A Sulphydryl Oxidase from Chicken Egg White*

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A dimeric glycoprotein containing one FAD per ~80,000 M₀ subunit has been isolated from chicken egg white and found to have sulphydryl oxidase activity with a range of small molecular weight thiols. Dithiothreitol was the best substrate of those tested, with a turnover number of 1030/min, a K_m of 150 µM, and a pH optimum of about 7.5. Oxidation of thiol substrates generates hydrogen peroxide in aerobic solution. Anaerobically, the ferric en- ium ion is a facile alternative electron acceptor. Reduction of the oxidase with dithionite or dithiothreitol under anaerobic conditions yields a two-electron intermediate (EH₂) showing a charge transfer band (λ_max 560 nm; ε_max 2.5 mm⁻¹ cm⁻¹). Complete bleaching of the flavin and discharge of the charge transfer complex require a total of four electrons. Borohydride and catalytic photoreduction give the same spectral changes. EH₂, but not the oxidized enzyme, is inactivated by iodoacetamide with alkylation of 2.7 cysteine residues/subunit. These data indicate that the oxidase contains a redox-active disulfide bridge generating a thiolate to oxidized flavin charge transfer complex at the EH₂ level. Sulfite treatment does not form the expected flavin ad- duct with the native enzyme but cleaves the active site disulfide, yielding an air-stable EH₂-like species. The close functional resemblance of the oxidase to the pyri- dine nucleotide-dependent disulfide oxidoreductase family is discussed.

Sulphydryl oxidases catalyze the oxidation of sulphydryl groups to disulfides according to the following general reaction.

$$2RSH + O_2 \rightarrow RS-SR + H_2O_2$$

REACTION 1

They have been purified from a number of microbial and mammalian sources, but many questions concerning their mecha- nism and functional roles remain unresolved. Iron-dependent sulphydryl oxidases have been found in bovine milk (1–3), in kidney (4), and in pancreatic zymogen granules (5). Copper-containing sulphydryl oxidases have been described from kidney and small intestine (6) and skin (7, 8).

A second distinct class of sulphydryl oxidase is provided by FAD-dependent enzymes isolated from both rat seminal vesicles and fungal sources (9–11). These careful studies show a range of diverse subunit composition and substrate specificity but clearly implicate the flavin cofactor in catalysis (9, 10). The present work reports the discovery and characterization of a flavoprotein sulphydryl oxidase in chicken egg white.

Our study began with the observation that egg white con- tains not only riboflavin (in the form of riboflavin-binding pro- tein; Ref. 12) but also measurable levels of FAD (13). While the function of riboflavin-binding protein in the transport and stor- age of vitamin B₂ is well known (14), the reason for the presence of FAD in egg white was obscure. We first purified the FAD-containing protein by following its yellow color and sub- sequently identified it as a sulphydryl oxidase active with a wide range of thiol substrates. This paper presents strong evidence for the involvement of a catalytically important disul- fide as a second redox-active moiety in these flavin dependent sulphydryl oxidases. These enzymes therefore show marked functional similarities with the flavin disulfide oxidoreductase family of which glutathione reductase is a prototypical member (15).

EXPERIMENTAL PROCEDURES

Materials—Dithiothreitol, reduced glutathione, cysteine, β-mercap- toethanol, and high molecular weight SDS protein standards were from Sigma. Sodium dithionite was from the Virginia Smelting Company.

General Methods—Concentrations of egg white sulphydryl oxidase were reported with respect to enzyme-bound flavin using a molar extinction coefficient of 12.5 mm⁻¹ cm⁻¹ at 454 nm (see below). Visible and ultraviolet absorption spectra were obtained using Perkin-Elmer 552 or HP8452A instruments. Anaerobic procedures, using all-glass titration cuvettes, were as described earlier (16). The oxidase was photooxidized anaerobically from a 150-watt flood lamp using 1.2 µM 5-deazafla- vin and 5 mM EDTA. Maximal EH₂ formation occurred at 90 s, and full reduction required illumination for 6 min. Fluorescence measurements used a Turner model 111 fluorimeter or an Amico-Bowman Series 2 Luminescence Spectrometer. Unless otherwise stated, all buffers con- tained 0.3 mM EDTA. Enzyme samples were concentrated and washed using Amicon Centricon 30 ultrafiltration cells.

Assays—Sulphydryl oxidase was routinely assayed in a Clarke-type oxygen electrode (YSI 5331) and oxygen monitor (YSI 53) using 5 mM DTT⁺ in 3 M of 50 mM potassium phosphate equilibrated with air at pH 7.5, 25 °C. A background trace followed the nonenzymatic oxidation of DTT, and assays were started by the addition of enzyme. Thiol sub- strates were prepared fresh in distilled water and were standardized using DTNB. Concentrated solutions of glutathione were brought to pH 7 prior to standardization. The effect of small levels of disulfide con- tamination in these samples was evaluated using borohydride treat- ment as described earlier (9); no significant inhibition of the oxidase by disulfide was noted. Analysis of the stoichiometry of the reaction used 150 µM DTT in air-saturated buffer (50 mM phosphate buffer, pH 7.5, 25 °C, containing 240 µM dissolved oxygen). The addition of 90 mM oxidase led to a 140 µM decrease in the concentration of oxygen, and the addition of 20 µg of bovine liver catalase returned 72 µM oxygen to the solution. The following 100 mM buffers were used for measurement of the pH dependence: sodium acetate (pH 4–5.5), phosphate (pH 7–8 and 11), Tris chloride (pH 8–9), and CHES (pH 10).

Iodometric and spectrophotometric assays of the purified oxidase used 200 µM ferri- cernium ion and 200 µM DTT in 50 mM phosphate buffer, pH 6.0 containing 0.3 mM EDTA. The initial rates were monitored at 300 nm before the precipitation of ferrocene (17).

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1 The abbreviations used are: DTT, dithiothreitol; DTNB, 5,5'-dithio- bis(2-nitrobenzoic acid); EH₂ and EH₄, 2- and 4-electron-reduced en- zyme, respectively (without regard to protonation state); FPLC, fast protein liquid chromatography; CHES, 2-(cylohexylamino)ethanesul- fonic acid.
Egg White Sulfhydryl Oxidase—The rrdl strain of single comb White Leghorn chickens (riboflavin-binding protein deficient, see “Results”; Refs. 18 and 19) was maintained on the University of Delaware Farm, and 900 eggs were collected and stored at 4 °C. The egg whites were collected (17 liters), and an equal volume of 20 mM Tris buffer, pH 8.1, 4 °C was added. The mixture was homogenized by vigorous stirring with a motor-driven, Teflon-coated, ground glass homogenizer and the pH was adjusted to 8.1 with HCl. The suspension was centrifuged (9 min in a GS-1 rotor at 9000 rpm), and the clear supernatants were collected and combined while discarding the gelatinous off-white precipitates. This clarification was necessary for the success of the subsequent batch absorption step. Equilibrated DE52 (Whatman, equivalent to 180 g of DEAE-cellulose) was added to the supernatant, and the suspension was stirred gently for 30 min at 4 °C. The yellow ion exchange resin was allowed to settle, collected, and washed with 2 liters of 20 mM Tris, pH 8.1, in a funnel lined with four layers of cheesecloth. The addition of a second aliquot of resin to the supernatant yielded a paler yellow gel, suggesting that all of the flavin was not absorbed by the first batch treatment. However, inclusion of additional batches of gel adversely affects subsequent purification steps, so a single limiting amount of gel was used. The resin was then rinsed with 0.5 liters of 0.5 mM NaCl in 20 mM Tris buffer, pH 8.1, 4 °C, and the yellow eluate was collected and brought to 45% saturation in ammonium sulfate. The suspension was centrifuged (10,000 rpm, GSA rotor; 10 min), and the clear supernatants were collected and brought to 45% saturation in ammonium sulfate. The suspension was centrifuged (9 min in a GS-1 rotor at 9000 rpm), and the clear supernatants were collected and combined while discarding the gelatinous off-white precipitates. This clarification was necessary for the success of the subsequent batch absorption step. Equilibrated DE52 (Whatman, equivalent to 180 g of DEAE-cellulose) was added to the supernatant, and the suspension was stirred gently for 30 min at 4 °C. The yellow ion exchange resin was allowed to settle, collected, and washed with 2 liters of 20 mM Tris, pH 8.1, in a funnel lined with four layers of cheesecloth. The addition of a second aliquot of resin to the supernatant yielded a paler yellow gel, suggesting that all of the flavin was not absorbed by the first batch treatment. However, inclusion of additional batches of gel adversely affects subsequent purification steps, so a single limiting amount of gel was used. The resin was then rinsed with 0.5 liters of 0.5 mM NaCl in 20 mM Tris buffer, pH 8.1, 4 °C, and the yellow eluate was collected and brought to 45% saturation in ammonium sulfate. The suspension was centrifuged (10,000 rpm, GSA rotor; 10 min), and the clear yellow supernatant was brought to 55% ammonium sulfate. The yellow precipitate was recovered by centrifugation, redissolved in a minimum volume of 20 mM Tris buffer, pH 8.1, 4 °C, and diazylated versus the same buffer. The oxidase in 43% saturated ammonium sulfate was applied to an octyl-Sepharose column (1.5 × 15 cm). The yellow band absorbed to the top of the column was washed at 1 ml/min with 250 ml of 20 mM Tris buffer containing 43% ammonium sulfate. Sulfhydryl oxidase was eluted with a decreasing salt gradient formed by the addition of 20 mM Tris buffer to a sealed flask containing 100 ml of the 43% ammonium sulfate solution. The enzyme eluted in a narrow band, and fractions with a 280/454 nm absorbance ratio of less than 12–15 were combined, diazylated versus 20 mM Tris buffer, and concentrated by ultrafiltration. The enzyme was applied in batches to a Superose 12 HR 10/30 FPLC column equilibrated with 75 mM Tris buffer, pH 7.5, 25 °C, and fractions with absorbance ratios of less than 10.5 and between 10.5 to 12 were combined separately. Enzyme was concentrated by ultrafiltration and stored frozen in 20 mM Tris buffer, pH 8.1, 4 °C, containing 0.3 mM EDTA.

Identification of FAD in Egg White Sulfhydryl Oxidase—Samples of the oxidase in 20 mM Tris buffer, pH 8.1, were denatured either by boiling for 5 min in foil-covered plastic centrifuge tubes or using 58% MeOH. The precipitate was removed by centrifugation, and portions of the supernatant were diluted in water or 10 mM HCl for measurement of flavin fluorescence (20). Solutions of FAD, purified as in Massey and Swoboda (21), and FMN served as controls. The fluorescence of the released flavin increased 4.3-fold in 10 mM HCl, suggesting that sulfhydryl oxidase contained FAD (20). High pressure liquid chromatography of the released flavin on a C18 reverse-phase semipreparative column used a gradient of methanol and 25 mM potassium phosphate, pH 5.3, developed at 2 ml/min. 3 min at 10% methanol; 15-min linear gradient to 60% methanol; 5 min at 60% methanol; 5 min to 10% methanol. In this system, authentic FAD and the flavin released upon denaturation of the oxidase coelute.

Amino Acid Analysis—Samples of untreated and alkylated oxidase were desalted by ultrafiltration against distilled water, and 2 nmol aliquots were hydrolyzed in 6 M HCl for 24 h at 110 °C in the presence of 5% phenol. Portions were analyzed with a Beckman 6300 Automaticalyzer equipped with a Sica 7000 integrator (22). For the measurement of cysteine plus half-cystine content, the oxidase (2 μM) was denatured in 5.9 M guanidinium hydrochloride, incubated with 1 mM DTT for 2 h, and alkylated with 4 mM iodoacetamide for 3 h. Excess reagents were removed by dialysis against two changes of distilled water, and the alkylated protein was lyophilized prior to amino acid analysis. Tryptophan content was estimated spectrophotometrically using the procedure of Edelhoch (23, 24). The oxidase was denatured in 6 M guanidinium hydrochloride, and the resulting visible spectrum was compared with an equivalent amount of free FAD in denaturant (24). The difference spectrum, representing the contribution of the apoprotein, gave 16 tryptophan residues/subunit.

Carbohydrate Analysis—The oxidase was desalted as described above and analyzed for monosaccharide content by Dr. Jeffrey Rohrer, Dionex, Sunnyvale, CA. Samples (25 μg of protein) were hydrolyzed using trifluoroacetic acid for monosaccharides (25) or HCl for saccharic acid (26), and the hydrolysates were analyzed in duplicate using high pH anion exchange chromatography using a DX-500 BioLC chromatograph (Dionex, Sunnyvale, CA) equipped with an AS500 autosampler (Thermo Separation Products, San Jose, CA) with pulsed amperometric detection.

Characterization of the Oxidase—The apparent subunit molecular weight of the oxidase was determined using 10% SDS-polyacrylamide gels with a 4% stacking gel. Gels were stained with Coomassie Blue and scanned with a Bio-Rad Gel Doc 1000. Molecular weight standards were bovine serum albumin, Escherichia coli β-galactosidase, bovine liver glutamate dehydrogenase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, chicken egg ovalbumin, phosphorylase b, phosphofructokinase, and myosin from rabbit muscle.

Size exclusion chromatography of the native oxidase was performed in 100 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl at 20 °C using a Superose 12 HR 10/30 FPLC column. The apparent native molecular weight was deduced in comparison with the following standards: aldolase, foraminifera, medium chain acyl-CoA dehydrogenase, bovine (γ-globulin, chicken ovalbumin, bovine serum albumin, and thyroglobulin. A sample of the purified oxidase was applied to Immobilon-P from Millipore and shown to be a glycoprotein with the periodic acid-Schiff reaction (27).

RESULTS

Purification of the Oxidase—The egg white sulfhydryl oxidase was purified to homogeneity following the yellow color of the flavoprotein because its catalytic identity was initially unknown (see above). However, the large amount of yellow riboflavin-binding protein present in the eggs from normal chickens complicated this approach. Hence, we used egg white from a strain of chickens that lack a functional riboflavin-binding protein and therefore do not accumulate significant riboflavin in their eggs (13, 18, 19). This greatly simplified the early phase of this project. For consistency, we have continued to use the mutant egg white as a source of sulfhydryl oxidase, although egg white from normal chickens appears to contain comparable sulfhydryl oxidase activity (using dithiothreitol as a substrate; see “Experimental Procedures”; not shown).

The purification procedure is described under “Experimental Procedures.” The key step is the initial batch absorption of the oxidase using small amounts of anion exchange cellulose added to a large volume of diluted egg white (see “Experimental Procedures”). This step results in a dramatic initial purification (an approximately 1000-fold decrease in protein; Table 1). Difficulties in assaying the oxidase in unfraccionated egg white

| Step                        | Volume | Activity |
|-----------------------------|--------|----------|
| Diluted egg white           | 34.000 | —        |
| DE-52 batch absorption      | 245    | 106      |
| Ammonium sulfate precipitation | 17     | 450      |
| Octyl-Sepharose             | 47     | 64       |
| Gel filtration              | 2.6    | 16.6     |

— Cannot be reliably quantitated in egg white because of light scattering and difficulties in measuring activity in crude egg white. The FAD content of undiluted egg white is approximately 0.1 μM (Ref 13 and Footnote 3), corresponding to about 21 A454 units, assuming an extinction coefficient of 12.5 molar−1 cm−1 for the bound flavin.

| Purification of sulphydryl oxidase from chicken egg white | ml | units | units | ratio | units |
|----------------------------------------------------------|----|-------|-------|-------|-------|
| De-52 batch absorption                                   | 245| 6950  | 20    | 346   | 532   |
| Ammonium sulfate precipitation                          | 17 | 450   | 9.3   | 48    | 357   |
| Octyl-Sepharose                                          | 47 | 64    | 5.16  | 11.6  | 243   |
| Gel filtration                                           | 2.6| 16.6  | 1.6   | 10.3  | 79    |
and in the spectrophotometric measurement of flavin absorbance in this turbid solution preclude relevant entries in Table I. Ammonium sulfate fractionation of the absorbed proteins, followed by hydrophobic interaction and gel filtration steps, yields the pure oxidase. The enzyme is stable for months when stored at −20 °C.

Physical Characterization of the Oxidase—The oxidase exhibits a typical unresolved flavin spectrum with an absorbance maximum at 454 nm and a second peak at 364 nm (Fig. 1). The flavin was released from the protein by denaturation using methanol or boiling (see “Experimental Procedures”) and found to be FAD by its fluorescence and chromatographic properties (see “Experimental Procedures”). The extinction coefficient of this noncovalently bound FAD was 12.5 mM−1 cm−1 (by the guanidine hydrochloride method; Ref. 28). Egg white sulfhydryl oxidase shows less than 1% of the fluorescence of an equivalent concentration of FAD (pH 7.5, 25 °C) exciting at 450 nm.

SDS-polyacrylamide gel electrophoresis (10%) of the pure oxidase revealed a single, rather diffuse, band with an apparent molecular weight of ~93,000 (see “Experimental Procedures”). The enzyme was found to be a glycoprotein using the periodate–Schiff reagent (see “Experimental Procedures”) and by carbohydrate analysis (see Table II). The minimum molecular weight per FAD was calculated to be 67,000 based on amino acid content and about 80,000 including carbohydrate (Table II). This latter value is consistent with the apparent molecular weight of 93,000 observed on SDS-polyacrylamide gel electrophoresis, since glycoproteins typically run more slowly than expected. Gel filtration of the native sulfhydryl oxidase (see “General Methods”) gave an apparent molecular weight of 202,000. These data are consistent with a homodimeric structure for the oxidase with one molecule of FAD per subunit.

The native oxidase does not react significantly with DTNB at pH 7.5 (see “Experimental Procedures”). Similarly, the oxidized enzyme incubated with 1 mM iodoacetamide for 24 h under nondenaturing conditions at pH 7.5 showed only 0.3 molecules of S-carboxymethylcysteine/subunit. When the enzyme was denatured with 5.8 M guanidine hydrochloride before the addition of DTNB, 1.05 thiol residues/subunit were exposed. Essentially the same result (0.9 thiols) was obtained denaturing the enzyme first under anaerobic conditions to ensure that oxidation of free thiols by any enzyme that had yet to be inactivated had not occurred. Exhaustive reductive alkylation (see “Experimental Procedures”) gave a total of 5.4 molecules of S-carboxymethylcysteine/subunit. These data are consistent with a subunit containing two disulfide bridges and one free sulfhydryl group that is unreactive in the native oxidized enzyme (see below).

Catalytic Activity—Like the other sulfhydryl oxidases (9–11) the consumption of oxygen could be conveniently monitored in the oxygen electrode using air-saturated buffers at pH 7.5. The addition of limiting amounts of the dithiol DTT (to a final concentration of 150 μM) caused the uptake of 140 μM oxygen. Discharge of the hydrogen peroxide upon the addition of catalase led to the reappearance of 72 μM oxygen (not shown; see “Experimental Procedures”) in agreement with the expected stoichiometry for the sulfhydryl oxidases (2). Table III summarizes the turnover numbers and Km values for a series of common thiol reagents determined at pH 7.5. Despite a wide range of Km values (from 0.15 mM for DTT to 54 mM for β-mercaptoethanol), the maximal turnover numbers are rather similar. Further work will determine whether this reflects a common rate-limiting step after the initial reduction of the enzyme by thiol reagents (see below). When assay traces are recorded using a large excess of thiol over dissolved oxygen, e.g., 5 mM GSH or DTN (compared to 0.24 mM oxygen), almost linear rates are obtained for 90% of the trace. The Km for oxygen is thus less than 20 μM and cannot be reliably determined with these experiments.

The apparent pH dependence of the oxidation of dithiothreitol by the egg white oxidase is shown in Fig. 2. Deprotonation of a group with an apparent pK of 5.2 leads to an increase in activity. A small loss of activity at high pH with an apparent pK of 9.3 is observed (Fig. 2). Consequently, the oxidase is active over a rather broad pH range (greater than 75% of maximal activity is observed from pH values of 6–10).

The kcat/Km for oxygen with these thiol substrates is greater than 105 M−1 s−1 (assuming a Km of less than 20 μM), so there appears to be little doubt that oxygen is the physiological electron acceptor of this enzyme. However, we have found that sulfhydryl oxidase, like glucose oxidase (29) and several other flavoenzymes (17, 30, 31), can utilize the ferricenium ion as an alternate oxidant even in air-saturated solutions. Assays of sulfhydryl oxidase were performed spectrophotometrically at pH 6 (see “Experimental Procedures”) to minimize the nonenzymatic oxidation of dithiothreitol by the ferricenium ion.

\[
2\text{RS} + 2\text{Fc}^+ \rightarrow \text{RS-SR} + 2\text{Fc} + 2\text{H}^+
\]

**Fig. 1. Visible and UV spectrum of egg white sulfhydryl oxidase.** The spectrum was recorded in 100 mM phosphate buffer, pH 7.5, 25 °C containing 0.3 mM EDTA.

**Table II**

| Amino acid | Residue/FAD | Carbohydrate | Residue/FAD |
|------------|-------------|--------------|-------------|
| Aspartic acid | 37          | Fucose       | 1           |
| Threonine  | 31          | Galactose    | 11          |
| Serine     | 37          | GlN          | 4           |
| Glutamic acid | 78       | Gln          | 21          |
| Proline    | 42          | Mannose      | 7           |
| Glycine    | 34          | Sialic acid  | 15          |
| Alanine    | 65          |              |             |
| Cysteine (SCMC) | 5     |              |             |
| Valine     | 32          |              |             |
| Methionine | 7           |              |             |
| Isoleucine | 26          |              |             |
| Leucine    | 67          |              |             |
| Tyrosine   | 18          |              |             |
| Phenylalanine | 27     |              |             |
| Lysine     | 18          |              |             |
| Histidine  | 16          |              |             |
| Arginine   | 38          |              |             |
| Tryptophan | 16          |              |             |
The activity of the egg white sulfhydryl oxidase is essentially unchanged after a 20-h incubation with 1 mM iodoacetamide or iodoacetic acid (104 and 103% of control levels, respectively; see "Experimental Procedures"). Further, only about 0.2 thiols remained when reduced with sodium dithionite.

Fig. 2. pH dependence of the activity of sulfhydryl oxidase toward DTT. The activity of egg white sulfhydryl oxidase was measured in the oxygen electrode using 10 mM DTT in air-saturated buffers (see "Experimental Procedures"). The curve is the nonlinear least squares fit with limiting values for the turnover at low, intermediate, and high pH values of 0, 1050, and 740/min respectively and pK values of 5.2 and 9.3.

At a concentration of 200 μM DTT and 200 μM ferricenium ion, respectively, 540 mol of this one-electron oxidant were reduced per minute at 25 °C. Under anaerobic conditions, the turnover number increased to 760/min. Thus, the ferricenium assay provides a simple alternative to the oxygen electrode assay for the pure oxidase.

Reductive Titrations of Sulfhydryl Oxidase—Fig. 3 shows a dithionite titration of the egg white oxidase under anaerobic conditions at pH 7.5. Complete reduction of the enzyme flavin requires a total of 4.6 electrons (curve 1 to curve 5; see inset). The long wavelength band observed at intermediate dithionite additions (maximal at 2.3 electrons) shows an absorbance maximum at about 560 nm without the fine structure expected for a flavin blue radical (32). Rather, this species resembles the thiolate to flavin charge transfer complexes observed in the flavin disulfide oxidoreductase family (EH2, Scheme 1; Ref. 15). Further evidence to support the involvement of a redox-active disulfide in the egg white oxidase will be presented later. The fully reduced oxidase (curve 5) is rapidly reoxidized in the presence of air. Reduction of the enzyme after each aliquot of DTT (4.4 electrons; see inset) appeared at the same position as that generated by sodium dithionite or borohydride. Early spectra in the titration show an isosbestic point at 505 nm, corresponding to the conversion of oxidized enzyme to the EH2 state before the fully reduced form of the enzyme begins to accumulate. Full reduction required 2.2 equivalents of the two-electron donor, DTT (4.4 electrons; see inset).

Reduced glutathione effects essentially identical spectral changes (not shown), but an approximately 7-fold molar excess is required to reach maximal charge transfer complex formation, and about 100 mol of GSH are required for complete conversion to the EH2 state (Fig. 5). These differences presumably reflect the fact that GSH is a thermodynamically weaker reductant than DTT (−0.24 versus −0.31 V; Ref. 34).

Reactivity of Sulfhydryl Oxidase with Sulfite—Flavoprotein oxidases usually react with sulfite to generate N-5 flavin adducts in a reaction that leads to substantial bleaching of the flavin chromophore at 450 nm (35, 36). The egg white oxidase provides an interesting variation to this generalization (Fig. 6). Sulfite addition at pH 7.5 under aerobic conditions leads to the appearance of a long wavelength species very similar, but of weaker intensity, to that encountered in the reductive titrations of Figs. 3–5. Difference spectra show that the position of the long wavelength maximum is essentially the same between reductive and sulfite titrations (not shown). An important difference, however, is that the spectral changes induced by sulfite are stable in air. Thus, the enzyme was held on ice for 72 h after the completion of the titration shown in Fig. 6 without a significant decline in the long wavelength band (not shown). Data from all these spectrophotometric titrations (Figs. 3–6) are consistent with involvement of a redox-active disulfide in the catalysis by the oxidase (see below).

Further Evidence for a Catalytically Important Redox-active Disulfide—The activity of the egg white oxidase is essentially unchanged after a 20-h incubation with 1 mM iodoacetamide or iodoacetic acid (104 and 103% of control levels, respectively; see "Experimental Procedures"). Further, only about 0.2 thiols react in a 20-min incubation of the native oxidase with 0.5 mM DTNB in phosphate buffer, pH 7.5 (not shown). However, the oxidase became sensitive to alkylation with either iodoacetamide or iodoacetate when reduced with sodium dithionite.

The same long wavelength intermediate is observed during anaerobic titration of the oxidase with thiol reagents. Fig. 4 shows a titration with reduced dithiothreitol, and the inset shows the absorbance changes at 454 and 560 nm with equivalents of DTT added. The long wavelength band generated (maximal at 1.05 mol of DTT/mol of FAD) are omitted for clarity. The same long wavelength intermediate is observed during the catalysis by the oxidase (see below).

FIG. 3. Dithionite titration of sulfhydryl oxidase. The oxidase (13 μM in 100 mM phosphate buffer, pH 7.5; curve 1) was titrated anaerobically with 0.61, 1.13, 1.68, and 2.47 equivalents of sodium dithionite/mol of flavin (curves 2–5, respectively). Intermediate spectra are omitted for clarity. The inset plots absorbance changes at 560 and 452 nm (open and closed circles, respectively).

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560-nm-long wavelength band observed previously, but the intermediate decayed to an oxidized flavin spectrum after about 20 min. The reversion to an oxidized flavin spectrum is responsible for the lag phase seen in the inset (Fig. 7).

Curve 2 is after the addition of 2.4 electrons. Subsequent reduction of the flavin (curves 3–6) proceeds without the accumulation of significant semiquinone or long wavelength species (Fig. 7). After incubation of the oxidase for 12 h, the enzyme was allowed to reoxidize in air and assayed in the oxygen electrode. The iodoacetamide-treated reduced enzyme showed 2.3% of control activity. Comparable experiments with 1 mM iodoacetate gave analogous spectral changes with a residual activity of 3.5% (data not shown). Alkylation effects very small changes in the visible spectrum of the oxidase (not shown). There is a 1–2-nm blue shift in the main absorbance peak and a slightly increased absorbance below 400 nm for the iodoacetate-modified protein. In contrast, iodoacetamide-treated enzyme shows an almost undetectable blue shift in the 454-nm absorbance envelope. Neither of the carboxymethylated proteins regained significant activity after the removal of excess reagents by ultrafiltration.

Amino acid analysis of the oxidase alkylated with iodoacetamide in the absence of reduction shows essentially no S-carboxymethyl cysteine. Reduction as in Fig. 7 gave approximately 2.7 residues of S-carboxymethylcysteine/subunit of the oxidase.

**DISCUSSION**

Table IV summarizes properties of flavoprotein sulfhydryl oxidases purified to date. All contain FAD and are either monomeric or dimeric proteins. The enzymes show a range of specificities from those with a pronounced preference for glutathione (e.g. the *Penicillium* enzyme; Ref. 11) to those active with a comparatively broad range of substrates. All of the oxidases except the *Penicillium* enzyme significantly accelerate the reoxidation of reduced ribonuclease (Refs. 9 and 10; see below).

The data in this paper strongly suggest that the egg white sulfhydryl oxidase contains a redox-active disulfide bridge functionally equivalent to those found in the flavin disulfide...
with precedents set earlier (15, 38).

We arbitrarily depict the lower sulfur atom in Scheme I as reactive with external nucleophiles such as reduced glutathione or sulfite (see below). The upper thiol communicates primarily with the flavin prosthetic group and thus forms the charge transfer complex seen in Fig. 5.

The egg white oxidase gave pronounced long wavelength bands at the midpoint of reductive titrations for all reagents used (e.g. Fig. 3–5). Weak long wavelength bands were observed with both the rat seminal oxidase reduced with excess (10 mM) β-mercaptoethanol and the A. niger enzyme treated with 20 mM GSH. Similar substantially bleached flavin spectra with a small residual long wavelength (EH2) component were observed toward the completion of DTT and GSH titrations of the egg white enzyme (not shown). There can be little doubt that all of these enzymes show thiolate to flavin charge transfer complexes at the EH2 level (Scheme I, dotted lines) by analogy with precedents set earlier (15, 38).

The alkyl data of Table II are consistent with the alkylolation of both redox-active cysteine residues in EH2 as observed with thioredoxin reductase (Scheme I; Refs. 15, 39). Comparable experiments with lipoamide dehydrogenase, glutathione reductase, and mercuric reductase show monoalkylation of the N-terminal redox-active cysteine residue (40–42). Presumably in these cases, the charge transfer thiol is unreactive because it is inaccessible to relatively bulky and polar alkylating reagents (15).

The behavior of the egg white oxidase toward sulfite is unusual, although sulfitolysis of disulfides is well known (43, 44). The weaker intensity of the charge transfer band observed in Fig. 6 compared with those seen with dithionite or DTT might reflect a change in the environment and/or pK of the upper thiol in Scheme I accompanying polar modification of the lower thiol. Alternatively, the reaction with sulfite might occur at either upper or lower thiols (of which only the lower is shown in Scheme I). The failure of lipoamide dehydrogenase and glutathione and thioredoxin reductases to yield comparable adducts (35) might reflect their lower redox potential (and hence a greater resistance of their active site disulfides to sulftolysis). The A. niger oxidase gives a partial bleaching of the enzyme flavin, consistent with formation of a sulfite adduct of the flavin (10, 35). In contrast, the rat seminal enzyme was reported not to react with sulfite (9). The reasons for the differences in behavior between the flavoprotein sulfhydryl oxidases are not yet understood.

The marked similarities in behavior between the sulfhydryl oxidase and the pyridine nucleotide flavin disulfide oxidoreductases could reflect a common evolutionary origin for these enzymes (15). If the oxidase has evolved from this disulfide oxidoreductase family, it has not retained a functional pyridine nucleotide binding site; the enzyme shows no detectable spectral changes when mixed anaerobically with either 100 μM NADH or NADPH (see “Experimental Procedures”; data not shown). Similarly, the flavoprotein oxidases from seminal vesicles and Aspergillus fail to communicate with pyridine nucleotides (9, 10). In contrast, a possible convergent solution to the problem of efficient communication between exogenous thiols and flavin might have involved recruitment of a suitably placed disulfide bridge from a preexisting flavoprotein oxidase family of which glucose oxidase and alcohol oxidase are members (45). Divergent and convergent mechanisms might both participate in the evolution of the sulfhydryl oxidases.

A number of reports have suggested a role for sulfhydryl oxidases in protein disulfide bond formation, and an association of the enzyme with tissues active in protein secretion has been noted (2, 5, 9, 10, 46). Both the Aspergillus and the seminal enzyme were found to catalyze the reoxidation of reduced ribonuclease at apparently modest rates. The Penicil-
Egg White Sulfhydryl Oxidase

Sulfhydryl oxidase was reported to be unable to catalyze protein disulfide bond formation (11). Preliminary experiments with the egg white oxidase suggest that it effects rapid disulfide bond formation in reduced ribonuclease. Since egg white has an aggregate disulfide concentration of about 10–15 mM, a role for sulfhydryl oxidase in disulfide bond formation is plausible. Egg white proteins are synthesized directly in the ovipositor and it will be interesting to see whether these secretory cells themselves contain high levels of sulfhydryl oxidase activity. It should be noted that several other pathways for the net synthesis of disulfide bridges in proteins have been suggested including a microsomal hydroxylase (47) and a route involving the preferential transport of oxidized glutathione into the endoplasmic reticulum (48). Since egg white is a self-contained package of secreted proteins, it may prove useful in the study of the synthesis and isomerization of protein disulfides. In conclusion, the egg white oxidase provides a valuable complement to earlier studies on the seminal and microbial sulfhydryl oxidases. Further work will explore the mechanism, structure, and physiological role of these interesting but understudied enzymes.

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