Purification and characterization of catalase HPII from *Escherichia coli* K12

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Catalase (hydroperoxidase II or HPII) of *Escherichia coli* K12 has been purified using a protocol that also allows the purification of the second catalase HPI in large amounts. The purified HPII was found to have equal amounts of two subunits with molecular weights of 90 000 and 92 000. Only a single 92 000 subunit was present in the immunoprecipitate created when HPII antiserum was added directly to a crude extract, suggesting that proteolysis was responsible for the smaller subunit. The apparent native molecular weight was determined to be 532 000, suggesting a hexamer structure for the enzyme, an unusual structure for a catalase. HPII was very stable, remaining maximally active over the pH range 4–11 and retaining activity even in a solution of 0.1% sodium dodecyl sulfate and 7 *M* urea. The heme cofactor associated with HPII was also unusual for a catalase, in resembling heme *d* (a_2) both spectrally and in terms of solubility. On the basis of heme-associated iron, six heme groups were associated with each molecule of enzyme or one per subunit.

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Nous avons purifié la catalase (hydroperoxydase II ou HPII) d'*Escherichia coli* K12 à l'aide d'un protocole qui permet aussi la purification en grandes quantités de la seconde catalase HPI. La HPII purifiée contient des quantités égales de deux sous-unités dont les poids moléculaires sont 90 000 et 92 000. Seule la sous-unité de 92 000 est présente dans l'immunoprécipité formé quand l'antisérum contre HPII est ajouté directement à un extrait brut; la protéolyse serait donc responsable de la sous-unité plus petite. Nous avons déterminé que le poids moléculaire natif est de 532 000; l'enzyme aurait donc une structure hexamère, structure inhabituelle pour une catalase. HPII est très stable. Elle conserve son activité maximale à des pH allant de 4 à 11 et elle retient cette activité même dans une solution de dodécyl sulfate de sodium 0,1% et d'urée 7 *M*. Le cofacteur hème associé à l'HPII est aussi inhabituel pour une catalase en ce qu'il ressemble à l'hème *d* (a_2) tant au point de vue spectral qu'en termes de solubilité. Nous basant sur le fer associé à l'hème, nous savons que six groupes hémiques sont associés à chaque molécule d'enzyme, un par sous-unité.

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Introduction

Biochemical and genetical studies have revealed that *Escherichia coli* produces two catalases. One catalase, also called HPI, exists as an isoenzyme pair and is bifunctional possessing both catalase and peroxidase activities. HPI has been purified from *E. coli* B and characterized as a tetramer with subunits having a molecular weight of 84 000 (1). The synthesis of this enzyme is affected and possibly encoded by *katG* (2). The second catalase HPII is monofunctional and, as purified from *E. coli* B, exists as a tetramer with subunits having a molecular weight of 78 000 (3). The synthesis of this enzyme is affected by *katE* (4) and *katF* (5). A third catalase named HPIII was identified in *E. coli* (5), but this was later determined to be HPII.

The existence of two loci affecting the synthesis of HPII raised the question of whether the subunits were in fact identical as reported or different but coincidently with the same molecular weight. This paper describes the purification of HPII from *E. coli* K12 and its partial characterization having a subunit composition quite different from that previously reported and a heme cofactor that resembles heme *d*.

ABBREVIATIONS: HP, hydroperoxidase; SDS, sodium dodecyl sulfate.

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Materials and methods

Materials

Bactotryptone and yeast extract were obtained from Gibco. DEAE-Sephadex A-25 and the most common biochemicals were obtained from Sigma. Sepharose 4B and 6B were obtained from Pharmacia. Bio-Gel A-1.5 m, Bio-Gel HTP, and acrylamide were obtained from Bio-Rad. Ultrapure guanidine hydrochloride was obtained from BRL.

Strains and growth medium

The following strains were used: JA200/pLC36-19 (F^+ *recA trp thr leu*) carrying plasmid pLC36-19 that encodes HPI (6), UM120 (HfrH *thi katE*::Tn10) producing only HPI, UM202 (HfrH *thi katG*::Tn10) producing only HPII (7), and B23 (wild type) (8). For catalase preparations, all strains were grown in LB medium (9) containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per litre. Cultures were grown in 10-L batches with forced aeration and stirring for 16–18 h. Cells were collected by centrifugation and frozen as a cell paste. The normal yield was 40 g of paste from 10 L of medium.

Purification of HPII and HPI

The procedure followed was essentially that of Claiborne and Fridovich (1) used to prepare HPI, but with a number of

TABLE 1. Purification of catalases HPII from 40 g of cell paste of *E. coli* JA200/pLC36-19

Step	Volume, mL	Total protein, mg	Total units	Yield, %	Specific activity, U/mg	Purification, *n*-fold
1. Crude extract	230	6463	613 000	100	95	1.0
2. 2.5% Streptomycin sulfate	220	3520	605 000	99	172	1.8
3. $(NH_4)_2SO_4$ fractionation I						
40–50% Pellet	53	610	146 000	24	240	2.5
50–60% Pellet	50	910	179 000	29	197	2.1
4. $(NH_4)_2SO_4$ fractionation II						
40–50% Pellet	26	1001	104 000	17	104	1.1
50–60% Pellet	25	473	97 900	16	207	2.2
5. $(NH_4)_2SO_4$ fractionation III, of 50% pellet in step 4						
50–60% Pellet	15	132	50 000	8	379	4.0
6. $(NH_4)_2SO_4$ fractionation III, of 60% pellet in step 4						
50–60% Pellet	15	255	83 500	14	327	3.5
7. DEAE-Sephadex A-25 of 60% pellets in steps 5 and 6	7	35.7	110 800	18	3 100	32.7
8. Bio-Gel A-1.5 m	19	9.5	82 300	13	8 670	91.3
9. Bio-Gel HTP	2	3.6	53 300	9	14 820	156.1

technical modifications that would allow the simultaneous purification of HPI and HPII. All manipulations were carried out at 5°C. The crude extract was prepared using the Aminco French press at 20 000 psi (1 psi = 6.894 757 kPa) and a 2.5% streptomycin sulfate precipitation was carried out as described (1), except that 1 m*M* EDTA was present in the buffer. The subsequent ammonium sulfate precipitation was modified to separate HPII from HPI and also to achieve a greater increase in the specific activity of HPII. The first fractionation was carried out at 30, 40, 50, and 60% saturation with solid ammonium sulfate. At each step the solution was stirred gently for 30 min to ensure complete dissolution and precipitation. The precipitate was collected by centrifugation and additional ammonium sulfate was added to the supernatant. Except when UM202 was used, HPI was usually found in the 30 and 40% pellets and could be subjected to further purification by additional ammonium sulfate precipitations and elution from DEAE-Sephadex A-25. No further steps were required. HPII was found in the 50 and 60% pellets, and following dissolution the combined solutions were fractionated a second time with ammonium sulfate at 40, 50, and 60% saturation. The individual 50 and 60% pellets were subjected to one further ammonium sulfate fractionation from which HPII with a specific activity greater than 300 U/mg protein was obtained.

The pooled ammonium sulfate fractions were dialyzed for 4 h against 4 L of 50 m*M* potassium phosphate (pH 7.0) and charged on a 2.8 × 55 cm column of DEAE-Sephadex A-25 equilibrated in the same buffer. The column was washed until the absorbance of the eluate at 280 nm had dropped below 0.05. HPII was eluted with a linear gradient prepared by mixing 50 m*M* potassium phosphate (pH 7.0) with 0.5 *M* NaCl in the same buffer. Although the ammonium sulfate fractionation usually effected a complete separation of HPI and HPII, the two enyzmes were also separated on this column (5). The peak of HPII activity was pooled, concentrated by ultrafiltration (Amicon), and loaded on a 2.2 × 100 cm column of Bio-Gel A-1.5 m equilibrated with 5 m*M* potassium phosphate (pH 7.0). The peak of HPII activity was pooled and immediately applied, without further concentration or dialysis, to a 2.2 × 10 cm column of Bio-Gel HTP equilibrated with 5 m*M* potassium phosphate (pH 7.0). The column was eluted with a linear gradient prepared by mixing equal volumes of 5 and 400 m*M* potassium phosphate (pH 7.0). The HPII-containing eluate was concentrated by ultrafiltration, dialyzed for 4 h against 1 L of 50 m*M* sodium phosphate (pH 7.0), and stored at −20°C. A summary of the results from the purification of HPII from JA200/pLC36-19 is contained in Table 1.

Native molecular weight analysis

Three independent methods were used. The sedimentation equilibrium method of Yphantis (10, 11) in a Beckman model E ultracentrifuge at 20°C was used. Samples were dialyzed against 50 m*M* potassium phosphate (pH 7.0). The dialyzed samples were run at 0.2, 0.4, and 0.8 mg/mL in a six-sector cell with the dialyzate used as reference. A partial specific volume of 0.73 cm/g (3) was assumed.

The method of Hedrick and Smith (12) was also used in which the protein was electrophoresed as described by Davis (13), except in pH 8.1 Tris–HCl, on a series of gels of various acrylamide concentrations from 6 to 14%. The R_f of the protein relative to bromphenol blue tracking dye was determined for each different percentage gel. The retardation coefficient for each protein was determined from the slope of a graph of 100 log ($100R_f$) against the polyacrylamide concentration. The logarithm of the retardation coefficient was then plotted against the logarithm of the protein molecular weight.

The gels were stained for protein with Coomassie brilliant blue dye or for catalase and peroxidase activity as described below.

Lastly, the gel filtration method was used. A 0.7 × 110 cm column of Sepharose 4B was equilibrated with 10 m*M* Tris – 70 m*M* glycine (pH 8.1). The elution volumes V_d were determined by absorbance at 280 nm or by assaying for catalase activity and blue dextran was used to determine the void volume V_0.

Denatured molecular weight analysis

Two methods were used. In the first method the protein was mixed with an equal volume of sample buffer containing 0.1 *M* sodium phosphate (pH 7.0), 1% SDS, 0.14 *M* 2-mercaptoethanol, and 7 *M* urea and boiled for 2 min. This solution was loaded on a discontinuous slab gel composed of a 4% stacking gel at pH 6.8 and an 8% running gel at pH 8.8 with both gels containing 0.1% SDS as described by Laemmli (14) and Weber (15). Electrophoresis was at 2 mA/cm of gel length until the bromphenol blue marker dye reached the bottom of the gel, after which the gel was stained with Coomassie brilliant blue.

In the other method, gel filtration in the presence of guanidine hydrochloride was carried out as described by Mann and Fish (16), but on a 0.7 × 110 cm column of Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride in 50 m*M* sodium acetate (pH 4.8). Protein samples were denatured in 6 *M* guanidine hydrochloride in the presence of 0.1 *M* mercaptoethanol, after which iodoacetic acid was added for carboxymethylation (14) prior to loading on the column.

Visualization of catalase and peroxidase activity on polyacrylamide gels

Following electrophoresis on 8.5% polyacrylamide gels run as described by Davis (13), but with the separation gel prepared at pH 8.1 rather than pH 8.9, peroxidase activity was visualized by the method of Gregory and Fridovich (17) and catalase was stained by the method of Clare et al. (18), but using 20 m*M* H_2O_2 for better contrast.

Catalase assay and protein determination

Catalase activity was determined by the method of Rorth and Jensen (19) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min at 37°C. Protein was estimated by the methods outlined by Layne (20).

Hemochromogen characterization

Optical spectra were obtained using a Beckman DU8 spectrophotometer. The hemochromogen was extracted in acetone–HCl, demetalized, and methylated as described (21).

Immunoprecipitation

Preparation of the antiserum against HPII and isolation of the HPII immunoprecipitate for electrophoresis was carried out as previously described (2).

Results

Growth conditions

The growth phase and extent of aeration have both been shown to be important in determining the amount of HPII produced (7). Consequently, cultures were grown with forced aeration and simultaneous stirring to ensure optimum aeration into the stationary phase. In this way, HPII concentrations that were 15- to 30-fold higher than the basal level in middle log cells were obtained. Strain JA200/pLC36-19 was chosen because HPI, encoded by the plasmid, could also be purified in large amounts from the same batch of cells. Strain UM202, deficient in HPI synthesis, was used to determine the extent of purification in the absence of HPI and also to confirm that HPII with the same properties could be purified in the absence of HPI.

Purification

The procedure of Claiborne and Fridovich (1) for the purification of HPI was used with a number of modifications described above. This procedure was somewhat simpler than the protocol described by Claiborne et al. (3) for the purification of HPII and yielded both HPII and HPI in pure form. The key step in the protocol was found to be a repetition of the ammonium sulfate precipitations. They were rapid and yielded enzyme twofold purer than did a single precipitation, a fact critical to the success of the protocol. In addition, a complete separation of HPI and HPII was achieved with HPI appearing in the pellets of protein precipitated with ammonium sulfate at 30 and 40% saturation, and HPII appearing in the pellets precipitated at 50 and 60% saturation.

The two species of catalase could also be separated on the column of DEAE-Sephadex (5) with HPII eluting at a higher salt concentration. Because there was little difference in the elution volumes of HPI and HPII from Bio-Gel A-1.5 m and Bio-Gel HTP, it was important that their separation be achieved early in the protocol.

The purification and yields of HPII at each step in the purification from JA200/pLC36-19 are outlined in Table 1. The purity of HPII at each step in the purifications starting with UM202 or B23 was similar, except in the crude extract and streptomycin sulfate supernatant where there was less (B23) or no (UM202) HPI. Consequently, the final purification of HPII appeared to be greater in the latter two strains, although the final specific activity was the same. For example, in the case of UM202 where there was no HPI in the crude extract, a 464-fold purification of HPII was achieved with a final specific activity of 12 400 U/mg. Unlike HPI which was brown in color, the purified HPII was green.

The purified catalases from all three strains were subjected to electrophoresis on native and denaturing SDS–polyacrylamide gels to assess their purity. The activity stain on native gels revealed that catalase but no peroxidase activity migrated coincident with the main protein band for HPII (Fig. 1). For comparison, both catalase and peroxidase activities comigrated with the protein bands from HPI. Unexpectedly the denaturing

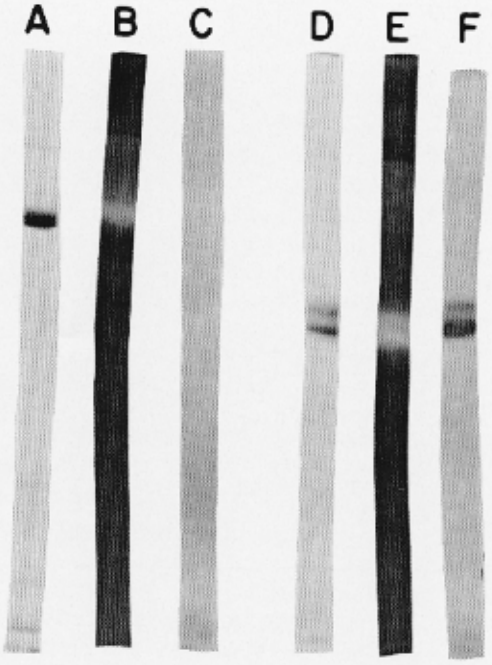

FIG. 1. Electrophoresis of purified HPII and HPI from JA200/pLC36-19 on native 8.5% polyacrylamide gels: (A and C) 12 μg of HPII; (B) 1.2 μg of HPII; (D and F) 15 μg of HPI; (E) 1.5 μg of HPI. Gels A and D were stained for protein; gels B and E were stained for catalase; and gels C and F were stained for peroxidase. HPII from UM202 and B23 migrated similarly.

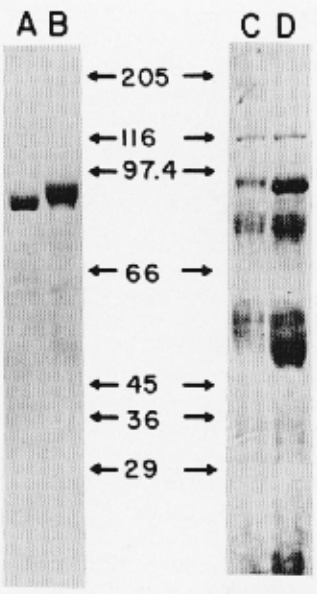

FIG. 2. Electrophoresis of purified HPII and HPI and immunoprecipitated HPII on denaturing 8% polyacrylamide gels: (A) 12.5 μg of HPI; (B) 14.0 μg of HPII; (C) immunoprecipitate from 0.8 mg protein of a crude extract from UM120; (D) immunoprecipitate from 0.8 mg protein of a crude extract from UM202. The numbers indicate the location of the following molecular weight standards ($\times 10^{-3}$); myosin, β-galactosidase, phosphorylase B, bovine plasma albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase. HPII from UM202 and B23 migrated similarly to HPII in B.

gel revealed two main subunit bands of about equal intensity for HPII (Fig. 2). If EDTA was not included in the buffer up to the ammonium sulfate stage, additional smaller bands could be observed in preparations of HPII, suggesting proteolytic digestion of the enzyme was occurring during the purification. When a crude extract of UM202 was immunoprecipitated with an HPII antiserum, a single band corresponding to the upper 92 000 band was present in the immunoprecipitate (Fig. 2). The immunoprecipitate of UM120 which contained no HPII because of the *katE*::Tn10 mutation did not contain any of this protein. The strong band at 50 000 was the immunoglubulin, while the faint bands present in the UM120 precipitate arose from the antiserum. The presence of the protease inhibitor leupeptin did not affect the subunit distribution shown in Fig. 2 for HPII. If proteolytic digestion was occurring, it did not have a significant effect on the enzyme specific activity even in preparations which contained a mixture of subunit sizes from 84 000 to 92 000.

Subunit size determination

A number of proteins of known molecular weights were electrophoresed on the same SDS–polyacrylamide gels as HPI and HPII and the R_f values relative to bromphenol blue dye were determined. The R_f of the two protein bands in HPII were compared to the standards (Fig. 3) revealing molecular weights of 90 000 and 92 000. The subunit size of HPI was 84 000. Because the values for HPII were significantly different from the molecular weight of 78 000 previously reported for HPII (3), they were confirmed by gel filtration on Sepharose 6B in the presence of guanidine hydrochloride. It was not possible to resolve the two subunits observed on gels, but a single peak of protein eluted with an apparent molecular weight of 91 000 (Fig. 4). HPI eluted with an apparent size of 81 000 (Fig. 4). The same subunit sizes were observed for HPII from both K12 strains and from B23, confirming that the discrepancies between these results and those of Claiborne *et al.* (3) were not a matter of strain differences.

Native size determination

Three independent methods were used to determine the apparent molecular weight of nondenatured HPII. The sedimentation equilibrium method revealed an apparent molecular weight of 532 000 (Fig. 5). Similar

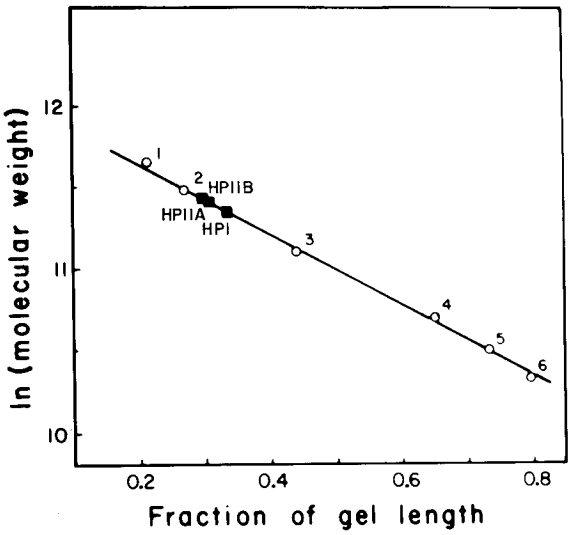

FIG. 3. Determination of the subunit molecular weights of purified HPII and HPI from denaturing polyacrylamide gels. Purified enzyme was electrophoresed as shown in Fig. 2 and the distances migrated were compared with the migration distances of a series of standards. The standards used were as follows: *1*, β-galactosidase; *2*, phosphorylase B; *3*, bovine plasma albumin; *4*, egg albumin; *5*, glyceraldehyde-3-phosphate dehydrogenase; *6*, carbonic anhydrase. The molecular weights determined were as follows: HPIIA, 90 000 ± 1000; HPIIB, 92 000 ± 1000; HPI, 84 000 ± 1000.

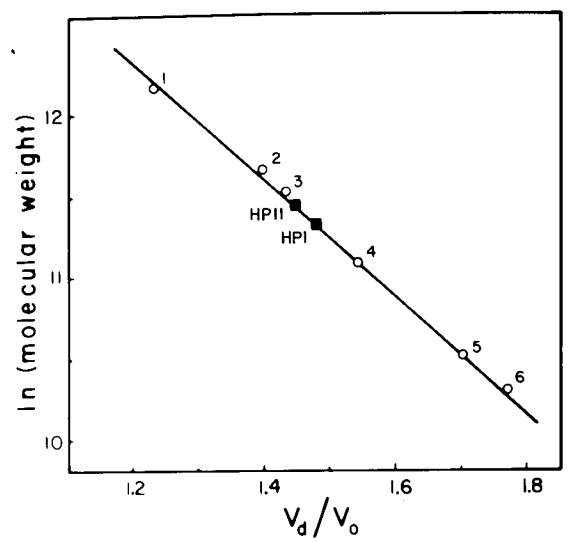

FIG. 4. Determination of the subunit molecular weights of purified HPII and HPI by elution from Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride. The standards used were as follows: *1*, myosin; *2*, β-galactosidase; *3*, phosphorylase B; *4*, bovine plasma albumin; *5*, alcohol dehydrogenase, 36 300; *6*, carbonic anhydrase. The molecular weights determined were as follows: HPII, 91 000 ± 3000; HPI, 82 000 ± 3000.

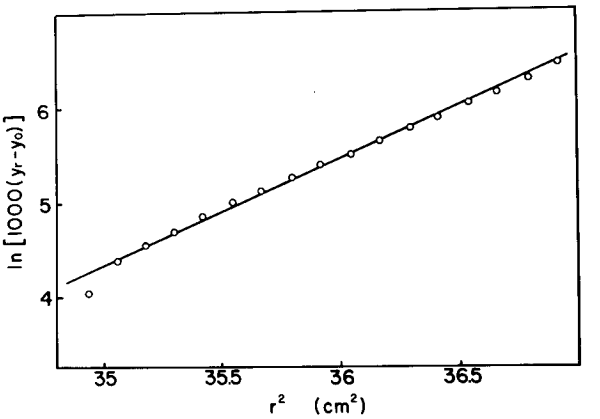

FIG. 5. Determination of nondenatured molecular weight of HPII by sedimentation equilibrium at 6000 rpm. The apparent molecular weight of 532 000 was calculated as outlined in Ref. 11.

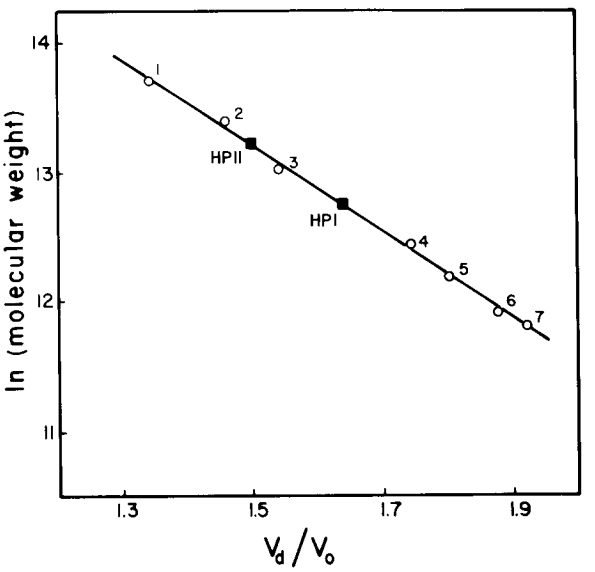

FIG. 6. Determination of nondenatured molecular weights of purified HPII and HPI by elution from Sepharose 4B equilibrated with Tris–glycine buffer. The standards used were as follows: *1*, apoferritin (dimer), 886 000; *2*, thyroglobulin (tetramer), 669 000; *3*, apoferritin (monomer), 443 000; *4*, bovine catalase, 240 000; *5*, α-amylase, 200 000; *6*, alcohol dehydrogenase, 150 000; *7*, bovine plasma albumin (dimer), 132 000. The molecular weights determined were as follows: HPII, 540 000 ± 20 000; HPI, 345 000 ± 15 000.

molecular weights were obtained at three different concentrations and at two different rotor speeds, 6000 and 6400 rpm. A second method involving gel filtration on Sepharose 4B revealed an apparent molecular weight of 540 000 (Fig. 6). A third method correlated the rate of migration with molecular weight on a series of different percentage gels, revealing an apparent molecular weight

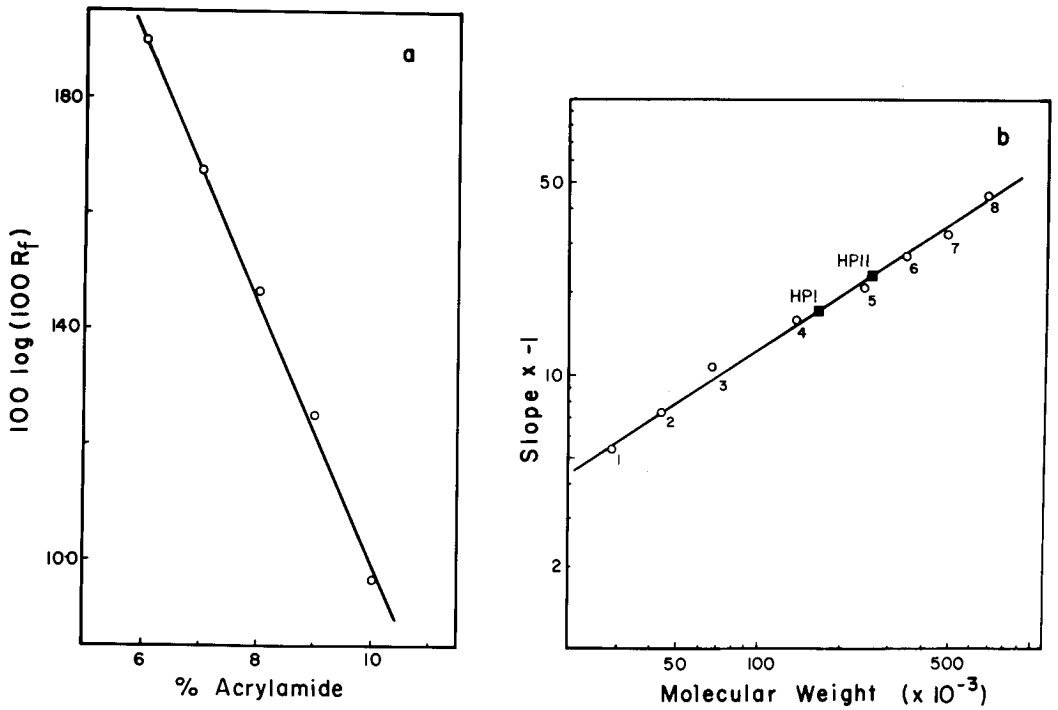

FIG. 7. Determination of nondenatured molecular weights of purified HPII and HPI by electrophoresis on native polyacrylamide gels. (*a*) Effect of different acrylamide concentrations on the mobility of purified HPII. The equivalent data for HPI are shown in Ref. 2. (*b*) Determination of the molecular weight of HPII from the slope determined in *a*. The proteins used as molecular weight standards were as follows: *1*, carbonic anhydrase; *2*, egg albumin; *3*, bovine plasma albumin (monomer); *4*, bovine plasma albumin (dimer); *5*, urease (dimer), 240 000; *6*, thyroglobulin (dimer), 335 000; *7*, urease (tetramer), 480 000; *8*, thyroglobulin (tetramer), 669 000. The molecular weights determined were as follows: HPII, 265 000 ± 15 000; HPI, 165 000 ± 5000.

of 265 000 (Fig. 7). The smaller sizes of HPII determined in the latter gel systems is similar to the discrepancy observed for HPI, which migrated as a dimer with a molecular weight of 165 000 on native polyacrylamide gels and a tetramer with a molecular weight of 345 000 on Sepharose 4B (Fig. 6) or during sedimentation equilibrium (1). Conditions on the polyacrylamide gels in the chaotropic Tris–glycine buffer may cause the parital breakdown of HPI to a dimer and HPII to a trimer, but catalase activity remains either because the partial components are active or because comigration of the components allows their reassociation and thus the formation of the active enzyme during staining.

pH optima and stability of HPII

Purified HPII retained maximal activity over a very broad pH range extending from pH 4 to 11. Poorly defined maxima were evident at these two extreme pHs and enzyme activity was still present at pH 3 and 13 (Fig. 8). This is quite different from the more normal pH curve determined for purified HPI which is also shown in Fig. 8. Meir and Yagel (22) have reported a slightly narrower, but still very broad, pH range of activity for crude extracts containing both HPI and HPII.

The purified enzyme was resistant to denaturation at room temperature in either 0.1% SDS or 7 *M* urea, with no reduction in activity being observed in either reagent. Incubation overnight at room temperature in a mixture of 0.1% SDS and 7 *M* urea did result in an 80%, but not complete, loss of activity. In fact when this mixture was subjected to electrophoresis on a SDS–polyacrylamide gel without boiling, all of the protein migrated slower than the subunit size range of 90 000 – 92 000. Another aspect of the extreme stability of HPII has been obtained by Meir and Yagel (personal communication) who observed that HPII in crude extracts retained its activity at temperatures up to 70°C.

Optical spectra of HPII and identity of the hemochromogen

The spectrum of purified HPII from *E. coli* K12 is shown in Fig. 9*a* and is very similar to the spectrum reported for HPII isolated from *E. coli* B (3). A Soret band was present at 406 nm (relative intensity, 5.32) and smaller peaks were evident at 590 (1.0), 630 (0.56), and

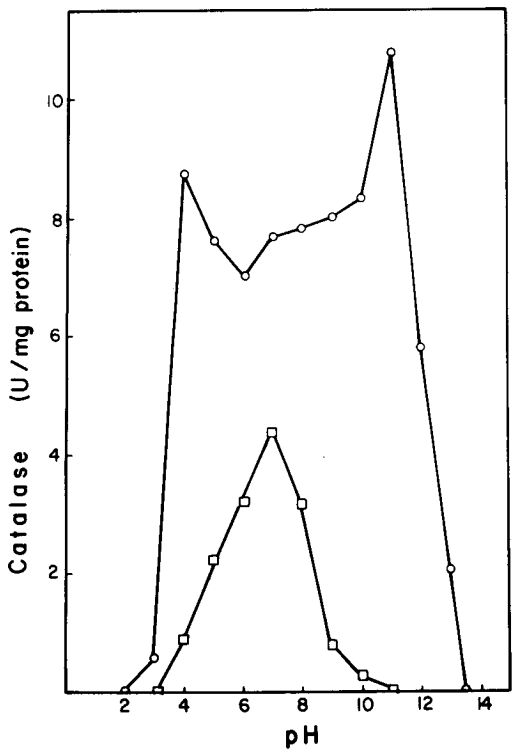

FIG. 8. Determination of activity of purified HPII (○) and HPI (□) as a function of pH. The enzyme was incubated in 50 m*M* potassium phosphate buffer at the various pH values for 1 min prior to commencing the assay by adding H_2O_2.

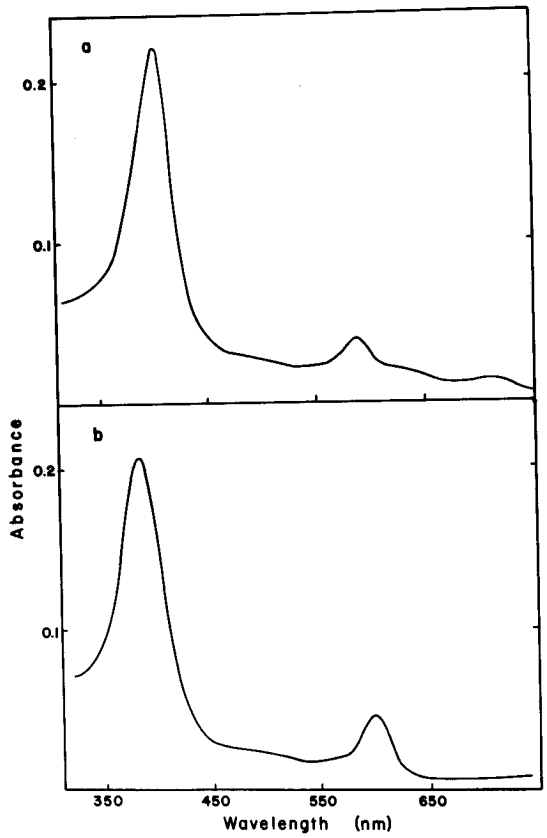

FIG. 9. Optical absorption spectra of purified HPII (*a*) and the hemochromogen extracted in acetone–HCl (*b*). HPII was present at 1 μ*M* concentration in 50 m*M* sodium phosphate (pH 7.0) in *a*. In *b*, 1 nmol of HPII was extracted with 0.015 *M* HCl in acetone and the spectrum was determined following removal of the precipitated protein.

715 nm (0.30). This spectrum was clearly different from that of HPI (1, 3), but similar in some respects to the spectrum of a catalase from *Neurospora crassa* (23, 24) which had an A_{400}/A_{590} equal to 4.89. The major differences were the location of the Soret band and the absence of the maximum at 630 nm in the *N. crassa* spectrum.

Following extraction with acetone–HCl, the hemochromogen from HPII exhibited a Soret band at 387 nm (5.02) which moved to 411 nm in pyridine and a smaller band at 603 nm (1.0) which was unchanged in pyridine (Fig. 9*b*; Table 2). The Soret band and 603-nm band were in the same locations in the spectrum of the heme isolated from *N. crassa* catalase, although a maximum at 565 nm was more prominent (23, 24). Demetalization of the HPII heme caused a small shift in the Soret band to 403 nm and a more significant shift in the 603 nm maximum to 642 nm. Following methylation, the HPII methylporphyrin in piperidine exhibited maxima at 655 (1.0), 578 (0.43), 542 (0.63), 501 (1.12), and 405 nm (9.94). These were very similar to the reported maxima for the *N. crassa* methylporphyrin.

On the basis of spectral evidence, the hemochromogen of *N. crassa* catalase was concluded to be similar to heme *d* (heme a_2; Ref. 25). A comparison of the absorption maxima locations for the heme and porphyrin groups from HPII, *N. crassa* catalase, and cytochrome *d* (a_2), as well as tetraphenylchlorin and octaethylchlorin, confirmed that the HPII heme was a dihydroporphyrin or chlorin type very much like heme *d* and very similar to the porphyrin from *N. crassa* catalase. Furthermore, spectral changes resulting from pH changes and changes in oxidation state, such as the peak at 603 nm being shifted to a diffuse peak at 660 nm in dilute NaOH or to 615 nm by the addition of dithionite (25), were consistent with the heme-*d*-like structure for the HPII heme. The polarity of the HPII heme was also similar to heme *d* as determined by its solubility in diethyl ether and this presented one major difference between the hemes from HPII and *N. crassa* catalase. The latter was not soluble in ether or benzene, a property attributed to a greater number of carboxylic acid residues as compared with heme *d*.

To quantitate the number of heme groups per enzyme

TABLE 2. Absorption spectra of catalase HPII heme and porphyrin methyl ester compared with the spectra of other related compounds

Compound	Wavelength maxima, nm (ratio of peak intensities)						Ref.
Catalase HPII heme	387 (5.02)	480 (0.49)	514 (0.42)	561 (0.35)	603 (1.0)	745 (0.16)	This paper
Fe(III)octaethyldihydroporphyrin	376 (3.68)	471 (0.33)	510 (0.23)	559 (0.25)	603 (1.0)	751 (0.11)	28
Heme *d* (heme a_2)	—	—	—	—	603 (1.0)	748 (0.2)	25
N. crassa catalase heme	412 (2.65)	483 (0.43)	528 (0.36)	565 (0.78)	602 (1.0)	—	24
Catalase HPII porphyrin methyl ester	405 (9.94)	501 (1.12)	542 (0.63)	578 (0.43)	655 (1.0)		This paper
Octaethyldihydroporphyrin	391 (2.58)	496 (0.18)	544 (0.02)	593 (0.06)	647 (1.0)		28
Tetraphenyldihydroporphyrin	419 (4.52)	517 (0.38)	542 (0.29)	598 (0.15)	652 (1.0)		28
Chlorin *d* (a_2)	405 (4.63)	503 (0.39)	540 (0.11)	—	650 (1.0)		25
N. crassa catalase porphyrin methyl ester	399 (4.42)	500 (0.34)	533 (0.11)	599 (0.11)	653 (1.0)		24

multimer, an indirect method was required because the identity and molar absorptivity of the heme were not precisely known. Consequently, the extracted heme was assayed for iron content and this value was compared with the protein concentration and the absorbance of the heme. This procedure revealed the presence of 5.4–6.0 iron atoms per enzyme based on a molecular weight of 540 000 in three different batches of enzyme. That each iron atom was associated with a heme group was confirmed by obtaining similar values from diethyl ether extracted heme from which any nonheme iron atom would have been excluded. A molar absorptivity ($E_{603-700\,nm}$) of 9055 was calculated for the extracted heme from HPII.

Discussion

The HPII catalase purified in this work is similar in several respects to the HPII purified by Claiborne *et al.* (3), including its spectrum, the presence of a heme exhibiting a similar spectrum, and the lack of any peroxidase activity. However, the native and subunit molecular weights of 532 000 and 92 000, respectively, determined in this work differ significantly from the 312 000 and 78 000 reported earlier (3). To verify the current data, three independent methods were used to determine the native molecular weights and two independent methods were used to determine the subunit sizes. Furthermore, they were confirmed in different preparations of the enzyme from two K12 strains and one B strain.

Proteolytic degradation of HPII during the purification procedure giving rise to the two subunit sizes was suggested by the appearance of even smaller subunit sizes in the absence of EDTA and by the presence of a single 92 000 subunit in the immunoprecipitate. The catalase from *N. crassa*, which was similar to HPII in some respects, was also sensitive to proteolysis (23). Unlike the *N. crassa* catalase, HPII did not have its specific activity significantly altered by the limited proteolysis. This evidence of degradation may explain the smaller subunit size of HPII reported earlier (3). Furthermore, proteolytically modified subunits may not associate as efficiently, giving rise to the smaller apparent native size also reported earlier (3).

Regardless of whether or not proteolysis is involved, a comparison of the native and subunit sizes suggests that HPII exists as a hexamer; six subunits of 92 000 molecular weight give rise to a native molecular weight of 552 000. This is an unusual structure for a catalase because all previously characterized catalases, whether from eucaryotes (23, 26) or procaryotes (1, 27), have been tetrameric. Further kinetic analysis of the enzyme is warranted. The presence of one heme-associated iron atom per subunit is consistent with the structure of most catalases excepting HPI (1), but the heme cofactor of HPII is not the usual protoheme IX found in most catalases. Instead the HPII heme bears a strong spectral

and polar resemblance to heme *d*. A catalase from *N. crassa* has also been reported to exhibit a green color and spectra similar to heme *d* (23, 24). Despite some differences between the spectra of HPII and the *N. crassa* catalase, it is likely that the HPII heme is very similar to the heme of the *N. crassa* catalase, possibly differing only in having fewer carboxylic acid residues than the more polar *N. crassa* heme.

Escherichia coli is unique in possessing two catalases, unlike any other previously characterized catalases. HPI is a tetrameric enzyme with the normal protoheme IX cofactor, but with larger than normal subunits and with a second enzymatic activity, that of a peroxidase. HPII is normal in possessing only a catalase activity, but is unusual in most other respects including its hexamer structure, the large subunit size, and the heme-*d*-like cofactor. Neither catalase is required for growth under normal laboratory conditions but, for two unique catalases to have evolved, there must be a strong selective advantage in the cell's natural environment.

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