THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 254, No. 10, Issue of May 25, pp. 4245–4252, 1979
Printed in U.S.A.

Purification of the *o*-Dianisidine Peroxidase from *Escherichia coli* B

PHYSICOCHEMICAL CHARACTERIZATION AND ANALYSIS OF ITS DUAL CATALATIC AND PEROXIDATIC ACTIVITIES*

(Received for publication, October 23, 1978)

Al Claiborne and Irwin Fridovich

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Extracts of aerobically grown *Escherichia coli* B exhibit both catalase and dianisidine peroxidase activities. Polyacrylamide gel electrophoresis demonstrates two distinct catalases which have been designated hydroperoxidases I and II (HP-I and HP-II) in order of increasing anodic mobility. HP-I has been purified to essential homogeneity and found to be composed of four subunits of equal size. Its molecular weight is 337,000, and it contains two molecules of protoheme IX per tetramer. Its amino acid composition is unusual, for so large a protein, in lacking half-cystine. HP-I is a very efficient catalase with an activity optimum at pH 7.5, a K_m for H_2O_2 of 3.9 mM, and a turnover number of 9.8×10^5 per min. It is also a broad specificity peroxidase capable of acting upon dianisidine, guaiacol, *p*-phenylenediamine, and pyrogallol. Dianisidine acted as a powerful reversible inhibitor of the catalatic activity of HP-I and as a suicide substrate when HP-I functioned in its peroxidatic mode.

Catalases and peroxidases, collectively referred to as hydroperoxidases (1), seem to exhibit a fundamental difference. Catalatic action thus appears to involve electron pair transfers, while single electron transfers are characteristic of the peroxidatic mechanism (2–4). Hydroperoxidases from eukaryotic sources have been extensively studied (5–10), but the corresponding bacterial enzymes have attracted comparatively little attention. Two bacterial catalases, one from *Micrococcus lysodeikticus* (11) and the other from *Rhodopseudomonas spheroides* (12), have been isolated. These were similar to the mammalian enzyme in being 240,000-dalton homotetrameric hemoproteins, but did differ somewhat in kinetic behavior (13). Two bacterial peroxidases, one a ferrocytochrome *c* peroxidase from *Pseudomonas fluorescens* (14) and the other an NADH peroxidase from *Streptococcus faecalis* (15), have also been isolated, the former of these being a hemoprotein and the latter a flavoprotein.

In 1974 Gregory and Fridovich (16), using a newly described activity stain, noted distinct catalase and peroxidase bands on polyacrylamide gel electropherograms of *Escherichia coli* extracts. Dianisidine peroxidase activity was also noted in these extracts and was measured, along with catalase and superoxide dismutase, during studies of adaptation to oxygen toxicity (17–23). During subsequent attempts to isolate and characterize the dianisidine peroxidase of *E. coli*, two electrophoretically distinct catalases were found, and one of these was seen to co-purify with the dianisidine peroxidase (24). The isolation and characterization of the latter enzyme (HP-I) is the subject of this report.

* This work was supported by research grants from the National Institutes of Health, Bethesda, Maryland, the United States Army Research Office, Research Triangle Park, North Carolina, and Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MATERIALS AND METHODS

E. coli B B_{12}^- (provided by D. H. Hall, Georgia Institute of Technology), American Type Culture Collection 29682, was cultured in 15-liter batches of nutrient broth/yeast extract (0.8% nutrient broth plus 0.5% yeast extract) in 20-liter Pyrex carboys at 37°C with moderate aeration. The air supply was filtered through 0.35-μ Ultipor (Pall Corp., Cortland, N. Y.) disposable filters. Cells were harvested with a chilled, air turbine driven, Sharples supercentrifuge, 14 to 15 h after inoculation with 1.0 liter of a late log culture. The cell paste (35 to 40 gm/15 liters of culture) was stored at −20°C until needed. Freezing the cells had no effect on the catalase and peroxidase activities of extracts prepared therefrom. Nutrient broth and yeast extract were obtained from the Baltimore Biological Laboratories.

Catalase and peroxidase were assayed as described in the Worthington enzyme manual (25), with but minor modifications. Decomposition of H_2O_2 was followed at 240 nm using $E = 43.6\ M^{-1}\ cm^{-1}$ (26), or in terms of oxygen evolution under a Clark electrode (Rank Brothers, Bottisham, Cambridge, England) connected to a Heath-Schlumberger model EU-205-11 recorder. Dianisidine dihydrochloride (3,3'-dimethoxybenzidine) was recrystallized (27) prior to use, and its oxidation was followed at 460 nm. Since the product of dianisidine oxidation is poorly soluble and tends to deposit upon surfaces, the cuvettes were washed with 6 N HCl/50% ethanol between uses. Unless otherwise specified, all assays were at 25°C. H_2O_2 (30%) was from Mallinckrodt and *o*-dianisidine from Sigma.

Peroxidation of glutathione (G—SH) was followed at 340 nm by coupling with the glutathione reductase reaction (28). Ferrocytochrome *c* was prepared by reduction of the ferri compound with ascorbate or dithionite, followed by dialysis (29), and its peroxidation was monitored at 550 nm. G—SH, horse heart cytochrome *c* (type III), glutathione reductase (yeast, type III), and sodium ascorbate were from Sigma, NADPH was from P-L Biochemicals, and sodium dithionite was from Baker.

Electrophoretic separations were performed on 4% polyacrylamide gels (30), and activity bands were stained by a modification of the method described previously (16). Having noted that gels buffered at pH 8.9 develop artifactual peroxidatic bands, we lowered the pH to 7.8 and eliminated this problem. Thus, gels were pre-electrophoresed against bicine/imidazole buffer at pH 7.8 (31), prior to application of the pH 5.5 stacking gel (32), and the actual electrophoresis of sample was performed with bicine/imidazole, pH 7.8, as the upper buffer. Bromphenol blue was used as the tracking dye. Acrylamide and bisacrylamide from Eastman were recrystallized prior to use. Horseradish peroxidase (HPOD grade) was from Worthington and 3,3'-diaminobenzidine tetrahydrochloride from Sigma.

Subunit molecular weight was determined by SDS[1] disc gel electrophoresis, as described by Weber and Osborn (33). Polyacrylamide gels, containing 3.3% acrylamide and 0.1% SDS, were loaded with 10 μg of protein sample, which had been treated with 1% SDS ± 1% 2-mercaptoethanol for 2 min at 100°C. Protein standards, with molecular weights in the range 53,000 to 318,000, were obtained from

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

Gallard-Schlesinger and were used to calibrate these gels. Protein bands were located by staining the electropherograms with Coomassie brilliant blue R (Sigma). SDS was obtained from Schwarz-Mann and 2-mercaptoethanol from Eastman.

The molecular weight of native enzyme was determined by sedimentation equilibrium (34) using a Beckman model E analytical ultracentrifuge equipped for temperature control and photoelectric scanning. Samples were dialyzed at 23°C against 50 mM potassium phosphate, pH 7.0. The dialyzed sample at 0.2 mg/ml, was placed in a double sector cell with the dialysate used as the reference. The equilibrium distribution of HP-I, in centrifugal fields at 6310 and 7200 rpm, was scanned at 280 nm and at 405 nm. The temperature was 16–17°C. The ratio A_{405}/A_{280} remained constant during centrifugal equilibration, indicating both the purity and the stability of the HP-I. Partial specific volume was calculated (35) to be 0.73 cc/gm.

Heme was determined as the pyridine hemochromogen, by the method of Falk (36), with spectra recorded against a pyridine-dithionite blank. Pyridine was from Matheson, Coleman & Bell and hemin from Sigma.

Proteins were desalted, reduced, and carboxymethylated, prior to amino acid analysis. After exhaustive dialysis against 0.1 M ammonium bicarbonate to remove guanidinium chloride, the samples were hydrolyzed in 6 N HCl/0.1% phenol, *in vacuo*, at 110°C for 24, 48, and 72 h. A Beckman 119 amino acid analyzer was employed in the procedure of Spackman *et al.* (37). Half-cystine was measured both as carboxymethylcysteine (38) and as cysteic acid, after performic acid oxidation (39). Tryptophan was estimated by the method of Edelhoch (40), after prolonged dialysis against 6 M guanidinium chloride, to remove heme. Guanidine hydrochloride was obtained from Schwarz/Mann. Protein was estimated by the method of Lowry *et al.* (41) with bovine serum albumin (Sigma) as the standard.

Other Materials—$(NH_4)_2SO_4$ (granular) was from Fisher, streptomycin sulfate from Sigma, Sephadex G-25 (coarse) from Pharmacia, DE52 from Reeve Angel Co., Bio-Gel A-1.5m agarose and HTP hydroxylapatite from Bio-Rad Laboratories, and imidazole (grade 1), Tris base, L-histidine·HCl, morpholinopropane sulfonic acid; and butylated hydroxyanisole from Sigma. Guaiacol was from Eastman, pyrogallol from Aldrich, *p*-phenylenediamine from Fisher, and catechol and dextrose from Matheson, Coleman & Bell. Glucose oxidase (grade 1) was a product of Boehringer Mannheim.

RESULTS

Purification of HP-I—Frozen *E. coli* (77.2 gm) was thawed overnight at 4°C and suspended in 400 ml of 50 mM potassium phosphate, pH 7.0, by stirring for 1 h. All steps were performed at 4°C. The suspension was passed through an Aminco French press at 20,000 p.s.i. and was then clarified by centrifugation at 27,300 × *g* for 60 min. Streptomycin sulfate was added to the supernatant fraction to 2.5%, and the mixture was stirred for 1 h. The precipitate was removed by centrifugation for 60 min at 27,300 × *g*, and the supernatant solution was dialyzed overnight against 12 liters of the neutral phosphate buffer.

After dialysis the solution was clarified by centrifugation, and solid $(NH_4)_2SO_4$ was added to 25% of saturation, while the pH was maintained at 7.0 by cautious addition of 10.0 N NaOH. After stirring for 1 h the precipitate was removed by centrifugation, and the supernatant was brought to 60% saturation with $(NH_4)_2SO_4$ at pH 7.0, as before. After stirring for 1 h the precipitate was collected by centrifugation and was dissolved in H_2O to a final volume of 130 ml. Residual $(NH_4)_2SO_4$ was partially removed by passage over a column (5.0 × 53.3 cm) of coarse Sephadex G-25, equilibrated with 50 mM potassium acetate, pH 5.5. The column was developed with 2.0 liters of this buffer, while fractions (16.3 ml) were collected. Catalatically active fractions were pooled, clarified by centrifugation, and diluted with H_2O to the conductivity of 50 mM potassium acetate, and the pH was adjusted to 5.5 with dilute acetic acid. This material (1800 ml) was again clarified by centrifugation and was passed onto a column (2.7 × 22.9 cm) of DE52 equilibrated with 50 mM potassium acetate, pH 5.5. The sample was washed onto the column with 300 ml of this buffer, and a 2.0-liter gradient (0.050 to 0.50 M potassium acetate, pH 5.5) was applied, followed by 300 ml of the 0.5 M potassium acetate buffer. Fractions (16.3 ml) were assayed for A_{280}, catalase, peroxidase, and conductivity.

Catalase and peroxidase activities eluted together (Fractions 151 to 182), and these fractions were pooled, clarified by centrifugation, and concentrated to approximately 2.0 ml over an Amicon PM-30 membrane. This sample was applied to a column (1.4 × 127 cm) of agarose A-1.5m equilibrated with 50 mM potassium phosphate, pH 7.0. The column was eluted with this buffer, while 3.9-ml fractions were collected and assayed for A_{280}, catalase, and peroxidase. As shown in Fig. 1*A*, catalase and peroxidase again eluted in concert (Fractions 21 to 27). These fractions were pooled, concentrated to less than 10 ml over an Amicon PM-30 membrane, and dialyzed 15 h against 10 mM potassium phosphate, pH 6.5, before being applied to a column (1.4 × 20.3 cm) of Bio-Gel HTP hydroxylapatite equilibrated with 10 mM potassium phosphate, pH 6.5. The sample was washed on with 60 ml of this buffer and eluted with 400 ml of a linear gradient (0.010 to 0.20 M potassium phosphate, pH 6.5) followed by 60 ml of the 0.2 M buffer. Fractions (3.9 ml) were assayed for A_{280}, catalase, peroxidase, and conductivity. As shown in Fig. 1*B*, catalase, peroxidase, and protein eluted in parallel (Fractions 34 to 43).

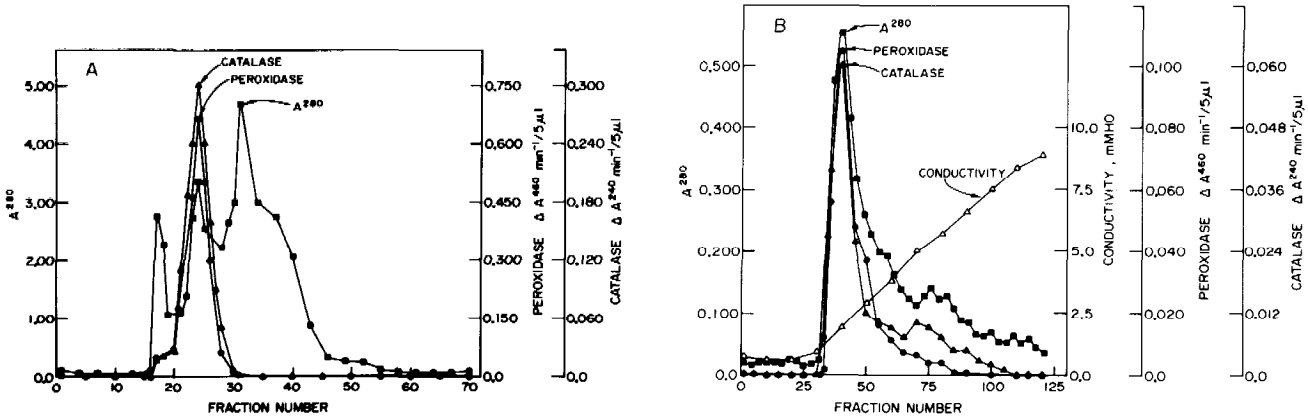


FIG. 1. Co-purification of catalase and *o*-dianisidine peroxidase activities from *E. coli* B. *A*, sample was applied to a column (1.4 × 127 cm) of Bio-Gel agarose A-1.5m in 50 mM potassium phosphate, pH 7.0, and was eluted with this buffer as described in the text under "Purification of HP-I." Absorbance at 280 nm (■) as well as catalase (▲) and peroxidase (●) activities are here plotted as a function of fraction (3.9 ml) number. Enzyme activities are reported for 5-μl aliquots diluted into 3.0-ml reaction mixtures. *B*, sample was applied to a column (1.4 × 20.3 cm) of Bio-Gel HTP hydroxylapatite in 10 mM potassium phosphate, pH 6.5, and was eluted with a linear gradient in this buffer, as described in the text under "Purification of HP-I." Absorbance at 280 nm (■) and conductivity (△) as well as catalase (▲) and peroxidase (●) activities are here plotted as a function of fraction (3.9 ml) number.

TABLE I

Purification of E. coli B hydroperoxidase I from 77.2-g cell paste

Step	Volume	Protein	Protein, total	Specific activity		Total units		Yield		Purification	
				catalase	peroxidase	catalase	peroxidase	catalase	peroxidase	catalase	peroxidase
	ml	*mg/ml*	*mg*	*units/mg*							
1. Crude extract	435	11.7	5,090	37.6	0.746	191,384	3,797.1	100	100	1.0	1.0
2. 2.5% Streptomycin SO_4, supernatant	415	8.7	3,611	47.5	0.767	171,523	2,769.6	90	73	1.3	1.0
3. Centrifuged dialysate	470	7.6	3,572	54.3	0.878	193,960	3,136.2	101	83	1.4	1.2
4. 0–25% $(NH_4)_2SO_4$, supernatant	495	5.7	2,822	48.3	0.675	136,303	1,904.9	71	50	1.3	0.9
5. 25–60% $(NH_4)_2SO_4$, resuspended	113	20.9	2,362	56.0	0.860	132,272	2,031.3	69	54	1.5	1.2
6. G-25 eluate	490	4.2	2,058	49.1	0.709	101,048	1,459.1	53	38	1.3	1.0
7. DE52 eluate	540	0.7	378	275.2	2.2	104,037	831.5	54	22	7.3	2.9
8. Bio-Gel A-1.5m eluate	31	2.2	68	743.9	7.2	50,583	487.4	26	13	19.8	9.7
9. Bio-Gel HTP eluate	31.2	0.46	14.47	1,486.5	15.2	21,510	219.9	11	6	39.5	20.4

These fractions were pooled and brought to 90% saturation with $(NH_4)_2SO_4$, and could then be stored for months at 4°C, without loss of activity. The results of this purification procedure are summarized in Table I. The values pertaining to dianisidine peroxidase activity are not precise, because initial rates were not well maintained in this assay. This was due to progressive inactivation of the enzyme by an intermediate of dianisidine oxidation. This phenomenon will subsequently be presented in greater detail. The protein concentrations in Steps 1 to 6 were determined by the Lowry method (41) and in Steps 7 to 9 from $E_{1\%}^{280} = 10.0$. When the protein content of the final product was assessed by the Lowry method the total purification was 70-fold, rather than the 39.5-fold shown in Table I. The ratio of absorbance at 405 nm to that at 280 nm was 0.55 for the purified enzyme. This may be compared with the ratio of 1.2 shown by the catalase from *R. spheroides* (12).

Homogeneity—Purified HP-I exhibited an electrophoretic mobility identical with that of the catalase isozyme seen on polyacrylamide electropherograms of crude extracts of glucose-grown (42) *E. coli*. This is shown in Fig. 2. Furthermore, the great majority of this material moved as a single protein component which exhibited both catalase and peroxidase activities. There was a very minor contaminant whose electrophoretic mobility was less than that of HP-I and which coincided with a second peroxidatic band. R_F was 0.48 for the major band and 0.32 for the minor band.

The subunit weight of HP-I was estimated to be 78,000 from SDS disc gel electrophoresis. These data are shown in Fig. 3. 2-Mercaptoethanol (1%) was necessary to reduce higher order aggregates to monomers. Nevertheless, some monomer was generated by treatment with SDS alone. Furthermore, subsequent amino acid analysis showed no half-cystine in HP-I. We conclude that covalent cross-links between subunits were absent. HP-I did migrate as a single component upon SDS disc gel electrophoresis after treatment with 2-mercaptoethanol.

HP-I was homogeneous in centrifugal fields generated at either 6310 or 7,200 rpm, and this was the case whether the equilibrium distribution of protein was scanned at 280 nm or at 405 nm. Representative Yphantis plots are shown in Fig. 4. Molecular weights calculated from the slopes of these lines and from $\bar{v} = 0.73$ cc/gm are 330,000 and 344,000. HP-I is thus revealed as a tetramer whose subunit weight is 84,000 and whose aggregate weight is 337,000.

Heme Content and Amino Acid Composition—The absorption spectrum of HP-I exhibits a Sorêt band at 407 nm, and when reduced in alkaline pyridine it yielded a hemochromogen whose spectrum was identical with that of protoheme IX.

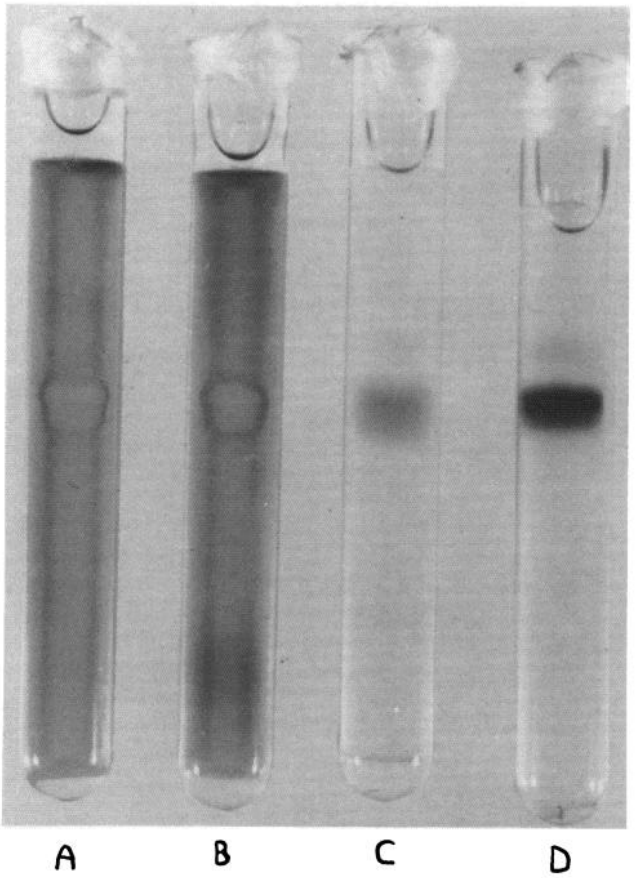


FIG. 2. Polyacrylamide gel electrophoresis of HP-I. *Gels A and B*, crude extract of *E. coli* B containing 10 units of catalase activity (*A*) and a comparable amount of activity in the form of purified HP-I (*B*). Gels were developed and stained for catalase activity, as described under "Materials and Methods." *Gels C and D*, purified HP-I was applied to gels which were developed and stained for peroxidase activity (*C*, 10 units of catalase activity applied) and protein (*D*, 25 μg applied). Peroxidase stains were accomplished by simply omitting horseradish peroxidase from the catalase activity staining procedure described under "Materials and Methods."

These spectra are given in Fig. 5. The protoheme content of HP-I was estimated from the spectrum of its pyridine hemochromogen, upon the basis of $E_{418.5} = 191.5 \times 10^3\ \text{M}^{-1}\ \text{cm}^{-1}$ (43). On this basis there are 2.07 molecules of protoheme IX per tetrameric molecule of HP-I. This low heme content is consistent with the A_{405}/A_{280} ratio of 0.55. HP-I thus has one-half the heme content and one-half the 405 nm absorbance of *R. spheroides* catalase. It does not appear that this is due to

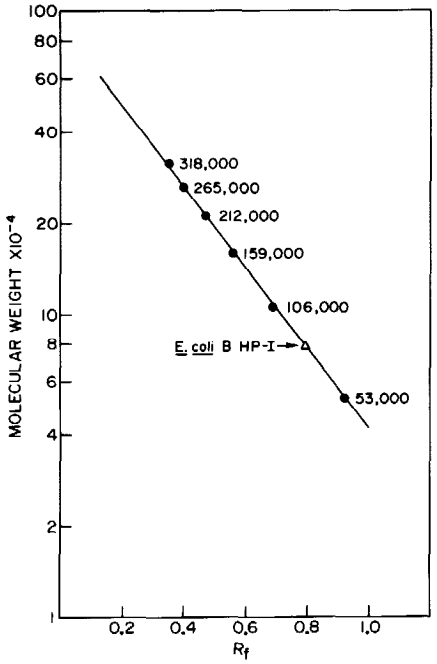


FIG. 3. Determination of subunit weight by SDS polyacrylamide gel electrophoresis. Purified HP-I was treated with SDS and 2-mercaptoethanol and electrophoresed in the presence of SDS. Protein standards of known molecular weight were similarly treated, as described under "Materials and Methods." Log molecular weight is here plotted as a function of mobility relative to the dye front (R_f).

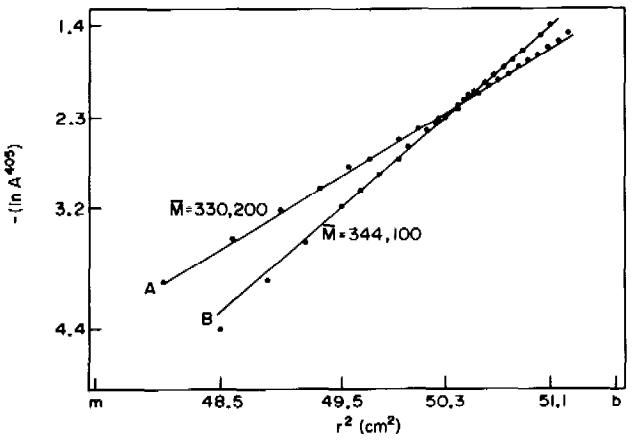


FIG. 4. Sedimentation equilibrium of purified HP-I. The enzyme was brought to sedimentation equilibrium at 6309 rpm (*Line A*) and at 7202 rpm (*Line B*), as described under "Materials and Methods." The distribution of the protein as judged from absorbance at 405 nm is presented according to Yphantis (34). Identical results were obtained when equilibrium distribution was based upon absorbance at 280 nm. *m* and *b* denote the meniscus and bottom of the cell, respectively.

loss of heme during isolation, since the mobility of this enzyme was not modified during purification, and the heme content was remarkably constant from preparation to preparation.

The amino acid composition of HP-I is presented and compared with that of bovine liver catalase in Table II. The surprising lack of half-cystine was confirmed by reduction-carboxymethylation and by performic acid oxidation procedures.

pH Optima and Effects of Nitrogenous Compounds—The activity *versus* pH profiles for the catalatic and peroxidatic activities of HP-I were similar, but the catalatic optimum was at 7.5, while the peroxidatic optimum was at 6.5. This is shown in Fig. 6. When Tris-HCl (10 mM) was used in place of potassium phosphate for buffering, in the range pH 7.5 to 8.0, the catalatic rate was elevated. This effect was due to stimulation by Tris, rather than to inhibition by phosphate, and other nitrogenous compounds, such as imidazole or histidine, also stimulated. Addition of 10 mM imidazole to a phosphate buffer at pH 7.5 nearly doubled the catalatic activity of HP-I, but had minimal effect on its peroxidatic activity. The different nitrogenous compounds appeared to be acting in the same manner, since maximal stimulation by imidazole (10 mM) precluded further stimulation by Tris or histidine.

Autoinactivation during Peroxidatic Turnover—Initial rates were not well maintained during the peroxidation of *o*-dianisidine by HP-I. Constantly declining rates were seen

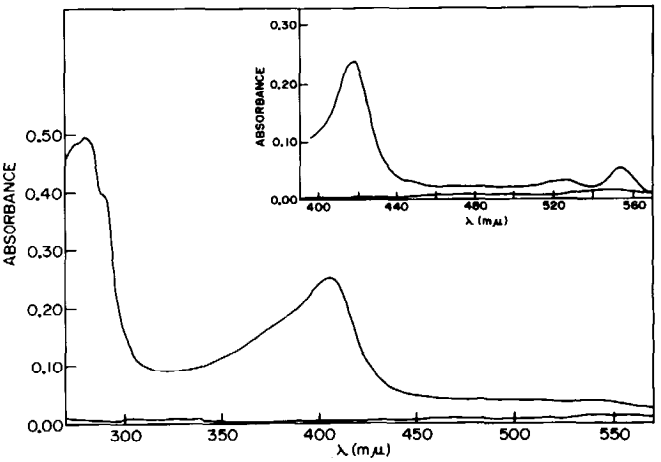


FIG. 5. Optical absorption spectra. The absorption spectrum of 8.5×10^{-7} M HP-I in 0.01 M potassium phosphate buffer at pH 6.5. *Inset*, the absorption spectrum of the pyridine hemochromogen derived from 6.2×10^{-7} M HP-I. The method of preparing this hemochromogen is presented under "Materials and Methods."

TABLE II

Amino acid compositions of E. coli B hydroperoxidase I and of beef liver catalase[a]

Details of the HP-I analysis are given under "Materials and Methods." Except where otherwise noted, values represent averages of 24-, 48-, and 72-h hydrolyses.

Amino acid	Residues per 335,000 daltons HP-I	Residues per[b] 250,000 daltons bovine liver catalase
Lysine	153.4	116
Histidine	35.6	89
Arginine	178.2	130
Aspartic acid	230.7	294
Threonine	110.5	96[c]
Serine	400.5[c]	97[c]
Glutamic acid	308.6	197
Proline	155.2	166
Glycine	242.8	155
Alanine	271.2	154
Half-cystine	0[d]	29[c]
Valine	184.8	141
Methionine	46.5	41
Isoleucine	94.2	79
Leucine	241.0	154
Tyrosine	60.4	86[c]
Phenylalanine	125.0	132
Tryptophan	167.9[e]	

[a] See Ref. 44.

[b] Values represent averages of 22- and 70-h hydrolyses unless otherwise noted.

[c] Values extrapolated to zero hydrolysis time to account for hydrolytic destruction.

[d] Determined both as carboxymethylcysteine and as cysteic acid.

[e] Determined spectrophotometrically after dialysis in 6 M guanidine·HCl to remove heme.

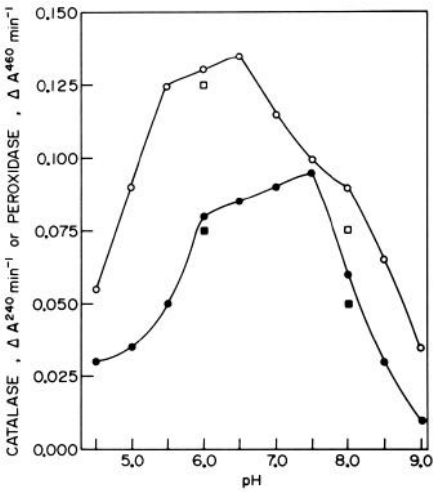


FIG. 6. Peroxidase and catalase activities of HP-I as a function of pH. The peroxidase activity of 1.3 nM HP-I upon 0.33 mM *o*-dianisidine plus 0.88 mM H_2O_2 was measured in 10 mM buffers at the indicated pH (○, □). Initial rates were estimated from the rates seen within ⅕ min of mixing to minimize errors due to the autoinactivation. The catalase activity of 2.4 nM HP-I acting on 18 mM H_2O_2 is presented as a function of pH (●, ■). In the pH range 4.5 to 6.0, buffering was achieved with potassium acetate; 6.0 to 8.0, potassium phosphate; 8.0 to 9.0, sodium pyrophosphate. Potassium phosphate, pH 6.0 (□, ●); potassium acetate, pH 6.0 (○, ■); sodium pyrophosphate, pH 8.0 (□, ■).

both with dialyzed crude extracts of *E. coli* and with homogeneous HP-I, under conditions where substrate depletion was not a factor. Addition of a second aliquot of enzyme, after the reaction initiated by the first had virtually ceased, gave a rate profile identical with that seen with the first aliquot. This rules out the accumulation of an inhibitor as the cause of the declination in rate. Bovine serum albumin, at 50 μg/ml, was without effect on the autoinactivation, and HP-I was stable at high dilution in the absence of the peroxidation reaction. This rules out inactivation due to surface denaturation. The enzyme was stable to dianisidine in the absence of H_2O_2 and to H_2O_2 in the absence of dianisidine. In the latter case its catalatic activity was expressed without obvious declination of rate. Inactivation of HP-I, during peroxidation of dianisidine, was not reversed by dialysis. When 3.8 nM HP-I was exposed to 0.33 mM dianisidine and 0.87 mM H_2O_2 in 10 mM potassium phosphate at pH 6.5 and 25°C, it exhibited a half-life for the inactivation process of 8 s. This rapid loss of activity during dianisidine peroxidation, which precluded steady state kinetic analysis, was not apparent when HP-I catalyzed the peroxidations of guaiacol (470 nm), pyrogallol (430 nm), *p*-phenylenediamine (500 nm), or catechol (415 nm). We conclude that inactivation was due to attack upon the enzyme by an intermediate in the oxidation of dianisidine.

Kinetics of the Catalatic Reaction—HP-I did not suffer rapid autoinactivation during its action on H_2O_2, and classical kinetic analysis was performed. From the data in Fig. 7, K_m for H_2O_2 was 3.9 mM at pH 7.5, and the maximum turnover number was 9.8×10^5 mol of H_2O_2 per mol of HP-I per min. Because of the dual catalatic and peroxidatic activities of HP-I, we had anticipated that catalatic depletion of H_2O_2 would interfere with measurements of dianisidine peroxidation. This proved not to be the case, because dianisidine powerfully inhibited the catalatic activity, both in the absence and in the presence of stimulation by nitrogenous compounds. Thus, *Line 1* in Fig. 8 presents the rate of dioxygen evolution due to the catalatic action of HP-I, and *Line 2* documents the stimulation caused by 10 mM imidazole. As shown by *Lines 3* and *4*, 0.5 mM dianisidine suppressed the catalatic activity, in the absence or in the presence of imidazole.

This inhibition of the catalatic action of HP-I, by dianisidine, was distinct from the autoinactivation seen during dianisidine peroxidation. Thus, it could be seen at levels of *o*-dianisidine too low to support irreversible inactivation, there was no indication of a progressive inactivation during the period of observation, and this inhibition was reversible by dialysis of the reaction mixtures. Prior conversion of *o*-dianisidine to its oxidation product, by incubation with horseradish peroxidase plus H_2O_2, completely eliminated its ability to inhibit the catalatic action of HP-I. The inhibitor is thus *o*-dianisidine or some intermediate of its oxidation, rather than its final oxidation product. Dianisidine increased both slopes and intercepts of reciprocal plots of kinetic data collected by

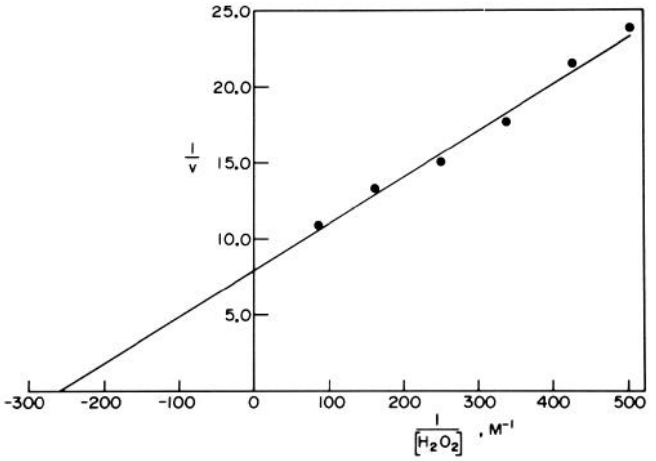


FIG. 7. Effect of H_2O_2 concentration on catalatic action of HP-I. HP-I at 2.9 nM was allowed to act upon H_2O_2 in 10 mM potassium phosphate at pH 7.5 and at 25°C. Rates were measured in terms of decrease in absorbance at 240 nm. Initial rate as a function of initial H_2O_2 concentration is here presented on reciprocal coordinates.

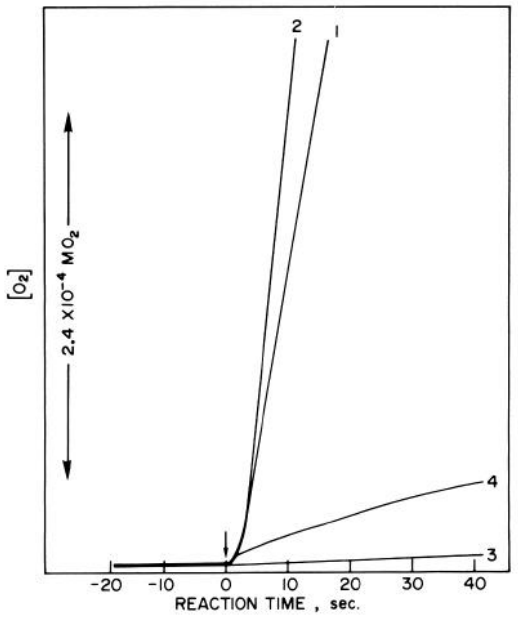


FIG. 8. Inhibition of the catalatic activity of HP-I by *o*-dianisidine and stimulation by imidazole. Catalatic activity of 2.4 nM HP-I, in 10 mM potassium phosphate at pH 7.5 and 25°C, was measured in terms of dioxygen evolution with a Clark electrode. *Line 1*, 35 mM H_2O_2; *Line 2*, 35 mM H_2O_2 plus 10 mM imidazole; *Line 3*, 35 mM H_2O_2 plus 0.5 mM dianisidine; *Line 4*, 35 mM H_2O_2 plus 0.5 mM dianisidine plus 10 mM imidazole. Reaction was initiated with H_2O_2 (*arrow*).

measuring the evolution of dioxygen during the action of HP-I on H_2O_2. These data are shown in Fig. 9. K_I dianisidine, calculated from its slope effect, is 1.1×10^{-7} M, while the corresponding $K_{I'}$ calculated from the intercept effect is 2.7×10^{-7} M.

Significance of the Peroxidatic Activity—If the peroxidatic activity of HP-I is to be considered physiologically significant there must be an effective and renewable electron donor within the cell. Glutathione and ferrocytochrome *c* might serve in this fashion and were considered as candidates for this role (45, 46). HP-I did not catalyze the peroxidation of G—SH. Indeed, because of its catalatic action, it protected G—SH against nonenzymic oxidation by H_2O_2. HP-I was able to catalyze the peroxidation of 1.5×10^{-5} M ferrocytochrome *c* by 0.8 mM H_2O_2 in a neutral phosphate buffer, but here too its catalatic activity was overwhelming, and the reaction quickly came to a halt due to depletion of H_2O_2. When the glucose oxidase reaction was used to generate a steady infusion of H_2O_2, HP-I did not catalyze the peroxidation of ferrocytochrome *c*, but rather strongly diminished its spontaneous oxidation by H_2O_2 by catalatically scavenging this oxidant. Nitrite was also examined as a potential electron donor for the peroxidase action of HP-I and gave results similar to those described for ferrocytochrome *c*.

Crude extracts of *E. coli* were examined for the presence of a peroxidase substrate. In one experiment the peroxidation of dianisidine by dialyzed and undialyzed extracts were compared. The glucose-glucose oxidase reaction was used as a source of H_2O_2, and the reaction was followed at 460 nm. If the extracts contained a dialyzable, nonchromogenic, peroxidase substrate, then one might anticipate a competition between that substrate and dianisidine. In that case the rate, as followed at 460 nm, should be more rapid with the dialyzed extract. As shown in Fig. 10, this is exactly what was observed. There is, however, another possible explanation for this result. The crude extracts might have contained a dialyzable substance capable of reacting with some intermediate or product of the oxidation of dianisidine. This could change the nature of the final oxidation product accumulated and thus change the rate of increase of absorbance at 460 nm. Butylated hydroxyanisole was selected, because of its anti-oxidant properties and because it is not directly peroxidizable by HP-I, as a model of such behavior. When butylated hydroxyanisole was present, it did modify the spectral changes accompanying the peroxidation of dianisidine and, in its presence, these

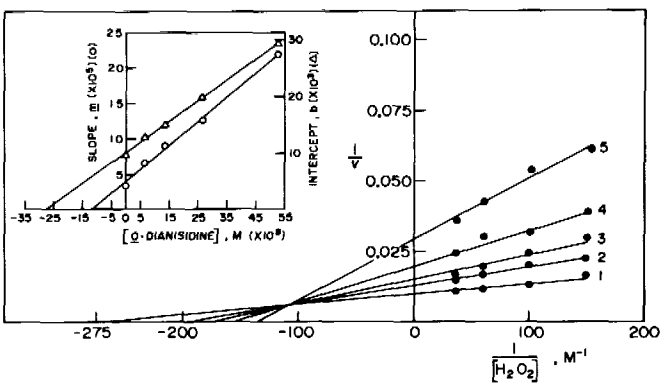


FIG. 9. Analysis of the inhibition by *o*-dianisidine of HP-I catalatic activity. Catalatic activity of 4.2 nM HP-I was measured as a function of $[H_2O_2]$ in 10 mM potassium phosphate at pH 7.5 and at 25°C, at the following concentrations of *o*-dianisidine: *Line 1*, none; *Line 2*, 67 nM; *Line 3*, 134 nM; *Line 4*, 267 nM, and *Line 5*, 527 nM. *Inset*, secondary plots of slopes (*m*) and intercepts (*b*) as functions of dianisidine concentration.

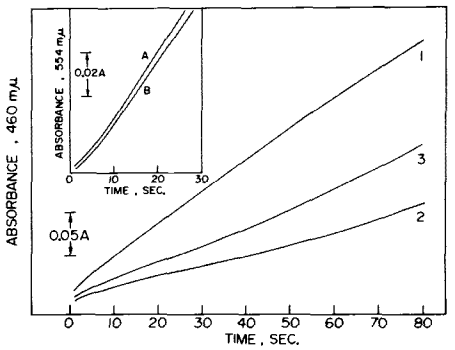


FIG. 10. Evaluation of the possible presence of competing substrates for dianisidine peroxidase activity in concentrated extracts of *E. coli* B. Extract was prepared from a 200-ml culture of *E. coli* B grown on nutrient broth-yeast extract to $A^{600} = 2.5$. The pellet, after thorough washing in cold neutral phosphate buffer, was resuspended in 20 ml of 0.01 M potassium phosphate, pH 7.5, and passed three times through the French press at 20,000 p.s.i. Without further centrifugation, the sample was divided into two parts, one of which was dialyzed for 10 to 12 h in 0.01 M potassium phosphate, pH 7.5, at 4°C. Final protein concentration measured on the dialyzed extract was 5.4 mg/ml by the Lowry method. Using the Aminco DW-2 UV-VIS spectrophotometer to minimize light scattering in turbid suspensions, 0.75 ml of sample was mixed with 1.8 ml of 10 mM potassium phosphate, pH 7.5, containing 2.3% glucose and 0.36 mM dianisidine. Rates were assayed at 460 nM upon addition of 4.5 μg of glucose oxidase and were repeated in duplicate. *Line 1*, 4 mg of dialyzed extract (Lowry protein); *Line 2*, 4 mg of undialyzed extract; *Line 3*, 2 mg each of dialyzed and undialyzed extracts. *Inset*, the same procedure was followed, except that butylated hydroxyanisole is present at 0.39 mM final concentration, and the rates are followed at 554 nm. *A*, 4 mg of dialyzed extract; *B*, 4 mg of nondialyzed extract.

changes were maximal at 554 nm. When dianisidine peroxidation was followed at this wavelength, in the presence of 0.39 mM butylated hydroxyanisole, there was no significant difference between dialyzed and undialyzed cell extracts. This is shown in the *inset* to Fig. 10. We conclude that extracts of *E. coli* do contain a dialyzable antioxidant, which can react with some product of dianisidine peroxidation, but do not contain peroxidizable substrates capable of competing with dianisidine.

DISCUSSION

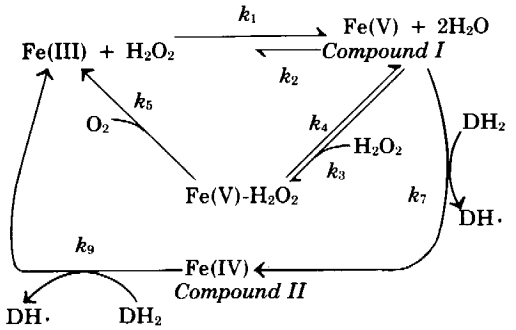
Soluble extracts of *E. coli* B contain two electrophoretically distinct hydroperoxidases. One of these, designated HP-I, has now been isolated. It is distinct from previously encountered hydroperoxidases in acting both as a catalase and as a broad specificity peroxidase. HP-I is composed of four subunits of equal size and has a molecular weight of 337,000. It contains only two molecules of protoheme IX per molecule of enzyme. This heme paucity explains its low A_{405}/A_{280} ratio. HP-I appears eminently suited for the catalatic scavenging of H_2O_2. Thus its K_m for H_2O_2 is 3.9 mM, and its turnover number is 9.8×10^5/min. Horse liver catalase, in comparison, exhibits K_m $H_2O_2 = 1.1$ M and a turnover number $= 2.3 \times 10^9$/min (47). If these two enzymes were compared at 3.9 mM H_2O_2, their turnover numbers would be 4.9×10^5/min and 8.0×10^6/min, respectively. Since HP-I did not catalyze the peroxidation of glutathione, ferrocytochrome *c*, or nitrite, and since crude extracts of *E. coli* did not contain dialyzable components capable of competing with dianisidine, we conclude that it acts predominantly as a catalase.

Nitrogenous compounds, such as imidazole or Tris, stimulated the catalatic activity of HP-I and did so by increasing V_{max}, while having no significant effect upon K_m H_2O_2. This recalls the stimulation of horseradish peroxidase by such compounds (48), and the mechanistic basis of these effects

remains unknown. Imidazole was without effect upon the optical spectrum of HP-I and thus gave no indication of binding to the active site of the resting enzyme. It might, however, interact with one of the enzyme intermediates encountered in the catalytic cycle.

Dianisidine had two remarkable effects upon HP-I. It powerfully and reversibly inhibited the catalatic activity, with K_I and $K_I' = 1.1$ and 2.7×10^{-7} M, respectively. When present at 1000-fold higher concentrations, in the presence of H_2O_2, it progressively and irreversibly inhibited its own peroxidation. It would thus appear that dianisidine can bind, reversibly and with very high affinity, to the active site and block catalatic turnover, and, furthermore, that some unstable intermediate of dianisidine peroxidation can attack and irreversibly modify the enzyme. Dianisidine thus acts as a suicide substrate for HP-I acting in the peroxidatic mode.

A minimal scheme can be constructed which accounts for these observations (Scheme 1).



SCHEME 1

If k_1 were larger than any of the other rate constants in the catalytic cycle, then during the steady state most of the enzyme would be present in the form of Compound I, and the overall rate would vary according to the Michaelis-Menten equation with the first power of the concentration of H_2O_2. This has been shown to be the case for liver catalase (47, 49, 50) and appears also to fit the behavior of *E. coli* HP-I. If stimulatory nitrogenous compounds were to interact with Fe(V)-H_2O_2 and accelerate k_5, they would speed the overall catalatic reaction without modifying the peroxidatic rate and without modifying the spectrum of the resting enzyme. If the affinity of Compound I for dianisidine were very much greater than the comparable affinity of Compound II, then low concentrations of dianisidine could inhibit the catalatic cycle by converting Compound I to Compound II. This inhibition would be reversed during dialysis because Compound II could be reconverted to resting enzyme, after removal of H_2O_2 by residual dianisidine. At much higher levels of dianisidine, sufficient to activate the k_9 reaction, intermediate oxidation products of dianisidine would be produced in quantity and would progressively inactivate the enzyme. The A-3 isozyme of horseradish peroxidase, like HP-I, has been reported to suffer irreversible inactivation during the peroxidation of dianisidine (51).

The peroxidatic activity of HP-I is very different from that of mammalian catalase. Thus the former activity can be seen even at relatively high concentrations of H_2O_2 and with a variety of electron donors, such as guaiacol, dianisidine, pyrogallol, and *p*-phenylenediamine. In contrast, only a few compounds, such as formate, methanol, ethanol, and nitrite, have been seen to serve as substrates for the liver catalase and then only at low steady state levels of H_2O_2 achieved by continual enzymic generation or by other means of gradual infusion (52, 53). The peroxidations by liver catalase have been shown by Chance (54–56) to proceed via two electron transfers from the donors to Compound I at a rate only 1/10,000 the rate of reaction with H_2O_2. This great discrepancy in relative rates explains why peroxidations by liver catalase can only be seen at vanishingly small concentrations of H_2O_2. The bacterial catalases described heretofore (12, 13) are similar to the liver enzyme in their kinetic preference for the catalatic mode. Native erythrocyte catalase does not catalyze the peroxidation of guaiacol, but the activity can be generated by dissociation of the enzyme with urea (57). This activity of the urea-treated mammalian catalase appears to be unrelated to the peroxidase activities of native *E. coli* HP-I.

We have previously reported that HP-I appears to be a precursor of HP-II (24) and have supported this suggestion by studies of the kinetics of induction of these enzymes (42). Work on the isolation and characterization of HP-II and on the precursor-product relationship between HP-I and HP-II is underway and will be reported subsequently.

Acknowledgments—We wish to thank Darrell R. McCaslin for performing the sedimentation equilibrium experiments and Douglas P. Malinowski and John L. Abernethy for the amino acid analyses.

REFERENCES

1. Saunders, B. C., Holmes-Siedle, A. G., and Stark, B. P. (1964) in *Peroxidase*, p. 124, Butterworths, London
2. Chance, B., and Fergusson, R. R. (1954) in *The Mechanism of Enzyme Action* (McElroy, W. D., and Glass, B., eds) pp. 389–398, Johns Hopkins Press, Baltimore
3. Nicholls, P., and Schonbaum, G. (1963) in *The Enzymes* (Boyer, P. D., Lardy, H. A., and Myrbäck, K., eds) Vol. 8, pp. 147–225, Academic Press, New York
4. Yamazaki, I. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed) pp. 535–558, Academic Press, New York
5. Hunter, M. J. (1955) *Methods Enzymol.* **2,** 791–794
6. Maehly, A. C. (1955) *Methods Enzymol.* **2,** 794–807
7. Maehly, A. C. (1955) *Methods Enzymol.* **2,** 807–813
8. Polis, B. D., Shmukler, H. W. (1955) *Methods Enzymol.* **2,** 813–817
9. Paul, K. G. (1963) in *The Enzymes* (Boyer, P. D., Lardy, H. A., and Myrbäck, K., eds) Vol. 8, pp. 227–274, Academic Press, New York
10. Saunders, B. C., Holmes-Siedle, A. G., and Stark, B. P. (1964) *Peroxidase*, pp. 53–57, Butterworths, London
11. Herbert, D., and Pinsent, J. (1948) *Biochem. J.* **43,** 193–202
12. Clayton, R. (1959) *Biochim. Biophys. Acta* **36,** 40–47
13. Chance, B., and Herbert, D. (1950) *Biochem. J.* **46,** 402–414
14. Lenhoff, H. M., and Kaplan, N. O. (1956) *J. Biol. Chem.* **220,** 967–982
15. Dolin, M. I. (1957) *J. Biol. Chem.* **225,** 557–573
16. Gregory, E. M., and Fridovich, I. (1974) *Anal. Biochem.* **58,** 57–62
17. Gregory, E. M., and Fridovich, I. (1974) *J. Bacteriol.* **117,** 166–169
18. Yost, F. J., and Fridovich, I. (1974) *Arch. Biochem. Biophys.* **161,** 395–401
19. Yost, F. J., and Fridovich, I. (1976) *Arch. Biochem. Biophys.* **175,** 514–519
20. Hassan, H. M., and Fridovich, I. (1977) *J. Bacteriol.* **129,** 1574–1583
21. Hassan, H. M., and Fridovich, I. (1977) *J. Bacteriol.* **130,** 805–811
22. Hassan, H. M., and Fridovich, I. (1977) *J. Biol. Chem.* **252,** 7667–7672
23. Hassan, H. M., and Fridovich, I. (1979) *Rev. Infect. Dis.*, in press
24. Claiborne, A. (1978) *Fed. Proc.* **37,** 1513
25. *Worthington Enzyme Manual* (1972) pp. 41–45, Worthington Biochemical Corp., Freehold, New Jersey
26. Hildebrandt, A. G., and Roots, I. (1975) *Arch. Biochem. Biophys.* **171,** 385–397
27. Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., and Butler, A. M. (1940) *J. Biol. Chem.* **134,** 319–330
28. Stults, F. H., Forstrom, J. W., Chiu, D. T. Y., and Tappel, A. L. (1977) *Arch. Biochem. Biophys.* **183,** 490–497
29. Wharton, D. C., and Tzagoloff, A. (1967) *Methods Enzymol.* **10,** 245–250

30. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **23,** 404–427
31. Bio-Rad Bulletin 1038, Bio-Rad Laboratories, Richmond, California
32. Maurer, H. R. (1971) *Disc Electrophoresis*, p. 45, Walter de Gruyter, New York
33. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412
34. Yphantis, D. A. (1964) *Biochemistry* **3,** 297–317
35. Cohn, E. J., and Edsall, J. T. (1965) *Proteins, Amino Acids and Peptides*, p. 375, Hafner Publishing Co., New York
36. Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, pp. 181–182, Elsevier Publishing Co., New York
37. Spackman, D. H., Stein, W. H., and Moore, S. (1958) *Anal. Chem.* **30,** 1190–1206
38. Crestfield, A. M., Moore, S., and Stein, W. H. (1963) *J. Biol. Chem.* **238,** 622–627
39. Hirs, C. H. W. (1956) *J. Biol. Chem.* **219,** 611–621
40. Edelhoch, H. (1967) *Biochemistry* **6,** 1948–1954
41. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
42. Hassan, H. M., and Fridovich, I. (1978) *J. Biol. Chem.* **253,** 6445–6450
43. Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, p. 240, Elsevier Publishing Co., New York
44. Schroeder, W. A., Sala, A., Fenninger, W. D., and Cua, J. T. (1962) *Biochim. Biophys. Acta* **585,** 611–613
45. Mills, G. C. (1957) *J. Biol. Chem.* **229,** 189–197
46. Altschul, A. M., Abrams, R., and Hogness, T. R. (1940) *J. Biol. Chem.* **136,** 777–794
47. Ogura, Y. (1955) *Arch. Biochem. Biophys.* **57,** 288–300
48. Fridovich, I. (1963) *J. Biol. Chem.* **238,** 3921–3927
49. Ogura, Y., Tonomura, Y., Hino, S., and Tamiya, H. (1950) *J. Biochem. (Tokyo)* **37,** 153–177
50. Ogura, Y., Tonomura, Y., and Hino, S. (1950) *J. Biochem. (Tokyo)* **37,** 249–267
51. Kay, E., Shannon, L. M., and Lew, J. Y. (1967) *J. Biol. Chem.* **242,** 2470–2473
52. Keilin, D., and Hartree, E. F. (1936) *Proc. R. Soc. London B Biol. Sci.* **119,** 141–159
53. Keilin, D., and Hartree, E. F. (1945) *Biochem. J.* **39,** 293–301
54. Chance, B. (1949) *J. Biol. Chem.* **179,** 1341–1369
55. Chance, B. (1950) *J. Biol. Chem.* **182,** 649–658
56. Chance, B. (1948) *Nature* **161,** 914–917
57. Scherz, B., Kuchinskas, E. J., Wyss, S. R., and Aebi, H. (1976) *Eur. J. Biochem.* **69,** 603–613