Properties of a Flavoprotein Sulfhydryl Oxidase from Rat Seminal Vesicle Secretion†

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ABSTRACT: Rat seminal vesicle secretion is a rich source of a flavoprotein oxidase that acts upon sulfhydryl compounds. The enzyme was obtained in homogeneous form as previously described [Ostrowski, M. C., Kistler, W. S., & Williams-Ashman, H. G. (1979) *Biochem. Biophys. Res. Commun. 87*, 171–176] and characterized with respect to prosthetic group, size, reaction stoichiometry, and substrate specificity. On the basis of its behavior during zone sedimentation, gel filtration, and electrophoresis in the presence of sodium dodecyl sulfate, it appears to be a monomeric enzyme of about 66 000 daltons. Acid denaturation liberates 1 mol of flavin adenine dinucleotide (FAD) per mol of enzyme. The reaction catalyzed was shown to be $2RSH + O_2 \rightarrow RSSR + H_2O_2$. Superoxide formation could not be demonstrated. Unlike many flavoprotein oxidases, the enzyme failed to form a bleached complex with sulfite. The enzyme accepts a variety of small sulfhydryl compounds as substrates, including glutathione, cysteine, dithiothreitol, and 2-mercaptoethanol. Michaelis–Menten kinetics were obtained with these substrates providing disulfide contamination was initially eliminated by treating thiols with borohydride. The K_M for glutathione was 4.4 mM with a V_{max} estimated as 660 μmol per min per mg of protein. The enzyme was capable of markedly enhancing the rate of renaturation of fully reduced ribonuclease. The physiological function of the enzyme is not yet clear, though several possibilities are discussed.

Chang & Morton (1975) reported that a variety of tissues and secretions of the male reproductive tract contain high levels of a sulfhydryl oxidizing activity. Studies of the activity in crude extracts from hamster epididymal fluid indicated that a variety of low molecular weight thiols could be oxidized and that the products of the reaction were a disulfide and hydrogen peroxide (Chang & Morton, 1975). In a subsequent study, Chang & Zirkin (1978) found in rats and hamsters that the highest levels of this activity were present in seminal vesicle secretion. In the course of our own studies of seminal vesicle proteins (Ostrowski et al., 1979a), we obtained a yellow protein byproduct that was subsequently shown to account for the bulk of the sulfhydryl oxidase activity of rat seminal vesicle secretion (Ostrowski et al., 1979b).

In the present report we describe a number of properties of the homogeneous oxidase purified from rat seminal vesicle secretion.

Experimental Procedures

Purification of the Sulfhydryl Oxidase. The enzyme was prepared from rat seminal vesicle secretion exactly as specified previously (Ostrowski et al., 1979b). The activity of the purified enzyme is stable for months when it is stored in 125 mM NaCl and 100 mM sodium acetate, pH 5.6, at a protein concentration of 1 mg/mL at 4 °C. Its stability under other conditions has not been systematically explored.

Sulfhydryl Oxidase Assay. 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 was altered slightly from that used previously (Ostrowski et al., 1979b) and contained 10 mM dithiothreitol and 20 mM sodium phosphate, pH 7.0. The total volume of the reaction was 1.6 mL, with the temperature maintained at 25 °C with stirring. Oxygen uptake in the absence of enzyme was negligible, and assays were performed with amounts of enzyme such that oxygen consumption was proportional to the amount of enzyme added and to the time of incubation. The initial concentration of oxygen in the assay solution was taken to be 0.21 μmol of oxygen per mL under the above conditions.

For kinetic studies it was necessary to ensure that all of the sulfhydryl substrate was in the reduced form. This was accomplished by the addition of sodium borohydride, final concentration of 20 mM (Jocelyn, 1972), to 200 mM substrate stock solutions buffered at pH 8.0 by 20 mM sodium phosphate. After 30-min incubation at room temperature, excess borohydride was destroyed by addition of acetone (12 μL per mL of substrate stock), followed by a second 30-min period of incubation. Final substrate concentrations were estimated with Ellman's reagent[1] [5,5′-dithiobis(2-nitrobenzoic acid)] (Ellman, 1959).

Stoichiometry and Product Determination. The ratio of sulfhydryl disappearance to oxygen consumption was measured in a reaction initially containing 20 mM dithiothreitol, 12 units/mL crystalline bovine liver catalase (Sigma C-30), and 1 unit of purified sulfhydryl oxidase in a final volume of 1.6 mL. When the oxygen tension had decreased 50 to 80% as measured by the Oxygraph, the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5% (w/v). After centrifugation (5 min, 800*g*, 4 °C), samples of the supernatant were assayed for free sulfhydryl using Ellman's reagent.

The production of a disulfide as a product of the reaction was demonstrated by using glutathione reductase. Glutathione at concentrations from 2 to 10 mM was incubated with 12 units/mL catalase and 10 units/mL sulfhydryl oxidase in a final volume of 2 mL at pH 7 and 35 °C in an open, 25-mL Erlenmeyer flask with shaking until greater than 90% of the glutathione was consumed as determined by assay with Ellman's reagent. The reaction was then stopped by the addition of trichloroacetic acid to a final concentration of 5%. After centrifugation (5 min, 800*g*, 4 °C), the supernatant was

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[1] Abbreviations used: Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); FAD, flavin adenine dinucleotide.

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neutralized with 1 M Tris base. Samples were then added to cuvettes containing final concentrations of 0.2 mM NADPH, 50 mM Tris-Cl, pH 8, 5 mM Na_2EDTA, and 2 units of yeast glutathione reductase (Sigma G-4759) in a volume of 3 mL. A decrease in absorbance at 340 nm was then followed until the completion of the reaction.

The stoichiometry of hydrogen peroxide production was determined by incubating dithiothreitol (2 mM) in the presence of 10 units/mL sulfhydryl oxidase at pH 7 at 35 °C. At various time intervals, aliquots were removed for sulfhydryl estimation by Ellman's reagent and for H_2O_2 measurement using the horseradish peroxidase–o-dianisidine method (Decker, 1977).

Preparation of Apoflavoenzymes and Reconstitution of Activity. The apo forms of D-amino acid oxidase and sulfhydryl oxidase were prepared by acid–ammonium sulfate precipitation (Strittmatter, 1961). All manipulations were carried out at 4 °C. A 1-mL sample of flavoprotein (2 mg/mL) in 0.1 M Tris-acetate and 1 mM Na_2EDTA, pH 8.1, was mixed in a centrifuge tube with 1 mL of 3 M KBr. One milliliter of saturated ammonium sulfate adjusted to pH 1.5 with H_2SO_4 was then added with swirling over a 20-s time interval. After 40 s an additional 4 mL of the acidified $(NH_4)_2SO_4$ was added quickly, and the tube was centrifuged at 20000*g* for 5 min. The pellet was redissolved in 2 mL of 0.1 M Tris-acetate and 1.5 M KBr, pH 8.1. The above precipitation scheme was repeated twice more. After the final precipitation, the pellet was dissolved in 1.5 mL of the same Tris-acetate–KBr solution and dialyzed for 12 h against two changes of 0.1 M sodium phosphate, pH 7.0 (for the sulfhydryl oxidase), or 0.1 M sodium pyrophosphate, pH 8.1 (for D-amino acid oxidase). The apoenzymes were quick frozen and stored at −20 °C until use.

Reconstitution of the holoenzymes was carried out according to the protocol of Massey & Swoboda (1963). Apoenzyme (30–50 μg) was incubated in the Oxygraph cell in the presence of flavin in the appropriate buffer (50 mM sodium pyrophosphate, pH 8.1, for D-amino acid oxidase; 20 mM sodium phosphate, pH 7.0, for sulfhydryl oxidase), containing 1 mg/mL bovine serum albumin for 30 min at 37 °C. The appropriate substrate was then added (30 mM D-alanine or 10 mM dithiothreitol), and oxygen consumption followed.

The flavin component of sulfhydryl oxidase was prepared by precipitation of protein at 4 °C by trichloroacetic acid at a final concentration of 5%. The denatured enzyme was removed by centrifugation, and trichloroacetic acid was eliminated from the supernatant by repeated extraction with ether. The flavin, which remained in the aqueous phase, could then be added to the reconstitution mixture for either apooxidase prepared above.

Reductive Denaturation and Renaturation of RNase A. Bovine pancreatic RNase A (20 mg, Sigma R 5125) was dissolved in 3 mL of freshly prepared 8 M urea (Schwarz/Mann, UltraPure) adjusted to pH 8.6 with 5% (v/v) methylamine just prior to use. 2-Mercaptoethanol (20 μg) was added, and the container was flushed with N_2, sealed, and allowed to stand at room temperature for 4.5 h. After this period the pH was adjusted to 3.5 with glacial acetic acid, and the enzyme was separated from denaturants by gel filtration through Sephadex G-25 using a column (1.5 × 30 cm) equilibrated and eluted with 0.1 M acetic acid. The extent of reduction of RNase was determined by using Ellman's reagent as described by Anfinsen & Haber (1961).

For renaturation of the reduced RNase, the protein was diluted to a concentration of 1 mg/mL with 0.1 M acetic acid, and the pH was adjusted to 7.0 with saturated Tris base (Haber & Anfinsen, 1962). Sulfhydryl oxidase in varying amounts was added to individual 2-mL samples of the preparation, and the mixtures were allowed to stand at room temperature. Duplicate samples were removed periodically for determination of sulfhydryl content (Ellman, 1959) and RNase activity, using yeast RNA as substrate at pH 5 (Kalnitsky et al., 1959).

Polyacrylamide Gel Electrophoresis. Gels containing sodium dodecyl sulfate were prepared and run according to the procedure of Laemmli (1970) using 10% (w/v) acrylamide and 0.09% methylenebis(acrylamide) in the small pore gel. Isoelectric focusing gels (2.5 × 120 mm) were polymerized from a solution with the following composition: 5% (w/v) acrylamide, 0.56% (w/v) methylenebis(acrylamide), 2% (w/v) ampholines (3.5 to 10 range, LKB), 5% (v/v) glycerol, 10^{-5} M riboflavin, and 0.1% (v/v) *N,N,N′,N′*-tetramethylethylenediamine. Polymerization was induced by photoirradiation. Samples were loaded in 50% (w/v) sucrose and overlaid with 20 μL of 20% sucrose containing 1% ampholines. The positive electrode buffer was 0.01 M phosphoric acid and the negative electrode buffer was 0.02 M NaOH. Electrophoresis was for 4 h at a constant power of 0.4 W/gel. After electrophoresis, a gel that contained no protein sample was cut into 5-mm sections that were soaked overnight in 1 mL of deionized water. The pH of the resultant solutions was then determined. Gels containing protein were soaked overnight in 12.5% (w/v) trichloroacetic acid, then stained for 1 h in 0.1% (w/v) Coomassie brilliant blue R, 10% (v/v) acetic acid, and 30% (v/v) 2-propanol, and destained by diffusion in 5% (v/v) acetic acid and 10% (v/v) ethanol. The stained gel was then scanned at 595 nm using a Gilford spectrophotometer equipped with a gel transport.

Sucrose Gradient Centrifugation. Linear 5 to 20% sucrose gradients in 0.038 M sodium phosphate, pH 7.0, were constructed. Such gradients are approximately isokinetic for proteins in a Beckman SW-41 rotor (Fritsch, 1973). Centrifugation was for 15 h at 40000 rpm at 15 °C in a Beckman L5-50 ultracentrifuge. Gradients were fractionated and monitored for UV-absorbing material with an ISCO Model 640 fractionator equipped with a Model UA-5 absorbance monitor.

Analytical Gel Filtration. Filtration through Sephadex G-200 was carried out at 4 °C using a column (1.5 × 80 cm) equilibrated and eluted with 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.0. Calibration standards were beef liver catalase, yeast hexokinase, beef heart lactate dehydrogenase, and bovine serum albumin. Elution positions for enzymes were determined by activity assays (Decker, 1977), while bovine serum albumin was located by absorbance at 280 nm.

Miscellaneous Methods. Spectra were obtained by using a Cary Model 219 recording spectrophotometer. Protein was estimated by the method of Lowry et al. (1951) referenced to crystalline bovine serum albumin. Measurements of pH were made with an Orion combination glass electrode and refer to final mixtures at room temperature unless otherwise stated. Biochemicals and proteins, unless otherwise stated, were obtained from Sigma. Benzenethiol was from Eastman. Impure glucose oxidase preparations were from Sigma (G 6125), while the purified enzyme was obtained from Boehringer (Grade 1).

Results

Identification of FAD as the Prosthetic Group. The seminal vesicle secretion thiol oxidase is obtained in slightly better than 50% yield by a three-step procedure involving centrifugation to remove saline-insoluble proteins, ammonium sulfate frac-

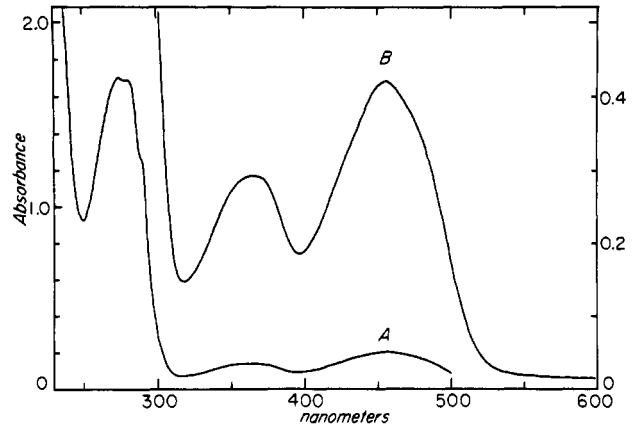

FIGURE 1: Spectrum of the sulfhydryl oxidase in 0.02 M sodium phosphate, pH 6.8: (A) 0.75 mg/mL, full scale = 2.0; (B) 1.5 mg/mL, full scale = 0.5.

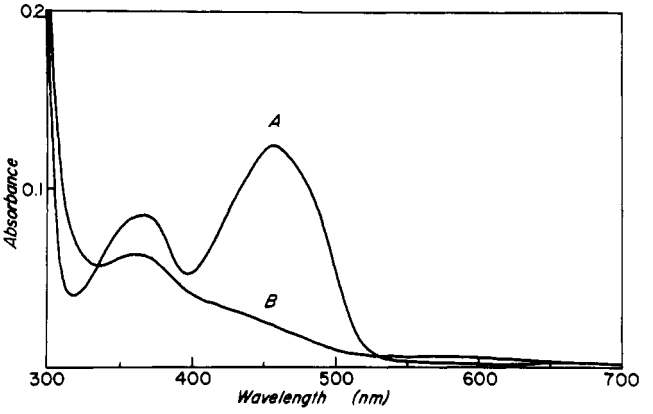

FIGURE 2: Spectrum of the sulfhydryl oxidase in 0.05 M sodium phosphate, pH 6.8: (A) oxidized form of the enzyme; (B) after addition of 10 mM 2-mercaptoethanol.

tionation, and chromatography on carboxymethyl-Sephadex (Ostrowski et al., 1979b). The spectrum of the purified enzyme near neutrality indicated that it was a flavoprotein but gave no evidence for the presence of heme or of nonheme iron–sulfur centers (Figure 1). The aerobic addition of thiol substrates for the enzyme in amounts significantly in excess of the concentration of dissolved oxygen resulted in the bleaching of the visible absorption bands (Figure 2). The slight increase in absorbance for the reduced enzyme in the 530 to 640 range was reproducible but varied somewhat in magnitude with the thiol used as reductant. Whether the long-wavelength absorbance indicates the presence of some partially reduced enzyme or, as suggested by a reviewer, a charge-transfer interaction between reduced flavin and product has not been rigorously explored. The addition of sodium sulfite at final concentrations as high as 0.1 M failed, however, to modify the spectrum of the enzyme (not shown), though many flavoprotein oxidases form complexes of altered spectral properties with this reagent (Massey et al., 1969a).

The flavin component of the sulfhydryl oxidase could be liberated by trichloroacetic acid precipitation of the apoenzyme or by boiling, thus indicating a labile, presumably noncovalent, association between the enzyme and its prosthetic group. Chromatographic studies of the liberated flavin had indicated it was not FMN and likely to be FAD (Ostrowski et al., 1979b). This identification was confirmed by establishing that the liberated flavin could restore activity to the apoenzyme of D-amino acid oxidase to the same extent as authentic FAD (Table I). Conversely, authentic FAD, but not FMN or

Table I: Reactivation of Apooxidases by Added Flavins[a]

enzymes	flavin added	act.
D-amino acid apooxidase (45 μg)	none	0
	FAD, 50 nmol	80
	FMN, 1 μmol	0
	Cl_3AcOH-extracted thiol oxidase flavin, 50 nmol	80
sulfhydryl apoοxidase (30 μg)	none	0
	FAD, 50 nmol	88
	FMN, 1 μmol	0
	riboflavin, 1 μmol	0

[a] D-Amino acid oxidase and sulfhydryl oxidase were converted to inactive, apo forms as described under Experimental Procedures. They were then incubated in the absence or presence of various flavins as described under Experimental Procedures, followed by determination of activity. Activity was calculated as a percentage of that obtained for an equivalent protein amount of native enzyme. The concentrations of FAD and of the flavin liberated from the sulfhydryl oxidase were estimated spectrophotometrically, $E_{450}^{1\%} = 11\,300$ (Morris & Redfearn, 1969). The final volume of the assay mixtures was 1.6 mL.

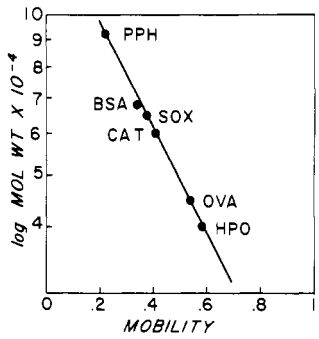

FIGURE 3: Mobility of the sulfhydryl oxidase and reference proteins during electrophoresis in the presence of sodium dodecyl sulfate. The gel was polymerized from a solution containing 10% acrylamide as previously described (Laemmli, 1970). Mobility is with reference to bromophenol blue. Reference proteins were phosphorylase (PPH), bovine serum albumin (BSA), catalase (CAT), ovalbumin (OVA), and horseradish peroxidase (HPO). The mobility of the sulfhydryl oxidase (SOX) is indicated.

riboflavin, was capable of restoring activity to the aposulfhydryl oxidase (Table I).

Physical Properties of the Sulfhydryl Oxidase. The mobility of the sulfhydryl oxidase during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicates that it contains a single class of polypeptide chain of about 66 000 daltons (Figure 3). Several lines of evidence indicate that the enzyme is in fact a monomeric protein. Under nondenaturing conditions, the enzyme sedimented almost indistinguishably from bovine serum albumin (4.5 S, 68 000 daltons) during ultracentrifugation through sucrose gradients (Figure 4). Furthermore, the sulfhydryl oxidase eluted just behind bovine serum albumin during gel filtration through Sephadex G-200 under nondenaturing conditions (not shown). Accordingly, all three methods agree in the assignment of a molecular mass of about 66 000 to the oxidase.

The stoichiometry of FAD to protein was determined in two ways. Titration of the apo form of D-amino acid oxidase with standard amounts of authentic FAD yields an activity vs. FAD curve that can be used to determine unknown amounts of FAD (Massey & Swoboda, 1963). The flavin liberated from known amounts of the sulfhydryl oxidase yielded a ratio of 1.05 mol of FAD per 66 000 g of enzyme. Similarly, using the absorbance of free FAD ($E_{450}^{1\%} = 11\,300$, Morris & Redfearn, 1969) the spectrum of known concentrations of the enzyme yields a ratio of 0.85 mol of FAD per mol of enzyme. These

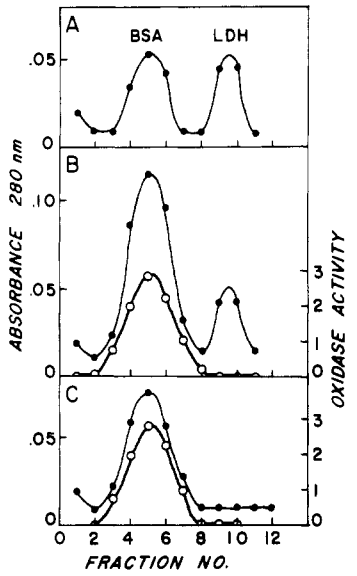

FIGURE 4: Zone sedimentation of the sulfhydryl oxidase and reference proteins in the ultracentrifuge. Conditions were as described under Experimental Procedures: (A) tube contained bovine serum albumin (BSA) and beef heart lactic dehydrogenase (LDH); (B) tube contained bovine serum albumin, lactic dehydrogenase, and the sulfhydryl oxidase; (C) tube contained the sulfhydryl oxidase. The three tubes were run in the same centrifuge run: absorbance (●); oxidase activity (enzyme units/fraction) (○); sedimentation from right to left.

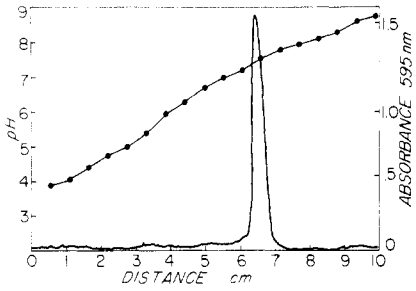

FIGURE 5: Isoelectric focusing of the sulfhydryl oxidase in a polyacrylamide gel. Conditions were described under Experimental Procedures: pH (●); absorbance (—).

data support the presence of a single FAD group per enzyme molecule.

The isoelectric point of the oxidase was found to be pH 7.45 after focusing in polyacylamide gels (Figure 5). The pH optimum of the enzyme is 7.0 using dithiothreitol as substrate at 35 °C in 0.02 M sodium phosphate. The activity drops sharply toward lower pH values, with only minimal activity detected below pH 5. The enzyme is active in the alkaline range at least to pH 10, though a substantial nonenzymatic oxygen uptake is evident at high pH. The oxidase activity is thermolabile, 50% of the initial activity being lost after 3.5 min of heating at 60 °C (protein concentration 1 mg/mL, pH 7.0, 20 mM $NaPO_4$).

Stoichiometry of the Reaction. After studying the sulfhydryl oxidase in crude extracts, Chang & Morton (1975) suggested the following formulation of the reaction catalyzed:

$$2R\text{–}SH + O_2 \rightarrow RSSR + H_2O_2$$

For stoichiometric measurements of sulfhydryl oxidation, it was necessary to include catalase in the reaction in order to destroy enzymatically generated H_2O_2, which otherwise can react nonenzymatically with thiols. Under these conditions, taking into account the effect of catalase on the apparent oxygen uptake, the expected ratio of thiol consumed to oxygen consumed (2:1) was obtained (Table II). By use of glutathione reductase it was possible to show that glutathione was quantitatively converted to glutathione disulfide in the presence of the sulfhydryl oxidase (Table III). Evidence for the formation of a disulfide product was also obtained with benzenethiol as substrate. The formation of diphenyl disulfide could be determined spectrophotometrically at 320 nm; in addition, characteristic precipitation of the sparsely soluble disulfide was observed as the reaction progressed (Aurback & Jakoby, 1962).

Table II: Stoichiometric Relationship between Oxygen Consumption and Thiol Disappearance[a]

expt	-SH consumed (μmol)	O_2 consumed (μmol)	μmol of SH/ μmol of O_2
1	0.891 ± 0.06	0.46 ± 0.03	1.93
2	1.21 ± 0.07	0.58 ± 0.01	2.09

[a] Oxygen uptake was measured in the presence of catalase using 2 mM dithiothreitol as substrate as described under Experimental Procedures. The reaction was terminated at desired points by the addition of trichloroacetic acid, and the thiol disappearance was quantitated by Ellman's reagent as described under Experimental Procedures. Oxygen uptake in the presence of catalase was exactly half that observed in its absence or in the presence of 10 mM azide. Accordingly, oxygen uptake values observed in the presence of catalase were multiplied by 2. The uncertainties represent the range of values in four separate determinations.

Table III: Stoichiometry of Disulfide Production[a]

initial glutathione/ mL (μmol)	glutathione consumed/ mL (μmol)	GSSG[b] formed/ mL (μmol)	GSSG/GSH
1.69	1.60	0.8	0.50
7.39	7.06	3.63	0.52
11.30	11.08	5.10	0.46

[a] Glutathione at the indicated concentration was incubated with the sulfhydryl oxidase, and the disappearance of thiol was compared to the appearance of oxidized glutathione by assay with glutathione reductase and NADPH as described under Experimental Procedures. [b] GSSG, oxidized glutathione; GSH, reduced glutathione.

Table IV: Stoichiometry of H_2O_2 Production[a]

time (min)	-SH consumed/ mL (μmol)	H_2O_2 formed/ mL (μmol)	H_2O_2 formed/ -SH consumed
2	0.78 ± 0.02	0.35 ± 0.02	0.45
4	1.37 ± 0.03	0.64 ± 0.04	0.47
8	2.38 ± 0.02	1.10 ± 0.02	0.46
16	3.89 ± 0.02	1.69 ± 0.02	0.43

[a] The sulfhydryl oxidase was incubated in the presence of 2 mM dithiothreitol, and at various intervals samples were removed for assay of thiol consumed and H_2O_2 produced as described under Experimental Procedures. The uncertainties represent the range of values found in three separate determinations.

Under the conditions described under Experimental Procedures, H_2O_2 production amounted to 85–90% of that expected for the quantity of thiol destroyed (Table IV). Attempts to demonstrate that oxygen was initially reduced in a one-electron process to generate the superoxide radical were not successful. Nitro blue tetrazolium may be reduced by certain flavoprotein oxidases such as xanthine oxidase both by oxygen-independent and oxygen-dependent pathways (Beauchamp & Fridovich, 1971). Reduction in the presence of oxygen is more rapid and is thought to proceed by way of superoxide, as the presence of superoxide dismutase sharply diminishes the rate of dye reduction (Beauchamp & Fridovich, 1971). Nitro blue tetrazolium (50 μM) was also reduced

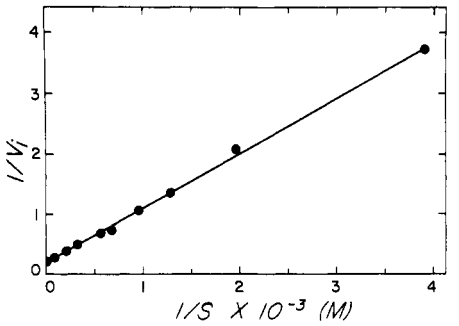

FIGURE 6: Variation of initial velocity of oxygen uptake with concentration of glutathione plotted according to Lineweaver and Burk. Velocity is expressed in terms of micromoles of O_2 consumed per minute.

Table V: Kinetic Constants for Various Substrates[a]

substrate	K_M (mM)	V_{max} (μmol of O_2 reduced per min per mg)	app mol act.[b]
glutathione	4.4	660	43 000
cysteine	9.1	1300	86 000
2-mercaptoethanol	32	600	39 000
dithiothreitol	0.7	940	62 000

[a] Reaction velocities were determined in the standard assay system using borohydride-reduced thiols. [b] Moles of product formed per minute per mole of enzyme, calculated from V_{max} values assuming 1 mol of enzyme is 66 000 g. Protein estimations may be inaccurate as they are referenced to bovine serum albumin.

rapidly under aerobic conditions by dithiothreitol in the presence of the thiol oxidase. However, the addition of purified superoxide dismutase (3.3 μg/mL) failed to diminish the rate of reduction. Addition of the same preparation of the dismutase did decrease significantly the photoinduced reduction of nitro blue tetrazolium in the presence of riboflavin, a known superoxide-dependent pathway (Beauchamp & Fridovich, 1971). Thus, if superoxide is generated by the reaction of the oxidase with oxygen, the radical species seems not to leave the enzyme surface.

Kinetic Properties of the Enzyme. We have not undertaken an extensive kinetic analysis of the enzyme, but we have examined the rates of the oxidation of a few substrates at the oxygen concentration in equilibrium with the atmosphere. A linear plot was obtained by the method of Lineweaver and Burk with reduced glutathione as substrate (Figure 6), and this is typical of the results obtained for several other thiols (summarized in Table V). Preliminary experiments indicated that high thiol concentrations (>20 mM) inhibited enzyme activity (Ostrowski et al., 1979b). However, it appears likely that this inhibition actually reflected disulfide contamination of the substrates. For example, oxidized glutathione at 1 mM inhibited the rate of oxidation of 10 mM glutathione by 50%. Pretreatment of substrates with sodium borohydride as described under Experimental Procedures eliminated such inhibition, and all kinetic data reflect assays done with borohydride-reduced substrates.

As a point of interest, 10 mM EDTA had no effect upon the rate of oxidation of dithiothreitol by the sulfhydryl oxidase.

Substrate Specificity. The sulfhydryl oxidase catalyzed the oxidation of all those low molecular weight thiols tested, including dithiothreitol, cysteine, glutathione, 2-mercaptoethanol, and benzenethiol. As will be shown below, reduced RNase A also appears to be a substrate for the enzyme. On the other hand, a variety of compounds, some of which are substrates for well-known flavoprotein oxidases, failed to stimulate oxygen uptake in the presence of the sulfhydryl oxidase at neutral pH. These included xanthine, NADH, NADPH, putrescine, benzylamine, D- and L-leucine, glucose, fructose, pyridoxal phosphate, and hypotaurine (seminal vesicle secretion is relatively rich in taurine; Kochakian & Marcais, 1974). Similarly, the oxidase tested negative for diaphorase activity with either NADH or NADPH in the presence of 2-methyl-1,4-naphthoquinone and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide. When NAD^+ or $NADP^+$ at 0.1 mM was added to standard assay mixtures at 25 °C, no reduction of the pyridine nucleotides was observed by monitoring for adsorbance increase at 340 nm.

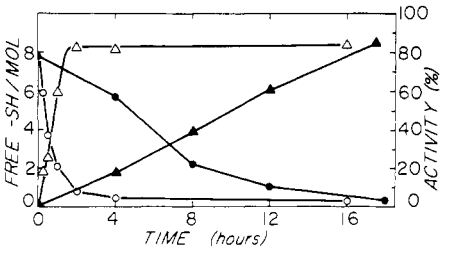

FIGURE 7: Reoxidation of reduced ribonuclease. Conditions were described under Experimental Procedures: free thiol groups in the absence (●) and presence (○) of the sulfhydryl oxidase; ribonuclease activity in the absence (▲) and presence (△) of the sulfhydryl oxidase. Activity is expressed as a percentage of that obtained from an equivalent amount of nondenatured RNase.

Reactivation of Reduced Ribonuclease. In order to determine whether the sulfhydryl oxidase would accept protein-bound cysteine residues as a substrate, we prepared fully reduced RNase (containing 7.8 reactive thiol groups per mol of enzyme) as described under Experimental Procedures. As is well-known (Haber & Anfinsen, 1962), RNase was capable of slow spontaneous reoxidation with recovery of better than 80% of its catalytic activity. This slow process could be dramatically shortened by the addition of an appropriate amount of the sulfhydryl oxidase (Figure 7). It is difficult to eliminate completely the possibility that trace amounts of 2-mercaptoethanol remained bound to RNase and that these molecules then performed a shuttle function to carry hydrogen equivalents from RNase to the sulfhydryl oxidase. However, in view of the rather high K_m of the oxidase for mercaptoethanol (Table V), the simplest interpretation of the results appears to be that the oxidase did act directly upon at least some of the cysteinyl residues of reduced RNase. Use of a higher ratio of oxidase to RNase than that employed in the experiment of Figure 7 led to a faster rate of disappearance of free thiol groups but to a lower recovery of RNase activity. For example, when a 100-fold higher concentration of sulfhydryl oxidase was present, free thiols were reduced to less than 1 per mol of RNase within 15 min, but the level of activity seen at that point (20%) failed to increase during the next 16 h (not shown).

In contrast to the results obtained with reduced ribonuclease, native bovine serum albumin, which has approximately one reactive cysteinyl residue (Peters, 1977), did not serve as a substrate for the oxidase. This was documented by the failure of the enzyme to alter the reactivity of albumin with Ellman's reagent and by the failure of the albumin to be converted to dimers as judged by its electrophoretic mobility in the presence of sodium dodecyl sulfate in the absence of reducing agents (not shown).

Discussion

The present study extends the characterization of the sulfhydryl oxidase from rat seminal vesicle secretion. The data

obtained indicate that it is a monomeric enzyme containing a single molecule of FAD as a prosthetic group and that it acts upon a variety of low molecular weight thiol compounds and probably also upon the cysteine residues of reduced ribonuclease. With the purified enzyme we have confirmed the reaction products and stoichiometry originally proposed by Chang & Morton (1975):

$$2RSH + O_2 \rightarrow RSSR + H_2O_2$$

A number of enzymes with sulfhydryl oxidase activity have been reported, but they remain a class of enzymes with a poorly understood physiological role. An azide-insensitive thiol oxidase activity was reported associated with fungal spores by Mandels (1956). Neufield et al. (1958) reported a fungal enzyme with specificity for the oxidation of sulfhydryl groups bonded to a carbon having sp^2 hybridization. Aurback & Jakoby (1962) later reported that this activity copurified with catechol oxidase and thus it may be an accidental property of the enzyme. No evidence was provided for the operation of a flavin in either of these fungal enzymes. More recently, Janolino & Swaisgood (1975) described the isolation of a nonflavoprotein, iron-containing sulfhydryl oxidase from bovine milk. This enzyme attacks low molecular weight thiols, such as glutathione, for which its apparent K_m is about 50-fold lower than that of the seminal vesicle oxidase. On the other hand, the specific activity of the milk oxidase is also nearly 50-fold lower with this substrate compared to that of the seminal vesicle enzyme. The milk sulfhydryl oxidase has been shown to catalyze the oxidative renaturation of ribonuclease (Janolino & Swaisgood, 1975) as well as chymotrypsinogen (Janolino et al., 1978).

In some instances established flavoproteins have been shown to have weak thiol oxidase activities that are apparently accidental and unrelated to the actual physiological function of the enzyme. For example, DeSa (1972) reported that a microbial putrescine oxidase showed weak oxidase activity with 2-mercaptoethanol, as well as other mono- and dithiols (DeSa, 1976). However, such activity is apparently dependent upon an alkaline pH. He has also shown that several flavoproteins, including glucose oxidase and L-amino acid oxidase, could be partially reduced to the semiquinone level by dithiols (DeSa, 1976). However, such reduction either does not occur or cannot form the basis for a full oxidation–reduction cycle at neutral pH. We have not observed that either enzyme will stimulate oxygen uptake in the presence of 10 mM dithiothreitol at pH 7 (M. C. Ostrowski and W. S. Kistler, unpublished experiments). Thiols have been reported to be oxidized by crude preparations of glucose oxidase (Young & Minno, 1972) and to lead to false positives in the use of such preparations for the identification of glucose (Kilburn & Taylor, 1969). We have confirmed such activity but found it to be azide sensitive and absent from more highly purified preparations of the enzyme (M. C. Ostrowski and W. S. Kistler, unpublished experiments). An additional microbial flavoprotein that seems to show weak thiol oxidase activity is a resorcinol hydroxylase from *Pseudomonas putida* (Ohta & Ribbons, 1976).

Free flavins can themselves catalyze thiol oxidation, but this requires alkaline conditions, presumably indicating a requirement for the thiolate anion (DeSa, 1976; Gascoigne & Radda, 1967). Under the conditions of the standard oxidase assay, free FAD (10 μM) showed no stimulation of oxygen consumption (M. C. Ostrowski and W. S. Kistler, unpublished experiments).

In view of the foregoing examples, it remains possible that the true physiological substrate for the seminal vesicle sulfhydryl oxidase is not a thiol and remains to be identified. It could even be that the form of the enzyme isolated from reproductive tract secretions has suffered proteolytic or other modifications, so that the substrate specificity of the enzyme has been altered. However, in view of its high activity at neutral pH against physiologically occurring thiols, such as glutathione, and because it can apparently act on protein-bound cysteine residues, we feel it is reasonable to consider the sulfhydryl oxidase activity a real rather than an adventitious capability of the enzyme. It should be pointed out on a number of grounds, for example, its flavoprotein nature, that the seminal vesicle oxidase is distinct from the glutathione oxidase activity recently reported to be associated with γ-glutamyl transpeptidase (Tate et al., 1979; Tate & Orlando, 1979) and from the disulfide exchange enzyme first reported by Goldberger et al. (1964).

The lack of demonstrable free superoxide as a product of the enzyme is typical for oxidases in contrast to the weak oxidase activity sometimes shown by dehydrogenases (Massey et al., 1969b). On the other hand, many flavoprotein oxidases react with sulfite to give a bleached spectrum (Massey et al., 1969a), but no such effect was seen with the seminal vesicle enzyme. It is interesting that the putrescine oxidase mentioned above shares this lack of reactivity with sulfite (DeSa, 1972).

The physiological utility of high sulfhydryl oxidase activity in the male reproductive tract and in seminal vesicle secretion in particular (Chang & Morton, 1975; Chang & Zirkin, 1978) is not immediately evident. The mechanism operating during the correct folding and oxidative generation of disulfide bonds in proteins remains incompletely understood (Freedman, 1979). In view of its effects upon reduced ribonuclease, the enzyme might serve to generate disulfide bonds in one or more of the major seminal vesicle secretory proteins (Ostrowski et al., 1979a), although little is known about the occurrence of such bonds in these entities. It is possible that the product of oxygen reduction, H_2O_2, has some utility as an antimicrobial agent in the various reproductive tract secretions. The prevention of microbial infection in such regions of stored energy sources opening to the urinary tract must be a serious one, but insufficient information is available to judge whether sufficient concentrations of low molecular weight thiol substrates would be available to provide significant H_2O_2 generation. It has been suggested that the oxidase serves to protect spermatozoa against the deliterious effect of low molecular weight thiols (Chang & Morton, 1975; Chang & Zirkin, 1978). Such effects indeed occur in the presence of high concentrations of thiols at alkaline pH values or in the presence of protein denaturants or detergents (Lung, 1972; Calvin & Bedford, 1971; Coelingh et al., 1969; Kistler et al., 1973; Calvin, 1976) but have uncertain relevance to physiological conditions. In fact, modest thiol concentrations are also known to have a protective effect upon sperm motility and metabolism under in vitro conditions (Morita & Chang, 1971; Mann, 1964). It is worth pointing out that seminal vesicle secretion is not in contact with stored spermatozoa and that any effect of the seminal vesicle enzyme directly upon sperm cells would be limited to the instant of ejaculation and the ensuing period in the female reproductive tract.

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