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Catalase of *Neurospora crassa*. 1. Induction, Purification, and Physical Properties†

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ABSTRACT: We have purified to homogeneity a catalase found in extracts of *Neurospora crassa* 5297a that had been induced for nitrate reductase in a nitrate-supplemented medium. The activity is stabilized in extracts by the protease inhibitor phenylmethanesulfonyl fluoride. The enzyme has an apparent molecular weight of 3.2×10^5 and is composed of four subunits of 8×10^4 molecular weight. The catalytic constant for H_2O_2 dismutation, at pH 6.8 and 25 °C, is 4.6×10^6 M^{-1} s^{-1}, and the activation energy is 7.2 ± 0.4 kcal/mol. The protein exhibits no peroxidase activity with guiacol as the substrate. The enzyme is not reducible by sodium dithionite. The inhibitory effect of KCN, NaN_3, NaF, Na_2SO_3, KNO_2, and KNO_3 on enzyme activity at pH 7 was studied; cyanide and azide were found to be strong inhibitors of activity. Though the protein is homogeneous according to ultracentrifugation and electrophoresis, the iron content averaged 3.4 atoms of Fe/molecule, suggesting that, in common with bovine liver catalase, the isolated protein does not carry a full complement of heme groups. The electronic spectrum exhibits maxima at 280 (1.1×10^5), 400 (8.2×10^4), 590 (1.7×10^4), and 712 nm (3.8×10^3) [absorbancy per mole of iron is given in parentheses]. The inducibility of the enzyme in the presence of nitrate, its molecular weight, and its electronic spectrum all distinguish this catalase from previously reported catalases. Antibodies raised to homogeneous samples of the enzyme were used in Ouchterlony and double-antibody radioimmunoassays to indicate that a minimum of 75% of the catalase induced in the presence of nitrate is identical with the newly identified catalase. Thus, the presence of nitrate in growth media for *N. crassa* initiates a series of events including synthesis of nitrate reductase, synthesis of catalase apoprotein, and probably the synthesis of the enzymes responsible for the production of the prosthetic group.

While attempting to purify the enzyme nitrate reductase from *Neurospora crassa*, we found that the addition of phenylmethanesulfonyl fluoride, a protease inhibitor, to buffers used in purification prevents inactivation of the enzyme (Jacob, 1976). This has led to the discovery and isolation of a green protein subsequently determined to be a catalase. This catalase is induced by nitrate as is nitrate reductase. Although catalases from a number of sources have been rather thoroughly studied, the inducibility as well as the unique color of the *N. crassa* enzyme prompted us to investigate it. The present communication describes its purification to homogeneity, subunit structure, and physical and catalytic properties and indicates that the enzyme contains an unusual heme prosthetic group.

Subramanian et al. (1968) previously reported that the level of catalase in nitrate-induced mycelia increased until a maximum was reached 7 h after transfer to the induction medium. It was suggested by these authors that induction was a consequence of increased intracellular peroxide produced by the interaction of oxygen with the flavin component of nitrate reductase. This explanation was supported by their report that *Neurospora* grown in ammonia medium containing peroxide also exhibited elevated catalase levels. We report here the results of immunochemical experiments to determine whether the catalase reported in this communication is the one responsible for the increased catalatic activity observed in nitrate-induced *N. crassa*.

Materials and Methods

Materials. Spores of *N. crassa* 5297a were obtained from the Fungal Genetics Stock Collection, Humbolt College, Arcata, CA. Bovine serum albumin, carboxypeptidase, glutamate dehydrogenase, phosphorylase *a*, beef liver catalase, and phenylmethanesulfonyl fluoride were obtained from Sigma. *N. crassa* nitrate reductase was purified by the method of Jacob (1976). Purified agar and Freund's adjuvants were obtained from Difco. Carrier free $Na^{125}I$ in 0.1 N NaOH, from New England Nuclear, and anti-rabbit IgG serum, obtained from immunized goats, were generous gifts from Dr.

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0006-2960/79/0418-2967$01.00/0

R. Hong. The reagents for polyacrylamide gel electrophoresis were purchased from Eastman Kodak, and dithiothreitol was from Calbiochem, Ampholine (pH range 3–9) from LKB Products, Sephadex G-200 from Pharmacia, DEAE-cellulose[1] (DE-52) from Whatman, and spectrophotometric grade pyridine from Mallinckrodt. Sodium dodecyl sulfate obtained from Eastman Kodak was recrystallized from chloroform before use. Ammonium sulfate used in step 4 of the enzyme purification procedure was "Enzyme Grade" from Grand Island Biological. All other chemicals were analytical reagent grade and used as supplied.

Culture Methods. Growth and induction of *N. crassa* was carried out at 30 °C in the dark in modified Fries basal media (Nicholas & Nason, 1954). *Growth medium* contained sodium tartrate (4.28 g/L), NH_4Cl (3.6 g/L), and KH_2PO_4 (1 g/L). *Induction medium* contained KH_2PO_4 (3 g/L), $NaNO_3$ (2 g/L), and sodium tartrate (1 g/L). In addition, both media contained (in g/L) the following: sucrose, 20; $MgSO_4$, 0.27; $CaCl_2 \cdot 2H_2O$, 0.13; NaCl, 0.1; sodium tetraborate, 8.8×10^{-5}; sodium molybdate, 8.8×10^{-5}; $FeCl_3 \cdot 6H_2O$, 9.6×10^{-4}; $ZnSO_4 \cdot 7H_2O$, 8.8×10^{-3}; $CuCl_2 \cdot 2H_2O$, 3.4×10^{-4}; $MnCl_2 \cdot 4H_2O$, 7.2×10^{-5}; and biotin, 5×10^{-6}. *Neurospora* was maintained by transfer and growth of conidia in 125-mL Erlenmeyer flasks containing 50 mL of Bacto-agar growth medium.

The conidia from one flask grown for at least 7 days were suspended in 50 mL of sterile distilled H_2O and used to inoculate 3 L of liquid growth medium contained in a 1-gal bottle fitted with a sparger and magnetic stir bar. After 1–2 days of growth with aeration and stirring, the contents were used to inoculate 15 L of liquid growth medium contained in a 30-L fermentor under a forced aeration of 0.3 ft^3/min. After 10–24 h of growth, the contents of the fermentor were used to inoculate 150 L of liquid growth medium in a 200-L fermentor. Growth was continued for 14–17 h under a forced aeration of 2 ft^3 of air/min. Determination of the dry weight of cells per liter of medium indicated that the growth of *Neurospora* was in late log phase at harvest. Mycelia were collected by filtration of the culture through several layers of cheesecloth and washed with distilled water; the excess water was removed by squeezing portions of the harvest in cheesecloth. The mycelia were induced for nitrate reductase by suspension in 150 L of induction medium in a second feremention under a forced aeration for 7 h and harvested as indicated earlier. Yields ranging from 5 to 7 kg (wet weight)/150 L of medium were obtained. The mycelia were frozen as small lumps in liquid nitrogen and subsequently stored at −15 °C.

Induction studies were performed on the mycelia from a 30-L fermentor. *Neurospora* was collected in early log phase by filtration through several layers of cheesecloth and subsequent washing with distilled water. It was then transferred to a Büchner funnel containing filter paper and stripped of excess water by suction. After a small portion of mycelia (designated "0-h mycelia") was frozen in liquid nitrogen, half of the remaining harvest was induced for nitrate reductase by suspension in 15 L of induction medium in a 30-L fermentor under a forced aeration of 0.3 ft^3/min. The other half of the mycelia was resuspended in NH_4^+ growth medium in a second 30-L fermentor under exactly the same conditions as the "induction" fermentor. Every hour 1 L of liquid culture was removed from both fermentors and filtered through a Büchner funnel. The mycelia retained on the filter paper were washed extensively with glass-distilled water and then immediately frozen in liquid nitrogen. The harvest time was kept to a minimum (<3 min). Mycelia were ground to a fine powder in a mortar and pestle precooled with liquid nitrogen. The powder was stored at −15 °C prior to extraction.

Analytical Methods. Iron and copper were determined by the method of Van De Bogart & Beinert (1967). Absorption spectra were measured at room temperature in cuvettes of 1-cm path length with a Cary Model 14 recording spectrophotometer, equipped with a 0–1.0 optical density slide-wire. Protein was determined by the microbiuret method with bovine serum albumin as the standard (Gornall et al., 1949).

Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed as described by Clarke (1964) at pH 8.1 (Tris–glycine) and 9.0 (Tris–borate). $NaDodSO_4$ slab gel electrophoresis was performed as described by Weber et al. (1972), and isoelectric focusing gels were run as described by Wrigley (1968).

$NaDodSO_4$ slab gel electrophoresis of cross-linked proteins (dimethyl suberimidate as the cross-linking reagent) was performed according to the procedure of Davies & Stark (1970), with the following modification. After the amidination with dimethyl suberimidate, the protein samples were denatured for 2 min at 100 °C in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol before electrophoresis. Twenty-five micrograms of each protein was applied to a 3.5% slab gel, and electrophoresis was performed at 110 mA for 6 h. Gels were stained with Coomassie blue according to Maurer (1971).

Assay of Catalase Activity. The rate of H_2O_2 disappearance was measured spectrophotometrically at 240 nm according to Bergmeyer (1974a). The reaction mixture contained, in a 1.0-mL total volume, 0.05 M potassium phosphate buffer (pH 6.8) containing 1.2 μL of 30% H_2O_2/mL (absorbance at 240 nm should be 0.500 ± 0.01) and an appropriate amount of enzyme. The assay was performed at 25 °C in quartz cuvettes of 1-cm path length. A unit of activity is defined as that amount of enzyme that catalyzes the destruction of 1 μmol of H_2O_2/min under the above assay conditions. Specific activity is defined as units per milligram of protein.

The determination of enzyme activity in the presence of various anions was measured by a modification of the above assay. The reaction mixture, composed of 0.1 mL of 10 mM anion and 0.8 mL of 0.1 M potassium phosphate buffer (pH 7.0), containing an appropriate amount of enzyme, was incubated for 2 min at 25 °C prior to the addition of 0.1 mL of H_2O_2 solution (12 μL of 30% H_2O_2 solution/mL of potassium phosphate buffer).

The determination of activity at various temperatures was performed with a glass-jacketed thermocouple immersed in the reaction mixture. The temperature was maintained with a jacketed cuvette compartment and a Lauda K-2/R circulating bath.

Amino Acid Composition. Amino acid analysis was performed on a Beckman Model 120 C according to single-column hydrolysate methodology.[2] Samples were hydrolyzed in 6 N HCl under vacuum for 24 and 48 h at 110 °C. An apoprotein sample was obtained from holoprotein by extraction of the heme with acetone containing 0.015 N HCl (Murphy et al., 1973). The precipitated apoprotein was collected by centrifugation, dissolved in 0.1 N NaOH, and hydrolyzed in the same manner as previously indicated for other samples. Cysteine and cystine were determined as cysteic acid after

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; cpm, counts per minute.

[2] Procedure used is found in Beckman A-TB-059A, Beckman Instrument Corp., Oct 1972.

performic acid oxidation (Moore, 1963). Tryptophan was estimated spectrophotometrically by the method of Edelhoch (1967) on an apoprotein sample obtained by dialyzing holoprotein for 2 days against a solution composed of hexamethylphosphoramide, *N*-methylformamide, and pyridine (7:2:1) and for 3 days against 6 M guanidine hydrochloride in water. This procedure yielded a heme-free protein solution.

Extinction Coefficient. A 0.5-mL sample of 7 mg/mL catalase was dialyzed against 0.1 M acetate buffer (pH 5.1) for 4 h, against 0.1 M NaCl solution for 4 h, and finally against six successive changes of 0.001 M NaCl solution, each lasting 6 h. The optical density of a 30-fold diluted sample of protein was determined at 400 and 280 nm. Triplicate 150-μL samples of protein and 0.001 M NaCl dialysate were placed in tared aluminum trays, and these were placed in a vacuum desiccator over NaOH. After evaporation, these trays were weighed every 6 h for 2 days. Heating the trays in vacuo to 100 °C with an infrared lamp for 2 h resulted in no additional weight loss. After constant weight was achieved, successive weighings were averaged and the difference between the weight of trays containing protein and dialysis solution was used to calculate the dry weight of the enzyme sample. The comparison of the dry weight of the enzyme sample to the absorbance obtained at 400 and 280 nm for the 30-fold diluted sample allowed the calculation of $E_{400}^{1\%}$ and $E_{280}^{1\%}$ of 8.86 and 11.6, respectively. Error in the weight of the protein samples due to Donnan equilibrium was minimized by dialysis at the isoelectric point of the protein (pH 5.1) before dialysis against NaCl solutions.[3]

Sedimentation Studies. Sedimentation velocity measurements were made at 50 740 rpm in a Beckman Model E ultracentrifuge by using Schlieren optics. Experiments were performed in single sector cells in an An-D rotor at 20 °C. Sedimentation coefficients were calculated as described by Schachman (1957).

Short-column sedimentation equilibrium experiments as described by Yphantis (1964) were performed in a Beckman Model E ultracentrifuge at 5 °C and 12 590 rpm by using Rayleigh interference optics. The results obtained with five fringes were averaged in constructing plots of $\log f$ vs. r^2. The molecular weight was determined from the slopes of these plots by the method of Yphantis (1964).

Immunization Procedures. Antisera were prepared by immunization of a male New Zealand rabbit with homogeneous *N. crassa* catalase. The catalase was dialyzed against a solution of 0.9% sodium chloride and 0.05 M potassium phosphate buffer, pH 7.4, designated "saline buffer", prior to injection. One milliliter of enzyme (2 mg/mL) was mixed with 1 mL of Freund's complete adjuvant and injected into the hind footpads and shoulder muscles of a single rabbit. Two weeks later the rabbit was given an intraperitoneal injection of 1 mL of enzyme (1.5 mg/mL) containing 4 drops of saturated alum, pH 7.2. Eight days after the second injection, 20 mL of blood was collected and allowed to clot and the serum was decanted and stored at 77 K. Preimmune serum had been withdrawn from the rabbit before any injection of catalase.

Immunodiffusion. Double diffusion in agar was performed by the method of Ouchterlony (1949).

Labeling N. crassa Catalase with ^{125}I. All dilutions were performed in 0.05 M sodium phosphate, pH 7.5. Homogeneous *N. crassa* catalase was labeled with ^{125}I by the chloramine-T method of McConahey & Dixon (1966). The following reagents were mixed: 0.5 mg of *N. crassa* catalase (1 mg/mL), 1 mCi of $Na^{125}I$ (0.1 mL), and 0.1 mL of chloramine-T (10 mg/mL). After the mixture was stirred for 5 min at 0 °C, the reaction was stopped by the addition of 0.1 mL of sodium metabisulfate (11 mg/mL) followed by 0.2 mL of bovine serum albumin (25 mg/mL) and 0.2 mL of KI (10 mg/mL). The ^{125}I-labeled enzyme was dialyzed three times against a solution composed of 0.9% sodium chloride, 0.1 M sodium borate, pH 8.0, and 0.02 M potassium iodide and then overnight against a solution containing 0.9% sodium chloride and 0.1 M sodium borate, pH 8.0. It was stored at 0 °C. The success of the radiolabeling experiment was confirmed by an autoradiogram of a dried slab gel containing enzyme subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis.

Radioimmunoassay. Radioimmunoassay was performed by the two-antibody method of Morgan & Lazarow (1963). Disposable borosilicate glass culture tubes (10 × 75 mm) were siliconized before use. A typical assay involved incubating 0.1 mL of antiserum (1:1000), 0–100 ng of unlabeled catalase (or crude extract containing an equivalent amount of catalase), and 0.150 mL of 0.1 M sodium borate, pH 8.0, containing 5% bovine serum albumin in a tube for 12 h at 0 °C. Ten nanograms of [^{125}I]catalase was then added, and the tube was incubated for 12 h more at 0 °C. The addition of 0.05 mL of normal rabbit serum (1:2) and 0.2 mL of goat anti-rabbit IgG serum was followed further by incubation for 12 h at 0 °C. The radioactivity of the sample was measured, the supernatant was removed by centrifugation, and the precipitate was washed with saline buffer followed by centrifugation. The supernatant was removed and the precipitate washed and centrifuged a second time. After the supernatant was removed, the radioactivity of the precipitate was determined in a Nuclear Chicago γ counter optimized for ^{125}I. The percent of ^{125}I precipitated = cpm (ppt)/cpm (sample) × 100.

Results

Purification of Catalase. Mycelia were ground for 3 min in liquid nitrogen in a 4-L stainless steel Waring blender operated at low speed. The powder was stored at −15 °C prior to extraction. All purification steps were performed at 0–4 °C.

Step 1. Extraction. Eight-hundred grams of powdered mycelia was suspended in 4 L of a solution of 0.1 M potassium phosphate, pH 7.3, 10^{-3} M phenylmethanesulfonyl fluoride, 10^{-3} M dithiothreitol, and 5×10^{-4} M EDTA ("buffer A").[4] After being stirred for 1 h, the suspension was centrifuged for 20 min at 20000*g* to yield a pellet, which was discarded, and a supernatant solution (crude extract).

Step 2. First $(NH_4)_2SO_4$ Fractionation. $(NH_4)_2SO_4$ (291 g/L of crude extract) was added, with stirring, to give a final concentration of 50% saturation. After an additional 30 min of stirring, the mixture was centrifuged for 20 min at 20000*g* to yield a supernatant, which was discarded, and a precipitate. The precipitate was dissolved in 100 mL of buffer A and dialyzed against buffer A for 18 h. The 50% $(NH_4)_2SO_4$ precipitate fraction from each batch was stored at −196 °C until 10 batches were processed to this stage.

Step 3. pH 5.0 Treatment. The 10 50% $(NH_4)_2SO_4$ precipitate fractions were combined and adjusted to pH 5.0

[3] Samples of dialyzed protein solution and dialysate were analyzed for Na^+ by atomic absorption spectrometry; this indicated that less than 2% of the weight of the sample could be attributed to counterions.

[4] Phenylmethanesulfonyl fluoride and dithiothreitol were included to stabilize the nitrate reductase which was isolated concurrently. The omission of the phenylmethanesulfonyl fluoride decreased yields of the catalase by four- to fivefold; the omission of dithiothreitol had *no* effect on yields, specific activity, or the electronic spectra, as checked in a parallel isolation carried through the fourth step of purification.

Table I: Purification of *N. crassa* Catalase

	vol (mL)	total protein (mg)	total activity (units)	specific activity (units/mg)	% recovery
crude extract	39200	89360	2.29×10^6	25.6	100
50% $(NH_4)_2SO_4$ ppt pH 5 treatment	1220	37400	1.49×10^6	39.8	65
35-50% $(NH_4)_2SO_4$ ppt	114	1208	6.16×10^5	510	27
pooled DEAE eluates	75	110	4.22×10^5	3850	18
Sephadex G-200 eluate	80	32	3.26×10^5	10200	14

by the dropwise addition, with constant stirring, of 1 M citric acid. The mixture was centrifuged for 20 min at 20000*g*, and the precipitate was discarded; the supernatant solution was designated the pH 5.0 supernatant fraction.

Step 4. Second $(NH_4)_2SO_4$ Fractionation. Solid $(NH_4)_2SO_4$, 194 g/L, was slowly added, with stirring, to the pH 5.0 supernatant fraction to give a final concentration of 35% saturation. After 1 h, the precipitate was removed by centrifugation and additional 97 g/L $(NH_4)_2SO_4$ was added to the supernatant solution to give a final concentration of 50% saturation. The precipitate collected after 1 h of stirring and subsequent centrifugation (20 min at 20000 *g*) was dissolved in 100 mL of a solution of 0.01 M potassium phosphate, pH 7.3, 10^{-3} M phenylmethanesulfonyl fluoride, 10^{-3} M dithiothreitol, and 4×10^{-4} M EDTA ("buffer B") and dialyzed against buffer B for 18 h.

Step 5. Cellulose Column Chromatography. The dialyzed 35–50% $(NH_4)_2SO_4$ precipitate fraction was applied to a DEAE-cellulose column (5 × 12 cm) previously equilibrated with buffer B. After being washed with 500 mL of buffer B, the column was eluted with a solution of 0.03 M potassium phosphate, pH 7.3, 10^{-3} M phenylmethanesulfonyl fluoride, 10^{-3} M dithiothreitol, and 5×10^{-4} M EDTA and 5-mL fractions were collected. The optical densities at 400 and 280 nm were determined on these fractions, and those having a purity index (ratio of absorbance at 400 and 280 nm) greater than 0.3 were combined to give the DEAE-cellulose eluate. The eluate was reduced in volume to 2 mL in an Amicon ultrafiltration cell by using a PM 30 membrane.

Step 6. Sephadex Column Chromatography. The concentrated DEAE-cellulose eluate was applied to a Sephadex G-200 column (2.5 × 90 cm) equilibrated with buffer A. The gel filtration, by using an upward flow, was at a rate of 10–12 mL/h; 2-mL fractions were collected. For a typical column run, a 400-nm absorbance peak appeared together with a 280-nm absorbance peak in the elution profile. A nearly constant 400/280-nm absorbance ratio was observed throughout the peak. Fractions of purity index equal to or greater than 0.75 were pooled and reduced, with an Amicon concentrator, to a volume of 2 mL containing 30–40 mg of protein. The purity index of enzyme obtained from a number of purifications varied between 0.73 and 0.80. Side fractions with lower purity indices were also pooled and concentrated for subsequent rechromatography or for experiments not requiring homogeneous protein.

From 8 kg of *N. crassa* (Table I) we obtained 32 mg with a total purification of 400-fold and a 14% yield of activity (with respect to the crude extract). The enzyme was found to be stable at 4 °C; the activity was unchanged after 1 month. It could also be stored at 77 K indefinitely without loss of enzymatic activity.

Purity of Enzyme. Rechromatography of Sephadex G-200 purified enzyme yielded a single peak of protein having a constant purity index throughout the peak. The enzyme exhibited a single protein band upon electrophoresis on 5% polyacrylamide gels at pH 8.1 and 9, after being stained with Coomassie brilliant blue. In unstained gels, a green band, due to the heme prosthetic group, was visible at the position corresponding to the stained band. Gels were also subjected to a heme stain (Baur, 1963) and a catalase stain (Gregory & Fridovich, 1974), and it was found that the protein band contained both the enzymatic activity and the heme.

The purified enzyme gave a single, symmetrical peak of protein when examined in the ultracentrifuge with Schlieren optics at 5 mg/mL protein concentration. The sedimentation coefficient, $s^{0.5}_{20,w}$, was found to be 12.3 S from the average of three ultracentrifuge runs at protein concentrations of 5 mg/mL.

Catalytic Properties. The "catalatic" activity of different preparations of enzyme varied from 9000 to 12000 units/mg. These preparations had purity indices ranging from 0.73 to 0.80. A plot of initial rate vs. enzyme concentration was found to be linear, and from this the standard velocity constant, k_0, was calculated to be 4.57×10^6 M^{-1} s^{-1}.

The enzyme was found to be inactive in a peroxidase assay (Bergmeyer, 1974b) by using guiacol (≤1 unit/mg).

pH Dependence. We found only a small effect of pH and type of buffer on the enzymatic activity. Below pH 4 the enzyme was inactivated within seconds of mixing as indicated by a rapid decrease in the initial rate of disappearance of substrate. A plateau in activity was observed over the pH range of 4–10 with slightly higher activities in the basic region. The type of buffer was observed to have a detectable effect on the activity, citrate consistently yielding a slightly lower value than phosphate at the same pH.

Temperature Dependence. The energy of activation, E_a, for the enzyme-catalyzed reaction was found to be 7200 ± 400 cal/mol from a plot of log k_0 vs. $1/T$.

Enzyme Inhibitors. The inhibitory effect of KCN, NaN_3, NaF, Na_2SO_3, KNO_2, and KNO_3 on enzyme activity was studied; 1 mM KCN and 1 mM NaN_3 were found to inhibit enzymatic activity 97 and 98%, respectively. KNO_2 and KNO_3 were moderate inhibitors at final concentrations of 1 mM, inhibiting 37 and 14%, respectively. The other anions did not give appreciable inhibition of the enzymatic activity.

Initial Rate vs. Concentration. A Lineweaver–Burk plot of $1/v$ vs. $1/[H_2O_2]$ yielded a Michaelis constant, K_m, of 0.025 M. In contrast to early reports of curved double-reciprocal plots, which were shown by Bonnichsen et al. (1947) to be attributable to enzyme inactivation, we found a linear plot when using H_2O_2 concentrations between 0.013 and 0.2 M. The present catalase shows no evidence of substrate inactivation in these experiments.

Spectral Properties of the Enzyme. The absorption spectrum of the green catalase is shown in Figure 1. It has maxima at 400 and 590 nm, with A_{400}/A_{590} equal to 4.89, and a weak maximum at 712 nm. The extinction coefficient of the enzyme was determined by measuring the dry weight of a catalase solution of known absorbance. The $E_{400}^{1\%}$ of 8.86 yields a molar extinction coefficient of 2.8×10^5 M^{-1} cm^{-1} at

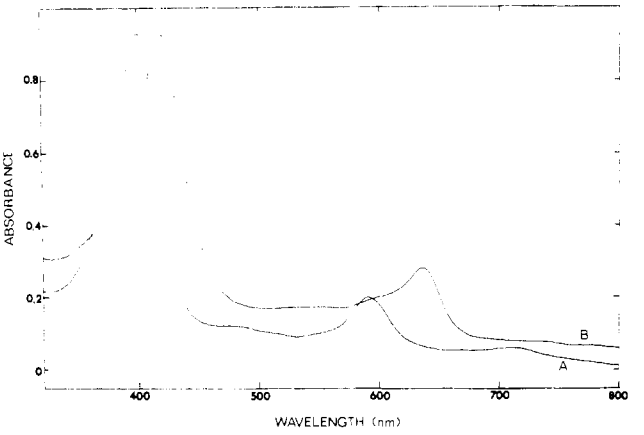

FIGURE 1: Electronic spectra of *N. crassa* catalase and its complex with KCN. Spectra of enzyme in buffer A were recorded vs. a blank containing only buffer. (A) 3.1 μM enzyme; (B) 4.0 μM enzyme in the presence of 2 mM KCN.

400 nm by using 320000 as the molecular weight (vide infra). It was found that protein concentrations measured by the microbiuret method were 35% higher than those determined by sample absorbance at 400 nm and $E_{400}^{1\%}$. Microbiuret determinations on two samples used in dry weight experiments also resulted in protein weights 25% higher than the dry weight values. This is most likely due to error in the biuret determination as a result of interference by the prosthetic group of the enzyme. For this reason, for all experiments in this paper involving homogeneous enzyme the concentrations were determined from the absorbance at 400 nm.

The enzyme is remarkably resistant to reduction, as has been reported for other catalases (Deisseroth & Dounce, 1970). Treatment with sodium dithionite, NADPH plus FAD, or sodium borohydride plus methyl viologen does not affect the absorption spectrum. Furthermore, the inability of sodium dithionite plus CO to alter the absorption spectrum suggests that the enzyme contains a ferric heme which is not reduced by sodium dithionite. Treatment of the enzyme with KCN, as shown in Figure 1, shifts the maxima in the absorption spectrum to 420 and 637 nm. The ready formation of a cyanide complex is further evidence of the presence of ferric heme in the enzyme.

The enzyme in pyridine–NaOH–dithionite, under conditions customarily used to determine pyridine hemochromes (Appleby & Morton, 1959), gives the spectrum shown in Figure 2. Three major absorbance bands appear at maxima of 602, 565, and 429 nm; three lesser bands appear at maxima of 527, 490, and 412 nm. Noteworthy is the unusually high ratio of the α-band absorbance (602 nm) to the Soret band absorbance (429 nm). We are aware of no reported spectrum of a pyridine hemochrome exhibiting these properties.

Amino Acid Composition. Table II presents the results of an amino acid analysis of catalase. The yields of hydrolyzed amino acids in all experiments were greater than 93%. Acid–acetone-precipitated protein was found to have the same composition as holoprotein. A comparison of the composition of *N. crassa* catalase with that of bovine liver catalase (Deisseroth & Dounce, 1970) indicated significant differences in residue content only for alanine, isoleucine, and proline. The partial specific volumes calculated from the composition data by the method of Cohn & Edsall (1943) yield values of 0.729 and 0.712 $cm^3\ g^{-1}$ for the *N. crassa* catalase and bovine liver catalase, respectively. The calculated value of bovine liver catalase differs significantly from the experimentally determined value which lies between 0.73 and 0.74 (Deisseroth & Dounce, 1970). Analysis for carbohydrate by the phenol–

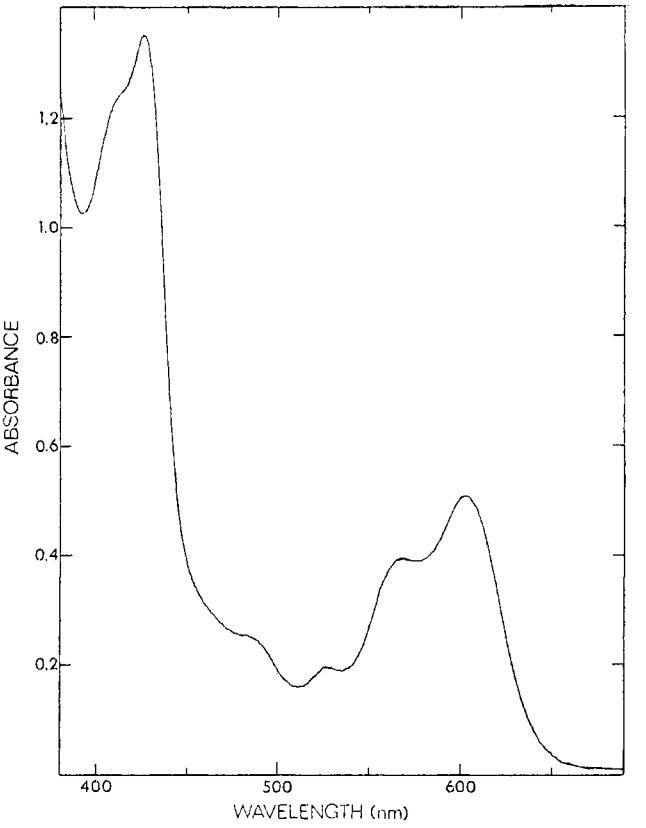

FIGURE 2: Electronic spectrum of pyridine hemochrome of *N. crassa* catalase. Final concentrations of components in buffer A are 8.0 μM enzyme, 0.05 N KOH, 2.5 M pyridine, and a few crystals of sodium dithionite.

Table II: Amino Acid Analysis of *N. crassa* Catalase[a]

amino acid	% of total residues	residues/ 320 000 mol wt
alanine	10.39	299
arginine	5.68	163
aspartic acid	10.77	310
half-cystine[b]	0.55	16
glutamic acid	10.91	314
glycine	7.13	205
histidine	3.54	102
isoleucine	5.50	158
leucine	5.86	169
lysine	5.55	160
methionine	1.98	57
phenylalanine	6.16	177
proline	5.50	158
serine	5.09	146
threonine	5.37	154
tryptophan[c]	0.99	28
tyrosine	2.47	71
valine	6.57	189

[a] Each value is the average of 24-h, 48-h, and apoprotein hydrolysates, except where noted. [b] Determined as cysteic acid following performic acid oxidation. [c] Determined on apoprotein by the spectrophotometric technique as described in the text.

sulfuric acid method (Ashwell, 1966) indicated that the *Neurospora* catalase contained 2–4% carbohydrate, based on glucose as standard. It seems probable that the partial specific volume for *N. crassa* catalase will also be found to be greater than the value calculated from its composition.

Molecular Weight. The molecular weight of *N. crassa* catalase was determined by the following three methods: sedimentation equilibrium by using the high-speed technique of Yphantis, Sephadex G-200 gel filtration, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Table III: Comparison of Molecular Weight of *N. crassa* Catalase Determined by Various Methods[a]

method	mol wt	
	$\bar{\nu} = 0.73$	$\bar{\nu} = 0.75$
sedimentation equilibrium	288 000	314 000
Sephadex G-200 gel filtration	314 000	340 000
$NaDodSO_4$-polyacrylamide gel electrophoresis	320 000–340 000[b]	

[a] Details of the experiments and calculations are presented in the text. [b] Determination was independent of the partial specific volume, $\bar{\nu}$.

Sedimentation Equilibrium. Enzyme solutions obtained from three purifications were examined at 12 590 rpm in a Beckman Model E ultracentrifuge. In all cases, a plot of log f vs. r^2 was linear. The average molecular weight calculated from these plots was 288 000 when a partial specific volume, $\bar{\nu}$, of 0.73 was used and 314 000 when a $\bar{\nu} = 0.75$ was used in the calculation.

Gel Filtration. Sephadex G-200 gel filtration chromatography as described by Ackers (1964) was used to obtain an average value of the Stokes radius for the *N. crassa* catalase of 59 Å. Bovine serum albumin, bovine liver catalase, and xanthine oxidase were used as standards on the Sephadex column. Siegel & Monty (1966) have pointed out that from the Stokes radius, a, and the sedimentation coefficient, s, the molecular weight of a macromolecule can be obtained by $M_r = (6\pi\eta Nas)/(1 - \bar{\nu}\rho)$, where M_r = molecular weight, η = viscosity of medium, N = Avogadro's number, $\bar{\nu}$ = partial specific volume, and ρ = density of medium. The molecular weight for *N. crassa* catalase was calculated to be 314 000 and 340 000 when partial specific volumes of 0.73 and 0.75, respectively, were used in the calculation.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. N. crassa catalase was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis after incubation in a solution of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 2 min at 100 °C. Gels exhibited one intense band accompanied by three very weak bands. A similar phenomenon has been noticed for other catalases by Seah & Kaplan (1973) when proteins were subjected to gel electrophoresis under denaturing conditions. The major species has a molecular weight of 80 000–85 000 as determined by interpolation from the plots of migration distances vs. molecular weights of the marker proteins bovine serum albumin, beef liver catalase, carboxypeptidase, glutamate dehydrogenase, and phosphorylase *a*. This suggests a molecular weight of 320 000–340 000 for the native tetrameric protein (see below). Table III lists the calculated molecular weights of catalase and the methods used to obtain it. Averaging the molecular weights obtained with the three methods yields a value of 320 000 for *N. crassa* catalase.

Subunit Structure. To obtain insight into their subunit composition, *N. crassa* catalase and bovine liver catalase were incubated with the cross-linking reagent dimethyl suberimidate prior to denaturation and electrophoresis. In both cases four bands were observed in the gels. Under appropriate conditions (lowered protein concentration) cross-linking between oligomers was found to be insignificant. Davies & Stark (1970) have shown that for oligomeric proteins composed of identical protomers, the correct number of protomers comprising an oligomer is clearly indicated by the number of principal bands detected. The evidence suggests that the *N. crassa* catalase is a tetrameric protein, as is bovine liver catalase. A plot of migration distance vs. the known molecular weights of the

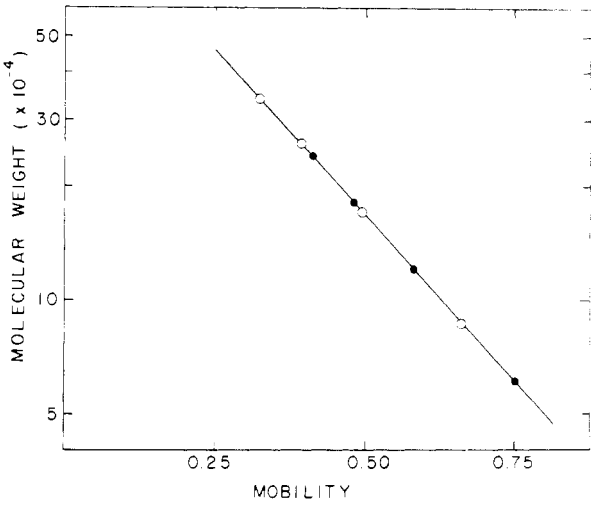

FIGURE 3: Determination of the molecular weight of the covalently linked species of *N. crassa* catalase. (●) Mobilities of the monomer, dimer, trimer, and tetramer of bovine liver catalase; (○) mobilities of the monomer, dimer, trimer, and tetramer of *N. crassa* catalase.

covalently linked species of bovine liver catalase is shown in Figure 3. From this plot the molecular weights of the covalently linked species of *N. crassa* catalase were found to be multiples of a protomer molecular weight of 85 000. The native tetramer thus has a molecular weight of 340 000 by this method, in agreement with the results reported above.

Prosthetic Group. Analysis of three enzyme samples for iron by the procedure of Van De Bogart & Beinert (1967) gave 3.4 g-atom of total iron/mol of enzyme when 320 000 was used as the molecular weight of catalase. Dividing the molar extinction coefficient determined at 400 nm by this value gives a determination of the extinction coefficient (per g-atom of iron) of 8.2×10^4 g-atom^{-1} cm^{-1}. On the assumption of 1 heme/iron, this value is comparable with molar extinction coefficients commonly cited for heme complexes.

Analysis of two enzyme samples for copper indicated less than 0.2 g-atom of total copper/mol of enzyme.

Isoelectric Focusing. Enzyme samples were electrofocused according to Wrigley (1968) in gels containing a pH 3–9 gradient. The most recent preparations focus as a single band, while some earlier preparations yielded a set of five bands contained in a 0.1 pH unit interval. In either case the isoelectric point of the protein thus determined is 5.0 ± 0.1. The origin of the earlier appearance of multiple bands during isoelectric focusing is not known. It is not due to physical alteration of the protein during gel formation as these bands were observed whether or not the enzyme was present in the acrylamide solution during polymerization.

Induction Experiments. N. crassa mycelia induced for different lengths of time in a 30-L stir jar containing nitrate induction medium were converted into crude extracts and assayed. The results confirmed the induction data obtained by previous investigators (Subramanian et al., 1968). Nitrate reductase is found to be maximally induced after 3 h and catalase after 8–10 h. The increase in catalatic activity of mycelia transferred to fresh NH_4^+-supplemented growth medium was slight. To check the identity of the induced catalase with that of the enzyme reported in this communication, antiserum against homogeneous *N. crassa* catalase was prepared (see Materials and Methods). Ouchterlony double-diffusion experiments confirmed the specificity of the antiserum for *Neurospora* catalase; bovine liver catalase was not cross-reactive, nor did preimmune serum give a precipitin band with catalase. Antiserum was used with ^{125}I-labeled *N.*

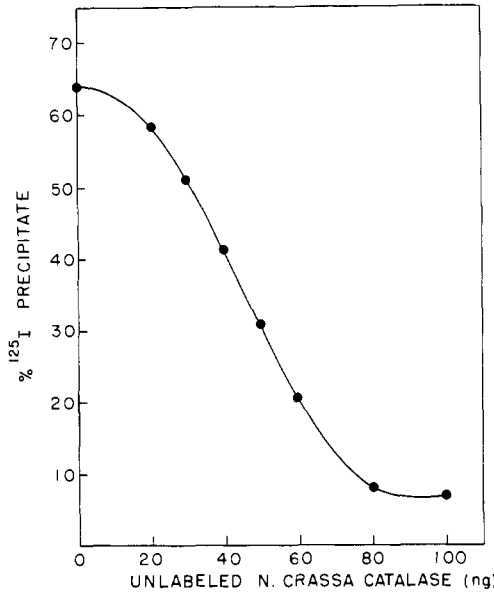

FIGURE 4: Assay curve for *N. crassa* catalase. The added unlabeled catalase was contained in 0.1 M sodium borate, pH 8.0, and 5% bovine serum albumin. The amount of tracer, dilution of the first antibody, and the time of incubation are given in Materials and Methods.

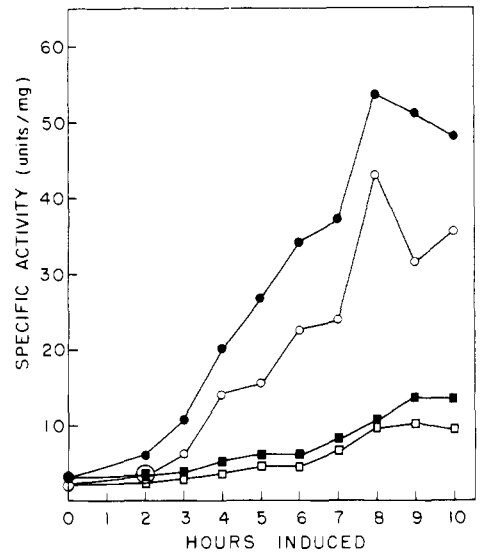

FIGURE 5: Induction of *N. crassa* catalase. For experimental details, see the text. (●) Catalase activity induced after transfer to nitrate-induction medium; (○) catalase level found by radioimmunoassay on mycelia transferred to nitrate-induction medium; (■) catalase activity induced after transfer to fresh NH_4^+ growth medium; (□) catalase level found by radioimmunoassay on mycelia transferred to fresh NH_4^+ growth medium. Conversions of catalase levels found by radioimmunoassay to equivalent enzymatic activities are described in the text.

crassa catalase in a radioimmunoassay of crude extracts from induced mycelia. A standard curve was obtained with varying amounts of unlabeled catalase. As shown in Figure 4, an increase in the amount of unlabeled catalase from 0 to 100 ng decreased the percent of [^{125}I]catalase present in the precipitate from 64 to 8%. Substitution of [^{125}I]IgG for [^{125}I]catalase in the assay resulted in less than 2% of [^{125}I]IgG in the precipitate; this confirmed the specificity of [^{125}I]catalase for antiserum used in these assays. Substitution of other proteins including bovine serum albumin, bovine liver catalase, and *N. crassa* nitrate reductase for the unlabeled *N. crassa* catalase did not result in decreases in the percent of [^{125}I]-catalase present in the precipitate. The specificity of this antiserum for *N. crassa* catalase thus allowed an estimation of the very small amount of antigen present in crude extracts of mycelia induced for different lengths of time in both nitrate-supplemented induction and NH_4^+-supplemented growth medium. Figure 5 presents a comparison of the "catalatic" activity measured on crude extracts and the *N. crassa* catalase level found by radioimmunoassay. Conversion of the amount of catalase in an extract, as determined by radioimmunoassay, to its equivalent enzymatic activity was made on the basis of a specific activity of 10 000 units/mg. Mycelia resuspended in ammonia-containing growth medium showed a much smaller increase in catalase activity as compared to mycelia induced in nitrate medium. Figure 5 clearly shows that the catalase responsible for most of the increase in enzymatic levels in crude extracts is the novel catalase reported in this paper. It also shows conclusively that this catalase is present in cells before induction, although at a much lower level. Radioimmunoassay on a crude extract that had additional *N. crassa* catalase added to it gave a value that was the sum of the values obtained on the extract and *N. crassa* catalase individually; this indicated that no agent was present which could interfere with the immunochemical estimation of the enzyme in crude extracts.

Discussion

In the course of purifying nitrate reductase from *N. crassa*, we observed a time-dependent inactivation of the enzyme. This could be prevented by the addition of excess bovine serum albumin or a protease inhibitor (Jacob, 1976). When a large-scale preparation using 1 mM phenylmethanesulfonyl fluoride was undertaken, a bright green side fraction was observed. This color proved to be due to the novel catalase reported here. Isolations performed in the absence of the protease inhibitor gave protein with identical spectral and enzymatic properties, though in much lower yields, indicating that the enzyme is not an artifact of the isolation procedure, but rather is extremely sensitive to proteases.

Catalase is widespread in nature, having been found in all aerobic organisms studied to date. Although there is extensive literature concerned with the subject, most of the work has been performed on the enzyme obtained from mammalian and bacterial sources where it is present in highest concentration. The enzymes from different species have been found to be quite similar in physical and structural properties. Except for an enzyme from baker's yeast (Seah et al., 1973), they all have molecular weights between 225 000 and 270 000. The amino acid analyses, sedimentation coefficients, subunit structures, enzyme activities, and spectral characteristics of the enzymes are all remarkably similar (Deisseroth & Dounce, 1970). In particular, they have all been found to contain protohemin as the prosthetic group.

We have isolated a catalase from *N. crassa* which, although in certain ways a typical catalase, is significantly different with respect to molecular weight and the nature of the prosthetic group. The identification of this enzyme as a catalase is based on the following lines of evidence. (a) The "catalatic" activity, although not as high as typical catalases, is still orders of magnitude larger than that of other hemoproteins. (b) The resistance to reduction by dithionite or other reducing agents is in agreement with this unique property observed for native catalases from every source studied to date. (c) The enzyme exists as a tetramer composed of apparently equivalent subunits as do other catalases. (d) The amino acid analysis, although different in certain respects, generally reflects the composition of known catalases. Particularly noteworthy is the finding of 16 cysteinyl residues/enzyme molecule, in agreement with the

value for beef liver catalase (Schroeder et al., 1969).

The enzyme is different from other catalases in a number of ways, however. Its molecular weight of 320 000 is substantially higher than that of any previously reported catalase; i.e., its monomer molecular weight of 80 000 is about one-third larger than that for typical catalases. The Stokes radius is 59 Å as compared to the reported value of 52 Å for beef liver catalase. Second, the electronic spectrum of *N. crassa* catalase is not similar to that of any isolated catalase nor is its pyridine hemochrome like that of any known example. This was an initial indication that, unlike all other catalases, this enzyme does not contain a protohematin. The extraction and identification of this group as a *chlorin* will be reported in a subsequent paper in this series (Jacob & Orme-Johnson, 1979).

A comparison of the kinetic properties of this enzyme with the known data for other catalases also reveals some general differences. The enzyme is a rather poor catalase at 25 °C as compared to the mammalian and bacterial enzymes. The velocity constant, k_0, of 4.57×10^6 M^{-1} s^{-1} is approximately sixfold smaller than the k_0 for horse liver catalase. The activation energies of 7200 and 1700 cal/mol for *N. crassa* catalase and horse blood catalase, respectively, are a related important difference. On the other hand, the *N. crassa* enzyme is similar to other catalases in its pH profile and inhibition by typical ferric heme ligands.

The iron content of the *N. crassa* enzyme was found to be 3.4 g-atom/mol of enzyme. On the assumption that the enzyme has one heme binding site per subunit, this is an indication that, as we have prepared the enzyme, not all subunits contain a heme prosthetic group. This phenomenon is not without precedent in the catalase field. Beef liver catalase isolated by traditional procedures is found to contain ~3 heme groups/molecule. This is due to an enzyme present in the beef supernatant fraction capable of converting heme to biliverdin. A similar process might be responsible for the <4 mol of heme/mol of *N. crassa* catalase. Greenfield & Price (1956) first succeeded in obtaining a 4-hematin catalase from rat liver by a procedure that eliminated enzymatic degradation of the heme. In addition, catalase from erythrocytes that lack the degradative enzyme is known to contain 4 hematins/molecule. The specific activities (a reflection of the heme content) determined on various preparations of *N. crassa* catalase may for similar reasons be lower than the maximum value expected for the native unaltered enzyme.

The result of the radioimmunoassays of crude extracts clearly indicates that the enzyme responsible for almost all of the catalatic activity in crude extracts is the same as the enzyme purified to homogeneity. This finding allows us to reject some alternative hypotheses for the enhanced catalatic activity of nitrate-induced cells. Although unlikely, a large increase in the relative amount of hemoproteins might have provided the increased activity. Alternatively, the increase could have been due to an entirely different catalase present in the cells. Between 65 and 80% of the catalatic activity found in crude extracts from induced cells could be attributed to the new catalase as measured by radioimmunoassay. The new catalase is present at ~8% of induced levels before induction and increases to ~16% of these levels after transfer to fresh ammonia-supplemented growth medium (noninducing conditions).

The physiological role of catalases in most organisms is still in doubt (Deisseroth & Dounce, 1970). The situation may be somewhat clearer in *Neurospora*. Nitrite reductase is not maximally induced until the sixth hour after transfer to nitrate induction medium (Garrett, 1972). Vega et al. (1975) have shown that the FAD-dependent NADPH inactivation of *N. crassa* nitrite reductase probably occurs as a result of the formation of H_2O_2. The inactivation was shown to be prevented by addition of catalase to incubation mixtures of nitrite reductase, FAD, and NADPH. Thus, it may be that the physiological role of the inducible *N. crassa* catalase is to provide protection for nitrite reductase against inactivation by peroxide. In any event, the present results lead us to suggest that the nitrate induction of catalase in *N. crassa* leads to the synthesis of a molecule which has a novel prosthetic group and which is physically and antigenically distinct from classical eucaryotic catalases (Bonnichsen, 1947; Deutsch & Seabra, 1955). This means that the presence of nitrate in the growth medium of this organism initiates a series of events that includes the synthesis of nitrate reductase, the synthesis of catalase apoprotein, and (it seems likely) the synthesis of the enzymes responsible for the formation of the curious prosthetic group.

Acknowledgments

We are deeply indebted to Professor Richard Hong and Janet Fallon for teaching us the double-antibody radioimmunoassay method and for many helpful discussions. Thanks are also due to Dr. Frances Webb for critical assistance during the sedimentation studies and to Dr. Thomas Rademacher for providing us with the immunization procedure. We thank the Fungal Genetics Stock Collection for spores of the strain used.

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Catalase of *Neurospora crassa*. 2. Electron Paramagnetic Resonance and Chemical Properties of the Prosthetic Group†

Gary S. Jacob‡ and W. H. Orme-Johnson*

ABSTRACT: The inducible catalase from *Neurospora crassa* 5297a when examined at 13 K exhibits electron paramagnetic resonance (9 and 34 GHz) with two sets of principal features at $g = 6.33$, 5.48, and 1.99 and 6.62, 5.18, and 1.99. Minor amounts of other resonances are seen near $g = 6$. Sodium formate converts these to a single species with features at $g = 6.51$, 5.34, and 1.99. This is consistent with the presence of two or more high-spin ferric porphyrin complexes in the untreated enzyme. The azide complex of this catalase yielded a single well-defined set of resonances at $g = 2.50$, 2.26, and 1.87, indicating that the high-spin forms of the enzyme are converted to a single low-spin compound under these circumstances. Integration of this signal indicated that at least 90% of the heme iron in the native enzyme is present as high-spin ferric complexes. In some preparations of the untreated enzyme small amplitude resonances at $g = 2.42$, 2.30, and 1.89 were observed; these appear to arise from inactive forms of the enzyme. Extraction of the heme group and subsequent removal of iron and conversion to the methyl ester yield a porphin with absorbance maxima at 653, 599, 533, 500, and 399 nm in a ratio of 2.92:0.33:0.33:1.00:12.9. The spectrum closely resembles that of the chlorin prepared by J. Barrett [(1956) *Biochem. J. 64*, 626] from cytochrome a_2 of *Escherichia coli* and is distinct from any reported porphyrin spectrum. The chromatographic properties of the catalase chlorin suggest the presence of approximately four carboxyl groups, whereas chlorin a_2 is reported to have two carboxyl groups. We were unable to reoxidize the presumed chlorin to a porphyrin by using 2,3-dichloro-5,6-dicyanobenzoquinone, a phenomenon observed previously with sterically hindered chlorins and phorbins [Woodward, R. B. (1961) *Pure Appl. Chem. 2*, 383]. Nonetheless, the evidence presented strongly suggests that, unlike previously studied catalases, the inducible catalase of *N. crassa* has as its prosthetic group a polar high-spin ferric dihydroporphyrin complex.

Catalase, the enzyme that converts hydrogen peroxide to oxygen and water, was first shown by Stern (1936) to contain protohematin as the prosthetic group of the enzyme. Since then, catalases from every organism studied have been found to contain this type of heme. Included are the enzymes from a diverse number of sources such as mammalian liver and erythrocytes (Stern, 1936; Herbert & Pinsent, 1948b; Bonnichsen, 1947; Nagahisa, 1962; Higashi et al., 1966), bacteria (Herbert & Pinsent, 1948a; Clayton, 1959), and yeast (Seah & Kaplan, 1973; Fujii & Tonomura, 1975). Catalases are known to be comprised of four subunits (Tanford & Lovrien, 1962; Schroeder et al., 1969; Kiselev et al., 1968) and contain four hematin prosthetic groups per molecule. Except for the enzyme from yeast, all catalases have molecular weights in the range of 225 000–270 000.

In the first paper of this series we reported a catalase isolated from *Neurospora crassa* that is different from other catalases in that the molecular weight is higher (320 000) and the enzyme contains a novel heme. These last results were based on spectrophotometric analyses of the native enzyme and of the pyridine hemochrome derived from it. The native enzyme is green in color and exhibits two absorption bands with maxima at 400 and 590 nm ($A_{400}/A_{590} = 4.89$). The spectrum of the pyridine hemochrome was also found to be unique. The

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0006-2960/79/0418-2975$01.00/0