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Activity, Peroxide Compound Formation, and Heme d Synthesis in *Escherichia coli* HPII Catalase

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Wild-type *Escherichia coli* HPII catalase (heme d containing) has 15% the activity of beef liver enzyme per heme. The rate constant for compound I formation with H_2O_2 is 1.3×10^6 M^{-1} s^{-1}. HPII compound I reacts with H_2O_2 to form O_2 with a rate constant of 1.8×10^6 M^{-1} s^{-1}. Forty percent of HPII hemes are in the compound I state during turnover. Compound I is reduced by ethanol and formate at rates of 5 and 13 M^{-1} s^{-1} (pH 7.0), respectively. Incubation of HPII compound I with ferrocyanide and ascorbate does not form a compound II species. Mutation of His128 to alanine or asparagine gives inactive protoheme proteins. Mutation of Asn-201 gives partially active heme d forms. Asn201Ala has 24%, Asn201Asp 10%, and Asn201Gln 0.4% of wild-type activity. Asn201His contains protoheme when isolated and converts this via protoheme compound I to a heme d species. Both distal heme cavity residues His128 and Asn201 are implicated in catalytic activity, compound I formation, and *in situ* heme d biosynthesis. HPII Asn201, like the corresponding residue in protoheme catalases, may promote H^+ transfer to His128 imidazole, facilitating (i) peroxide anion binding to heme and (ii) stabilization of a transition state for heterolytic cleavage of the O–O bond.

Key Words: **HPII; peroxide; catalatic reaction; catalase peroxide compound; heme transformation; protoheme; heme d; site-directed mutagenesis; *E. coli* catalase; reaction rates; heme pocket; distal residues.**

Hydroperoxidases, the enzymes that metabolize hydrogen peroxide, include the catalases and peroxidases. Metmyoglobin and methemoglobin also react with hydrogen peroxide but four to five orders of magnitude more slowly than do hydroperoxidases (1). This peroxide reactivity is controlled by the protein (2). A suitably positioned distal histidine (also present in myoglobin and hemoglobin) and a second distal residue (arginine in peroxidases, asparagine in catalases) are important for compound I formation and heterolytic peroxide cleavage.

The heme iron in these proteins is usually oxidized by hydrogen peroxide to a ferryl [FeIV=O] form. The second oxidizing equivalent produces either a protein radical—as in metmyoglobin (3–5), hemoglobin (6), and yeast cytochrome c peroxidase (7, 8)—or a porphyrin radical—as in plant peroxidases (9) and eukaryotic catalases (10). Thus, the reaction of beef liver catalase with H_2O_2 produces ferryl iron plus porphyrin π-cation radical or compound I. This is reduced in a single two-electron step to the ferric state or by a one-electron reaction to give the inactive peroxide compound II (11), in which the iron remains ferryl but the porphyrin radical has been lost.

As originally shown by Kirkman and co-workers, mammalian catalases (12), yeast catalases A and T (13), and the catalases of some prokaryotes (12, 14, 15) all bind one NADPH molecule per subunit, whose function appears to be protection against hydrogen peroxide inactivation and prevention of compound II formation.

The major catalase from *Escherichia coli*, HPII,[3] is evolutionarily homologous with the eukaryotic enzyme and with enzymes from other prokaryotes that contain protoheme as a prosthetic group. HPII is a monofunc-

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[3] Abbreviations used: HPII, hydroperoxidase II (catalase) of *E. coli;* WT, HPII wild type; CatPHFeIII(HOH), ferricatalase; CatPH$^{\bullet+}$FeIV=O, catalase compound I; CatPHFeIV=O, catalase compound II; catalatic reaction, the process of dismutation of $2H_2O_2$ to O_2 and $2H_2O$.

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tional catalase with one heme d per subunit (M_r 84,200) associated in a tetrameric structure (16). The heme d is a doubly *cis*-hydroxylated form (17) whose pyrrole ring III has been modified by the hydroxyl group attached to the carbon atom carrying the propionate side chain being linked to the latter via a lactone bridge (18) (see Fig. 7A under Discussion). HPII catalase does not bind NAD(P)H (13).

The crystal structure of HPII (16) places His128 just above pyrrole ring III (the hydroxylation site) and Asn201 in close proximity (see Fig. 7B under Discussion). These residues are counterparts of His74 and Asn147 in beef liver catalase, which have been postulated to play a role in H_2O_2 dismutation (19). HPII also catalyzes heme d formation from protoheme *in situ* using hydrogen peroxide as oxidant (18). Site-directed mutagenesis showed that the distal His128 is absolutely required for heme conversion.

We investigated the role of two distal heme cavity residues, histidine 128 and asparagine 201, in catalytic activity, in compound I formation, and in heme d synthesis, and we describe here the behavior both of wild-type HPII and of the mutants His128Ala, His128Asn, Asn201Ala, Asn201Asp, Asn201His, and Asn201Gln. This paper also reports the rates of compound I formation with hydrogen peroxide and of compound I reduction by ethanol and formate, compares the reactivities of protoheme and heme d forms, and proposes that transformation of protoheme to heme d occurs via a protoheme compound I. A previous study characterized the binding and inhibitory action of cyanide on free enzyme and some of the same mutants (20).

MATERIALS AND METHODS

Materials

Escherichia coli HPII catalases were isolated in the Department of Microbiology, University of Manitoba, according to Loewen and Switala (17). Site-directed mutants were prepared as described by Loewen *et al.* (18). Concentrations of HPII were estimated using an extinction coefficient per hematin (21) of 118 mM^{-1} cm^{-1} at 405 nm. The lyophilized protein was dissolved in potassium phosphate buffer and centrifuged to remove insoluble material. Fisher Scientific supplied the 30% hydrogen peroxide. KH_2PO_4, KCN, and cysteine were products of BDH Chemicals. K_2HPO_4 and 95% ethanol were from Caledon Laboratories. Potassium ferrocyanide and sodium ascorbate (BDH Chemicals) were prepared as 10 mM stock solutions in distilled water, kept frozen until use.

Methods

(a) Steady-state catalase assay. The dismutation of hydrogen peroxide was followed spectrophotometrically (22) using an extinction coefficient for hydrogen peroxide at 240 nm of 39.4 M^{-1} cm^{-1} (23). Measurements were carried out with an Aminco DW-2 spectrophotometer linked to a Compaq 286 device with Olis software and hardware. Data were analyzed using the Olis fitting routines for various types of exponential reaction. HPII and mutant concentrations were in the range of 1–10 nM. Pseudo-first-order rate constants for the samples examined were calculated as $k' = \ln 2/t_{1/2}$, where $t_{1/2}$ is the time taken for the absorbance level of H_2O_2 to decay half its initial value. Overall rate constants were calculated as $k = k'/$[catalase hematin].

(b) Steady-state spectral changes. During the steady-state catalysis of the substrate the ratio of free enzyme [CatPHFeIII(HOH)] to compound I [$CatPH^{\bullet+}FeIV{=}O$] is a constant determined by the rate constants k_1 (app) and k_2 (app) according to Eqs. [1]–[3]:

$$[CatPHFeIII(HOH)]/[CatPH^{\bullet+}FeIV{=}O] = k_2\,(app)/k_1\,(app) \quad [1]$$

as we have

$$CatPHFeIII(HOH) + H_2O_2 \xrightarrow{k_1\,(app)} CatPH^{\bullet+}FeIV{=}O + H_2O \quad [2]$$

$$CatPH^{\bullet+}FeIV{=}O + H_2O_2 \xrightarrow{k_2\,(app)} CatPHFeIII(HOH) + O_2 + H_2O \quad [3]$$

From the steady-state concentration of compound I and the measured kinetics of peroxide compound formation, k_2 (app) can be calculated (Eq. [1]). Alternatively, the rate of combination of catalase compound I and hydrogen peroxide may be obtained from Eq. [4], where k' is the overall rate constant for the catalatic reaction, p is the steady-state concentration of compound I, and e is total enzyme hematin concentration (cf. Ref. 24):

$$k_2\,(app) = k'/2(p/e). \quad [4]$$

In the catalatic steady state the Soret band of beef liver catalase compound I has a 12–15% lower intensity than that of the native enzyme. If 100% compound I is prepared using peracetate or an alkyl peroxide, the compound I Soret band is only 50% that of the ferric enzyme. The steady-state compound I concentration (p/e) is thus 25–30% and k_2 (app) is therefore three times k_1 (app).

To evaluate the corresponding steady state of HPII and to calculate values of k_2 (app) and k_1 (app) (Eqs. [1] and [4]), enzyme samples (hematin concentrations 3 to 5 μM) were titrated with hydrogen peroxide. In some cases the proportion of free catalase remaining in the system was determined by the cyanide "back titration" method of Chance (25). These measurements were carried out with a diode-array spectrophotometer (Beckman Instruments DU7400 uv/vis).

(c) Transient-state kinetics. The sequential stopped-flow apparatus and the associated computer system were from Applied Photophysics (UK): SX-17MV. In the conventional stopped-flow mode the apparatus uses two syringes, delivering a total of 0.1 mL/shot into a flow cell with a 1-cm light path. The fastest time for mixing two solutions was of the order of 1.5 ms. Each trace consisted of 400 data points. The enzyme concentrations for the kinetic experiments were 0.8 and 1.6 μM HPII and the H_2O_2 concentrations were at least 10 times in excess to ensure first-order kinetics. Temperature was set to 25°C. Three determinations of rate constants were performed for every substrate concentration and the mean value was used in calculation of the second-order rate constants. Second-order rate constants were calculated from the slope of the line defined by a plot of k_{obs} versus substrate concentration.

The reaction of HPII with hydrogen peroxide, k_1 (app), was followed at 405 nm. Reactions of compound I (produced by incubation of ferric-atalase with hydrogen peroxide) with either ethanol or formate [the peroxidatic reaction of Keilin and Hartree (26, 27)] were measured at 405 nm using the sequential stopped-flow method. The delay time (50–100 ms) in the aging loop was calculated from the reaction of the free enzyme with H_2O_2 at $[H_2O_2]$ = 2–5 times [hematin]. Ethanol and formate concentrations were 0.1–5 mM.

All experiments were performed at 25°C in 50 mM potassium phosphate buffer, pH 7.4.

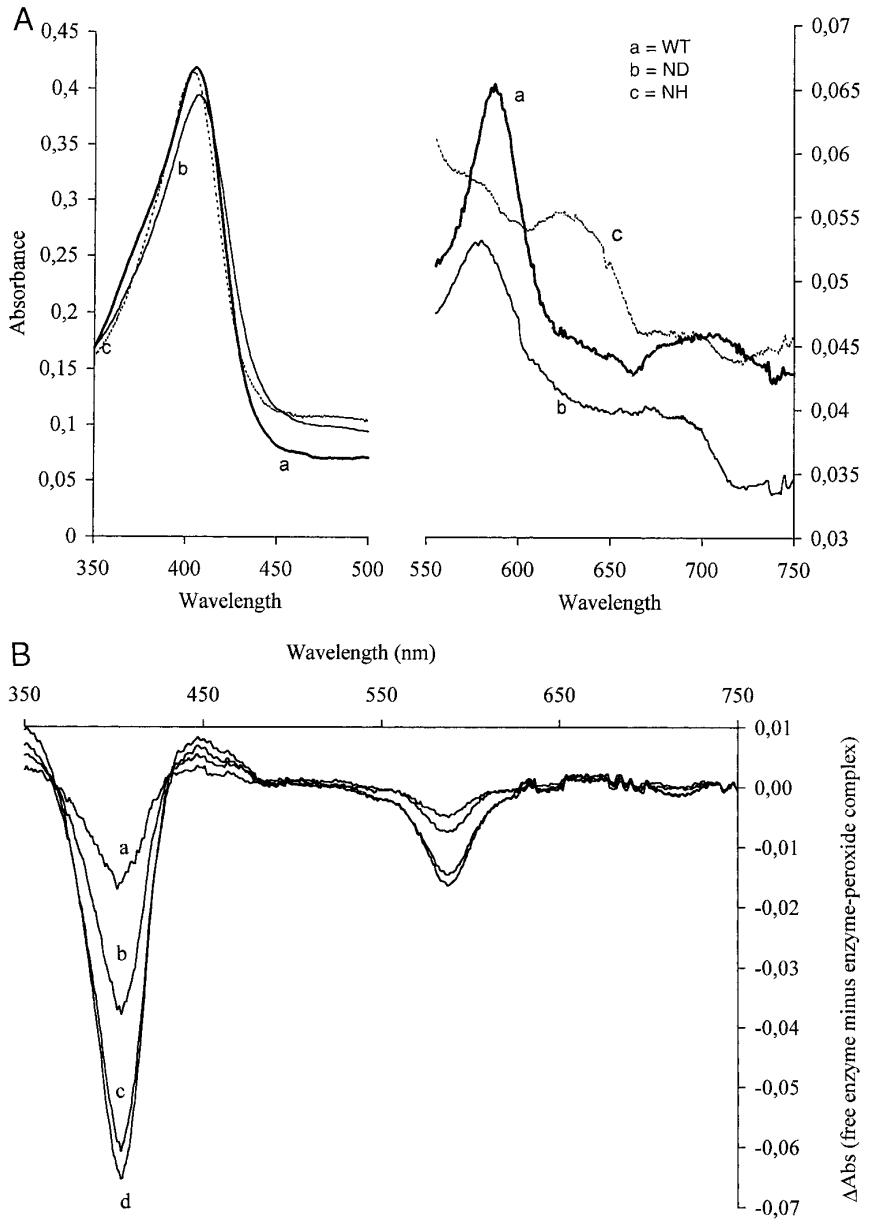

FIG. 1. Absolute and peroxide difference spectra of wild-type and mutant HPII catalases. (A) Absolute spectra of wild-type enzyme (4.2 μM) and the mutants N201D (4.0 μM) and N201H (3.9 μM) in 50 mM potassium phosphate buffer, pH 7.4, at 25°C. (B) Difference spectra of wild-type HPII peroxide complexes. Conditions as in (A). Hydrogen peroxide was added discontinuously to the resting enzyme [1.4 μM (a), 2.8 μM (b), 4.3 μM (c), and 5.7 μM (d)] and steady-state spectra were recorded immediately after addition using the Beckman 7400 diode array spectrophotometer.

(d) Molecular visualization. The structures of the HPII wild-type and mutant enzymes were visualized with a Silicon Graphics Iris (Unix-based) computer using crystal structure coordinates as in Bravo *et al.* (16) and the molecular modeling program Quanta.

RESULTS

Ultraviolet–Visible Spectra

Figure 1A shows the spectra of wild-type HPII and the mutants of interest. Wild-type HPII has a set of absorption bands displaced about 90 nm toward the red end of the spectrum compared to those characteristic of protohematin enzymes, indicating a d type heme as prosthetic group. Mutation of the distal His128 to Ala or Asn gave inactive enzymes containing mainly protoheme, whereas substitution of the Asn201 residue by Ala, Asp, or Gln was accompanied by retention of the red-shifted spectra. Of the Asn201 mutants only Asn201His showed a typical protohematin spectrum.

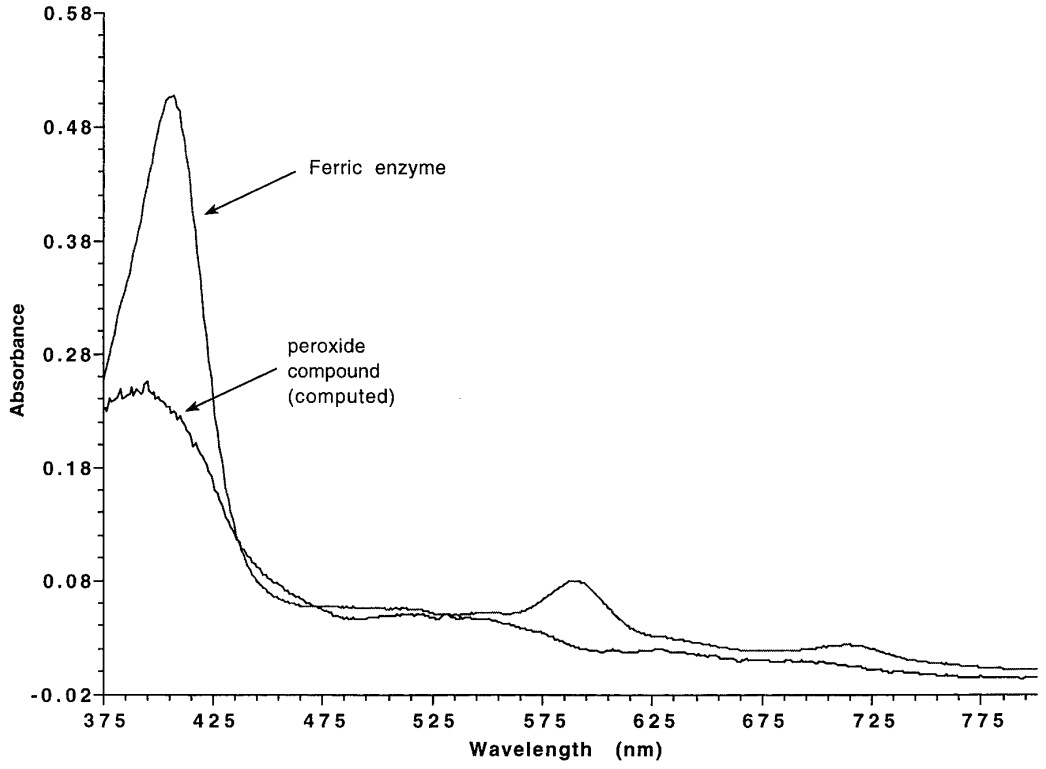

FIG. 2. Absolute spectra of wild-type HPII catalase and its primary hydrogen peroxide compound. HPII (wild-type) enzyme (~4 μM hematin) was treated with excess (>1 mM) H_2O_2 at pH 7 and 25°C. When oxygen evolution had ceased (i.e., free $[H_2O_2] \rightarrow 0$), potassium cyanide was added and the proportion of free enzyme present was calculated from a parallel experiment in the absence of cyanide (see Methods and Ref. 25). The expected spectrum of 100% compound I was then calculated and displayed. Other conditions are as in Fig. 1.

Titration of wild-type enzyme with hydrogen peroxide resulted in a maximal ~15–20% decrease in absorbance (hypochromicity) at both 405 and 590 nm (compound I formation). Figure 1B shows typical difference spectra (steady-state compound I minus free enzyme) which contain isosbestic points between native enzyme and compound I at 367 and 433 nm. These hypochromicities fit a steady-state compound I concentration between 30 and 40% of total hematin, assuming that 100% compound I formation would give a Soret absorbance ~50% that of the ferric enzyme. This was confirmed by experiments using the cyanide back titration method of Chance (25), results of which are illustrated in Fig. 2. This shows the calculated absolute spectrum of HPII peroxide compound I obtained by dividing the difference spectrum by the fractional occupancy and then adding this difference to the spectrum of the original enzyme. Fractional occupancy (p/e) was determined by the proportion of cyanide complex (20) formed by the steady-state mixture compared to that formed in the absence of peroxide ($1 - p/e$).

The results of similar experiments with HPII mutants are summarized in Table I. His128Ala and His-128Asn gave no spectral changes upon H_2O_2 addition, but Asn201 mutants typically showed a decrease in absorbance at 405 nm of between 10 and 20%, indicating a steady-state compound I concentration of between 20 and 40%. With Asn201His, titration with hydrogen peroxide leads to a maximum hypochromicity of 10–15% at 405 nm which is then followed by peak shifts (indicating heme conversion; see below).

TABLE 1

Kinetic Constants for the Catalatic Reaction of HPII Wild-Type and Mutant Catalases

Catalase	k (M^{-1} s^{-1})	% WT	Heme	k_{cat} (s^{-1})	Steady-state comp. I (%)
HPII wild type	1.3×10^6	100	d	26,700	30–40
H128N	$\ll 0.1$	—	b	—	—
H128A	$\ll 0.1$	—	b	—	—
N201 A	3.6×10^5	28	d	9,200	30–40
N201D	1.9×10^5	15	d	7,100	30–35
N201 Q	5.3×10^3	0.4	d	ND	ND
N201H protoheme	1.2×10^4	0.9	b	ND	20–30
N201H heme d_{cis}	6.4×10^4	5	d	ND	ND

Note. Conditions: 50 mM potassium phosphate buffer, pH 7.4, varying concentrations of hydrogen peroxide (from 1 to 20 mM), and varying HPII hematin concentrations over the nanomolar range, at 25°C (see Methods). ND, not determined.

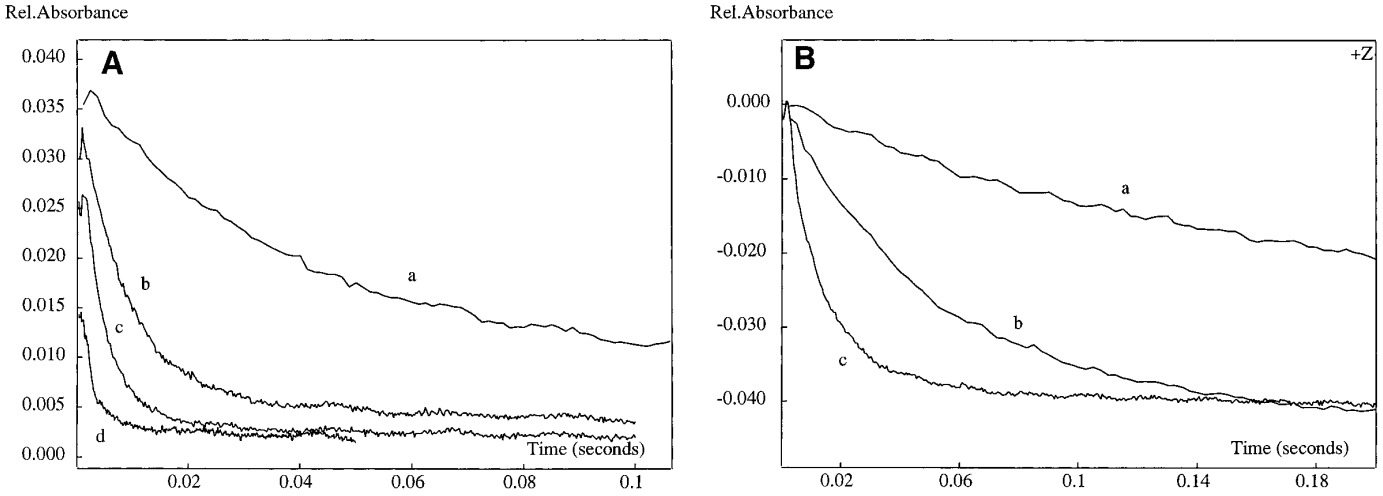

FIG. 3. Kinetics of the reactions of wild-type HPII and N201D mutant catalases with hydrogen peroxide. (A) Reaction of HPII wild type (1.6 μM) with (a) 15 μM, (b) 35 μM, (c) 125 μM, and (d) 250 μM H_2O_2. (B) Reaction of N201D mutant (1.7 μM) with (a) 15 μM, (b) 35 μM, and (c) 250 μM H_2O_2. The medium contained 50 mM potassium phosphate buffer, pH 7.4, 25°C. Time courses at 405 nm were monitored in the stopped-flow system (see Methods).

Catalatic Activity

Catalatic activity of HPII (wild type), measured at 240 nm (Methods), was proportional to enzyme (hematin) concentration with an overall rate constant of 1.3 $\times$ 10^6 M^{-1} s^{-1} at low [H_2O_2] levels, ~15% of the beef liver catalase activity. At high peroxide levels the reaction rate lost proportionality to [H_2O_2] and a k_{cat} value of about 27,000 s^{-1} was calculated. The apparent "K_m" was therefore ~20 mM H_2O_2 (see Discussion).

Some of the kinetic constants for HPII and its mutants are contained in Table I. His128Ala and His-128Asn are catalytically inactive, whereas mutation of Asn201 gave partially active forms (Table I). Both wild type and mutants are sensitive to hydrogen peroxide concentrations higher than 100 mM. At these concentrations there is a progressive inhibition of the enzyme (not shown), although spectral shifts were not detectable.

The rates of compound I formation of HPII and its Asn201 mutants were measured directly by stopped-flow spectroscopy. A typical series of time traces in the Soret region at various concentrations of hydrogen peroxide is shown in Fig. 3A; Fig. 3B shows the same experiment with the N201D mutant. In all cases the time courses were fitted as single exponential functions and apparent second-order rate constants calculated by plots of k_{obs} vs [H_2O_2]. Both for wild-type HPII and for its Asn201 mutants k_1 (app) values were reduced approximately in proportion to the reduction in overall rates (Table I). As Asn201Ala, Asn201Asp, and Asn201Gln also showed similar hypochromicities in the Soret region upon H_2O_2 addition to that of HPII wild type (Table I), we conclude that k_2 (app) values for the mutants decrease to the same extent as do k_1 (app) values (Eqs. [1]–[4]).

Peroxidatic Activity

Figure 4 shows a typical series of time traces of the reaction of HPII compound I with formate at 405 nm, using the sequential stopped-flow method (Methods). Similar traces were obtained with ethanol as hydrogen donor (not shown). The kinetics are of first-order processes whose calculated pseudo-first-order rate constants are proportional to donor concentration (Table II). Both ethanol and formate oxidations by WT compound I are much slower than the corresponding reactions with beef liver enzyme. The same experiments performed with Asn201 mutants again indicated a prominent role for asparagine in orienting the electron donor. All these mutants react more slowly than does the wild-type enzyme (Table II).

Absence of Compound II in HPII Catalase

WT and mutant enzymes never showed any shifts in their spectra which would indicate compound II formation. Neither potassium ferrocyanide, a one-electron reductant of compound I in beef liver catalase [discussed by Deisseroth and Dounce (28)], used in a concentration range from 10 μM to 10 mM, nor incubation with millimolar ascorbate ever produced a species similar to the secondary peroxide compound of beef liver catalase (results not shown).

Heme Conversion

HPII N201H contained protoheme and showed no more than 1% of the activity of HPII wild type. Its

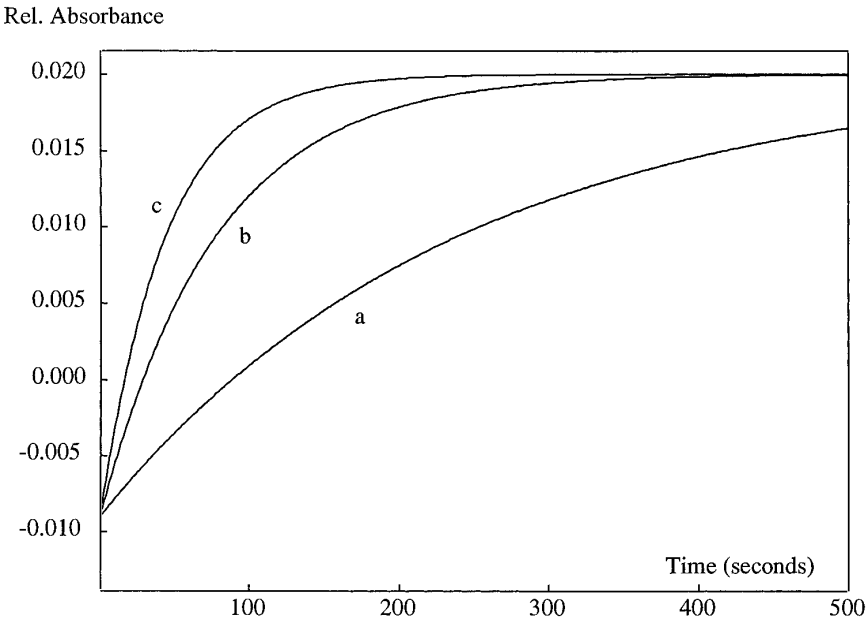

FIG. 4. Sequential traces for the reaction of HPII wild-type catalase compound I with formate. HPII wild-type (1.6 μM heme) compound I was formed with H_2O_2 as in Fig. 3 and then reacted with (a) 0.1 mM, (b) 1 mM, and (c) 2 mM sodium formate. Compound I formation occurred within the first few milliseconds. The medium contained 50 mM potassium phosphate buffer, pH 7.4. Time courses at 405 nm were monitored in the stopped-flow system at 25°C.

spectrum was shifted to that of a heme d species in the presence of hydrogen peroxide or hydrogen peroxide-generating systems such as ascorbate plus molecular oxygen (cf. Ref. 18). This conversion is accompanied by an increase in catalytic activity, as shown in Table I.

Figures 5A and 5B compare the overall kinetics of hydrogen peroxide degradation by WT enzyme with the kinetics of the mutants N201D and N201H. In contrast to the WT and N201D catalases, which exhibit typical pseudo-first-order kinetics for H_2O_2 dismutation, the rate constant for H_2O_2 degradation by N201H increases with time; this indicates that the enzyme was converted during the reaction course from a partially active to a more active form.

During the first few minutes the Soret band of N201H during its reaction with hydrogen peroxide (and also some peaks in the visible range) had approximately 10–14% less intensity than in the resting state. This suggested formation of a protoheme compound I which slowly shifts to the corresponding heme d form (Fig. 6). The protoheme form, with peaks at 403, 500, 538, and 632 nm, was transformed to a heme d species with a Soret band at 407 nm and a major peak in the visible region at 578 nm. As shown previously (20), the cyanide spectra of these two different heme forms also characterize them as protoheme low-spin and heme d low-spin complexes, respectively.

TABLE II

Transient State Rate Constants for the Reactions of HPII Wild-Type and Mutant Catalases and Peroxide Compounds with Hydrogen Peroxide and with Peroxidatic Donors

Catalase	k_1 (app) (M^{-1} s^{-1})	k_2 (app) (M^{-1} s^{-1})	Formate (M^{-1} s^{-1})	Ethanol (M^{-1} s^{-1})
HPII wild type	1.8×10^6	3×10^6	13	5
N201 A	6×10^5	9×10^5	3	1
N201D	2.9×10^5	4.4×10^5	0.9	0.2
N201 Q	7×10^4	1×10^5	ND	ND

Note. Conditions: 50 mM potassium phosphate buffer, pH 7.4, and 25°C (see Methods and Table 1). k_1 (app), the rate constant for reaction of ferric enzyme with peroxide; k_2 (app), the rate constant for reaction of compound I with peroxide; and k_2'(app), the rate constant(s) for reaction of compound I with other hydrogen donors (formate and ethanol) were all calculated from Eqs. [1] and [4] using the steady-state compound I concentrations given in Table I. ND, not determined (very low activity).

DISCUSSION

E. coli HPII contains heme d (the lactone form is shown in Fig. 7A) instead of protoheme, but in an analogous pocket at a similar depth in the protein to the heme in beef liver enzyme (16), as illustrated schematically in Fig. 7B. The present paper describes some effects of single-point mutations of residues on the distal side of the heme, analogs of which are known to be important for dismutation of H_2O_2 by beef liver catalase (19). Replacement of the distal histidine with alanine

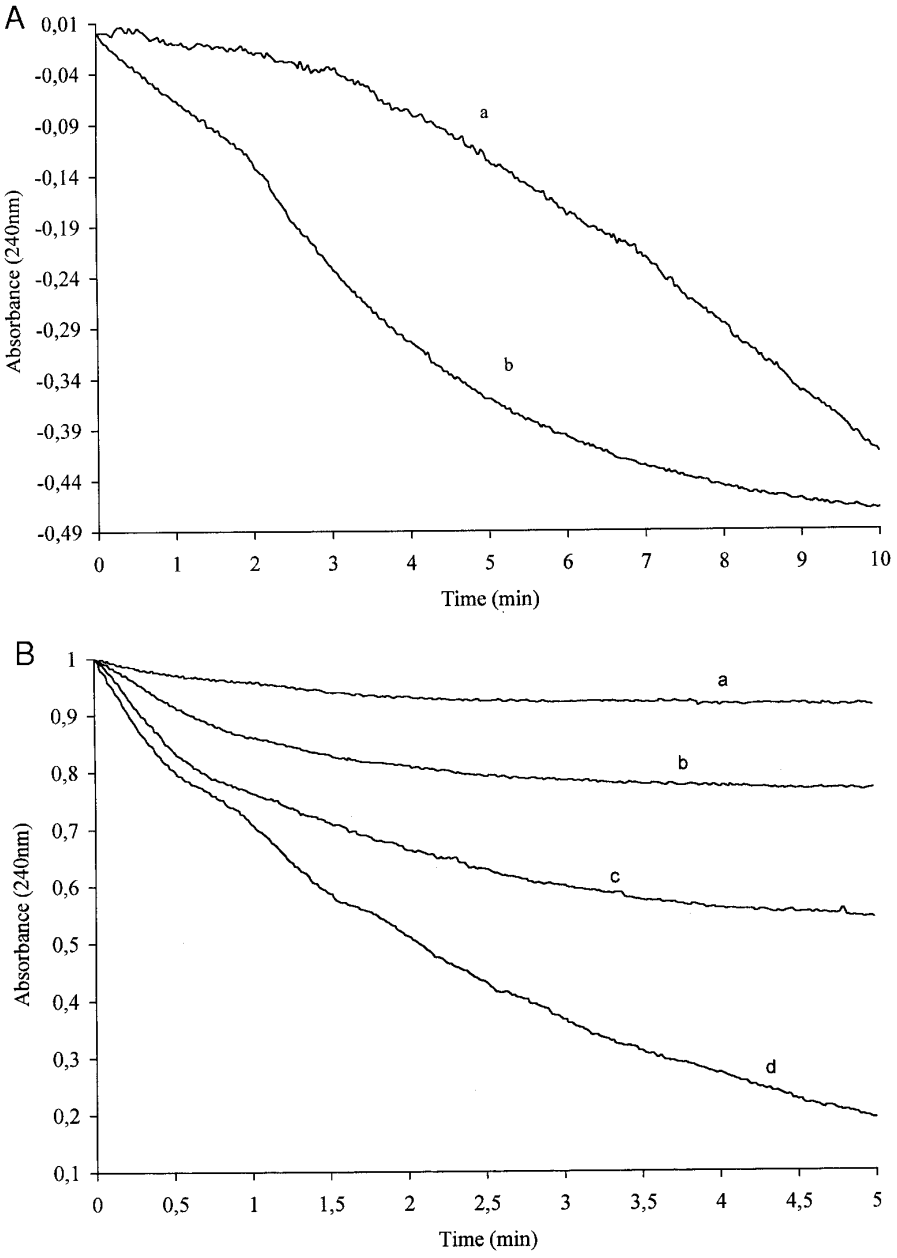

FIG. 5. Hydrogen peroxide degradation by mutant enzymes. (A) N201H kinetics. Catalytic decay of 45 mM H_2O_2 catalyzed by (a) 73 nM and (b) 220 nM HPII N201H mutant enzyme at pH 7.4, 25°C. Measurements were carried out at 240 nm. (B) N201D kinetics. Catalytic decay of (a) 4.8 mM H_2O_2, (b) 9.5 mM H_2O_2, (c) 19.5 mM H_2O_2, and (d) 47.6 mM H_2O_2 catalyzed by the N201D mutant enzyme. Reactions were started by addition of 19 nM (hematin) catalase. Other conditions as in (A) and Methods.

or asparagine results in mutant HPII enzymes H128A and H128N which contain only protoheme, whereas replacement of N201A, N201Q, and N201D all contain heme d species. One distal asparagine mutant, N201H, contains protoheme as isolated (13, 18).

The overall reaction rate constant (k') for various catalases is given by Eq. [5], where k_1 and k_2 are the rates of formation and reduction of compound I (see Eqs. [2] and [3] above).

$$v = \frac{2k_1k_2es}{\{k_1 + k_2\}} = k'es \qquad [5]$$

Values of k' typically fall between 0.6 and 2.0×10^7 M^{-1} s^{-1} (29). With a k' value of 1.3×10^6 M^{-1} s^{-1} HPII has about 15% the activity of beef liver catalase, and both k_1 and k_2 are reduced in magnitude, preserving some 30–40% of compound I in the steady state. Equation [5] is valid for beef liver catalase up to molar levels

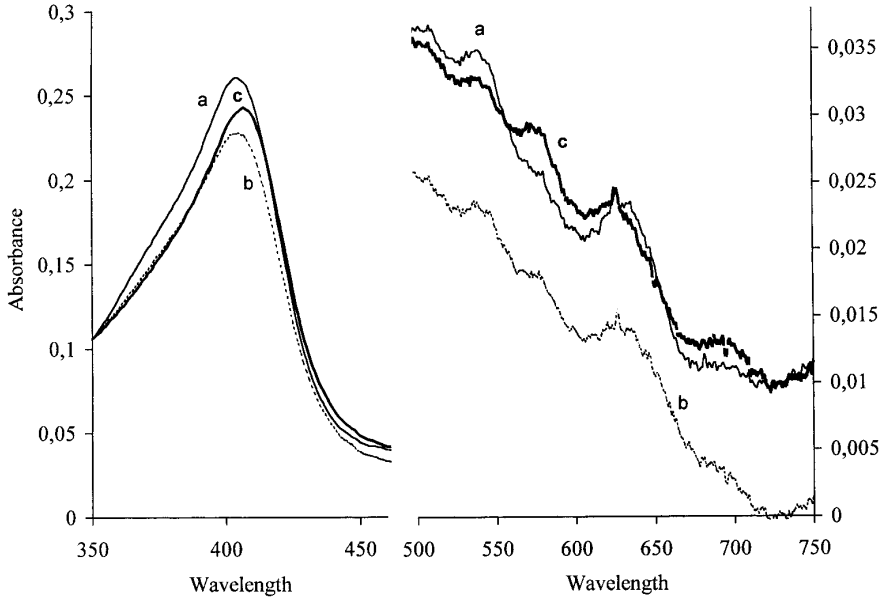

FIG. 6. Heme conversion of the HPII N201H mutant enzyme by hydrogen peroxide and ascorbate. N201H mutant enzyme (2.5 μM hematin) was incubated with 50 mM hydrogen peroxide in the presence of 1 mM ascorbate. Addition of H_2O_2 plus ascorbate to ferricatalase (a) induces a steady-state protoheme compound I spectrum (b) which is visible for 5 to 10 min. Finally the bands in the Soret and visible region shift (c), forming a species indicating either heme d itself or a precursor form. The medium was 50 mM potassium phosphate buffer, pH 7.4, at 25°C.

of peroxide [an apparent K_m for H_2O_2 of 1.1 M was reported at 20°C by Ogura (30), corresponding to a k_{cat} of 10^7 s^{-1}], but HPII apparently has a much lower maximal turnover (27,000 s^{-1}) and a correspondingly smaller K_m for hydrogen peroxide (~20 mM).

The initial step in the formation of compound I is the entrance of H_2O_2 into the distal side of the heme crevice. The peroxide is sterically constrained to move between the His74 and Asn147 residues (cf. Fig. 7B) before interacting with the heme iron. These two residues "preorient" the substrate and then interact with it via hydrogen bonding (15). Replacement of the critical histidine ligand in HPII gave (protoheme) enzymes which were catalytically quite inactive. His128 may be essential for proton abstraction from the peroxide as well as for transfer of hydrogen to the distal peroxide oxygen

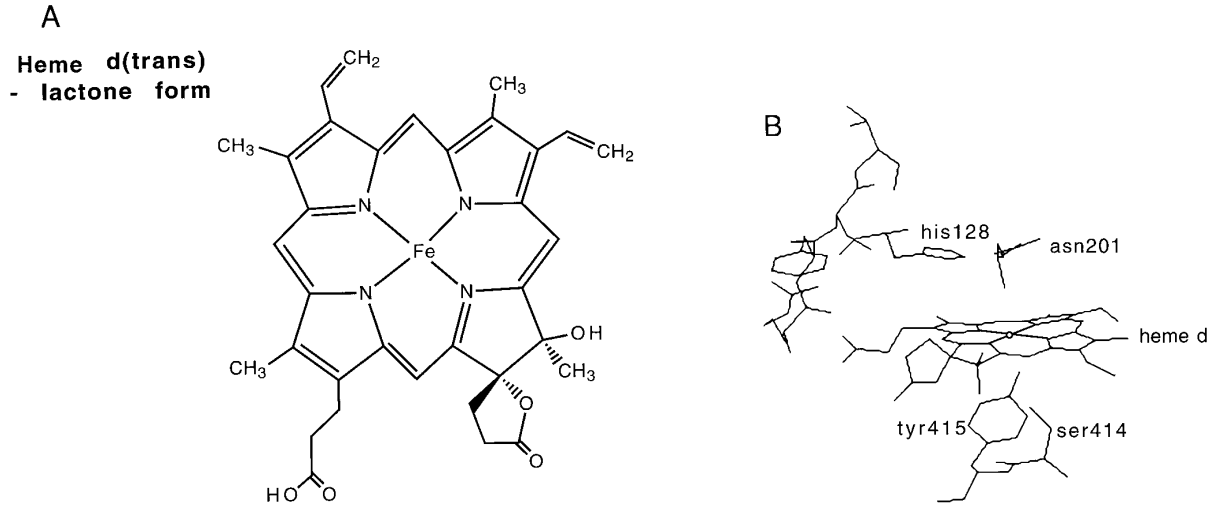

FIG. 7. Proposed structure of heme d and its location in the HPII active site. (A) Lactone or "spiro" form of heme d (16). Compare with the structures postulated by Timkovich and Bondoc (35). (B) Proposed arrangement of residues in the heme pocket, derived from the HPII crystal structure of Bravo *et al.* (16) (obtained from a Quanta image visualized with a Silicon Graphics Iris Unix-based computer).

during O–O cleavage, when a molecule of water is formed.

Changing the possible "essential" asparagine residue (N201) yielded mutants ranging in overall activity from 24% to nearly undetectable compared to the wild type. Although the asparagine thus may not be essential, it may nonetheless act via hydrogen bonding to orient hydrogen peroxide. As N201Q showed almost no activity, whereas N201A had ~24% and N201D ~10% of wild type, N201 has an obvious stereochemical role, incorporation of an extra CH_2-group hindering peroxide binding to the active site.

In contrast to beef liver catalase where in steady state only 25–30% of the hemes are in the compound I form during the steady state (31), HPII has at least 40% of the enzyme in this form (Fig. 2). This percentage remains the same for mutants showing much lower overall activity than the wild-type enzyme. Both rate constants k_1 and k_2 (Eqs. [2] and [3]) are therefore affected by distal residue modifications.

The observed differences in activity compared with beef liver catalase may be correlated with differences not only in the heme pocket (Fig. 7) but also involving the peroxide entry "channel." This channel leading from the exterior to heme d contains unique phenylalanine and tryptophan residues (16) that may occlude the diffusion pathway. Thus, ethanol and formate oxidations by HPII compound I are much slower than with beef liver catalase. The reaction of HPII with cyanide as a heme ligand is also slow (20).

Beef liver catalase has two phases of catalatic activity, termed α and β by George (32). In the β state the enzyme exists in a less active form, the rate and extent of inhibition being dependent upon peroxide concentration (32, 33). Chance interpreted the change in terms of compound II formation (34) and this analysis was extended by Nicholls and Schonbaum (24). *E. coli* HPII compound I, on the other hand, reacts with peroxides exclusively in a two-electron process. No compound II or compound III formation has been seen. HPII also does not bind NADPH, whose role is thought to be prevention of compound II formation and inactivation by H_2O_2 (13, 14). Nevertheless, HPII also shows inhibition at high peroxide levels, but this inhibition does not appear to involve compound II formation. Its mechanism is unclear and it may not be reversible.

It has been suggested by Timkovich and Bondoc (35) that protoheme is first bound to HPII apoenzyme and that heme hydroxylation is catalyzed by HPII itself utilizing one of the first H_2O_2 molecules to react catalytically. A role for His128 in the conversion has been demonstrated by Loewen *et al.* (18), but Asn201 is not an absolute requirement.

Heme d formation from the protoheme species of N201H occurs during the first few turnovers via protoheme compound I, which can be observed spectroscopically in the initial steady state. This is followed by conversion to a heme d species and formation of a more active enzyme. The details of this heme modification for HPII are still unknown. A serine residue (located ~2 Å below pyrrole ring III; see Fig. 7B) substitutes for an alanine in beef liver catalase and may assist in the hydroxylation to form heme d (cf. Ref. 16).

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