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Haemoprotein *b*-590 (*Escherichia coli*), a Reducible Catalase and Peroxidase: Evidence for its Close Relationship to Hydroperoxidase I and a 'Cytochrome a_1 *b*' Preparation

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A reducible hydroperoxidase, haemoprotein *b*-590, has been purified 16-fold from a soluble fraction of *Escherichia coli* K12, grown anaerobically with glycerol and fumarate. The M_r of the native protein, determined by gel filtration, was 331 000 although a minor, smaller species with a M_r of 188000 was also detected; both had catalase activities. Based on the subunit M_r, determined from SDS gel electrophoresis to be 75000, the above species are tentatively identified as tetramers and dimers, respectively. The isoelectric point of both species was 4·4. The absorption spectrum of the isolated haemoprotein is typical of ferric, high-spin haem. The A_{405}/A_{280} ratio never exceeded 0·27, a value half of that obtained for *E. coli* hydroperoxidase I. On reduction with dithionite, the γ, β, and α bands were at 441, 559 and 590 nm respectively, the α-band being unusually distinct. Treatment of the reduced form with CO gave a sharp prominent γ-band at 426 nm and caused significant shifts of the α and β bands to shorter (574 and 545 nm) wavelengths.

The pyridine haemochrome spectra showed the haem to be protohaem IX; the spectra were featureless between 580 and 630 nm, thus excluding the presence of haem *a*. However, some features of the difference spectra of the haemoprotein were reminiscent of cytochrome a_1, notably the maxima in reduced *minus* oxidized spectra at 444 and 593 nm and the peaks and troughs in CO difference spectra at 426 and 446 nm respectively. The haemoprotein had high catalase activity: V_{max} was 2·3 × 10^6 mol H_2O_2 (mol haem)$^{-1}$ min^{-1} and the K_m was 11 mM. At 10 mM-H_2O_2 the first order rate constant was 0·3 × 10^7 M^{-1} s^{-1}. The haemoprotein was also a peroxidase with *o*-dianisidine or 2,3′,6-trichloroindophenol as substrates; for the latter substrate, the K_m was 0·18 mM. It is concluded that haemoprotein *b*-590 strongly resembles the hydroperoxidase I purified by Claiborne & Fridovich (*Journal of Biological Chemistry* **254**, 4245–4252, 1979) and that a similar haemoprotein was mistaken for a cytochrome a_1 *b* complex by Barrett & Sinclair (*Abstracts of the 7th International Congress of Biochemistry*, Tokyo, H-107, p. 907, 1967).

INTRODUCTION

Reduced suspensions, or subcellular fractions, of *Escherichia coli* frequently contain a pigment with an absorbance band between 585 and 595 nm, which, because of its superficial similarity to a terminal cytochrome oxidase in *Acetobacter* spp., has been called 'cytochrome a_1' (for reviews, see Poole, 1983 and Poole *et al.*, 1985*a*). The nature and function of this haemoprotein have

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Abbreviation: TCIP, 2,3′,6-trichloroindophenol.

remained mysterious, although it appears to be synthesized coordinately with membrane-bound cytochrome *d* (one of two oxidases terminating O_2-dependent respiratory chains in *E. coli*). Indeed, a spectrally similar component is detectable in varying amounts in the solubilized and purified cytochrome *bd* oxidase complex (Miller & Gennis, 1983; Kita *et al.*, 1984).

'Soluble' fractions obtained by high-speed centrifugation of ultrasonically disrupted *E. coli* grown anaerobically with glycerol and fumarate, also contain a haemoprotein with some spectral features, notably a band in the reduced form near 595 nm, similar to the a_1-like component of membranes and of the purified *bd* complex. No cytochrome *d* is present in such supernatants. The partially purified a_1-like component has high catalase and peroxidase activities (Baines *et al.*, 1984). Significantly, haem extraction of either the crude soluble preparation or whole cells yields no haem *a*, but on the basis of the presence of only protohaem IX (the prosthetic group of cytochromes *b*) and similarities in the spectrum of the a_1-like component with certain high-spin *b*-type haem proteins, the name 'haemoprotein *b*-590' has been proposed in accordance with recommended enzyme nomenclature (Poole *et al.*, 1984).

In view of the catalatic and peroxidatic activities of haemoprotein *b*-590, we have explored the possible relationship between this enzyme and previously described hydroperoxidases in *E. coli*. (The term hydroperoxidase is taken to describe both catalases and peroxidases, which have fundamentally similar modes of action. *E. coli* possesses probably three electrophoretically distinct hydroperoxidases (HP-I, HP-II, HP-III), but only the first two have been well-characterized biochemically. Both are tetrameric proteins, having subunit M_r values in the range 78000 to 84000. HP-I is an efficient catalase as well as catalysing peroxidation of a range of substrates, whereas HP-II has higher catalase activity but negligible peroxidase activity. Both show γ absorption peaks at 407 nm in the native state but have not been fully characterized optically.

In this paper, we report the purification of haemoprotein *b*-590 and describe those features of its structure and function that allow direct comparison with the hydroperoxidases previously reported in *E. coli*. We also suggest that a partially characterized preparation, previously described as a cytochrome 'a_1 *b*' (Barrett & Sinclair, 1967), and having high peroxidase activity, consisted largely of haemoprotein *b*-590.

METHODS

Organism and growth conditions. Escherichia coli strain A1002 (NCIB 11825) was grown anaerobically in the medium described previously containing 0·5% (w/v) glycerol and 50 mM-fumarate, supplemented with molybdate and selenite (Baines *et al.*, 1984). An unshaken starter culture (200 ml) was inoculated into 20 l of medium, slowly stirred, and sparged with O_2-free N_2 at 1·3 l min^{-1}. Apparent absorbance was measured in a Varian 634 spectrophotometer and the culture harvested after about 22 h when the OD_{600} reached approximately 1·0 (1 cm cuvettes, 1 nm band width) in the early stationary phase of growth.

Cell disruption and enzyme purification. Cells were harvested in the 6 × 1 l rotor of an MSE Coolspin centrifuge (5000 *g*, 15 min, 4 °C) yielding approx. 1·7 g (wet wt) cells l^{-1}. All subsequent steps were done at 4 °C. The cells were washed once in a buffer that contained 50 mM-Tris/HCl, 2 mM-$MgCl_2$ and 1 mM-EGTA, pH 7·4, centrifuged at 12000 *g* for 10 min and the pellets stored at −25 °C until required. Thawed cells were washed once and resuspended in the same buffer, to which a few flakes of pure deoxyribonuclease had been added. The weight of buffer used equalled (for sonication) or was double (for the French press) the wet weight of cells. Cells were disrupted either by sonication in <50 ml batches as described by Scott & Poole (1982) and Poole & Haddock (1974) but using a Branson Sonifier B12 Cell Disruptor operating at 80 to 100 W and with a probe of diameter 1 cm, or by three passages through a French pressure cell (constructed in the CSIRO workshops) at 9 t pressure on the 2·8 cm diameter piston of this cell (146 MPa). A high speed supernatant ('S_1') was obtained essentially as described by Poole & Haddock (1974) and brought slowly to 40% saturation with solid $(NH_4)_2SO_4$. The precipitate was resuspended and dialysed as described by Baines *et al.* (1984). Dialysis tubing was previously boiled in 0·01 mM-EDTA, pH 7·4, in water (twice) and stored in the presence of a thymol crystal. The retentate was clarified by centrifugation at 22 000 *g* for 30 min and the dark-brown supernatant was concentrated fivefold using an Amicon Model 402 ultrafiltration cell and YM30 membrane. The concentrate was quickly frozen in liquid N_2 and stored until required.

Three column chromatography steps were adopted for purification of haemoprotein *b*-590.

(*a*) *Gel filtration on Sephacryl S400.* The thawed material was applied at 2 ml min^{-1} to a column (5 × 50 cm) of Sephacryl S400, prepared as described by the manufacturers, and equilibrated with 50 mM-Tris/HCl (pH 8·0). The

column was eluted with this buffer while 21 ml fractions were collected and assayed for catalase activity and cytochromes. A_{403} and A_{277} were monitored using an LKB Uvicord III with 3 mm flow cell. Fractions judged rich in catalase and haemoprotein on the basis of the absorbance difference ($A_{446} - A_{480}$) in the CO + reduced *minus* reduced difference spectra were pooled, concentrated approximately fivefold by ultrafiltration and frozen in liquid N_2.

(*b*) *Anion exchange chromatography on DEAE-Sepharose CL6B.* Thawed material from the previous step was applied to a column (bed dimensions 2·5 × 21·5 cm) of DEAE-Sepharose CL6B equilibrated with 50 mM-Tris/HCl (pH 8·0). The sample was loaded at 1·3 ml min^{-1}; all haemoprotein was observed to be adsorbed to the top 3 cm of the column. Elution, begun after collection of 70 ml, was with a linear gradient (1 litre) of 0 to 1 M-NaCl in the same buffer. Fractions (approximately 14 ml) were analysed as in (*a*) and those rich in catalase and haemoprotein *b*-590 pooled and concentrated on a YM30 membrane. Conductivity was measured using a Radiometer conductivity meter, type CDM 2D.

(*c*) *Second gel filtration on Sephacryl S400.* The concentrated material was applied with a Pharmacia sample applicator SA-5 to a column (bed dimensions 1·6 × 58·5 cm) of Sephacryl S400 equilibrated with 50 mM-Tris/HCl, pH 8·0. The sample (2% of the bed volume) was loaded at 0·3 ml min^{-1} and eluted with the starting buffer. Fractions (2·8 ml) were collected and analysed as in (*a*), but to minimize use of material in spectral analysis, absolute spectra were recorded in a single cuvette and difference spectra generated by a computer (see below). Selected fractions were pooled, concentrated on a YM30 membrane and rapidly frozen in liquid N_2 before storage at −25 °C.

Determination of native M_r. This was done by gel filtration chromatography on Sephacryl S400 (*c* above). The column was calibrated using the proteins provided in a high M_r gel filtration calibration kit (Pharmacia). The proteins were ferritin (2 mg ml^{-1}), catalase (3 mg ml^{-1}) and aldolase (1 mg ml^{-1}), dissolved in starting buffer containing 1·8% (w/v) sucrose. The void volume was determined with blue dextran (2 mg ml^{-1}). K_{av} values were calculated as described by the manufacturer and in the legend to Fig. 2.

Preparative isoelectric focusing. The enzyme could be obtained, though in low percentage yield, directly from the material precipitated by 40% $(NH_4)_2SO_4$ using preparative flat-bed electrofocusing in a granulated gel. The material, resuspended in a small volume of 50 mM-Tris/HCl, pH 8·0, was dialysed overnight against 10 mM-MES, pH 5·5, centrifuged for 30 min at 24000 *g*, and the supernatant dialysed for a further 4 to 7 h against 1 mM-MES, pH 5·5, to reduce the salt concentration and bring the pH to that of the ampholyte. A white precipitate, removed from this dialysate by centrifugation at 140000 *g* for 30 min, was found to contain, typically, only 17% of the starting haemoprotein *b*-590 and to be enriched in contaminating *c*-type cytochromes. The volume of the supernatant was reduced to approximately 3 ml using a YM30 membrane in an Amicon micro-ultrafiltration cell (Model 8MC) and immediately applied to the loading well, placed 5 cm from the cathode, in an LKB 2117 Multiphor apparatus. The pH gradient was not preformed and the procedure closely followed that of Fuchsman & Appleby (1979) and LKB Application Note 198. The gel slurry (100 ml), before evaporation to a preset limit, contained 4% (w/v) Ultrodex (LKB) and 5% (v/v) ampholyte (Pharmalyte, pH range 4·0 to 6·5, Pharmacia). Electrode strips were soaked in 0·1 M-phosphoric acid (anode) or 0·1 M-NaOH (cathode). Electrofocusing was for 14 to 16 h at 8 W constant power and 4 °C. Regions of the gel containing haemoproteins were identified by their colour, removed with a spatula and the protein eluted with water (0·75 vol.) in a small funnel fitted with a nylon mesh to retain the gel. After measuring the pH of each eluate at 4 °C, an equal volume of 50 mM-Tris/HCl, pH 8·0, was added.

Analytical isoelectric focusing. This was done on a Pharmacia flat-bed apparatus using pre-prepared LKB Ampholine plates in the isoelectric focusing range pH 4·0 to 6·5. Staining for haem, or, more correctly, peroxidase activity, used a modification of the method of Reid & Ingledew (1980). Gels were soaked in a 2% (w/v) solution of *o*-toluidine in ethanol/water (90:10, by vol.) for 30 s, then transferred to 3% (v/v) H_2O_2 in 50 mM-potassium phosphate buffer, pH 7·0. An intense blue staining of haem-containing regions coupled with O_2 generation at the same sites appeared within a few minutes. Protein was stained subsequently with Coomassie Brilliant Blue.

Subunit M_r *determinations.* These were made by SDS disc gel electrophoresis by the modification of Laemmli's (1970) procedure described in Sigma Technical Bulletin no. MWS-877L.

Enzyme assays.

(*a*) *Catalase.* This was assayed at room temperature, pH 7·0, by following the decomposition of H_2O_2 at 240 nm in a Cary 14 spectrophotometer. Routine assays, e.g. of column fractions, were done as described by Aebi (1974), using an initial H_2O_2 concentration of 10 mM and following the reaction for 15 to 60 s. Such results are given as first-order rate constants, k (s^{-1}). Alternatively, for estimation of K_m and V_{max} values, catalase was assayed as described in the Worthington Enzyme Manual (1972), and the H_2O_2 concentration varied as shown in Results. The absorption coefficient used for H_2O_2 at 240 nm was 43·6 × 10^{-3} mM^{-1} cm^{-1} (Hildebrandt & Roots, 1975).

(*b*) *Peroxidase.* Dianisidine peroxidase was assayed as described in the Worthington Enzyme Manual (1972), with minor modifications. *o*-Dianisidine (3,3′-dimethoxybenzidine) was recrystallized (Talbot *et al.*, 1940) and prepared as a 0·5% (w/v) stock solution in methanol. The final concentrations in the assay were 0·86 mM-H_2O_2 and 0·26 mM-*o*-dianisidine. Interference from deposition of the product of dianisidine peroxidation was overcome by

using disposable cuvettes. The absorbance increase at 460 nm due to oxidation of the dye was markedly non-linear; initial rates were therefore measured over the first 12 s. The decomposition of H_2O_2 was related to absorbance increase at 460 nm by measuring the increases incurred by various amounts of H_2O_2 in the presence of excess enzyme (Worthington Enzyme Manual, 1972); thus, an absorption coefficient (ε) at 460 nm for H_2O_2 decomposition of $11{\cdot}3 \times 10^3$ M^{-1} cm^{-1} was obtained.

Peroxidase activity with 2,3',6-trichloroindophenol (TCIP) as electron donor was assayed using a method based on that of Lenhoff & Kaplan (1956). An aqueous solution of the dye (1 mM) was reduced for 2 min with a few grains of $NaBH_4$ and then filtered through a Whatman no. 1 filter into a tube stored in ice, where it was stable for a few hours. To each of two cuvettes were added dye (2·5 ml) and 0·36 M-potassium phosphate buffer, pH 7·5 (0·4 ml). When bubbling stopped, 0·1 ml H_2O_2 (30 mM in 50 mM-potassium phosphate, pH 7·5) was added to both cuvettes, and then enzyme was added to the sample cuvette. The oxidation of the reduced dye was observed by following the increase in absorption at 575 nm and was related to H_2O_2 decomposition by titration of limiting amounts of H_2O_2 to a reaction containing excess enzyme; thus an absorption coefficient (ε) at 575 nm for H_2O_2 decomposition of $3{\cdot}6 \times 10^3$ M^{-1} cm^{-1} was obtained. Unlike *o*-dianisidine peroxidase, the initial linear rates were well maintained.

Guaiacol peroxidase activity was assayed using a modification of the method of Sinclair (1966) at pH 7·5 and 470 nm. Final concentrations were 50 mM-potassium phosphate (pH 7·5), 10 mM-guaiacol and 1 mM-H_2O_2.

Spectrophotometric analysis. Absorption spectra were obtained with a Hitachi-Perkin Elmer model 557 spectrophotometer, interfaced to a PDP11/03 computer and Hewlett Packard model 7221-B plotter. Room temperature spectra were obtained in 1 cm, semi-micro, black-sided, stoppered cuvettes; other conditions are given in Figure legends. Absolute spectra were obtained by first scanning, in the split-beam mode, two cuvettes containing buffer, and exploiting the spectrophotometer's capability of subtracting this baseline from subsequent scans. Difference spectra were plotted either by subtraction of two selected absolute spectra in the computer or, for routine analysis of fractions, after 'memorizing' in the spectrophotometer a baseline obtained by scanning two cuvettes, each containing an oxidized or reduced preparation.

Spectra at 77 K were obtained in cuvettes of 2 mm path length in cryogenic cell model 0801 for the above spectrophotometer. To ensure reproducible and significant enhancement of sample absorbance at 77 K, samples were diluted with an equal volume of 60% (v/v) ethylene glycol, resulting in a polycrystalline matrix on rapid freezing (Vincent *et al.*,1982). Difference spectra were generated by subtraction of pairs of spectra; e.g., the spectrum of the difference between an oxidized haemoprotein sample and buffer was 'memorized' in the spectrophotometer and subtracted from the spectrum of the difference between a reduced sample and buffer, thus giving a reduced *minus* oxidized spectrum. This procedure allowed the recording of all the baseline-corrected 77 K difference spectra in Results using purified haemoprotein in only three cuvettes. Other instrument settings are given in the Figure legends.

Haem determination. To 0·6 ml of purified haemoprotein *b*-590 in a stoppered cuvette was added an equal volume of a reagent that contained 0·4 M-NaOH and 4·2 M-pyridine. The contents were mixed by inversion and half were transferred to a similar cuvette. A baseline (autoxidized vs autoxidized) was recorded before adding a few grains of $Na_2S_2O_4$ to the sample cuvette and recording the reduced *minus* oxidized difference spectrum from 650 to 500 nm. On adding one crystal of $K_3(FeCN)_6$ to the reference cuvette, no change was observed, indicating no self-reduction of the haem. The concentrations of protohaem IX and of contaminating *c*-type haem were calculated as described by Appleby (1978). The spectrum of the reduced form of the pyridine haemochrome was also scanned with buffer as the reference and after bubbling the reduced form with CO for 30 s.

Protein. This was determined using the Bio-Rad dye-binding assay based on that of Bradford (1976) using dry bovine serum albumin as standard.

Chemicals. MES and Tris buffers (Ultrol grade) were from Calbiochem, deoxyribonuclease from Worthington Diagnostic Systems, Sephacryl S400 and DEAE-Sepharose CL6B from Pharmacia, bovine serum albumin and *o*-toluidine from Sigma, ammonium sulphate (Ultrapure) from BRL and *o*-dianisidine (reagent grade) and guaiacol (reagent grade which was redistilled) were from BDH. Potassium phosphates for buffer preparation were reagent grade from Merck. All other chemicals were laboratory or reagent grade. Glass distilled water was further purified by passage through a Millipore 'Milli-Q' system.

RESULTS

Purification of haemoprotein b-590

High-speed supernatants (S_1) from *E. coli* suspensions, disrupted either by sonication (Baines *et al.*, 1984) or in the French pressure cell, exhibited identical absorbance spectra for both the dithionite-reduced and the dithionite-reduced plus CO states and contained approximately 80% of the haemoprotein *b*-590 detectable in intact cells, as measured at 446–480 nm in CO difference spectra. The French press was adopted for its speed in treating large volumes of cell suspension.

Table 1. *Purification of haemoprotein b-590 from anaerobically grown E. coli*

Step	Volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Haemoprotein Units*	Units mg^{-1}	Yield	Purification
High speed supernatant (S_1)	332	31·4	10425	38·8	0·0037	100	1
Precipitation with 40% $(NH_4)_2SO_4$	123	29·6	3648	32·6	0·0089	84·0	2·4
Chromatography on Sephacryl S400	235	9·2	2162	20·8	0·0096	53·6	2·6
Ion exchange on Sepharose CL6B	76·7	4·32	331·7	13·25	0·0400	34·1	10·8
Second chromatography on Sephacryl S400 and ultrafiltration	2·4	47·7	115	6·89	0·060	17·8	16·2

* ΔA, determined in CO difference spectra, 446 − 480 nm, path length 1 cm.

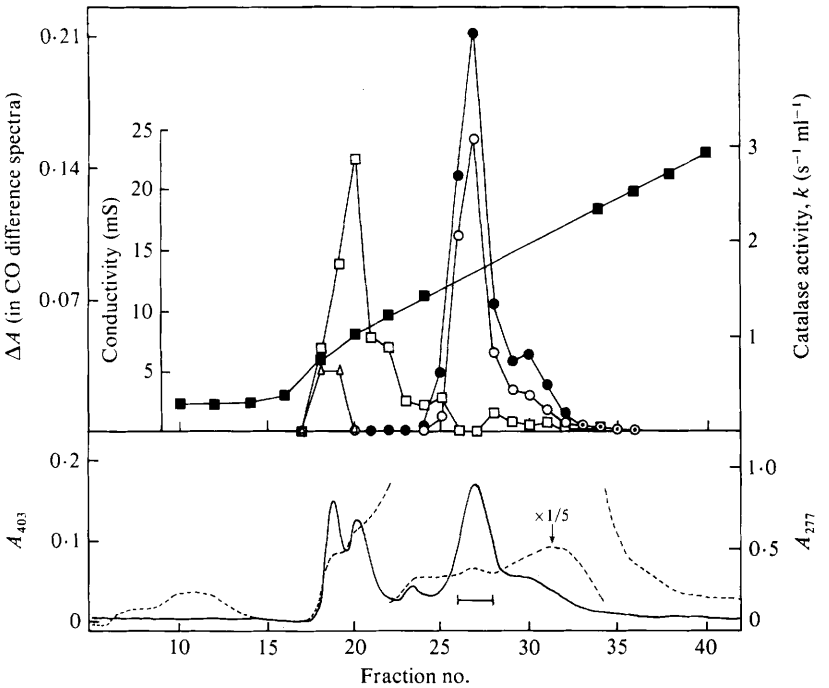

Fig. 1. Co-purification of catalase activity and haemoprotein *b*-590 by ion-exchange chromatography. A sample (25 ml) was applied to a column (2·5 × 21·5 cm) of DEAE-Sepharose CL6B equilibrated with 50 mM-Tris/HCl, pH 8·0, and eluted with a linear gradient of 0 to 1·0 M-NaCl in this buffer (see conductivity, ■). CO difference spectra (i.e. CO + reduced *minus* reduced; room temperature, 1 cm path length) of the fractions (13 ml) were used to quantify haemoprotein *b*-590 (●, 446−480 nm), CO-binding cytochrome *c* (□, 413−433 nm) and a CO-binding pigment characterized by its trough in such spectra (△, 436−480 nm). Catalase activity (○), A_{403} (——) and A_{277} (– – –) are also plotted as a function of fraction number. The bar indicates the fractions pooled for further purification.

Addition of $(NH_4)_2SO_4$ to 40% saturation typically precipitated 29 to 35% of total protein and 67 to 84% of the haemoprotein *b*-590 (Table 1). Although the remainder of the haemoprotein was precipitated between 40 and 70% saturation, such fractions were heavily contaminated with *c*-type cytochromes. Thus, of the *c*-type cytochromes present in S_1, the following were detected in the 40 to 70% $(NH_4)_2SO_4$ fraction: total *c*-type cytochromes (552·5–542 nm in reduced *minus* oxidized spectra), 82%; CO-binding *c*-type cytochrome (553–538 nm in CO difference spectra), 70%; and a haemoprotein detectable by a trough at about 436 nm in CO difference spectra, approximately 95%. This last compound was probably masked by the presence of haemoprotein *b*-590 in the 0 to 40% $(NH_4)_2SO_4$ fraction.

The first large-scale Sephacryl S400 gel filtration of the dialysed and concentrated $(NH_4)_2SO_4$ precipitate gave two major peaks of haem, as judged by A_{403} (elution profile not shown), which were pooled. A minor peak of lower K_{av} contained a heterogeneous mixture of cytochromes and negligible catalase activity. The peak of catalase activity was coincident with the peak of haemoprotein *b*-590 and A_{403}; a shoulder on the A_{403} profile at higher K_{av} values was due to CO-binding cytochrome *c*.

Ion exchange chromatography of the pooled fractions from gel filtration (see Methods) clearly separated (Fig. 1) CO-binding cytochrome *c* (eluted at 5 to 11 mS) from haemoprotein *b*-590 and catalase activity (eluted at 12 to 16 mS). Cytochrome *c* peroxidase activity (Baines *et al.*, 1984) also eluted coincident with catalase (not shown). The pooled fractions showed a > fourfold increase in purity of haemoprotein *b*-590 over the loaded material (Table 1).

A second chromatography step on Sephacryl S400 gave a bimodal profile of A_{277} (Fig. 2); one peak coincided with both the peak of catalase activity and A_{403}. Fractions 25 to 33 showed no traces of *c*-type cytochrome in reduced *minus* oxidized or CO difference spectra.

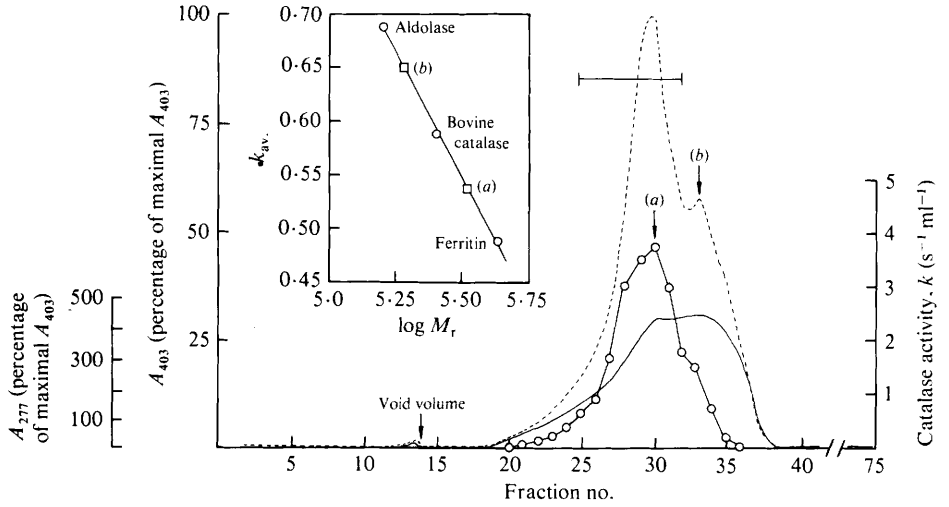

Fig. 2. Gel filtration of haemoprotein *b*-590 on Sephacryl S400. A sample (2·4 ml) was applied to a column (1·6 × 58·5 cm) of Sephacryl S400 equilibrated with 50 mM-Tris/HCl, pH 8·0. Catalase activity (○), A_{277} (——) and A_{403} (– – –) are plotted as a function of fraction (2·8 ml) number. The inset shows M_r determination for the native enzyme migrating at the peak of catalase activity (*a*) or as a shoulder (*b*). log M_r of haemoprotein *b*-590 and protein standards are plotted as a function of $K_{av} = V_e - V_0/V_t - V_0$, where V_e = elution volume for the protein, V_0 = column void volume (= elution volume for Blue Dextran 2000) and V_t = total bed volume. The M_r values for the standards were taken to be 158000 (aldolase), 230000 (bovine catalase) and 440000 (ferritin), giving M_r values for haemoprotein *b*-590 of 331000 (*a*) and 188000 (*b*). The recovery of haemoprotein *b*-590 (assayed in CO difference spectra) was 87·9%. The bar indicates fractions pooled for subsequent studies.

Determination of M_r

The M_r of the native protein was determined during final chromatography on Sephacryl S400 using appropriate M_r markers as described in Methods. Protein in the fraction containing the peak of catalase activity (Fig. 2) was found to have a M_r of 331000. A shoulder of activity that coincided in the elution profile with a second protein peak had an apparent M_r of 188000. However, the estimated apparent M_r values of the protein were variable; two preparations less pure than that shown in Fig. 2 ($A_{403}/A_{280} < 0{\cdot}23$) migrated with apparent M_r values of about 280000 (results not shown).

The subunit M_r was estimated to be 75000 from SDS gel electrophoresis. A minor band running at an apparent M_r of 53000 was also detected (results not shown). It is suggested that the predominant form of the enzyme purified is a tetramer (M_r = 331000) while the minor species (M_r = 188000), which retains considerable catalase activity, is probably a dimer.

Isoelectric point

Preparative isoelectric focusing of the crude material precipitated at 40% saturation with $(NH_4)_2SO_4$ gave threee major, well-separated coloured bands, with isoelectric points (pI) of 4·4 (brown), 5·0 (yellow) and 5·2 to 5·4 (tan). The first of these was haemoprotein *b*-590, contaminated with *c*-type cytochrome, which was evident only as a small shoulder at 420 nm in the absolute spectrum of the reduced sample. Sinclair (1966) found a pI of 4·1 for the peroxidase component of the 'cytochrome a_1b' using starch gel electrophoresis. Contamination by cytochrome *c* was significantly lower than that in the final preparation of Sinclair (1966), obtained by chromatography on Sephadex G200 and $(NH_4)_2SO_4$ precipitation, where a prominent peak at 421 nm was observed. The CO difference spectrum of the band at pI 4·4 revealed no contaminating CO-binding pigments (results not shown). The recovery of haemoprotein *b*-590 from the focusing gel was typically 24% and only limited quantities of

protein (< 200 mg) could be loaded. The method does, however, provide a rapid preparation of small amounts of haemoprotein. The bands at pI 5·0 and 5·2 to 5·4 contained cytochrome *c*; the reduced form of the former, and to a lesser extent of the latter, bound CO. Subsequent studies, to be reported elsewhere, of the broad band at about pI 5·3 revealed two close bands of similar intensity.

Analytical electrofocusing of purified haemoprotein *b*-590 obtained by gel filtration and ion exchange chromatography showed a single band at pI 4·4.

Absolute absorption spectra of the purified protein

As isolated, the purified haemoprotein has a γ-band peaking at 408·5 nm, a weak absorption shoulder between 500 and 550 nm and a further band peaking close to 632 nm (Fig. 3), suggesting high-spin ferric haem as in metmyoglobin (Wood, 1984).

In the absence of complex-forming ligands, reduction by dithionite elicited dramatic changes in the spectrum (Fig. 3). Complete reduction was not immediate; 20 to 30 min incubation with the reductant was required (not shown). The γ-band of the reduced form is broad (peak at 441 nm); in the α,β region, a broad band peaking at 559 nm has a distinct shoulder at 590 nm and a weak absorbance centred at about 632 nm. We proposed earlier (Poole *et al.*, 1984) that the 590 nm band is the α-band of a ferrous high-spin *b*-type haemoprotein; it is unusual but not unprecedented (e.g. tryptophan 2,3-dioxygenase) for the α-band to be so distinct. The 559 nm band is the β-band as is the dominating 556 nm band of ferrous myoglobin (Wood, 1984).

When a dithionite-reduced sample is bubbled with CO, the CO complex (low-spin) has a sharp γ-band at 426 nm (Fig. 3). The α- and β-bands are shifted 16 and 14 nm, respectively, to shorter wavelengths, with a slight trough between them, and the peak heights are more nearly equal. A band at 635 nm of unknown origin is also evident; it is possibly due to degraded protohaemoprotein, as found in other catalases.

These spectra are broadly similar to those presented for a partially purified preparation (Baines *et al.*, 1984). The spectra presented for HP-I (Claiborne & Fridovich, 1979) did not include the region > 560 nm and the effect of reductants and ligands was not described, but the spectra show a γ-band with a peak at 407 nm and a weak absorbance near 540 nm indicative of high-spin haem. The A_{405}/A_{280} ratio of the present preparation ($\approx$0·22; Fig. 3, inset) is significantly lower than that of HP-I (0·55) and other microbial catalases. Values obtained in other preparations of haemoprotein *b*-590 never exceeded 0·27. The absolute spectra of the pyridine haemochrome species were similar to those for myoglobin (Wood, 1984) and indicative of low-spin haem. The reduced pyridine haemochrome showed peaks at 418·5, 525·5 and 556 nm, identical to reduced pyridine protohaemochrome; addition of CO gave peaks at 417, 536·5 and 567·5 nm (not shown). The spectra of reduced and CO-liganded forms of the pyridine haemochrome were featureless between 580 and 630 nm, indicating the absence of *a*-type haems.

Difference absorption spectra of the purified protein

In the spectrum of the difference between the dithionite-reduced and native (ferric) forms at room temperature (Fig. 4*a*), the α-, β- and γ-bands are all shifted 3 to 5 nm to longer wavelengths compared to the absolute spectrum of the reduced form. At 77 K (Fig. 4*b*), the γ- and α-bands appear further red-shifted (to 445·5 and 594·5 nm respectively), whilst the β-band is blue-shifted to 562 nm. The 77 K spectrum and its first derivative (Fig. 4*c*) suggest the presence of a band at about 550 nm; its origin is unclear but a minor amount of *c*-type haem may be present (see below). The reduced *minus* oxidized spectrum of the alkaline pyridine haemochrome (Fig. 4*d*) is typical of the low-spin pyridine protohaemochrome IX and similar to that of low-spin *b*-type cytochromes. The α-peak is at 556 nm and the β-band at 524·5 nm. Measurement of absorbance values at wavelength pairs appropriate for proto- and *c*-type haem showed that cytochrome *c* could be present at up to 5% of the *b*-haem concentration (Appleby, 1978). The difference spectrum of the pyridine haemochrome was featureless between 580 and 630 nm, again confirming the absence of *a*-type haem.

The CO-reduced *minus* reduced difference spectrum at room temperature (Fig. 5*a*) shows a

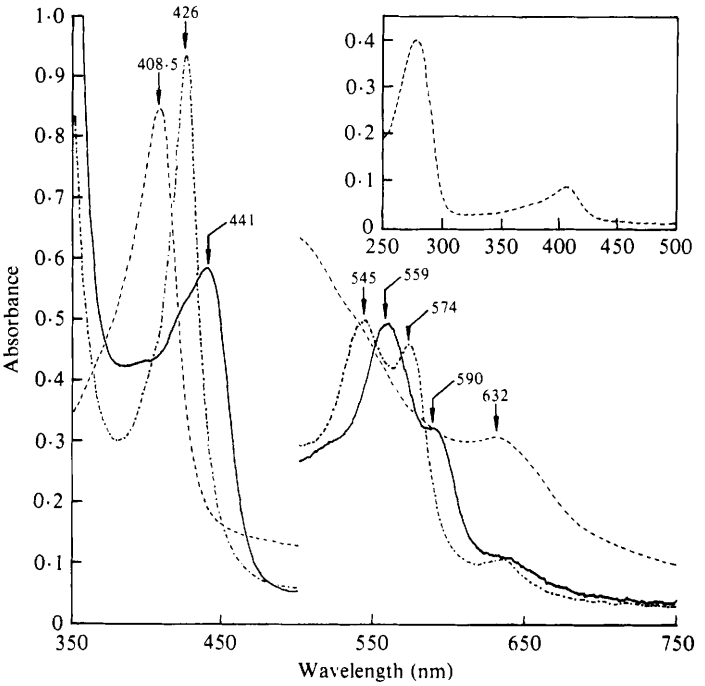

Fig. 3. Absorbance spectra were recorded of a sample of purified haemoprotein *b*-590 (peak fraction, Fig. 2), containing 5·68 μM protohaem. A baseline was recorded (not shown) using two cuvettes (1 cm path length) each containing 50 mM-Tris/HCl, pH 7·5, at 25 °C; the baseline was then 'memorized' in the spectrophotometer. Replacement of the 'sample' cuvette contents with haemoprotein *b*-590 gave the absolute spectrum of the ferric 'native' protein (---). A few grains of $Na_2S_2O_4$ were added and the spectrum of the reduced form recorded after 30 min (——). CO was slowly bubbled into the cuvette for 30 s, any foam being dispersed by touching with a glass rod smeared with Dow-Corning antifoam A. After a further 30 s, the cuvette was scanned again (–·–·). The spectral band width was 1·0 nm, the scan rate 1 nm s^{-1} and spectra were recorded on the computer data disc at 0·5 nm intervals. From 500 to 750 nm, the spectra of all these states are expanded fivefold on the absorbance axis. The inset shows a scan of the ferric protein in a cuvette of 1 mm path length. The ratio A_{407}/A_{277} is 0·22.

number of peaks and troughs. The 597 nm trough approximately coincides with the α-band of the reduced form, whilst the absorption maximum of the CO complex is at 578 nm. The 560·5 nm trough is attributed mainly to the β-band of the uncomplexed protein, but is also influenced by the trough at 563 nm between the α- and β-bands of the CO complex (Fig. 3*c*). The peak at 539 nm is the β-band of the CO complex. The γ-band of the complex is at 426 nm, indicative of a *b*-type haemoprotein (Wood, 1984), and the trough at 446 nm is caused by the disappearance from the difference spectrum of the γ-band of the uncomplexed reduced haemoprotein. From the absorption difference (A_{426}–A_{446}) between the γ-peak and γ-trough of the CO-reduced *minus* reduced spectrum (see Fig. 5a), a difference absorption coefficient (Δε) of 131 mM^{-1} cm^{-1} was calculated, a lower value than for some other high-spin *b*-type haemoproteins (Wood, 1984). For the wavelength pair, 446–480 nm, a difference absorption coefficient (Δε) of 60 mM^{-1} cm^{-1} was obtained. This value should be useful in quantifying *b*-590 in preparations containing other CO-binding pigments such as *o*- and *c*-types, whose peaks in such spectra would interfere at 426 nm but have less effect at 446 nm.

At 77 K (Fig. 5*b*), the α- and β-bands are displaced by up to 2 nm to lower wavelengths whereas in the γ-region, the trough is displaced almost 4 nm in the opposite direction. There was little band sharpening at low temperature and even the α-band of the CO complex at 578 nm (which is characteristically sharpened in myoglobin spectra) was little changed; its band width (at half peak height, measured from a baseline drawn between the neighbouring troughs)

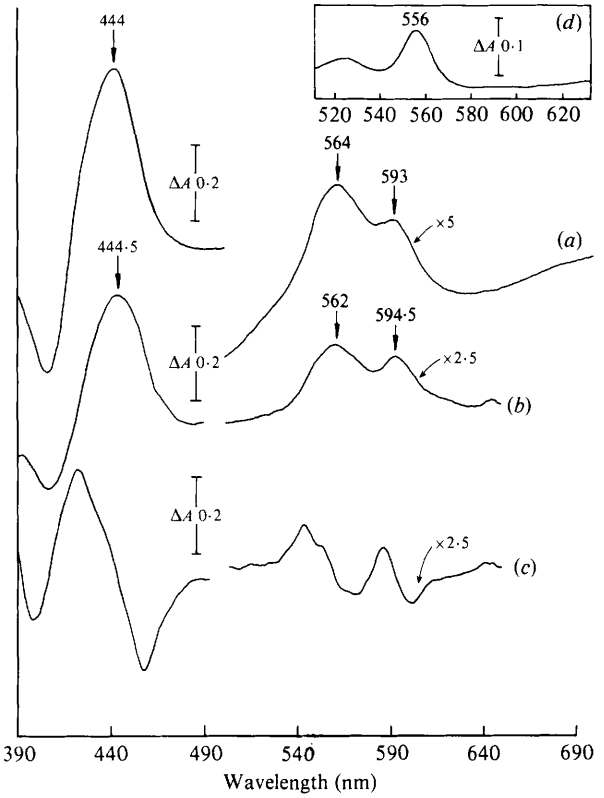

Fig. 4. Reduced *minus* oxidized difference spectra of purified haemoprotein *b*-590. Spectrum (*a*) is the computed difference between the spectra of native (ferric) and dithionite-reduced samples recorded at room temperature (1 cm path length) and shown as absolute spectra in Fig. 3. These samples were diluted with an equal volume of 60% (v/v) ethylene glycol containing $Na_2S_2O_4$, where appropriate, for the spectrum (*b*) recorded at 77 K (2 mm path length) and its first derivative (*c*). Spectrum (*d*) (inset) is the reduced *minus* oxidized difference spectrum of the pyridine haemochrome, prepared as described in Methods. The spectral band width was 1·0 nm throughout and the scan speed was 1 nm s^{-1} (*a*, *d*) or 0·5 nm s^{-1} (*b*, *c*). The response time was 'fast' for spectra (*a*) and (*d*) and 'auto' for (*b*) and (*c*).

decreased from 20 nm at room temperature (Fig. 5*a*) to 17 nm at 77 K (Fig. 5*b*). The first derivative spectrum (Fig. 5*c*) reveals no new features attributable to the contaminating cytochromes, apart from an undulation near 550 nm, possibly due to a trace of CO-binding *c*-type haem (see above).

These features of the CO-reduced *minus* reduced difference spectrum (Fig. 5*a*) are very similar to those reported from carboxymyoglobin. Two further indications of the high-spin state in haemoprotein *b*-590 may be obtained from this spectrum (Wood, 1984). Firstly, by inserting a line (see dashed line, Fig. 5*a*) between the (α,β) trough at 597 nm and the weak trough at about 510 nm in the room temperature CO difference spectrum, the trough at 560·5 nm is observed above this line, but close to it, suggesting that the reduced state is high-spin. Secondly, the ratio of absorbance difference between the γ-peak (426 nm) and trough (446 nm) to α-peak (578 nm) and trough (560·5 nm) (i.e. $A_{426}-A_{446} : A_{578}-A_{560\cdot5}$) in the CO-reduced *minus* reduced spectrum (see Fig. 5*a*) is 22·7: values above 30 or below 10 are typical of high- and low-spin states, respectively, and intermediate values indicate a mixture.

Kinetics of the catalatic reaction

The kinetics of most catalases are abnormal, in the sense that (i) the enzyme cannot be saturated with substrate within feasible concentration ranges (up to 5 M-H_2O_2), and (ii) the

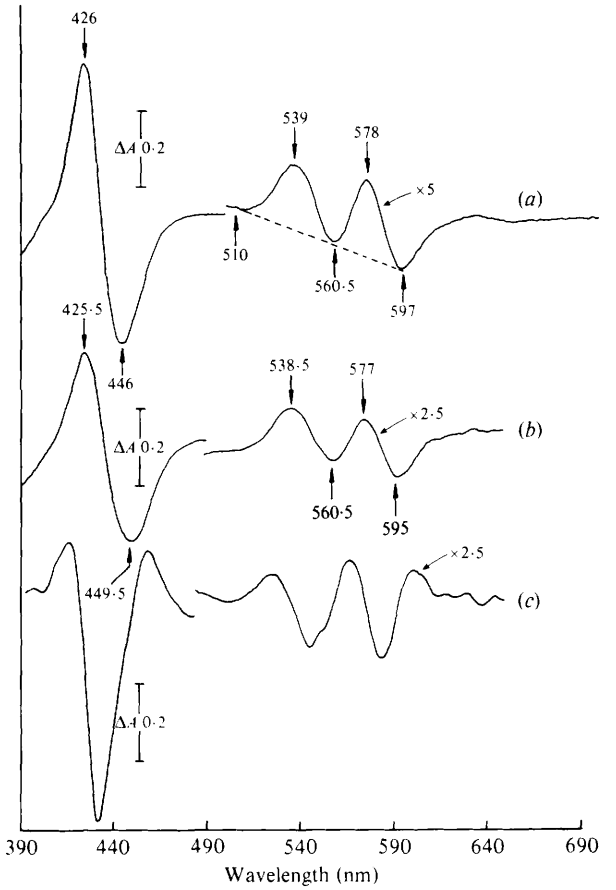

Fig. 5. CO + reduced *minus* reduced difference spectra of purified haemoprotein *b*-590. Spectrum (*a*) is the computed difference between the spectra of a dithionite-reduced sample and the same sample bubbled with CO, recorded at room temperature (1 cm path length) and shown as absolute spectra in Fig. 3. These samples were diluted with an equal volume of 60% ethylene glycol, containing $Na_2S_2O_4$, for the spectrum (*b*) recorded at 77 K (2 mm path length) and its first derivative (*c*). Spectral conditions were as for the corresponding spectra in Fig. 4.

mammalian enzyme is inactivated at H_2O_2 concentrations above 0·1 M. However, at H_2O_2 concentrations up to approximately 20 mM, *E. coli* haemoprotein *b*-590 did not suffer rapid inactivation and the initial linear rates of H_2O_2 decomposition were used to estimate values for K_m and V_{max} from Lineweaver–Burk plots (not shown). Using seventeen H_2O_2 concentrations between 1·8 and 18 mM (correlation coefficient, *r*, 0·98) the K_m was found to be 11 mM and the maximum turnover rate (V_{max}) 2·3 × 10^6 mol H_2O_2 (mol haem)$^{-1}$ min^{-1}. The K_m of this preparation is thus higher than the values (3·7 to 4·1 mM) given by Claiborne & Fridovich (1979) and Loewen & Triggs (1984). The V_{max} is also higher than the value of 0·49 × 10^6 mol H_2O_2 (mol haem)$^{-1}$ min^{-1} for HP-I, assuming that their haemoprotein contained 2 mol of haem per mol. Using the Aebi (1974) assay, the specific activity of the purified enzyme, expressed as a first order rate constant and at an initial H_2O_2 concentration of 10 mM, was 0·3 × 10^7 M^{-1} s^{-1}. This is 10-fold lower than the value for catalase purified from human erythrocytes (see Aebi, 1974).

Kinetics of the peroxidatic reaction

During enzymic peroxidation of *o*-dianisidine, initial rates were not well-maintained, a phenomenon attributed by Claiborne & Fridovich (1979) to destruction of the enzyme by

formation of an unstable peroxidase intermediate. The pH-activity profile in phosphate buffer (not shown) was similar to that described by these authors (who reported a pH optimum of 6·5), with a broad optimum between pH 6·1 and 6·6 and a rate, expressed as ΔA_{460}, in the presence of 1 mM-enzyme (as haem) of 0·058 min^{-1}. The value obtained by Claiborne & Fridovich (1979) is 0·052 min^{-1}, assuming 1 mol of their preparation to contain 2 mol haem. The peroxidase activity of haemoprotein *b*-590 was 3850 mol H_2O_2 decomposed (mol haem)$^{-1}$ min^{-1} with *o*-dianisidine as substrate. The peroxidation of TCIP was slower, 14·3 mol H_2O_2 (mol haem)$^{-1}$ min^{-1}, but was not subject to suicide inhibition and gave linear rates suitable for kinetic analysis. Varying the H_2O_2 concentration between 1 and 20 mM gave a Lineweaver–Burk plot (correlation coefficient, *r*, 0·97) that showed a K_m for this activity of 0·18 mM and a V_{max} of 19·2 mol H_2O_2 decomposed (mol haem)$^{-1}$ min^{-1}. The K_m is higher than the value of 0·048 mM for HP-I and/or II given by Loewen & Triggs (1984) with *o*-dianisidine as substrate. Guaiacol peroxidase activity was undetectable under the conditions specified [$\Delta A_{470} < 0{\cdot}005$ min^{-1} (0·01 nmol haem)$^{-1}$].

DISCUSSION

Catalases and peroxidases, collectively referred to as hydroperoxidases, are responsible for removing deleterious alkyl and hydrogen peroxides from living cells and tissues. Their activities are fundamentally similar; peroxidases reduce peroxide in a single-electron transfer from a reduced donor (such as cytochrome *c*), whilst catalases disproportionate peroxides, the oxidative power of one peroxide molecule being used to oxidize another in an electron pair transfer. *E. coli* possesses three electrophoretically distinct hydroperoxidases (for a survey, see Poole & Ingledew, 1986), only two of which have been well characterized. HP-I is constitutive, being present even in anaerobically grown cells, whereas HP-II (of higher anodic mobility) is synthesized during growth in which electron transfer occurs to a terminal electron acceptor such as oxygen or nitrate (Hassan & Fridovich, 1978). HP-I and HP-II are of similar M_r (337000 in the case of HP-I) and subunit composition (4 subunits of 78000), but whilst both exhibit high catalase activities, only HP-I has any appreciable peroxidatic activity. The absorption spectrum of the untreated (presumably ferric) HP-II is unusual in showing a band at 591 nm in addition to that at 407 nm (Claiborne *et al.*, 1979). HP-III (Loewen *et al.*, 1983) does not have peroxidase activity and is electrophoretically and immunologically distinct from HP-I and HP-II (Loewen & Triggs, 1984). It is thus to HP-I that the protein purified by us, haemoprotein *b*-590, bears the strongest resemblance. It is a soluble protein and was purified from anaerobically grown cells, although a spectrally similar protein is found in *E. coli* grown under various conditions, both aerobically and anaerobically (H. D. Williams and R. K. Poole, unpublished).

Similarities between haemoprotein *b*-590 and HP-I extend also to the cytochrome 'a_1b' purified by Sinclair (1966) and Barrett & Sinclair (1967), although spectral data are very incomplete for HP-I, and no kinetic description of the enzyme activities of the 'a_1b' fraction is available. Haemoprotein *b*-590 and HP-I have similar M_r values as do their subunits in SDS-polyacrylamide gels. By analogy with most other catalases, and consistent with these M_r determinations, each protein is believed to consist of four identical subunits. Both contain only protohaem IX. The haem content of 'cytochrome a_1b' was not reported. A striking difference, however, is the apparent haem content. HP-I contains two molecules of haem per tetramer, consistent with the low A_{405}/A_{280} ratio (0·55). The ratio in the present work (0·27) indicates only one haem per tetramer. Like Claiborne & Fridovich (1979), we observed that the ratio for haemoprotein *b*-590 was similar in all preparations; in our preparation, but not theirs, it never exceeded 0·27. It is well known that in mammalian catalase two of the four haems are degraded to a high-spin Fe(III)-biliverdin complex (for references see Reid *et al.*, 1981). Microbial catalases appear to contain between 2 and 3·4 mol protohaem per tetramer (Jacob & Orme-Johnson, 1979; Nies & Schlegel, 1982; Jouve *et al.*, 1984).

Despite the low haem content, haemoprotein *b*-590 is an active catalase and peroxidase. The V_{max} of the catalase activity is more than fourfold higher on a haem basis than for HP-I but the K_m is also higher. Like HP-I, it is a broad specificity peroxidase with a slightly acid pH optimum

and the kinetics of *o*-dianisidine peroxidation are markedly non-linear. The K_m for H_2O_2 is about 60-fold lower in the peroxidatic mode of action than the catalatic one, a relationship similar to that reported by Loewen & Triggs (1984). All studies on the peroxidatic reactions are frustrated by the very rapid catalatic decomposition of H_2O_2 which will influence the ability to detect peroxidation of a given substrate. Nevertheless, there is some agreement between the peroxidatic capabilities of the three preparations, although we could not detect activity with guaiacol. The use of alkyl peroxides that are not destroyed by catalase (e.g. Baines *et al.*, 1984) may reveal further similarities.

The 'cytochrome a_1b' preparation from 'soluble' fractions of aerobically or anaerobically grown cells of *E. coli* (Sinclair, 1966; Barrett & Sinclair, 1967) closely resembled haemoprotein *b*-590 in its spectral properties. The ferric form had bands at 405 and near 640 nm, the reduced form at 442, 560 and 596 nm and the reduced + CO complex showed maxima at 425, about 540 and at 575 nm (compare with Fig. 3). The 'a_1b' preparation also contained a cytochrome *c*, however. This preparation had five times the TCIP-peroxidatic activity of the crude membrane and some catalase activity (Sinclair, 1966). It was reported that the preparation also had peroxidatic activity towards guaiacol and cytochrome *c* but that the H_2O_2 was rapidly depleted by catalatic action.

The bands near 590 and 560 nm, respectively, in the reduced state led Sinclair (1966) and Barrett & Sinclair (1967) to suppose that their preparation contained a_1- and *b*-type haems. Recent work from our laboratory, however, has shown that the 590 nm band is not due to *a*-type haem but is the unusually prominent α-band of a high-spin *b*-type haemoprotein. The 560 nm band is not, as previously supposed, the α-band of a low-spin cytochrome *b*, but the β-band of the same high-spin haemoprotein (Baines *et al.*, 1984; Poole *et al.*, 1984). The present work confirms our previous finding that haem *a* is not present in suspensions of whole or broken cells that exhibit the a_1-like band. Reduction of HP-I was not reported by Claiborne & Fridovich (1979), possibly because the haem of 'classical' catalases is not dithionite-reducible and so further comparison in this respect is not possible.

This work demonstrates the catalatic and peroxidatic capabilities of haemoprotein *b*-590. It has long been suspected that a cytochrome a_1-like pigment in *E. coli* binds CO, and several workers have commented that such a pigment could function as a third terminal oxidase. However, neither photochemical action spectroscopy (Castor & Chance, 1959) nor stopped-flow kinetic studies (Haddock *et al.*, 1976) support this proposal. The one report (Edwards *et al.*, 1981) claiming 'cytochrome a_1' in *E. coli* to be a terminal oxidase describes a band in the photochemical action spectra at 592 nm. It is clear that the haemoprotein described in the present paper cannot account for that band, the nearest absorption maximum of the ferrous CO-liganded haemoprotein being at 574 nm. However, an oxidase role is still possible for the a_1-like haemoprotein that remains membrane-associated after cell disruption and fractionation and copurifies with the cytochrome *bd* complex. The absolute spectrum of the CO-liganded form of the membrane a_1-like haemoprotein has not been described but potentiometric resolution of the reduced *minus* oxidized difference spectrum of the cytochrome *bd* complex reveals a component closely resembling the protein described in the present paper, with maxima at 594 and 562 nm and a trough at 645 nm. Although it is premature to assume that this a_1-like component and haemoprotein *b*-590 are identical, it is interesting that a membrane-bound hydroperoxidase, detected immunologically, has a similar subunit M_r and pI to the soluble haemoprotein *b*-590.

Finally, we refrain from the danger of suggesting that all bacterial a_1-like haemoproteins resemble *b*-590. Indeed, a recent classification (Poole *et al.*, 1985*b*) recognizes four classes of a_1-like pigments. In class I are the membrane-bound 'true' cytochromes a_1 that function as oxidases. In class II is the cytochrome a_1c_1 complex from *Nitrobacter agilis* that acts as a nitrite dehydrogenase. Haemoprotein *b*-590 is included in class III where we propose many 'a_1-like' pigments should be placed. For example, recent work (C. A. Appleby & R. K. Poole, unpublished) suggests that a soluble, cytochrome a_1-like pigment in *Rhizobium japonicum*, previously called cytochrome P-428 (from the absorbance maximum of the CO-liganded, reduced form) and regarded as a possible terminal oxidase (Appleby, 1969), should be included in this class. The protein has been purified from soluble fractions and resembles haemoprotein *b*-

590 in its spectral properties and catalatic and peroxidatic activities. In class IV is the membrane- and cytochrome *d*-associated a_1-like haemoprotein. Further work is required to investigate the relationship with haemoprotein *b*-590. An interesting possibility is that this class IV a_1-like component plays a role in decomposing H_2O_2 generated during O_2 reduction by the cytochrome *bd* oxidase complex.

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