Aspergillus niger Sulfhydryl Oxidase†

Rebecca S. de la Motte and Fred W. Wagner*
Department of Biochemistry, University of Nebraska—Lincoln, Lincoln, Nebraska 68583-0718
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ABSTRACT: A procedure for the isolation of a sulfhydryl oxidase from an *Aspergillus niger* cell suspension involved three major steps and yielded enzyme preparations exhibiting a single but diffuse protein-containing zone when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with a subunit molecular weight estimated to be 53 000. Sedimentation equilibrium experiments indicated a native molecular weight of 106 000. Analyses for sugar residues showed that the enzyme is a glycoprotein, containing 20.3% neutral hexose and 1.9% aminohexose by weight. This enzyme catalyzed the conversion of reduced glutathione (GSH) to its disulfide form, with concomitant consumption of O_2 and release of H_2O_2. The ratio of GSH consumed to H_2O_2 produced was determined to be 2:1. At 25 °C, the optimum pH for the oxidation of GSH was 5.5. Under these conditions, the enzyme had a Michaelis constant of 0.3 mM for GSH. Other low molecular weight thiol compounds (cysteine, dithiothreitol, and 2-mercaptoethanol) were also oxidized, but the Michaelis constants for these substrates were substantially higher than that for GSH under identical conditions of temperature and pH. The rate of reactivation of reductively denatured ribonuclease A was enhanced by the presence of sulfhydryl oxidase, indicating that the latter is capable of oxidizing protein-associated thiol groups. The UV–visible spectrum of sulfhydryl oxidase solution had absorbance maxima at 274, 364.5, and 442.5 nm and was otherwise characteristic of the spectra of known flavoproteins. Addition of GSH to the enzyme solution at pH 5.5 caused bleaching of the visible band, although bleaching was not observed when the same concentration of GSH was combined with free flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) under the same conditions. The enzyme reacted with sodium sulfite to form a bleached complex, with a dissociation constant of 3.0×10^{-3} M under the conditions employed. The flavin component could be separated from the protein by boiling for 5 min and had the same fluorometric properties as similarly treated authentic FAD. The apoenzyme, prepared by treatment with 5 M guanidine hydrochloride, could be reconstituted to about 60% by incubation with excess FAD but regained no activity when incubated with excess FMN. Stoichiometric measurements showed that the enzyme preparation contained approximately 1.7 mol of flavin/mol of protein.

Oxidases capable of catalyzing the de novo synthesis of disulfide bonds have been reported regularly for 30 years, but it is only in the last 10 years that data pertaining to their physiological roles have emerged. Recent studies indicate that such enzymes may be involved in the interconversion of xanthine dehydrogenase and xanthine oxidase (Clare et al., 1981), the posttranslational modification of immunoglobulin M (Roth & Koshland, 1980), and the maintenance of galactose oxidase in its active form (Olsen, 1975). Despite the implications these studies have for the importance of sulfhydryl oxidases in the metabolic management of thiols, few researchers have undertaken to characterize such enzymes completely.

In 1972, Young and Nimmo reported that commercially available *Aspergillus niger* glucose oxidase preparations possessed the ability to oxidize reduced glutathione (GSH)[1] under conditions resulting in quantifiable consumption of molecular oxygen and production of hydrogen peroxide. This activity exhibited Michaelis–Menten kinetic properties, was not affected by dialysis, and could be destroyed by heating at 90 °C for 10 min. Since the ratio of the rate of oxygen consumption in the presence of saturating glucose to that in the presence of saturating GSH increased with the purity of the glucose oxidase in tested preparations, these researchers postulated that an enzyme contaminant was responsible for the observed GSH oxidizing capability. Preliminary work in our laboratory with *A. niger* cell extract confirmed this postulate and indicated that the enzyme was a flavin oxidase which could be prepared in reasonable yield from the crude material.

To our knowledge, an attempt has not been made to study a flavin-dependent sulfhydryl oxidase with the objective of elucidating its catalytic mechanism. Most flavin oxidases derive two reducing equivalents from one substrate molecule, whereas a sulfhydryl oxidase requires two reducing substrate molecules. This offers the researcher the ability to control the extent to which a catalytic event achieves completion to a greater degree than is possible with other flavin oxidases. Thus, it seems likely that a mechanistic study of a flavin-dependent sulfhydryl oxidase could provide some unique insights into the general nature of flavin-mediated oxidation.

For these reasons, we have undertaken to purify and characterize the sulfhydryl oxidase from *A. niger*. In this paper, we describe a procedure for the purification of the enzyme from *A. niger* cells. We also show that the enzyme is a dimeric flavin oxidase which binds one molecule of FAD

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* Address correspondence to this author at the Department of Biochemistry, University of Nebraska—Lincoln, Lincoln, NE 68583-0718.

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GSH, reduced glutathione; GSSG, glutathione disulfide; NADH, nicotinamide adenine dinucleotide (reduced); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); SDS, sodium dodecyl sulfate; SOX, sulfhydryl oxidase; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid.

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per protein subunit and catalyzes the conversion of thiol compounds to their corresponding disulfides according to the equation:

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

where R–SH may be either a thiol compound of low molecular weight or a protein. Further, we present the results of our investigations of some physical and chemical properties of the enzyme.

Experimental Procedures

Purification. A. niger mycelia[2] were received as a viscous suspension in 20% (w/v) NaCl. Cells were separated from the brine by vacuum filtration through Miracloth. This procedure yielded 850–950 mL of brine per liter of cell suspension. Various attempts to disrupt the recovered cells revealed that most of the sulfhydryl oxidase activity had been liberated into the brine prior to its separation from the cells, so the enzyme was purified from the yellow-brown brine extract, and the cells were discarded after filtration.

Proteins were separated from the brine filtrate by precipitation with methanol. In a typical precipitation, 270 mL of brine filtrate at 0 °C was mixed with 180 mL (40% v/v) of technical-grade methanol at −20 °C. This mixture was allowed to stand in an ice bath for 30 min and then was centrifuged for 20 min at 16300*g*. The pellets were discarded, and the supernatant fluid was combined with 450 mL (total 70% v/v) of methanol at −20 °C. The resulting suspension was allowed to stand for 10 min in an ice bath and then was centrifuged for 15 min at 16300*g*. The yellow supernatant fluid was discarded, and the centrifuge bottles were shaken and inverted to remove as much residual fluid as possible. A total of 80 mL of 40 mM sodium acetate, pH 5.5, was added to the centrifuge bottles, and the pellets were gently disrupted and allowed to soak in the buffer overnight at 4 °C. Undissolved material was then dispersed into the liquor, and the suspension was centrifuged again for 10 min at 27000*g*. The supernatant fluid was collected, and solid ammonium sulfate was added to 85% saturation at 25 °C. The suspension thus obtained could be stored at 4 °C for at least a month without any significant loss of sulfhydryl oxidase activity.

For further purification, six batches of the ammonium sulfate precipitated enzyme were combined and centrifuged at 4 °C for 15 min at 16300*g*. The sedimented material was dissolved in 10 mM sodium citrate, pH 4.0, so that the total volume of the solution did not exceed 40 mL. This solution was desalted by passage over a 2.5 × 35 cm Sephadex G-25 column equilibrated and eluted with 10 mM sodium citrate buffer, pH 4.0. The protein-containing peak was collected, and to it was added sufficient acetone at −20 °C to make the solution 30% (v/v) in acetone. This mixture was allowed to stand on ice for 15 min and then was centrifuged for 15 min at 27000*g*. The dark brown pellet was discarded, and the yellow supernatant fluid was passed in its entirety over a hydroxylapatite (Bio-Rad DNA grade Bio-Gel HTP) bed packed in a 50-mL syringe barrel plugged at the bottom with glass wool. All colored material in the acetone supernatant bound to the hydroxylapatite. The column was washed successively with approximately 2 volumes of each of the following: 30% acetone/70% 10 mM sodium citrate buffer, pH 4.0; 25% acetone/75% buffer; and 20% acetone/80% buffer. Finally, the hydroxylapatite was washed with 15% acetone/85% buffer until the eluate was colorless. All solvents were prepared by combination of degassed buffer with acetone which had been vacuum filtered through a Millipore GVWP membrane and were allowed to stand at 4 °C for several hours prior to chromatography. The hydroxylapatite was suspended in the acetone-free buffer, and the suspension was degassed before the material was packed in the syringe barrel. The column was equilibrated in the 30% acetone/70% buffer mixture immediately before the sample was applied. These measures were required to prevent the formation of gas bubbles in the column during loading and elution. All operations were performed at 4 °C. Flow rates were not greater than 1 mL/min during any step in the chromatography.

Most of the sulfhydryl oxidase activity was resident in the material eluted with the last solvent. This bright yellow solution was concentrated to about 40 mL by pressure filtration in a stirred cell over an Amicon YM-10 membrane, if necessary, and solvent exchange was accomplished by passage of the solution through a 2.5 × 35 cm Sephadex G-25 column equilibrated and eluted with 40 mM acetate, pH 5.5. The protein-containing peak was collected and applied to a 2.5 × 8 cm column of Whatman DE-52 DEAE-cellulose which had previously been equilibrated in 40 mM acetate buffer, pH 5.5. The column was developed with a 500-mL linear gradient of increasing total acetate concentration (40–300 mM), pH 5.5.

Fractions containing 8 mL each were collected after initiation of the acetate gradient, and those exhibiting the highest sulfhydryl oxidase specific activities (approximately fractions 8–18) were pooled. The purified product was stored at 4 °C as an 85% ammonium sulfate suspension. For later experiments, aliquots of the suspension were removed in the required amounts, centrifuged, dissolved in the desired buffer, and desalted by passage over a Pharmacia PD-10 (Sephadex G-25) column equilibrated in the same buffer.

Assays. Determination of sulfhydryl oxidase activity was based upon depletion of O_2 from assay mixtures containing thiol compounds, as measured with a Yellow Springs Instruments oxygen monitor (Model 5300) equipped with polarographic Clark-type electrodes. In a typical assay, 2.9 mL of a mixture consisting of a thiol compound in an appropriate buffer was equilibrated with stirring to 25 °C. After the vial was sealed with the electrode, a volume of 0.1 mL of enzyme solution was injected into the assay mixture. The rate of oxygen depletion was constant during the first 30 s of the assay; thus, the initial rate of O_2 depletion was taken to be sulfhydryl oxidase activity under the prevailing conditions. One unit of sulfhydryl oxidase was defined as that amount of enzyme which would deplete 1 μmol of O_2 per minute under the conditions of the assay. Unless otherwise indicated, the assay mixture contained 8 mM GSH in 40 mM acetate buffer, pH 5.5, at 25 °C.

In some experiments, depletion of reactive sulfhydryl from sulfhydryl oxidase assay mixtures was measured by reaction of residual substrate with DTNB. Specifically, an appropriate volume of assay mixture was diluted 10-fold with 0.2 mM DTNB in 20 mM sodium phosphate buffer at pH 8.0. This solution was allowed to stand at room temperature for at least 2 min, after which time its absorbance at 412 nm was measured against a blank prepared by substituting buffer for the sulfhydryl-containing solution in the assay procedure. Sulfhydryl concentration was calculated from the molar absorptivity of the liberated thiolate anion at 412 nm, 13 600 M^{-1} cm^{-1} (Ellman, 1959), in the case of protein substrates, or by comparison to a standard curve prepared from solutions of known sulfhydryl content, in the case of GSH.

[2] Generously supplied by the Finnish Sugar Co., Ltd., Helsinki, Finland, and Finnsugar Biochemicals, Inc., Schaumberg, IL.

Stoichiometry of the Reaction. The ratio of GSH consumed to H_2O_2 produced in the course of the catalysis was determined by subjecting preincubated mixtures of GSH and sulfhydryl oxidase to simultaneous assays for reactive sulfhydryl and H_2O_2. For these determinations, 0.5 mL of enzyme solution was added to 5 mL of 0.8 mM GSH in 40 mM sodium acetate buffer, pH 5.5, and incubated at 37 °C for 15 min. Residual sulfhydryl content was then determined as described in the preceding section. Hydrogen peroxide was quantitatively determined by its reaction with a peroxidase-coupled colorimetric reagent. In these analyses, 0.5 mL of the sulfhydryl oxidase/GSH preincubation mixture was diluted into 4.5 mL of the colorimetric reagent, which was prepared immediately before use by combination of the following, per 50 mL of reagent: 19 mL of 0.2 M sodium phosphate buffer, pH 8.0; 1 mL of 0.4 mg/mL (590 purpurogallin units) horseradish peroxidase[2] in 50 mM sodium phosphate buffer, pH 7.5; 20 mL of 1.25 mM 4-aminoantipyrine; 10 mL of 0.5% (w/v) phenol. The reaction mixture was incubated for 20 min at 37 °C, whereafter its absorbance at 500 nm was measured against a blank in which buffer had been substituted for the test material. For preparation of a standard curve for this assay, a nominally 30% (w/v) stock solution of H_2O_2 was standardized by titration in cold 1 M H_2SO_4 with potassium permanganate (Winterton, 1959). Solutions of known peroxide concentrations between 0.1 and 1 mM were prepared from the stock solution and subjected to the assay procedure. These solutions were preincubated in the presence of sulfhydryl oxidase, in the same amount and under the same conditions as were employed in the sample preparation, in order to compensate for possible effects of the enzyme on either the peroxidase or the colorimetric reagents.

Reductive Denaturation and Renaturation of Ribonuclease. Ribonuclease A (Sigma, type IIIA) was reductively denatured in the presence of 8 M urea and 2-mercaptoethanol according to the procedure of Anfinsen and Haber (1961), with approximately 40 mg of ribonuclease dissolved in 6 mL of urea solution. Urea was twice recrystallized from hot ethanol, dissolved immediately before use, and the solution was passed over a 1 × 4 cm Dowex-1 column. Sulfhydryl oxidase was tested for its ability to oxidize the reduced ribonuclease as described by Janolino and Swaisgood (1975), except that the pH of the ribonuclease incubation mixtures was adjusted to 7.0, the concentration of ribonuclease was 0.45 mg/mL, and the concentrations of sulfhydryl oxidase were 0.11, 0.055, or 0.028 mg/mL. Ribonuclease assays were performed by measuring the decrease in absorbance of a yeast RNA solution (Sigma, type XI) at 258 nm, as described by Kalnitsky et al. (1959).

Gel Electrophoresis. Enzyme purity was monitored by SDS–polyacrylamide gel electrophoresis in a Model V161 Bethesda Research Laboratories vertical slab gel electrophoresis system. All gels and buffers were prepared according to the method of Laemmli (1970), with stacking gels of 5% acrylamide and running gels of 10% acrylamide. Samples were diluted 2:3 with the sample buffer and boiled for 2 min before application to the gels. After electrophoresis, protein bands were stained by the silver nitrate method of Morissey (1981) or by incubation with a Coomassie brilliant blue staining solution.

For subunit molecular weight estimation, the same electrophoresis system was used, with running gel acrylamide concentrations of 7.5%, 10%, and 12%. Molecular weight markers (Sigma Chemical Co.) consisted of the proteins carbonic anhydrase, egg albumin, bovine albumin, phosphorylase *b*, β-galactosidase, and myosin.

Amino Acid Analysis. Vapor-phase acid hydrolysis of sulfhydryl oxidase (5.25 μg per sample) was performed in a vial fitted with a Teflon valve according to the procedure of Hirs (1967a) for 24, 48, and 72 h. Amino acid standards (Pierce, Standard H) were also subjected to the hydrolysis procedure for each of the times mentioned, to provide a measure of the loss of serine, threonine, and tyrosine during the hydrolysis. Excess HCl was removed under vacuum at room temperature. Samples and standards were derivatized with phenyl isothiocyanate and analyzed according to the PICO-TAG methodology of Waters Associates, Inc., on a Waters HPLC system consisting of a 3.9 mm × 15 cm stainless-steel PICO-TAG column, two M-510 pumps, and a U-6K injector. The effluent stream was monitored at 254 nm by a Waters Model 481 LC spectrophotometer. An Apple II computer was used with CHROMATOCHART, a software product of Interactive Microware, Inc. (State College, PA), to record and process data. Cysteine was determined as cysteic acid by treatment of the enzyme with performic acid prior to hydrolysis (Hirs, 1967b). Tryptophan was estimated spectrophotometrically as described by Edelhoch (1967).

Carbohydrate Analysis. Total neutral hexose content was measured by the Winzler (1958) orcinol–sulfuric acid method, as modified by Francois et al. (1962), with mannose as a standard. Aminohexose content was determined by an Elson–Morgan reaction (Rondle & Morgan, 1955; Kraan & Muir, 1957). The sulfhydryl oxidase sample was prepared for this analysis by hydrolysis in 4 M HCl at 100 °C for 4 h, followed by neutralization with NaOH. Determination of 6-deoxyhexose was performed according to the procedure of Dische (1962) except that thioglycolic acid was substituted for cysteine hydrochloride. Assays for sialic acid were performed as described by Warren (1959) after hydrolysis of the enzyme at 80 °C for 60 min in 0.1 N H_2SO_4.

Ultracentrifugal Studies. Sedimentation equilibrium experiments were performed by the high-speed method of Yphantis (1964), using a Spinco Model E analytical ultracentrifuge equipped with an AN-J rotor and Rayleigh interference optics. Experiments employed approximately 0.4 mg/mL enzyme in 50 mM sodium phosphate and 0.2 M NaCl, pH 6.0. After 40-h centrifugation at 18 000 rpm and 4 °C, when equilibrium had been achieved, the interference pattern was recorded photographically. Fringe displacement as a function of axial distance was measured from the photographic plates with a Scherr Tumico (St. James, MN) optical comparator, Model 22-0338.

Flavin Analysis and Stoichiometry. The sulfhydryl apooxidase was prepared by a modification of the guanidine hydrochloride method of Moore et al. (1978). One volume of sulfhydryl oxidase in 0.1 M sodium citrate, pH 3.0, was mixed with 2 volumes of 8 M guanidine hydrochloride in the same buffer. After 5-min incubation on ice, the mixture was applied to a Sephadex G-25 column, which was equilibrated and eluted with 0.1 M sodium phosphate, pH 7.0. All operations were carried out at temperatures of 0–4 °C. The degree of separation was established by examining the absorbance spectrum of the protein-containing fraction. When recovery of the flavin from the guanidine hydrochloride preparation was desired, the pooled flavin-containing fractions were repeatedly batch-extracted with CM-cellulose in order to remove guanidinium ion.

In reactivation experiments, freshly prepared apoenzyme was combined with flavin solution and 0.1 M sodium phosphate buffer, pH 7.0, in the volume proportions 1:1:2. This mixture was incubated for 14–16 h at 0 °C. After the incubation

Table I: Purification of Sulfhydryl Oxidase

fraction	volume (mL)	act. (units)	sp act. (units/mg of protein)	recovery (%)
(A) crude cell filtrate	1620	7857	7.5	100
(B) methanol precipitate	440	7920	8.0	101
(C) $(NH_4)_2SO_4$ pellet	50	6150	8.4	78
(D) G-25 eluate	56	6160	9.4	78
(E) acetone supernatant	80	8320		106
(F) hydroxylapatite eluate	80	6160		78
(G) G-25 eluate	59	3245	73.2	41
(H) DEAE-cellulose eluate	95	2812	114.6[a]	36

[a] The Bradford reaction was found to give an underestimate of sulfhydryl oxidase concentration in pure preparations; the specific activity of the purified material was 73.4 units/mg of protein, as calculated from its molar absorptivity at 274 nm.

period, the enzyme was separated from excess free flavin by passage through a Pharmacia PD-10 column equilibrated and eluted with 40 mM acetate buffer, pH 5.5.

The flavin component was also extracted from the enzyme by two other methods. Dialyzed sulfhydryl oxidase was boiled for 5 min with protection from light and cooled quickly by immersion in ice–water. Soluble protein was removed from the supernatant fluid by passage over an Amicon YM-10 ultrafiltration membrane under 40 psi nitrogen pressure. Alternatively, 0.5 mL of sulfhydryl oxidase in distilled deionized H_2O was made 15% in TCA by addition of 0.315 mL of 40% trichloroacetic acid in three equal aliquots, with 5-min incubation time between each addition. The solution was centrifuged after standing for 30 min at room temperature. Trichloroacetic acid was removed from the supernatant liquid by repeated extraction with chloroform.

Quantitative determinations of the concentrations of FAD and FMN solutions were based upon the molar absorptivities of these compounds at 450 nm, 11 300 and 12 200 M^{-1} cm^{-1}, respectively (Loach, 1970). FAD and FMN were qualitatively distinguished from one another by the fluorometric procedure of Faeder and Siegel (1973), with the use of a Perkin-Elmer MPF-44A fluorescence spectrophotometer. Flavin concentrations in standards and sample preparations were 1×10^{-5} to 2×10^{-5} M.

Miscellaneous Methods. All spectra were recorded against air on a Cary 219 UV–visible double-beam spectrophotometer interfaced with an Apple II computer. Spectral calculations were performed with software provided by Cary. Protein concentrations in crude preparations were estimated by the method of Bradford (1976) with bovine serum albumin (fraction V, Sigma) as a standard. Concentrations of purified enzyme preparations were calculated from the molar absorptivity at 274 nm.

Results

Purification. Sulfhydryl oxidase was readily purified from brine filtrate of *A. niger* cells by the procedure described under Experimental Procedures, as shown in Table I. From an initial volume of 1620 mL of crude filtrate (approximately 1 g of total protein), 38 mg of pure sulfhydryl oxidase was recovered. The purified material exhibited a single, rather diffuse protein-containing zone when SDS–polyacrylamide gel electrophoresis was performed.

The greatest degree of purification took place during the acetone precipitation and hydroxylapatite chromatography steps. These procedures took advantage of the unusual solubility and stability of the sulfhydryl oxidase in water–acetone mixtures, as compared with the other major protein constituents of the extract. The chromatography on hydroxylapatite resulted in a higher degree of purity and a greater final percent recovery than did bulk fractionation of the extract with acetone at concentrations higher than 30% (v/v).

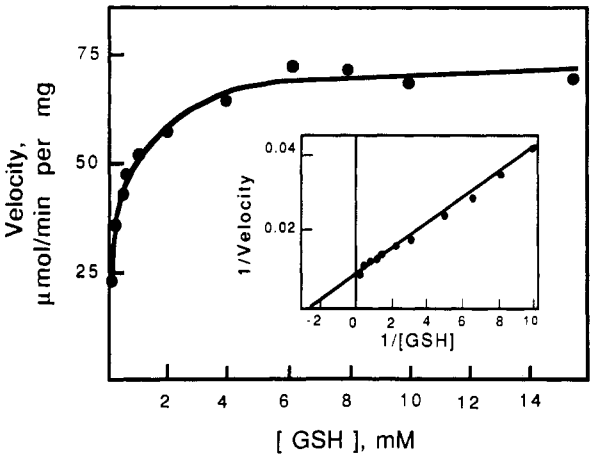

FIGURE 1: Activity of sulfhydryl oxidase as a function of GSH concentration. Assays were performed at pH 5.5 and 25 °C in 40 mM sodium acetate, with 0.166 mg/mL sulfhydryl oxidase. Velocity is expressed as activity units per milligram of enzyme. Inset: Double-reciprocal plot of the saturation data.

Table II: Kinetic Constants for Various Substrates[a]

substrate	$K_{m(app)}$ (mM)	$k_{cat(app)}$ (min^{-1})	$k_{cat(app)}/K_{m(app)}$ ($\times 10^{-5}$ min^{-1} M^{-1})
glutathione	0.3	7875	262.5
cysteine	43.0	9975	2.32
dithiothreitol	66.0	7140	1.08
2-mercaptoethanol	340	8715	0.25

[a] Assays were performed under the standard conditions defined in the text. Protein concentrations were calculated from the absorbance of the enzyme solutions at 274 nm. The concentration of oxygen in all assays was that of the air-saturated substrate solution.

The anomalous percent recovery values obtained during the acetone fractionation steps (see Table I) were reproducible and apparently resulted from a solvent activation effect. The nature of this effect was not investigated.

Although the first few steps in the purification contributed little to the overall increase in specific activity, they were to some extent required for the efficacy of the acetone fractionation steps. Replacement of the early steps by dialysis and concentration resulted in a less pure product after chromatography on hydroxylapatite (data not shown).

Since catalase, a major constituent of *A. niger* extracts, was present at varying levels in the early stages of the purification and suppressed the apparent sulfhydryl oxidase activities of those fractions to differing degrees, the activities shown in Table I were estimated by extrapolating plots of apparent activity vs dilution factor to infinite dilution.

Kinetic Properties and Substrate Specificity. A. niger cell extract and the purified enzyme both depleted O_2 from solutions containing various thiol compounds. Rates of O_2 depletion by the purified enzyme were measured at selected concentrations of GSH, cysteine, dithiothreitol, and 2-mercaptoethanol. Double-reciprocal plots prepared for each substrate tested were linear over a substrate concentration range of at least 1 order of magnitude. Figure 1 shows the saturation curve obtained when the substrate was GSH, with the double-reciprocal plot inset. Kinetic constants were calculated from the double-reciprocal plot by linear regression fitting of the data points. The values obtained for each substrate are given in Table II.

Sulfhydryl oxidase had maximal and nearly constant activity between pH 5.0 and 5.5 when GSH was the substrate. Activity

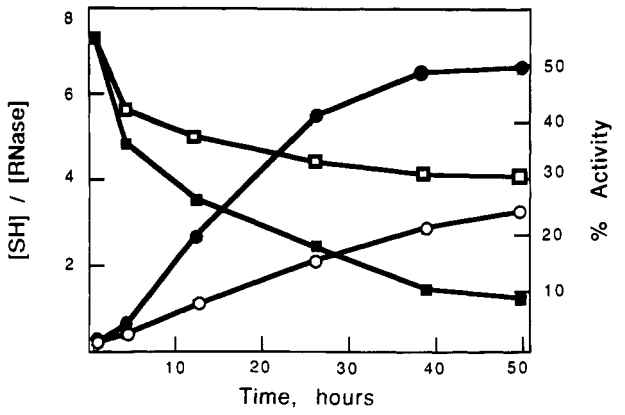

FIGURE 2: Reactivation of reductively denatured ribonuclease A. Reactive sulfhydryl content was measured by reaction with DTNB in the presence (■) and absence (□) of 0.11 mg/mL sulfhydryl oxidase. Restoration of ribonuclease activity in the presence (●) and absence (○) of sulfhydryl oxidase was measured by recording the increase in absorbance at 258 nm of a yeast RNA solution.

decreased slowly as the pH was increased and declined more rapidly as the pH was decreased. The enzyme had no activity at pH 2.5 and very little at pH 10.0. When sulfhydryl oxidase was incubated for 24 h at various values of pH but assayed under the standard conditions (pH 5.5), activity remained constant while the pH was between 3.0 and 8.5 in the incubation mixtures, but declined sharply in solutions with pH values more extreme than these.

The enzyme was tested for activity toward substrates of other known oxidases, including D-glucose, D-fructose, L-lysine, D-lysine, benzylamine, xanthine, putrescine, catechol, NADH, and NADPH. In each case, the enzyme failed to stimulate depletion of O_2 from assay mixtures containing the test compounds at concentrations of 10 mM in 40 mM sodium acetate at pH 5.5. In other tests, NAD and NADP did not act as electron acceptors in the presence of sulfhydryl oxidase and 4 mM GSH, as indicated by the lack of measurable increase in absorbance of these solutions at 340 nm.

The rate of reactivation of reductively denatured ribonuclease A was enhanced by the presence of sulfhydryl oxidase (Figure 2), which indicated that the enzyme is capable of catalyzing the oxidation of protein-associated thiol groups. Reactive sulfhydryl content of the ribonuclease preparation after reduction was 7.5 mol of SH/mol of ribonuclease, which represented 94% of complete reduction. Residual activity was 2% of that observed in a native control. After 50 h of incubation with the highest concentration of sulfhydryl oxidase, in which experiment ribonuclease was present in 31.9-fold molar excess, the ribonuclease preparation had regained 50% of its native activity. In the same period, the control mixture containing acetate buffer in place of sulfhydryl oxidase solution regained only 24% of its native activity. Reactive sulfhydryl content declined to 1.4 mol of SH/mol of ribonuclease in the presence of the sulfhydryl oxidase, but only to 4.2 mol of SH/mol of ribonuclease in the control mixture. Other incubation mixtures containing half and one-fourth this quantity of sulfhydryl oxidase exhibited smaller degrees of ribonuclease reactivation.

Product and Stoichiometry Determination. The oxidized product of the reaction with GSH as substrate was established to be glutathione disulfide by the incorporation of a GSH solution preincubated with the sulfhydryl oxidase into an assay for the NADPH-coupled enzyme glutathione reductase. A significant decrease in absorbance at 340 nm was recorded when a preincubation mixture containing GSH and native sulfhydryl oxidase was combined with a solution of NADPH and glutathione reductase; no such decrease occurred when boiled sulfhydryl oxidase solution was substituted for the native enzyme, or when active sulfhydryl oxidase was combined with GSSG and NADPH in the absence of glutathione reductase.

Table III: Chemical Composition of Sulfhydryl Oxidase

residue	g/100 g of protein[a]	residues per 53 000	nearest integer
amino acids			
Asx	10.93 ± 0.49	50.4	50
Glx	7.73 ± 0.44	31.7	32
Ser	6.42 ± 0.38	39.1	39
Gly	4.14 ± 0.41	38.5	39
His	1.13 ± 0.25	4.4	4
Arg	6.42 ± 0.31	21.8	22
Thr	6.20 ± 0.25	32.5	33
Ala	3.95 ± 0.07	29.4	30
Pro	4.19 ± 0.49	22.9	23
Tyr	2.87 ± 0.35	9.3	9
Val	6.53 ± 0.25	35.0	35
Met	1.47 ± 0.28	5.9	6
Ile	3.69 ± 0.44	17.3	17
Leu	4.31 ± 0.24	20.2	20
Phe	2.54 ± 0.38	9.2	9
Lys	2.45 ± 0.17	10.1	10
Trp[b]	1.39 ± 0.09	4.0	4
Cys[c]	0.54 ± 0.04	2.8	3
other			
hexose	20.3	66.3	66
aminohexose	1.9	6.2	6
FAD[b]	1.25	0.84	1

[a] Amino acid percent weights are averages of results from nine data sets. Values for acid-labile amino acids were corrected by comparison to standards subjected to the hydrolysis conditions. Increases in the concentrations of the hydrophobic residues were not observed for long hydrolysis times. Carbohydrate percent weights were determined in duplicate, as described in the text. [b] Determined independently by spectrophotometric analysis. [c] Determined as cysteic acid.

Reduction of O_2 in the course of the reaction resulted in production of H_2O_2, as was shown by the coupling of the GSH oxidation with the peroxidase colorimetric system described under Experimental Procedures. Quantitative determinations of this type, in conjunction with assays for residual reactive sulfhydryl groups in enzyme/GSH solutions, gave a ratio of GSH consumed to H_2O_2 produced of approximately 2:1. Results from three independent determinations were 0.758 ± 0.025 mmol of GSH depleted and 0.412 ± 0.013 mmol of H_2O_2 produced, for a ratio of GSH depleted to H_2O_2 produced of 1.84 ± 0.06.

Composition Studies. After concentrations of serine, threonine, and tyrosine were corrected for destruction by comparison to standards subjected to the hydrolysis procedure, there were no significant differences in the amino acid composition of sulfhydryl oxidase as calculated directly from the analysis of 24-, 48-, and 72-h hydrolysates. Also, analyses of hydrolysates from three different preparations of the enzyme gave the same results. Accordingly, amino acid composition data obtained from the different preparations and hydrolysis times were averaged. Carbohydrate analyses, performed in duplicate, showed the enzyme to consist of 21.0% neutral hexose and 1.9% hexosamine by weight but to contain no 6-deoxyhexose or sialic acid. The complete results of the composition studies are given in Table III.

Molecular Weight Studies. SDS–polyacrylamide gel electrophoresis of sulfhydryl oxidase resulted in values of subunit molecular weight of 51 000, 53 000, and 54 000 from gels with acrylamide concentrations of 7.5%, 10%, and 12%, respectively, giving an average of 52 700.

The native molecular weight was calculated from sedimentation equilibrium data. Plots of the natural logarithm

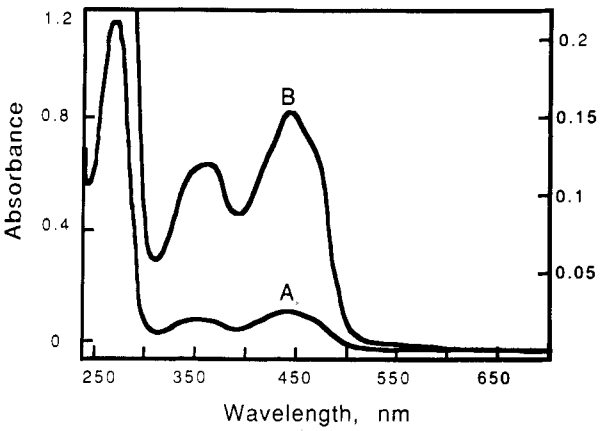

FIGURE 3: Spectrum of purified sulfhydryl oxidase in 40 mM sodium acetate, pH 5.5 (A) 0.8 mg/mL sulfhydryl oxidase; (B) detail of spectrum (A).

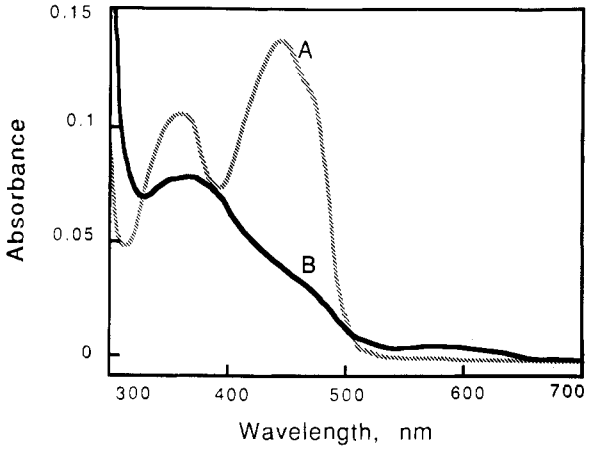

FIGURE 4: Spectra of purified sulfhydryl oxidase in 40 mM sodium acetate, pH 5.5, in the absence (A) and presence (B) of 20 mM GSH. The protein concentration was 0.71 mg/mL in both cases.

of fringe displacement vs the square of axial distance from two optical comparator data sets were linear, and molecular weights (M_r) of 107 400 and 104 200 (average 106 000) were calculated from their slopes with the equation $M_r = 2RT\text{-(slope)}/[(1 - \bar{v}\rho)\omega^2]$, where ω is the angular velocity, ρ is the solvent density, $\bar{v}$ is the partial specific volume of sulfhydryl oxidase, T is the temperature, and R is the gas constant. The partial specific volume of sulfhydryl oxidase was taken to be 0.72, as calculated from the enzyme's amino acid composition.

Spectral Studies. The UV–visible spectrum of the purified sulfhydryl oxidase had absorbance maxima at 442.5, 364.5, and 274 nm (Figure 3), which is consistent with the enzyme being a flavoprotein. The visible band had slight shoulders to the left and right of the maximum, at about 415 and 460 nm, respectively. The molar absorptivities at the three maxima were determined by recording spectra of enzyme solutions in water, drying the solutions in vacuo over P_2O_5, and weighing the residue on a Cahn electrobalance. The $E^{0.1\%}$ values obtained as averages of two determinations with different batches of sulfhydryl oxidase were 1.470, 0.167, and 0.197 cm^{-1} mg^{-1} mL at 274, 364.5, and 442.5 nm, respectively, with no more than a 3% difference between the averaged values. The corresponding molar absorptivities, assuming a molecular weight of 106 000, were 1.56×10^5, 1.74×10^4, and 2.09×10^4 cm^{-1} M^{-1}.

Addition of 20 mM GSH in sodium acetate, pH 5.5, to sulfhydryl oxidase in the presence of O_2 resulted in partial attenuation of the 442.5- and 364.5-nm bands and a slight long-wavelength increase in absorbance (Figure 4). Spectral

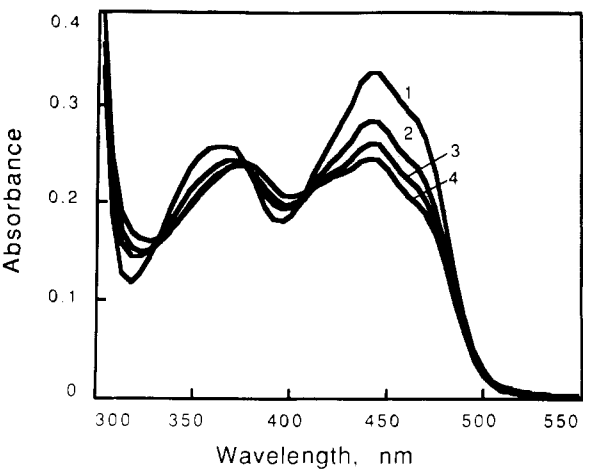

FIGURE 5: Bleaching of the visible band of the sulfhydryl oxidase spectrum by reaction with sodium sulfite at pH 6.0. Each spectrum was recorded 10 min after the addition of a small aliquot of sodium sulfite to a cuvette containing 1.8 mg/mL sulfhydryl oxidase in 50 mM sodium phosphate. Sulfite concentrations after each addition were 0, 0.99, 2.44, and 152 mM.

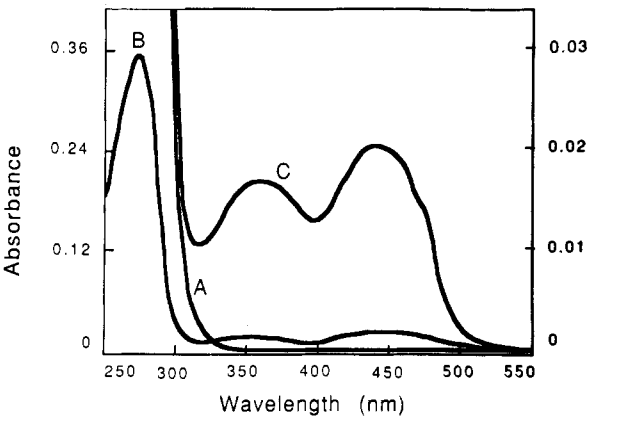

FIGURE 6: Spectrum of sulfhydryl oxidase (A) after removal of the native flavin moiety by treatment with 5 M guanidine hydrochloride and (B) after incubation of the apoenzyme with excess FAD at pH 7.0 for approximately 14 h. Spectrum (C) shows details of (B).

changes were not observed when the same amount of GSH was combined with free FAD or FMN under the same conditions. Sodium dithionite reduced both the sulfhydryl oxidase and the free flavin solutions.

Most flavin oxidases, but not flavin dehydrogenases, react with sodium sulfite to form a bleached flavin–sulfite complex (Massey et al., 1969). The spectral change incurred by incubation of sulfhydryl oxidase with sodium sulfite at varying concentrations is shown in Figure 5. The dissociation constant $K_d = [SOX][SO_2^-]/[SOX\text{-}SO_2^-]$ for the sulfhydryl oxidase flavin–sulfite complex was calculated from the first few spectra under the assumption that spectrum 4 (Figure 5) represented full conversion of the enzyme to the sulfite complex and that the initial excess of sulfite was so large that its concentration was essentially unchanged at equilibrium. The first assumption was justified by the fact that spectrum 4 (152.8 mM sulfite) was identical with the spectrum recorded when the sulfite concentration was just 42.8 mM (not shown). These calculations gave values of 3.04×10^{-3} and 2.95×10^{-3} M for K_d, from spectra 1 and 2, respectively.

Flavin Analysis and Stoichiometry. Treatment of the enzyme with guanidine hydrochloride followed by particle exclusion chromatography resulted in complete resolution of the flavin component and the protein, as was indicated by the failure of the visible and near-UV absorbance bands to appear

Table IV: Reconstitution of Sulfhydryl Apooxidase[a]

	act. (units/mg)	act. (%)	$A_{442.5}/A_{274}$	FAD bound (%)
native SOX	9.7	100	0.112	100
apoSOX	0.0	0	0.0	0
apoSOX + FAD	6.3	65	0.067	60
apoSOX + FMN	0.0	0	*b*	*b*

[a] Activity and flavin binding parameters for sulfhydryl oxidase treated as in Figure 6. The ratio $A_{442.5}/A_{274}$ was taken as a measure of flavin binding, with the assumption that the native and apo forms of the enzyme had the same molar absorptivities at 274 nm. [b] Not determined.

Table V: Analysis of Flavin Resolved from Sulfhydryl Oxidase[a]

method of resolution	F_{base}/F_{acid}	act. (units/mL)[b]
boiling	0.21	0.35
trichloroacetic acid treatment	1.47	0.0
guanidine hydrochloride treatment	1.48	0.0
authentic FAD	0.23	0.58
authentic FMN	1.38	0.0

[a] Samples of flavin resolved from sulfhydryl oxidase were prepared and subjected to fluorometric analysis as described in the text. The quantities F_{base} and F_{acid} are proportional to the fluorescences of the flavin solutions at pH 7.7 and 2.6, respectively. Concentrations of flavin standards and flavin samples were not identical. [b] Activities are those of sulfhydryl apooxidase samples after 14-h incubation at 0 °C in the presence of resolved flavins or standards.

in the spectrum of the recovered protein (Figure 6). Flavin-depleted preparations did not have measurable activity toward GSH. Incubation of the apoenzyme with excess FAD at neutral pH, followed by gel filtration for removal of unbound flavin, restored activity to the enzyme and resulted in recovery of the spectral bands at 442.5 and 364.5 nm (Figure 6). The reconstitution was incomplete, but the restored activity was essentially proportional to restored flavin, as can be seen by comparing the specific activity and the ratio $A_{442.5}/A_{274}$ of the reactivated enzyme to those of the native control (Table IV). Incubation with FAD for times longer than 14 h failed to change either of these parameters further. Incubation of the apooxidase with excess FMN under a variety of conditions (pH values ranging from 3 to 9 in the presence of various buffer salts) did not result in any detectable restoration of activity toward GSH under standard assay conditions.

Fluorometric analysis of the flavin constituent gave results which are summarized in Table V. At values of pH below 3, FAD exhibits strong fluorescence at 530 nm when excited at 450 nm. At neutral to basic pH, FAD is weakly fluorescent owing to quenching by its adenine moiety. FMN and other nonadenylated flavins, on the other hand, fluoresce strongly under both conditions of pH (Bessey et al., 1949; Cerletti & Siliprandi, 1958). Thus, the ratio of fluorescence at basic pH to that at acidic pH distinguishes adenylated from nonadenylated flavins. The data in Table V show that the flavin preparation method which involved denaturation of the sulfhydryl oxidase by boiling resulted in the liberation of a flavin with fluorometric characteristics identical with those of FAD, whereas the other two methods gave a nonadenylated flavin as their products. When the isolated flavins were recombined with sulfhydryl apooxidase, only the product of treatment by boiling was capable of restoring activity to the enzyme (Table V).

Assuming the native flavin constituent to be FAD and the molecular weight of the enzyme to be 106 000, the flavin to protein stoichiometry of sulfhydryl oxidase was established to be 1.68 mol of FAD/mol of enzyme by recording the spectrum of a known concentration of native sulfhydryl oxidase, liberating the flavin by boiling as described under Experimental Procedures, and recording the spectrum of the boiled solution. These experiments showed the enzyme to be slightly hyperchromic relative to free FAD; the molar absorptivity of the bound flavin was 12 400 cm^{-1} M^{-1}, as compared to 11 300 cm^{-1} M^{-1} for free FAD.

Since flavin oxidases containing essential metal ions are known to exist, sulfhydryl oxidase was examined for changes in activity upon treatment with the chelating agents. No activity decreases were observed when the enzyme was combined with and assayed in the presence of either 1 mM *o*-phenanthroline or 1 mM 8-hydroxyquinoline in 0.05 M phosphate buffer, pH 7.0, even after overnight incubation with the chelating agents.

Discussion

In this study, we have demonstrated that the ability of *A. niger* glucose oxidase preparations to catalyze the oxidation of GSH is in fact the catalytic activity of a sulfhydryl oxidase of *A. niger*. The enzyme catalyzes the conversion of low molecular weight thiol compounds to their corresponding disulfides and has a similar action on reduced ribonuclease.

Several enzymes with similar catalytic activities have been reported. In 1956, Mandels showed that the spores of the fungus *Myrothecium verrucaria* contained a sulfhydryl oxidase which catalyzed the oxidation of GSH, cysteine, and homocysteine with concomitant reduction of O_2 to H_2O. Thiooxidase activity was found in the cell-free culture filtrates of the fungi *Piricularia oryzae* and *Polyporous versicolor* (Neufeld et al., 1950). This oxidase failed to utilize GSH and cysteine as substrates but oxidized ethylenic-linked thiol groups in a wide range of compounds. Thiooxidase isolated from *P. oryzae* (Aurbach & Jakoby, 1962) was shown to be responsible for the *m*-polyphenol oxidase activity that had been observed in crude *P. oryzae* extracts. In 1975, Olsen isolated a sulfhydryl oxidase from the culture fluid of an organism believed to be *Dactylium dendroides*. This Cu metalloenzyme was found in mycelium extracts which also contained galactose oxidase. The purified sulfhydryl oxidase was shown to be capable of reactivating reductively denatured galactose oxidase, as well as protecting the latter enzyme against inactivation by a low molecular weight inhibitor present in the culture fluid.

Sulfhydryl oxidases have also been obtained from mammalian sources. An Fe-dependent sulfhydryl oxidase which had activity toward GSH, cysteine, dithiothreitol, 2-mercaptoethanol, and reduced ribonuclease A was purified from bovine milk (Janolino & Swaisgood, 1975). The enzyme also restored reduced chymotrypsinogen to a form capable of undergoing conversion to active chymotrypsin (Janolino et al., 1978) and catalyzed the conversion of xanthine dehydrogenase to xanthine oxidase (Clare et al., 1981). Milk extracts from other sources, including human, have been seen to exhibit sulfhydryl oxidase activity (Isaacs et al., 1984) as have the homogenates of kidney and pancreas tissues from various mammals (Clare et al., 1984). An enzyme isolated from the skin of young rats (Takamuri et al., 1980) catalyzed the oxidation of dithiothreitol, dithioerythritol, D-penicillamine, and L-cysteine, but GSH and 2-mercaptoethanol were very poor substrates.

There is no evidence that any of the aforementioned enzymes are flavoproteins. However, an intense sulfhydryl oxidase activity discovered in rat epididymal fluid (Chang & Morton, 1975) was later demonstrated to be the activity of an FAD-dependent enzyme (Ostrowski et al., 1979; Ostrowski & Kistler, 1980). This sulfhydryl oxidase had as its best substrates dithiothreitol, GSH, and cysteine; like the skin and

bovine milk enzymes, it was capable of reactivating reduced ribonuclease A.

Despite the large number of clearly distinct sulfhydryl oxidases that have been discovered, there is a dearth of information concerning the physiological role of this class of enzymes. It has been suggested that these enzymes may have a part in the formation of disulfide bonds during or after the cellular synthesis of proteins (Janolino & Swaisgood, 1975). This possibility is supported by evidence that an enzyme which catalyzes posttranslational immunoglobulin M assembly is a sulfhydryl oxidase (Roth & Koshland, 1980). Another hypothesis is that sulfhydryl oxidases may protect other enzymes or biological tissues against adventitious reductive damage (Chang & Morton, 1975); the effect of the *D. dendroides* sulfhydryl oxidase on galactose oxidase may provide an example of this.

As has been mentioned, several of the sulfhydryl oxidases previously investigated have tested positive for the ability to enhance the reactivation of reductively denatured ribonuclease A, which implies that these enzymes are capable of accepting protein-associated thiol groups as substrates. Whether this indicates that protein-associated thiols represent the native substrates in these cases, or whether it merely means that these enzymes are not highly specific for their native substrates, is not clear. Unfortunately, differences in experimental protocols, sources of ribonuclease, degrees of reactivation in control mixtures, and methods of data presentation make it impossible to compare the avidities of these various oxidases for protein substrates on the basis of published results. However, the fact that they exhibit some degree of activity toward reduced ribonuclease distinguishes them as a class from the sulfhydryl interchange enzymes, which can only reactivate reduced ribonuclease in the presence of low molecular weight thiols, or ribonuclease which has been "scrambled" with respect to its disulfide linkages by full reduction and fast forced reoxidation.

Incubation overnight at pH 3.0 failed to destroy *A. niger* sulfhydryl oxidase activity. The acid stability of the enzyme may be related to the fact that the flavin moiety is difficult to remove from the enzyme without irreversible protein denaturation. Acid ammonium sulfate precipitation, extended dialysis against high concentrations of KBr at low pH, incubation with 8 M urea, and treatment with various chaotropic salts all failed to give any resolution of flavin from protein. In addition, the fluorometric data suggest that guanidine hydrochloride method used to prepare the apoenzyme was effective, at least in part, through its ability to convert the native adenylated flavin to an unadenylated form.

A. niger sulfhydryl oxidase exhibited the reactivity with sulfite which is characteristic of most flavin oxidases, but not flavin dehydrogenases, and is believed to be correlated directly with the enzyme's ability to undergo oxidation by molecular oxygen (Massey et al., 1969). The dissociation constant for the sulfhydryl oxidase flavin–sulfite complex was on the same order of magnitude as those reported by Massey et al. (1969) for D-amino acid oxidase and oxynitrilase, but the spectral changes accompanying sulfite binding to sulfhydryl oxidase were much less extreme than those seen for any of the reactive enzymes investigated by these workers. The absorbance of sulfhydryl oxidase at 442.5 nm was only 25% reduced by complete conversion to the sulfite complex, and the 364.5-nm band showed a shift rather than an attenuation. It is notable that the flavin sulfhydryl oxidase from rat epididymal fluid failed to react with sulfite at concentrations as high as 0.1 M (Ostrowski & Kistler, 1980) as judged by the absence of changes in the enzyme's visible spectrum.

The molecular weight determinations performed in this study suggest that *A. niger* sulfhydryl oxidase is dimeric, with a subunit molecular weight of about 53 000. It is therefore reasonable to expect that the flavin to protein stoichiometry should be integral with respect to this molecular weight. However, the data pertaining to flavin stoichiometry gave a value of 1.68 mol of FAD/mol of protein, or 0.84 FAD molecule per subunit. There are several possible reasons for this: the apoenzyme may be incompletely assembled into holoenzyme by the organism; the flavin moiety may be a derivative of FAD, rather than FAD itself; or the molecular weight estimate may be prejudiced by the assumption that 0.72 represents a reasonable partial specific volume. Macromolecular carbohydrates, in general, have lower partial specific volumes than do proteins (Smith, 1967), and it is well-known that SDS–polyacrylamide gel electrophoresis may fail to give good estimates of the molecular weights of glycoproteins. Understanding the cause of the nonintegrality of the flavin stoichiometry awaits the purification of sufficient material for an accurate pycnometric determination of the enzyme's partial specific volume.

Young and Nimmo (1972) reported that the sulfhydryl oxidase activity found in impure *A. niger* glucose oxidase preparations satisfied the Michaelis–Menten kinetic equation. We have found that double-reciprocal plots of saturation data when GSH is the substrate for the purified enzyme are linear when the substrate concentrations lie between 0.2 and 20 mM. An examination of the kinetic equations describing the velocity of an enzyme which couples two identical substrate molecules shows that in most mechanistic interpretations, double-reciprocal plots should be parabolic or hyperbolic rather than linear, but will approximate linearity (over the substrate concentration ranges employed in our experiments) if there are two substrate binding events which have very different apparent Michaelis constants. The linearity observed in these studies may indicate that the substrates tested here are high-efficiency substrates with respect to one binding event, but low-efficiency substrates with respect to another binding event. Identification of classes of substrates with these properties would be a powerful tool for the elucidation of this enzyme's catalytic mechanism, since they would allow for the spectral and kinetic study of individual steps in the catalyzed reaction. This advantage, not offered by flavin oxidases which utilize a single reducing substrate molecule per catalytic event, provides a unique opportunity for the study of flavin-mediated oxidations.

Registry No. SOX, 9029-39-4; GSH, 70-18-8; FAD, 146-14-5; RNase A, 9001-99-4; $HO(CH_2)_2SH$, 60-24-2; L-cysteine, 52-90-4; dithiothreitol, 3483-12-3.

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Inhibition of the Catalytic Subunit of cAMP-Dependent Protein Kinase by Dicyclohexylcarbodiimide†

Jean Toner-Webb‡ and Susan S. Taylor*
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
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ABSTRACT: The hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD) has been shown to inhibit the catalytic (C) subunit of adenosine cyclic 3′,5′-phosphate dependent protein kinase (EC 2.7.1.3) in a time-dependent, irreversible manner. The rate of inactivation was first order and showed saturation kinetics with an apparent K_i of 60 μM. Magnesium adenosine 5′-triphosphate (MgATP) was capable of protecting against this inhibition, whereas neither a synthetic peptide substrate nor histone afforded protection. Mg alone afforded some protection. When the catalytic subunit was aggregated with the regulatory subunit in the holoenzyme complex, no inhibition was observed. The inhibition was enhanced at low pH, suggesting that a carboxylic acid group was the target for interaction with DCCD. On the basis of the protection studies, it is most likely that this carboxylic acid group is associated with the MgATP binding site, perhaps serving as a ligand for the metal. Efforts to identify the site that was modified by DCCD included (1) modification with [^{14}C]DCCD, (2) modification by DCCD in the presence of [^{3}H]aniline, and (3) modification with DCCD and [^{14}C]glycine ethyl ester. In no case was radioactivity incorporated into the protein, suggesting that the irreversible inhibition was due to an intramolecular cross-link between a reactive carboxylic acid group and a nearby amino group. Differential peptide mapping identified a single peptide that was consistently lost as a consequence of DCCD inhibition. This peptide (residues 166–189) contained four carboxylic acid residues as well as an internal Lys. Two of these carboxyl groups, Asp-166 and Asp-184, are conserved in all protein kinases, including oncogene transforming proteins and growth factor receptors, and thus are likely to play an essential role.

The active form of the catalytic subunit of adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase is a monomeric protein with a molecular weight of 38 500. The enzyme shows an ordered mechanism of binding where the binding of magnesium adenosine 5′-triphosphate (MgATP) precedes peptide binding (Whitehouse et al., 1983). Synthetic peptides have been used to map the requirements for peptide recognition. These requirements include two basic residues, most frequently arginines in physiological substates, which precede the site of phosphorylation (Kemp et al., 1977). The phosphate acceptor is serine or less frequently threonine.

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* Author to whom correspondence should be addressed.
‡ Present address: Department of Biological Chemistry, University of California, Irvine, Irvine, CA 92717.

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