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Escherichia coli Flavohemoglobin Is an Efficient Alkylhydroperoxide Reductase*

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***Escherichia coli* flavohemoglobin (HMP) is shown to be capable of catalyzing the reduction of several alkylhydroperoxide substrates into their corresponding alcohols using NADH as an electron donor. In particular, HMP possesses a high catalytic activity and a low K_m toward cumyl, linoleic acid, and *tert*-butyl hydroperoxides, whereas it is a less efficient hydrogen peroxide scavenger. An analysis of UV-visible spectra during the stationary state reveals that at variance with classical peroxidases, HMP turns over in the ferrous state. In particular, an iron oxygen adduct intermediate whose spectrum is similar to that reported for the oxo-ferryl derivative in peroxidases (Compound II), has been identified during the catalysis of hydrogen peroxide reduction. This finding suggests that hydroperoxide cleavage occurs upon direct binding of a peroxide oxygen atom to the ferrous heme iron. Competitive inhibition of the alkylhydroperoxide reductase activity by carbon monoxide has also been observed, thus confirming that heme iron is directly involved in the catalytic mechanism of hydroperoxide reduction. The alkylhydroperoxide reductase activity taken together with the unique lipid binding properties of HMP suggests that this protein is most likely involved in the repair of the lipid membrane oxidative damage generated during oxidative/nitrosative stress.**

"Hemoglobin-like" proteins represent an increasingly growing family of globins whose genes are widespread among prokaryotic and eukaryotic microorganisms (1, 2). These proteins are structurally related to vertebrate hemoglobins and myoglobins in that their architecture is based on the typical globin fold and the heme is linked to the polypeptide chain through a proximal histidine residue. Two main classes of hemoglobin-like proteins have been identified, namely single domain bacterial hemoglobins (comprising the so-called truncated hemoglobins) and flavohemoglobins in which the globin domain is fused with a ferredoxin reductase-like FAD and NAD binding module. The "functional annotations" of these proteins are still controversial, and different physiological roles have been proposed that span from simple oxygen delivery to terminal oxidases (facilitated diffusion) to more complex enzymatic activities linked to oxidative and/or nitrosative stress cell responses (1, 2).

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The flavohemoglobin from *Escherichia coli* (HMP)[1] has been the object of a large number of investigations to unveil its physiological role in the framework of bacterial resistance to nitrosative stress. HMP expression has been demonstrated to respond to the presence of nitric oxide (NO) in the culture medium, and an explicit mechanism has been proposed that involves NO scavenging and its reduction to N_2O under anaerobic conditions (3). In contrast to (or together with) the anaerobic NO reductase activity, HMP has also been shown to be able to catalyze the oxidation of free NO to nitrate (nitric-oxide dioxygenase activity) both *in vivo* and *in vitro* in the presence of oxygen and NADH (4–7). Alternatively, an NO denitrosylase function has been proposed in which, at low oxygen tensions, HMP turns over in the ferric state with the intermediacy of an iron-bound nitroxyl anion that is subsequently transformed into nitrate in the presence of oxygen (8). It remains to be established which of these diverse enzymatic activities correspond to a physiologically relevant process.

A second set of functional hypotheses has been inferred on the basis of the structural features of the active site of HMP (9) and other closely related proteins. HMP, similar to the single chain hemoglobin from *Vitreoscilla* sp. (10) and the flavohemoglobin from *Alcaligenes eutrophus* (11), displays a structural geometry of the active site that is strongly reminiscent of that of typical peroxidases. In particular, the x-ray crystal structures of all of the above mentioned proteins show that the proximal heme pocket is characterized by a network of hydrogen-bonding interactions that comprises the Nϵ atom (hydrogen-bonding donor) of the proximal histidine, thus conferring an anionic character to the imidazole ring (9, 11). This feature allows a clear cut structural distinction between genuine hemoglobins in which the integrity of the ligand in the *trans* position needs to be preserved and peroxidases in which electron donation to the *trans* ligand is at the core of the catalytic activity (electron push effect). The spectroscopic signature of the peroxidase-like character of the active site is provided by resonance Raman measurements on the fully reduced, deoxygenated HMP derivative whose spectrum displays a very high iron histidine-stretching frequency, in line with the values observed for typical peroxidases (12). Subsequently, FTIR measurements conducted on the cyanide-bound ferric species confirmed the structural analogies between HMP and peroxidases highlighting the strong electron donation to the iron-bound ligand (13). Nevertheless, an extensive screening of possible peroxidase and/or monooxygenase activities toward a number of typical peroxidase and/or cytochrome P-450 sub-

[1] The abbreviations used are: HMP, *Escherichia coli* flavohemoglobin; NO, nitric oxide; GC, gas chromatography; HPLC, high pressure liquid chromatography; ESI, electrospray ionization.

strates has been unfruitful (14).[2] As a matter of fact, ferric HMP appears to be inert toward hydrogen peroxide and displays a complex ligand binding behavior toward typical ferric heme substrates (14).

Lastly, a number of experimental observations carried out on highly similar bacterial hemoglobins have revealed different possible scenarios. In particular, the presence of a tightly bound phospholipid within the heme pocket of *A. eutrophus* flavohemoglobin initially suggested the participation of the protein to lipid membrane transport and/or processing (15). This finding has been completely overlooked in subsequent investigations until very recently where HMP as well as the single chain bacterial hemoglobin from *Vitreoscilla* sp. (VHB) and even the truncated hemoglobin from *Mycobacterium tuberculosis* (HbO) have been reported to be preferentially localized in contact with the bacterial inner membrane and capable of reversible binding to liposomes obtained from bacterial lipid extracts (16, 17). HMP has also been shown to bind unsaturated and/or cyclopropanated fatty acids with high affinity within the heme pocket (14). Along the same line, the three-dimensional structures of the flavohemoglobins from *A. eutrophus* (FHP) and *E. coli* and of the single chain hemoglobin from *Vitreoscilla* sp. (VHB) pointed out that lipid binding contact regions are conserved (9).

All together, the ensemble of experimental observations just mentioned provides a puzzling picture whose elucidation might be achieved by merging membrane binding properties with the peroxidase-like character and eventually with the NO-induced expression of HMP. In this work, ferrous HMP has been demonstrated to possess a genuine alkylhydroperoxide reductase activity, thus suggesting that HMP itself and possibly other members of the hemoglobin-like protein family are involved in the reduction of lipid membrane hydroperoxides.

EXPERIMENTAL PROCEDURES

HMP has been expressed and purified as reported previously (13) with an additional chromatographic step on a lipid avid hydroxy-alkoxy-propyl dextran resin (Type X, Sigma). The protein solution (8 ml, 40–80 μM) was passed through a 2.2 × 6-cm column containing the hydroxy-alkoxy-propyl resin at 20 °C in 20 mM phosphate buffer at pH 7.0 under a flow rate of 0.05 ml/min. This last chromatographic step ensured complete removal of physiologically bound phospholipids (14, 15). Protein concentration was measured spectrophotometrically using the molar absorptivity at 421 nm of 156,000 M^{-1} cm^{-1} for the ferric cyanide adduct.

Kinetic measurements were carried out by mixing anaerobic protein solutions containing 250 mM NADH and 1 mM EDTA with solutions containing different concentrations of *tert*-butyl, cumyl, linoleic acid hydroperoxides (13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid), or hydrogen peroxide (Sigma) in an Applied Photophysics rapid mixing apparatus equipped with a diode array device (Leatherhead, United Kingdom). Stock solutions of *tert*-butyl, cumyl, and linoleic acid hydroperoxide were prepared in 50% water/ethanol mixtures, degassed in a tonometer, and kept at 4 °C in the dark before use. Solutions containing HMP and NADH and solution containing hydroperoxides were accurately degassed in a tonometer and anaerobically transferred to stopped-flow syringes. The syringes containing HMP and NADH were allowed to stand for 5 min before mixing to ensure the consumption of residual oxygen. Double mixing experiments were also carried out in the same stopped-flow apparatus. NADH (250 μM) reduced HMP (8 μM) was first mixed with H_2O_2 in the pre-mixing chamber, and after a 2-s delay, it was mixed with 20–1000 μM CO (oxygen-free) solutions. Alternatively, slower kinetic time courses at low protein concentrations (below 1 μM) were carried out by mixing the NADH-reduced protein with the alkylhydroperoxide solutions directly in a tonometer equipped with a 1-cm quartz cuvette and a side arm reservoir. In CO inhibition experiments, a few microliters of a CO gas-saturated solution were added to the tonometer with a microsyringe and equilibrated for 20 min with the aqueous phase before adding the substrate to yield an equilibrium CO concentration of 1–20 μM. Time courses were followed at 340 nm (absorption maximum of NADH) on a Jasco V-570 spectrophotometer. All of the experiments were carried out in 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA at 25 °C. In the case of linoleic acid hydroperoxide, reaction kinetics were carried out in the presence of either 0.1% Triton X-100 or 20% v/v ethanol/water mixture.

A complete quantitative analysis of the reaction product was carried out for cumyl hydroperoxide. A 5-ml solution containing 5 μM HMP, 5 mM NADH, and 2.2 mM cumyl hydroperoxide in 0.1 M phosphate buffer was equilibrated under nitrogen for 1 h at 25 °C. A protein-free blank was also prepared under the same conditions. The reaction was quenched by the addition of 5 ml of ethyl acetate, and the organic phase was recovered and split in two aliquots. The first sample was dried under vacuum and resuspended in methanol for HPLC analysis, and the second sample was directly used for GC analysis. In both HPLC and GC analyses, 2 mM benzyl alcohol was used as an internal standard to quantify the cumyl hydroperoxide reaction products. The methanol solutions (blank and sample) were injected onto an Agilent 1100 Series HPLC system equipped with a Supelcosil LC-18DB (25 cm × 2 μm) reverse phase HPLC column. The products were eluted with 30% v/v methanol/water solution at a flow rate of 0.5 ml/min and monitored at 256 nm. Product elution peaks were identified by comparison with authentic standards under identical elution conditions. GC analysis was carried out using a Varian CP-3800 gas chromatographic apparatus equipped with a CP-Sil 5 CB column (30 cm × 0.25 mm).

For ESI mass spectrometry measurements, linoleic acid hydroperoxide or its reaction products were extracted as follows. The reaction mixture (1 ml) containing 2 μM HMP in 0.1 M phosphate, pH 7.0, and 1 mM EDTA and 20% v/v ethanol, 140 μM linoleic acid hydroperoxide, and 500 μM NADH was allowed to stand 15 min under a nitrogen gas atmosphere at 25 °C. At the end of the reaction, the solution was treated with 2 ml of ethyl acetate, vortexed, and centrifuged at 6000 rpm for 15 min. Two blank solutions were prepared in which either NADH or HMP was omitted. The ethyl acetate extracts were dried under vacuum and resuspended in 80% ethanol/water containing 0.5% v/v ammonia. The ethanolic solutions were analyzed by infusion (5 μl/min) in an electrospray ion trap mass spectrometer (model LCQ, ThermoFinnigan, San Jose, CA). Linoleic hydroperoxide standards dissolved in the same solvent were used to optimize the ESI source parameters. The mass spectra were collected over the mass range of 70–700 a.m.u. in negative ion mode.

RESULTS

The hydroperoxide reductase activity of HMP was screened by using H_2O_2, *tert*-butyl hydroperoxide, cumyl hydroperoxide, and linoleic acid hydroperoxide as substrates. The reaction products analyzed by HPLC, GC, or ESI mass spectrometry revealed transformation of the alkylhydroperoxide species into their corresponding alcohols. A complete quantitative analysis of the reaction products carried out for cumyl hydroperoxide is shown in Fig. 1. The ethyl acetate extracts obtained from the reaction mixture in the absence (blank) and in the presence of HMP, respectively, were analyzed by HPLC. Elution profiles are shown in *panel A* (blank) and *panel B*. Benzyl alcohol (peak at 15.1 min) was used as an internal standard to quantify the reaction products. The elution profile of the blank in *panel A* demonstrates that stock solutions of cumyl hydroperoxide (peak at 42.1 min, 89%) contained both acetophenone (peak at 28.7 min, 3%) and cumyl alcohol (peak at 36.7 min, 8%) as contaminants. Data shown in *panel B* indicate a complete transformation of cumyl hydroperoxide into cumyl alcohol (peak 36.7 min), whereas the peak at 28.7 min reflects the small amount of acetophenone whose molar absorptivity at 256 nm is approximately 30-fold higher with respect to that of cumyl alcohol already present in the blank. Accordingly, GC mass data, performed in order to take into account the possible formation of other volatile reaction products, revealed full conversion of cumyl hydroperoxide into cumyl alcohol in the reaction mixture (data not shown).

ESI mass spectrometric data (Fig. 2) revealed a similar trend in the case of linoleic acid hydroperoxide. Mass spectra of the ethyl acetate extracts of the blank solutions (in which either protein or NADH was absent) yielded a major peak at

[2] A. Bonamore, P. Gentili, A. Ilari, M. E. Schininà, and A. Boffi, unpublished results.

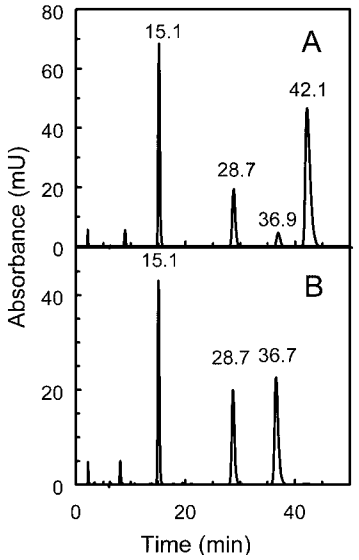

FIG. 1. **HPLC elution profiles of the reaction products obtained from the reaction of *E. coli* flavohemoglobin and cumyl hydroperoxide.** *A*, the elution profile (at 256 nm) of a reaction blank obtained from ethyl acetate extraction of a solution containing NADH (5 mM) and cumyl hydroperoxide (2 mM) in 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA (reaction was carried out under N_2 atmosphere, 1-h incubation at 20 °C). *B*, the elution profile obtained from the same reaction mixture in the presence of 10 μM HMP. The peaks are assigned as follows: 42.1 min, cumyl hydroperoxide; 36.9 min (*A*) or 36.7 min (*B*), cumyl alcohol; 28.7 min, acetophenone; and 15.1 min, benzyl alcohol (internal standard). Acetophenone is present as a contaminant in the stock solution (3%) and is recovered in a similar amount in both reaction mixtures of *A* and *B*. Note that the molar absorptivity of acetophenone at 256 nm is 30-fold higher than that of cumyl alcohol.

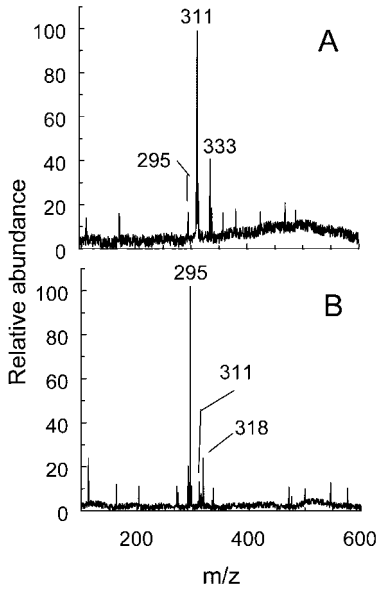

FIG. 2. **ESI mass spectra of the reaction products obtained from the reaction of *E. coli* flavohemoglobin and linoleic acid hydroperoxide.** *A*, the spectrum of a reaction blank obtained from an ethyl acetate extraction of a solution containing NADH (0.5 mM) and linoleic acid hydroperoxide (140 μM). The major peak at 311 a.m.u. corresponds to linoleic hydroperoxide anion. *B*, the spectrum obtained from the same reaction mixture in the presence of 2 μM HMP. The major peak at 295 a.m.u. corresponds to linoleic alcohol. Experiments were carried out in 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA (reaction was carried out under N_2 atmosphere, 15-min incubation at 20 °C).

311 a.m.u. (corresponding to the molecular weight of linoleic acid hydroperoxide anion), whereas the dominant feature was at 295 a.m.u. (−16 a.m.u. with respect to the 311 peak) in the

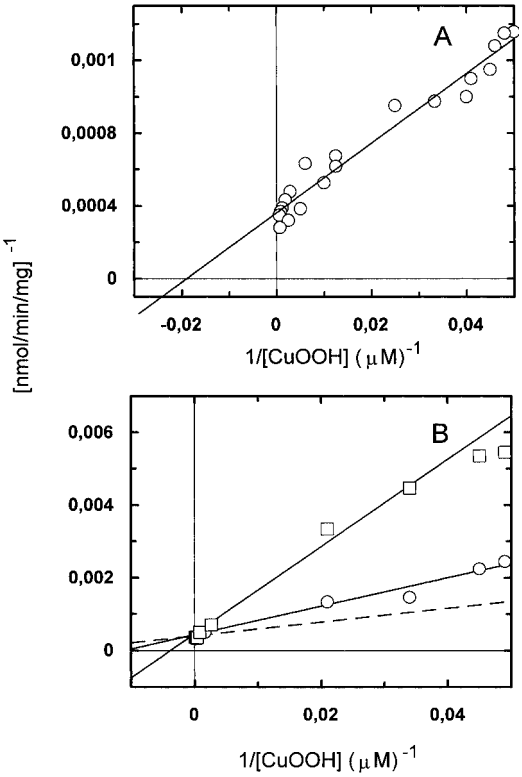

FIG. 3. **Double reciprocal plots of *E. coli* flavohemoglobin-alkylhydroperoxide reductase activity *versus* cumyl hydroperoxide concentrations.** The activities were measured under N_2 atmosphere in the presence of 0.2–1 μM protein, 250 μM NADH in 0.1 M phosphate buffer at pH 7.0. The *straight line* represents a linear fitting to the experimental data. *B*, CO inhibition of the alkylhydroperoxide reductase activity *versus* cumyl hydroperoxide. The *dashed line* corresponds to the linear fit reported in *A*. The *continuous lines* represent the linear fits to the data obtained in the same reaction mixture in the presence of 1 μM (○) and 10 μM (□) CO.

reaction mixture containing both protein and NADH. Spurious peaks at 334 and 317 a.m.u. (+22 with respect to 311 and 295 a.m.u., respectively, attributed to Na^+ ion adducts) are apparent in the blank and in the complete reaction mixture, respectively.

Reaction kinetics were monitored on the different substrates by means of UV-visible absorption spectroscopy in both spectrophotometric experiments at low protein concentrations (0.2–1 μM) and in stopped-flow rapid kinetic measurements at higher protein concentrations (4–6 μM). HMP activities determined upon increasing amounts of alkylhydroperoxides or H_2O_2 to degassed HMP solutions in the presence of excess NADH (250 μM) are depicted in Fig. 3 (*panel A*). Double reciprocal plots of Fig. 3 are the result of several separate experiments carried out on different protein preparations. Data linearization and fitting yielded the set of steady state kinetic parameters reported in Table I. The highest activity was observed for cumyl and linoleic acid hydroperoxides, whereas the reduction of *tert*-butyl hydroperoxide and hydrogen peroxide was 2- and 3-fold slower, respectively (Table I). It should be pointed out that reaction with linoleic acid hydroperoxide is strongly solvent-dependent. Data obtained in the presence of 0.1% Triton X-100 were fully reproducible and displayed nearly zeroth-order kinetics. In contrast, experiments carried out in 20% ethanol/water mixture in which substrate is soluble up to 2–3 mM concentration at 25 °C exhibited a slightly slower rate and displayed nearly exponential time courses.

Carbon monoxide inhibition of the hydroperoxide reduction reaction was also determined for cumyl hydroperoxide. The

TABLE I
E. coli flavohemoglobin steady state kinetic constants for hydroperoxide substrates

Data were obtained in 0.1 M phosphate buffer, pH 7.0, containing 250 μM NADH and 1 mM EDTA at 25 °C.

Hydroperoxide	K_M	V_{max}[c]
	μM	*nmol/min/mg*
H_2O_2	260 ± 28	789 ± 102
tert-Butyl	76 ± 15	1125 ± 135
Cumyl	55 ± 15	2564 ± 155
Linoleic	26 ± 14[a]	1876 ± 188[a]
	115 ± 33[b]	872 ± 215[b]

[a] 0.1% Triton X-100.
[b] 20% etanol/water mixture.
[c] For comparison with data from other authors, activities can be redimensioned "per mol" using the molecular mass of 43,000 g mol^{-1} for HMP.

data reported in Fig. 3*B* indicate a clear competitive inhibition mechanism by CO with an apparent K_i of ~0.2 μM (calculated from the apparent K_m^{app} values according to a simple Michaelis-Menten formalism).

Rapid kinetic experiments carried out by mixing degassed HMP solutions (8 μM) in the presence of excess NADH (320 μM) with cumyl hydroperoxide (1 mM) solutions are depicted in Fig. 4*A*. The kinetic spectral profiles were characterized by a fast decay at 340 nm ($t_{½}$ = 3 s) because of NADH oxidation while HMP remains in its fully reduced (ferrous heme, reduced flavin) deoxygenated derivative. Upon depletion of NADH-reducing equivalents (5–6 s), HMP undergoes complete oxidation (ferric heme, oxidized flavin). A similar trend is observed with linoleic acid hydroperoxide, whereas the formation of a spectroscopically distinct contribution characterized by an absorption peak at 420 nm was noticed under stationary state conditions in the reaction with *tert*-butyl hydroperoxide (data not shown). The species characterized by the 420-nm peak was the dominant feature in the reaction of ferrous HMP with hydrogen peroxide (Fig. 4*B*). An analysis of the time-dependent spectral profiles of Fig. 4 allowed the identification of the absorption spectra relative to each species (Fig. 5). The spectral profile 3 obtained from the data of Fig. 4 was thus attributed to an oxo-ferryl heme iron adduct by analogy with the spectra of typical Compounds II of peroxidases and must be distinguished from the ferrous heme iron dioxygen adduct (profile 4). The oxo-ferryl adduct was relatively stable (seconds to minutes) under reducing conditions (250 μM NADH) and 50–250 μM hydrogen peroxide concentrations, whereas it rapidly decayed to a mixture of ferric heme and unidentified heme degradation products in the presence of H_2O_2 in larger excess (>2 mM). To confirm the oxo-ferryl spectrum assignment, the following set of controls was carried out. (i) The species obtained in the presence of H_2O_2 and NADH was mixed with CO-saturated solutions (1 mM) in a double mixing experiment (see "Experimental Procedures"), and no spectral changes were observed. (ii) The same compound could not be reduced with sodium dithionite.

DISCUSSION

The results here presented demonstrate that HMP is capable of a very efficient alkylhydroperoxide reductase activity toward a set of synthetic substrates, namely *tert*-butyl, cumyl, and linoleic acid hydroperoxides (Figs. 1 and 2). Interestingly, the hydroperoxide reductase activity is higher toward cumyl, *tert*-butyl, and linoleic acid hydroperoxides than toward H_2O_2, thus indicating a preference for highly hydrophobic substrates (Table I). This finding must be analyzed in parallel with recent investigations in which HMP was also shown to interact with bacterial lipid membranes and to be able to bind specifically phospholipids and/or fatty acids within the heme pocket (14, 15). Taken together, these observations pave the way to a functional role for HMP and possibly other members of the bacterial hemoglobin family. HMP is involved in the repair of peroxidized membrane phospholipids, thus reducing hydroperoxides into their corresponding alcohols.

The alkylhydroperoxide reductase activity is widespread among diverse bacterial species and is thought to represent a major functional response of the cell to oxidative stress (18). In *E. coli*, oxidative stress involves among others the activation of

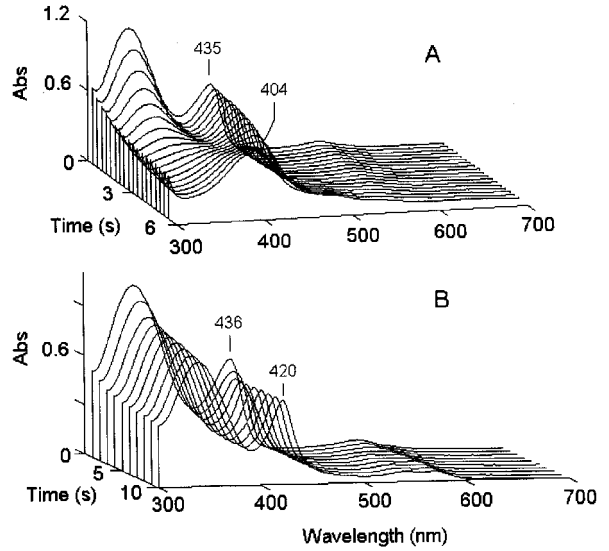

FIG. 4. **UV-visible absorption spectral changes accompanying hydroperoxide reductase activity in *E. coli* flavohemoglobin.** Spectra were collected with a diode array device after mixing a solution of ferrous HMP (4 μM) containing 250 μM NADH with a solution containing 1 mM cumyl hydroperoxide (*A*) or 1 mM H_2O_2 (*B*). The measurement has been carried out in 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA at 20 °C. Solutions were degassed and kept under nitrogen before use.

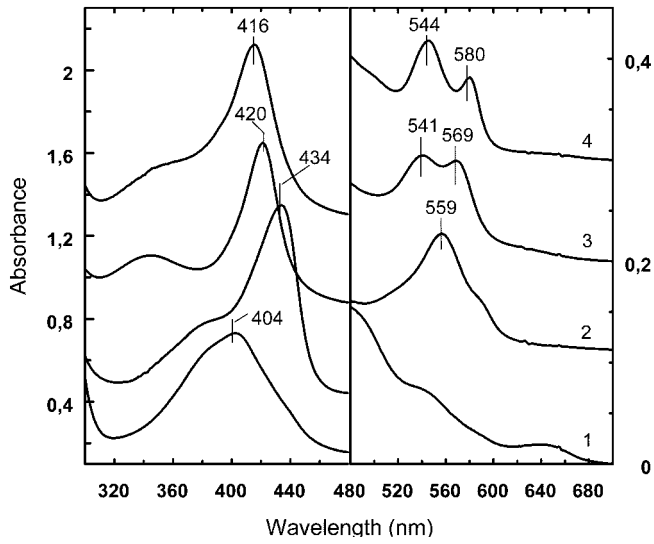

FIG. 5. **UV-visible absorption spectra of *E. coli* flavohemoglobin intermediates in its reaction with alkylhydroperoxides.** The spectrum of ferric HMP (1) has been taken at the end (10 s) of the reaction of HMP with cumyl hydroperoxide. NADH-reduced deoxygenated HMP spectrum (2) was collected before mixing with cumyl hydroperoxide. The spectrum of the HMP Compound II intermediate (3) obtained as a transient species from the experiment carried out with H_2O_2 (see Fig. 3*B*) can be compared with the spectrum of fully reduced dioxygen HMP adduct (4). Spectra *2–4* have been corrected by subtracting the contribution of NADH. All of the spectra have been taken at pH 7.0 in 0.1 M phosphate buffer.

OxyR, a hydrogen peroxide sensor and transcriptional activator that elicits the expression of a number of proteins and enzymes involved in hydrogen peroxide scavenging and/or repair of peroxidized substrates (19). Among these, the 2-Cys peroxiredoxin/flavoprotein complex (ahpCF) has been shown to be able to reduce a broad class of peroxides spanning from hydrogen peroxide to unsaturated and aromatic hydrocarbon hydroperoxides and thus has been considered as a key enzyme in the bacterial alkylhydroperoxide reductase activity (18–20). Nevertheless, the structural diversity of peroxidized compounds possibly formed within the cell under oxidative stress is difficult to reconcile with the presence of a single enzyme capable of recognizing specifically the hydroperoxide moiety within complex molecular frames such as a DNA strand or a phospholipid acyl chain. So far, phospholipid-specific alkylhydroperoxidases have been identified in a number of prokaryotic and eukaryotic organisms (21, 22). Along the same line, recent observations demonstrated that AhpCF peroxiredoxin complex plays a pivotal role in removing hydrogen peroxide in *E.* coli cells under stationary phase conditions (in virtue of a lower K_m value with respect to catalases, whereas it is less efficient as a lipid hydroperoxide scavenger (23).

HMP-catalyzed conversion of cumyl hydroperoxide to cumyl alcohol (currently used as a standard for comparing the alkylhydroperoxide reductase activity among different enzymes) occurs at a rate (2564 nmol/min/mg) that is at least two orders of magnitude higher than that measured for the *E. coli* AhpCF complex under similar experimental conditions (Ref. 18 and references cited therein) and ~2-fold higher than that measured for the most efficient *Amphibacillus xylanus* peroxiredoxin reductase under saturating conditions of AhpC (24). Such a high activity corresponds to a turnover number (mol of substrate per mol of protein) of 110 min^{-1} (see Fig. 2 and Table I). CO inhibition experiments (Fig. 3, *panel B*) demonstrate that the activity is heme-linked and is not a spurious effect because of the presence of the highly reactive flavin prosthetic group within the protein. The residual activity observed in HMP in the presence of CO is in fact fully compatible with a competitive inhibition mechanism in which substrate binding is limited kinetically by the rate of CO release from the heme iron.

Simple inspection of the time-dependent absorption spectra offers several interesting insights into the mechanism of the alkylhydroperoxide reductase activity. An analysis of the spectroscopic data of Figs. 4 and 5 together with previous experimental observations on lipid binding to HMP permits an unequivocal elucidation of the early steps in the mechanism of alkylhydroperoxide reduction and establishes differences and analogies with classical heme-based peroxidase enzymes. HMP is capable of recognizing the alkylhydroperoxide moiety because of the highly hydrophobic distal heme pocket, and in the case of peroxidized phospholipids containing unsaturated fatty acids, HMP has been inferred to be able to adjust the hydrocarbon chain kink in correspondence of the *cis* double bond above the heme iron (14, 15). Thereafter, the binding of the hydroperoxide to the ferrous heme iron occurs with the concomitant two-electron reduction and cleavage of the dioxygen bond and consequent formation of a peroxidase-like Compound II and an alkyl alcohol. It is important to note that this first step does not imply electron transfer from the flavin to the heme iron but rather a direct two-electron iron oxidation. The fate of the ferryl-oxo compound thus generated is less obvious. Reduction of the ferryl-oxo compound to water and ferric heme with concomitant flavin oxidation and release of the hydroxyphospholipid to the membrane may be hypothesized. Most intriguingly, the active form of the protein capable of binding a new molecule of substrate is the ferrous derivative and not the ferric one. In fact, at variance with classical peroxidases, no alkylhydroperoxide and/or H_2O_2 binding to the heme iron can be detected when the heme iron is in the ferric state. Consistently, ferric HMP is unable to perform monooxygenations typically catalyzed by peroxidases and/or cytochrome P-450 on a broad variety of substrates (14).

At present, a direct *in vivo* determination of the HMP-dependent alkylhydroperoxide reductase activity still needs to be explored. Nevertheless, a number of experimental observations on HMP expression in response to oxidative stress conditions provide convincing evidence for a genuine physiological role of the protein in the repair of oxidative damage. In particular, it was observed that HMP expression, controlled by the fumarate nitrate regulator transcription factor, is enhanced under conditions of prolonged oxidative stress such as those imposed by administrating paraquat and other agents generating oxygen reactive species to the culture medium (4, 25). This finding is consistent with HMP being transcribed in a later phase after the oxidative pulse. In fact, whereas the fast response to oxidative stress is governed by enzymes that are directly under the control of the transcription factor OxyR (18, 27), the late response to oxidative stress involves a web of interactions that ultimately lead to HMP transcription (25). Thus, enzymes involved in hydrogen peroxide scavenging (AhpCF and KatG/E gene products) or DNA protection (Dps gene product) are rendered available as a first barrier against peroxidation, whereas HMP and possibly other substrate-specific enzymes may play a role in the repair of diverse peroxidized species. In this framework, the link between the reported NO-induced (fumarate nitrate regulator-mediated) expression of HMP (3) and oxidative stress may reflect another facet of the complex and intimately correlated responses of the bacterial cell to NO and oxygen reactive species. NO induction of enzymes involved in the oxidative stress response is in fact well documented (28), and explicit mechanisms that envisage an increase of the alkylhydroperoxide reductase activity in the presence of NO have been proposed (29). In particular, nitroperoxide species formed at a diffusion-limited rate by the reaction of NO with superoxide anion is known to be a strong promoter of membrane lipid peroxidation (30). Accordingly, NO-induced HMP expression under low oxygen tensions might well allow a direct (fumarate nitrate regulator-mediated) mechanism for lipid hydroperoxide reduction, whereas peroxiredoxins actively scavenge nitroperoxide anions (29).

On the basis of the considerations just presented, HMP and other bacterial hemoglobins would be key enzymes in maintaining the cell membrane integrity at the aerobic/anaerobic interface. Such a hypothesis also provides a plausible explanation for the reported high growth performance of *E. coli* overproducing bacterial hemoglobins under microaerobic environments (31). Nevertheless, other catalytic options must also be considered in further studies. In fact, lipid-hydroperoxide reductase activity might be part of a specific reaction pathway that leads to the formation of lipid hydroperoxide-derived active compounds whose functions are yet to be established. It is worth mentioning that in chloroplasts, for instance, unsaturated fatty acid hydroperoxides are converted to signaling molecules by specific cytochrome P-450-like proteins such as hydroperoxide lyase and allene oxidase (26). Unsaturated fatty acid processing by heme-based cyclo-oxygenase enzymes is also at the basis of the eicosanoids pathway in eukaryotic cells. Intriguingly, cyclo-oxygenases have been proposed to be activated by NO (or by nitroperoxide) to trigger the catalytic cycle.

In the same way, bacterial hemoglobins might be involved in the (possibly NO-driven) processing of phospholipids in the framework of a more complex physiological response.

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