor Starter Grant from the National Foundation/March of Dimes.

REFERENCES

1) Chang, T.S.K. and Morton, B. Biochem. Biophys. Res. Commun., 66, 309-315 (1975).
2) Chang, T.S.K., and Zirkin, B.R. Biol. Reprod., 17, 745-748 (1978).
3) Ostrowski, M.C., Kistler, M.K., and Kistler, W.S. J. Biol. Chem., 254, 383-390 (1979).
4) Laemmli, U.K. Nature, 227, 680-685 (1970).
5) Gornall, A.G., Bardawill, C.J., and David, M.M. J. Biol. Chem., 177, 751-756 (1949).
6) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. J. Biol. Chem., 193, 265-275 (1951).
7) Williams-Ashman, H.G., Wilson, J., Beil, R.E., and Lorand, L. Biochem. Biophys. Res. Commun., 79, 1192-1198 (1977).
8) Kilgour, G.L., Felton, S.P., and Huennekens, F.M. J. Amer. Chem. Soc., 79, 2254-2256 (1957).
9) Janolino, V.G., and Swaisgood, H.E. J. Biol. Chem., 250, 2532-2538 (1975).
10) Abraham, P., Swaisgood, H.E., and Horton, H.R. Federation Proc., 37, 1341 (Abstract #399) (1978).
11) Aurbach, G.D., and Jacoby, W.B. J. Biol. Chem., 237, 565-568 (1962).
12) Goldberger, R.F., Epstein, C.J., and Anfinsen, C.B. J. Biol. Chem., 239, 1406-1410 (1964).
13) Freedman, R.B., and Hawkins, H.C. Biochem. Soc. Trans., 5, 348-357 (1977).
14) Bray, R.C. The Enzymes (3rd Edtn. P.D. Boyer, ed.), Vol. 12, pp 300-419 Academic Press, New York (1975).
15) Fridovich, I. Annu. Rev. Biochem., 44, 147-159 (1975).
16) Misra, H.P. J. Biol. Chem., 249, 2151-2155 (1974).
17) Bedford, J.M., Calvin, H.I., and Cooper, G.W. J. Reprod. Fert., Suppl., 18, 199-213 (1973.
18) Marushige, Y., and Marushige, K. J. Biol. Chem., 250, 39-45 (1975).
19) Mahi, C.A., and Yanagamichi, R.J. Reprod. Fert., 44, 293-296 (1975).
20) Kistler, W.S., Geroch, M.E., and Williams-Ashman, H.G. J. Biol. Chem., 248, 4532-4543 (1973).
21) Mann, T. The Biochemistry of Semen and of the Male Reproductive Tract (Wiley, 1964).
22) Li, T-K. Biol. Reprod., 12, 641-646 (1975).