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The Role of the Hybrid Cluster Protein in Oxidative Stress Defense*

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Hybrid cluster proteins (HCP) contain two types of Fe/S clusters, namely a $[4Fe\text{-}4S]^{2+/1+}$ or $[2Fe\text{-}2S]^{2+/1+}$ cluster and a novel type of hybrid cluster, [4Fe-2S-2O], in the as-isolated state. Although first isolated from anaerobic sulfate-reducing bacteria, the analysis of the genomic sequences reveals that genes encoding putative hybrid cluster proteins are present in a wide range of organisms, aerobic, anaerobic, or facultative, from the Bacteria, Archaea, and Eukarya domains. Despite a detailed spectroscopic and structural characterization, the precise physiological function of these proteins remained unknown. The present work shows that the transcription of the *Escherichia coli hcp* gene is induced by hydrogen peroxide, and this induction is regulated by the redox-sensitive transcriptional activator, OxyR. The *E. coli hcp* mutant strain exhibits higher sensitivity to hydrogen peroxide, a behavior that reverts to the wild type phenotype once a plasmid carrying the *hcp* gene is reintroduced. Furthermore, the purified HCPs from *E. coli* and *Desulfovibrio desulfuricans* ATCC 27774 show an alternative enzymatic activity, which under physiological conditions exhibited K_m values for hydrogen peroxide (~0.3 mM) within the range of other peroxidases. Altogether, the results reveal that HCP is involved in oxidative stress protection.

Hybrid cluster proteins (HCP),[2] first isolated from the sulfate-reducing bacteria *Desulfovibrio vulgaris* (1) and *Desulfovibrio desulfuricans* (2), contain an unusual iron-sulfur cluster, which in the as-isolated (partially oxidized) state is a mixed oxygen-iron-sulfur cluster, [4Fe-2S-2O], the so-called hybrid cluster, and a cubane $[4Fe\text{-}4S]^{2+/1+}$ cluster (3) or a dinuclear $[2Fe\text{-}2S]^{2+/1+}$ cluster (4). HCPs are widespread among the three life domains, as genes encoding for orthologs of HCP are observed in a wide range of distinct organisms such as enterobacteria, clostridia, *Bacteroides*, Cyanobacteria, *Bacillus* sps., methanogens, and protozoa, *e.g. Entamoeba* (5).

In *Escherichia coli* HCP was detected by immunoblotting in cells grown anaerobically with nitrate or nitrite (4). In accordance, the transcription of *hcp* from *E. coli* (6, 7), *Salmonella enterica* serovar *typhimurium* (8), *Shewanella oneidensis* (9), and *D. vulgaris* (10) was found to be elevated in response to nitrogen oxides such as nitrate, nitrite, or *S*-nitrosoglutathione. Interestingly, it was observed in the microarray profile of *Erwinia chrysanthemi* that the transcription level of *hcp* is highly increased upon plant infection (11). The expression profile of the colonizer of the human urinary tract *E. coli* strain 83972 in patients carrying urinary infections also showed up-regulation of *hcp* (12).

Moreno-Vivian and collaborators (13) reported the increase of anaerobic tolerance to hydroxylamine of *E. coli* cells overproducing *Rhodobacter capsulatus* HCP. However, the optimal conditions of the oxygen-sensitive hydroxylamine reductase activity of *R. capsulatus* HCP (13), as well of the *E. coli* HCP (14), could only be found at pH 9. The anaerobically purified *D. desulfuricans* HCP also exhibited a very high K_m value for hydroxylamine at neutral pH (15). Other studies showed that disruption of *hcp* in *E. coli*, *Morganella morganii* (4), and *S. enterica* (8) did not exhibit abnormal behavior, and it caused no change in growth behavior in anaerobic media using nitrate or nitrite as respiratory substrates. In addition, *D. desulfuricans* HCP was expressed in equivalent amounts in sulfate- or nitrate-grown cells (15). Altogether, these results strongly suggest that HCP has a distinct physiological function, possibly related to other types of stress (16). In fact the presence of the gene in a large number of organisms not involved in the biological nitrogen cycle led us previously to suggest its involvement in oxidative stress response (15). In this study, we have discovered that *E. coli hcp* is induced by hydrogen peroxide, is involved in oxidative stress protection, and is regulated by the peroxide regulator OxyR.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Assays—E. coli K-12 (ATCC 23716) was used as the wild type strain. Cells were grown in LB or minimal salts medium (17) with casamino acids, pH 7, aerobically in flasks filled with one-fifth of their volume or anaerobically in rubber seal-capped flasks that, once filled with medium and closed, were extensively bubbled with nitrogen. Cells were cultivated at 37 °C, with the aerobically grown cultures shaken at 150 rpm, and in all cases the growth process was started with a 1% inoculum of an overnight LB grown aerobic culture. For the sensitivity stress conditions, growing cells (A_{600} between 0.1 and 0.3) were treated with H_2O_2 at the concentrations and times indicated for each experiment. Untreated cultures were incubated in parallel over equal periods of time. Sensitivity was determined either by following the A_{600} of liquid cultures or by serial dilution of the bacterial suspension with

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[2] The abbreviations used are: HCP, hybrid cluster protein; ABTS, 3-ethylbenzthiazoline-6-sulfonic acid.

phosphate-buffered saline followed by plating on LB agar and incubation overnight at 37 °C.

RNA Extraction and Reverse Transcription-PCR Analysis—Cells of wild type and Δ*oxyR* strains were grown aerobically or anaerobically in LB to an A_{600} of 0.3 and exposed to 3 mM H_2O_2 for 5 min or left untreated. Total RNA was extracted using an RNA kit from Roche Applied Science and treated with DNase I. After confirming the absence of any residual DNA, reverse transcription-PCR reactions were performed with 50–500 ng of RNA using primers that amplified an internal product of 589 bp for *hcp*. The *E. coli gap* gene, which does not vary in expression upon treatment with hydrogen peroxide (18), was used to guarantee that equal amounts of RNA were compared.

Construction of E. coli hcp Deletion Strain and Complementation Analysis—Strain LMS0873 defective in the *hcp* gene (K-12 *hcp*::Cm^r) was produced according to the Datsenko method (19) by replacing a 1.5-kb fragment of the gene (from 43 bp after the starting codon to 41 bp before the end of the gene) by a chloramphenicol resistance cassette.

For the complementation analysis, plasmids pWB208 carrying the *hcp* gene (4) and pUC18 were individually transformed into the LMS0873 strain (*E. coli hcp* mutant). Single colonies were grown overnight in LB supplemented with chloramphenicol and ampicillin. The overnight cultures were used to subculture LB and grown to an OD of 0.25, at which point cultures were submitted to 3 mM hydrogen peroxide for 2 h or left untreated and analyzed by serial dilution plating.

Gels Stained for Peroxidase Activity—For activity staining gels, samples were run in a 7.5% gel under native conditions. Immediately after completion of electrophoresis, the gel was stained in a solution containing H_2O_2 and 3,3′-diaminobenzidine followed by potassium ferricyanide and ferric chloride staining as described previously (20).

Enzymatic Assays—Peroxidase activity was determined aerobically at room temperature following the decrease in absorbance of sodium ascorbate at 290 nm (ϵ_{290} = 2.8 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7, 250 μM–5 mM hydrogen peroxide, 0.2 mM sodium ascorbate, and *E. coli* HCP (5 μM) or *D. desulfuricans* HCP (2 μM). Appropriate controls, without protein, were performed for each assay. The kinetic constants, K_m and V_{max}, were determined from fitting the hyperbolic data with a nonlinear regression routine from GraphPad Prism4. Other different substrates for peroxidase activity were also tested: 0.3 mM ABTS (ϵ_{415} = 36 mM^{-1} cm^{-1}), 5 mM guaiacol (ϵ_{470} = 26.6 mM^{-1} cm^{-1}), and 0.4 mM NAD(P)H (ϵ_{340} = 6.22 mM^{-1} cm^{-1}).

Catalase activity was measured in 50 mM potassium phosphate buffer, pH 7, using 10.5 mM hydrogen peroxide (ϵ_{240} = 43.6$M^{-1}cm^{-1}$). Visible spectra and peroxidase activity assays were performed on a Shimadzu UV-1700 spectrophotometer.

Production of E. coli Recombinant HCP and D. desulfuricans HCP—The entire coding region of *E. coli hcp* was amplified by PCR from *E. coli* K-12 ATCC 23716 genomic DNA using primers that incorporated NdeI and HindIII restriction sites. The amplified gene was cloned into pET-28A(+) (Novagen, VWR Int., Lisbon, Portugal), which allows the introduction of a His tag into the N terminus of HCP, and sequencing confirmed the integrity of the gene. Overexpression of the recombinant protein was achieved in *E. coli* BL21Gold(DE3) cells (Stratagene, Cultek, Madrid, Spain) transformed with the constructed plasmid pET-HCP and grown as described previously (21). *E. coli* HCP was isolated from cells broken in a French press and centrifuged at 8000 × *g* for 8 min, and the supernatant was loaded onto a His spin trap (GE Healthcare), and pure HCP, as judged by SDS-PAGE, was eluted with 500 mM imidazole, pH 7.4, and dialyzed. Purification of *D. desulfuricans* ATCC 27774 HCP was achieved as reported elsewhere (3). The protein concentration was assayed by the bicinchoninic acid method using bovine serum albumin as the standard (22). EPR spectra were obtained on a Bruker EMX spectrometer equipped with an Oxford Instruments continuous flow helium cryostat.

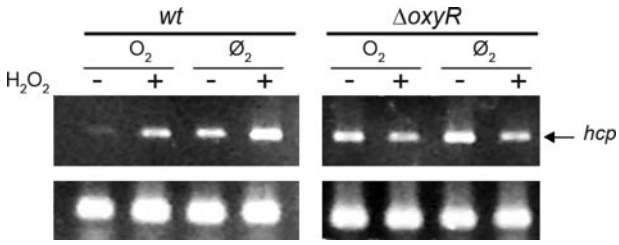

FIGURE 1. ***E. coli* hcp is induced by hydrogen peroxide in an OxyR-dependent mode.** mRNA levels of *hcp* assayed in cells of *E. coli* K-12 (*wt*, wild type) (300 ng RNA) or in *E. coli* cells lacking *oxyR* (Δ*oxyR*) (500 ng RNA), grown aerobically (O_2) or anaerobically ($Ø_2$) in LB to an A_{600} of 0.3 and treated with 3 mM for 5 min (+) or collected without H_2O_2 treatment (−). The *lower band*, present in all reactions, shows the amplification product of the internal standard gene, *gapA* (see "Experimental Procedures").

RESULTS

E. coli hcp Is Induced by Hydrogen Peroxide in an oxyR-dependent Mode—In previous studies, *E. coli* HCP could be detected only in cells grown under anaerobic conditions using nitrate or nitrite as electron acceptors (4). In the present work, we found that *hcp* was expressed in cells of *E. coli* grown aerobically or anaerobically in the absence of any electrons acceptors (Fig. 1), with a higher transcriptional level under fermentative conditions. Interestingly, the expression of *hcp* was found to be regulated at the transcription level by hydrogen peroxide, as judged by the increased levels of *hcp* mRNA detected in H_2O_2-treated cells grown under aerobic or fermentative conditions (Fig. 1).

To investigate whether expression of *hcp* is induced by oxidative stress in a OxyR-dependent fashion, the mRNA *hcp* level was analyzed in a strain lacking the peroxide response regulator OxyR (18). The induction of *hcp* by hydrogen peroxide was found to be abolished in the *oxyR* mutant compared with the parental strain, under aerobic and anaerobic conditions (Fig. 1). These results show that a functional OxyR is essential for induction of *hcp* upon oxidative stress. Analysis of the *E. coli hcp* promoter region shows a sequence resembling the consensus OxyR-binding sequence ($ATAGN_7CTATN_7ATAGN_7CTAT$) (23) located at −105 to −65 bp upstream of the *hcp* start codon (24).

Deletion of hcp Causes an Oxidative Stress Phenotype—The increase in the mRNA *hcp* level upon addition of hydrogen peroxide to cells of *E. coli* suggests its involvement in the oxidative stress response. For further analysis, an *E. coli*

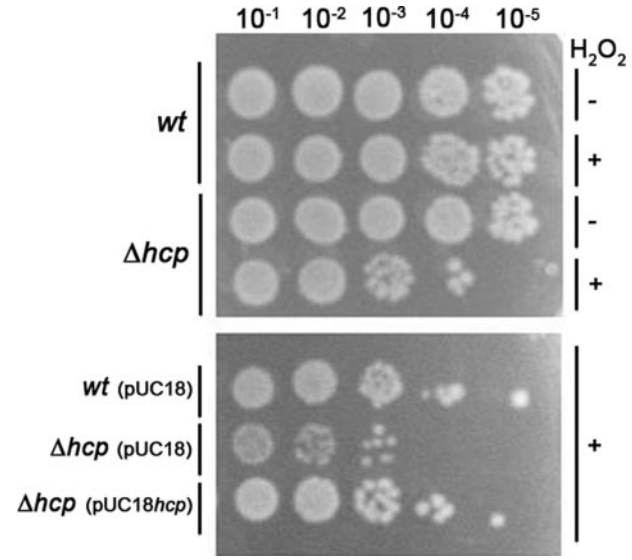

FIGURE 2. **Deletion of *hcp* increases the hydrogen peroxide sensitivity of *E. coli*, and HCP from expression of plasmid-borne pWB208 restores the wild type phenotype.** Cells of *E. coli* K-12 (*wt*, wild type), LMS0873 (Δ*hcp*), *E. coli* K-12 transformed with pUC18 (*wt* (pUC18)), LMS0873 transformed with pUC18 (Δ*hcp* (pUC18)), and LMS0873 transformed with plasmid pUC18 carrying the *hcp* gene cluster (Δ*hcp* (pUC18*hcp*)), grown in LB at an A_{600} of 0.2 were treated with 3 mM H_2O_2 for 2 h. Cell viability was assayed by plating serial dilutions (10^{-1}-10^{-5}) on LB plates. For the same dilution, the difference of viability between cells of wild type/Δ*hcp* and wild type/Δ*hcp* carrying pUC18 is due to the slower growth of the latter.

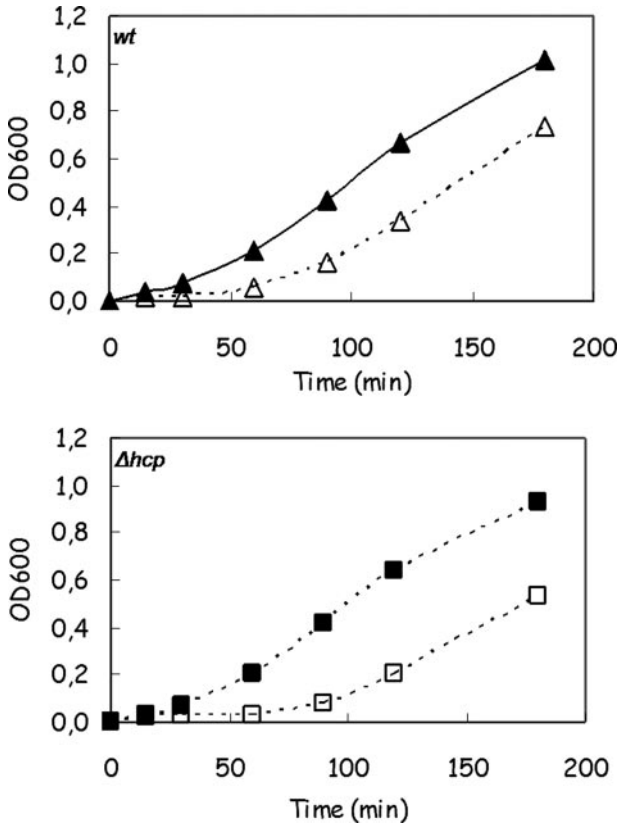

FIGURE 3. **The growth of *E. coli hcp* mutant is inhibited by hydrogen peroxide under anaerobic conditions.** *E. coli* K-12 wild type (*wt*, *triangles*) and the strain mutated in *hcp* (Δ*hcp*, *squares*) were grown anaerobically in minimal medium (*filled symbols*) and submitted to 2.5 mM H_2O_2 (*open symbols*) as described under "Experimental Procedures."

strain defective in *hcp* was constructed, and the sensitivity to hydrogen peroxide was tested. It was observed that under aerobic or fermentative conditions the mutant showed no major growth differences relative to wild type *E. coli*. The two strains were exposed for 2 h to 3 mM H_2O_2 in liquid cultures grown aerobically in LB followed by transfer of aliquots onto solid medium. The results (Fig. 2) showed that Δ*hcp* exhibits lower viability (around 2 orders of magnitude) after treatment with H_2O_2 compared with the wild type strain. Since the expression of *hcp* increases when cells are shifted to anoxic conditions, the hydrogen peroxide sensitivity of the mutant was also analyzed under anaerobic fermentative conditions. Under these conditions, a significant growth arrest of the Δ*hcp* cells treated with hydrogen peroxide was also observed (Fig. 3). On the contrary, no differences in viability were detected between the wild type and mutant when cells were treated with paraquat, a superoxide generator (data not shown).

The *hcp* disruption mutant could be complemented by a clone containing the *hcp* gene. When exposed to hydrogen peroxide the complemented strain grew to a final culture density similar to that of the parent strain, under both aerobic (Fig. 2) and anaerobic conditions (data not shown), thus showing that the higher sensitivity of the Δ*hcp* strain to oxidative stress is indeed caused by the absence of the *hcp* gene.

Peroxidase Activity of HCP—Because HCP contains a cluster with μ-oxo-bridges, which are lost upon reduction (15), and as a first attempt to find an enzymic function for HCP, we assayed the peroxidase and catalase activities of *E. coli* and *D. desulfuricans* HCPs. These two proteins have a high amino acid identity of 40 and 59% similarity; therefore, *D. desulfuricans* HCP was also tested to further corroborate the results obtained for *E. coli* HCP.

The peroxidase activity of *E. coli* and *D. desulfuricans* HCPs was initially probed by activity stained gels using the purified proteins. Both HCPs exhibited peroxidase activity in gels using the double staining method (Fig. 4). For spectrophotometric enzymatic peroxidase activity determination, ascorbate, guaiacol, ABTS, NADH, and NADPH were used as substrates. Only with ascorbate was a measurable activity observed, with a V_{max} of 0.2 μmol H_2O_2·min^{-1} mg^{-1} and a K_m value for H_2O_2 of 0.3 mM for *E. coli* HCP, a V_{max} of 0.04 μmol H_2O_2·min^{-1} mg^{-1} and a K_m for H_2O_2 of 0.3 mM for *D. desulfuricans* HCP, at pH 7, and with K_m for H_2O_2 calculated as described under "Experimental Procedures." Although the V_{max} values are much lower than those reported for heme-containing peroxidases, the K_m values for H_2O_2 are within the range of values measured for canonical peroxidases (Table 1), and no catalase activity was detected. In addition, other kinetic parameters determined for *E. coli* and *D. desulfuricans* HCPs, respectively, were k_{cat} of 9.9 min^{-1} and 2.2 min^{-1} and k_{cat}/K_m of 3.7×10^4 M^{-1} min^{-1} and 7.9×10^3 M^{-1}min^{-1}. This suggests that, as for many other peroxidases, the true physiological substrate is not known, thus explaining the low activity observed. Furthermore, the physiological reductant for either HCP was not available, further hindering a correct enzymatic assay.

To check the possible effect of hydrogen peroxide on HCP, the EPR spectra of *D. desulfuricans* HCP were recorded upon

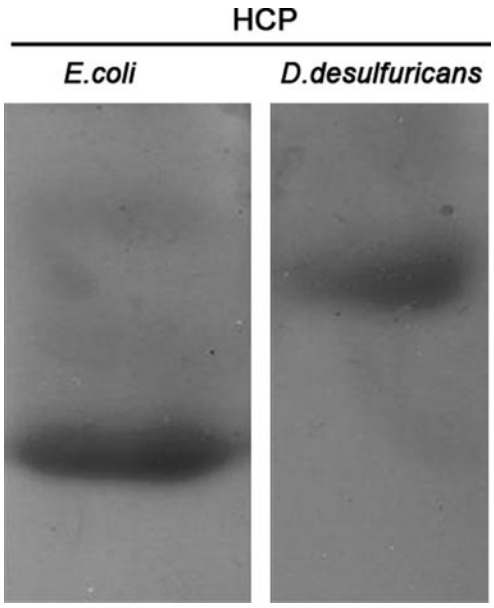

FIGURE 4. **Peroxidase activity of HCP.** Peroxidase activity of HCP from *E. coli* and from *D. desulfuricans* was detected in a native gel stained by the double staining method.

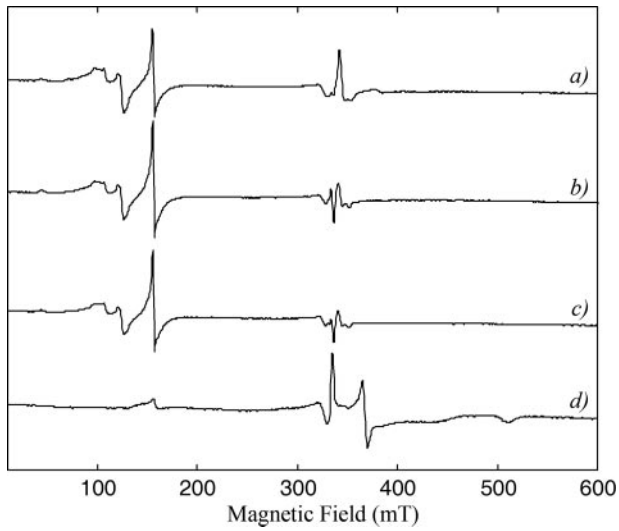

FIGURE 5. **EPR spectra of *D. desulfuricans* HCP purified anaerobically (174 μM) and air-oxidized (*a*), after adding 1 mM H_2O_2 (*b*) and 2 mM H_2O_2 (*c*), and after reducing sample *c* with sodium dithionite (*d*).** EPR conditions: temperature, 12 K; microwave power, 2.4 milliwatts; microwave frequency, 9.41 GHz.

TABLE 1

Comparison of K_m for various peroxidases

This is an adaptation from Koga *et al.* (29).

Source	K_m for H_2O_2
	mM
E. coli HCP	0.3
D. desulfuricans HCP	0.3
Flavobacterium meningosepticum	0.0095
E. coli o-dianisidine peroxidase	3.9
Halobacterium halobium	0.077
Bacillus stearothermophilus	1.3
Arthromyces ramosus	0.19
Plant horseradish peroxidase	0.24

treatment of the HCP protein under different conditions; this enabled us to monitor the characteristic fingerprints of the hybrid and cubane clusters in the oxidized or reduced states: as-isolated protein, incubated with a 6–10-fold molar excess of H_2O_2, which was subsequently reduced; and enzyme reduced with sodium dithionite followed by treatment with hydrogen peroxide. The spectra revealed that H_2O_2 did not damage the protein, and the typical fingerprints for the cubane and hybrid cluster centers were observed after treatment with hydrogen peroxide (Fig. 5). All features of both the hybrid and cubane clusters were identical to those reported for this enzyme in the oxidized and reduced states (*e.g.* Refs. 1–5).

DISCUSSION

When exposed to oxidative stress *E. coli* induces the OxyR regulon (25). Our work shows that *hcp* is under the positive control of OxyR, as has also been observed for a large number of hydrogen peroxide-inducible genes (24). *E. coli hcp* was previously shown to be regulated by Fnr (fumarate-nitrate regulator) (6, 8) and by the nitrate/nitrite regulators NarL and NarP (6), whereas *in silico* analysis predicted the control of the *hcp-hcr* operon by the nitrite-sensitive repressor NsrR (26). Hence, *E. coli hcp* seems to be under the control of multiple regulatory proteins that coordinate the *hcp* expression in response to different stimuli.

In *Clostridium perfringens* disruption of the *hcp* gene yields a strain that under anaerobic conditions shows an increased resistance to hydrogen peroxide and decreased resistance to the superoxide-generating compound, plumbagin (16). Although these results already suggest a role of HCP in oxidative stress, they are the opposite to the behavior reported here for the *E. coli* Δ*hcp* strain, which is more sensitive to anaerobic hydrogen peroxide stress than the parental strain and shows no differences upon exposure to the superoxide releaser agent paraquat.

The reactivity of H_2O_2 with HCP in the presence of ascorbate can be rationalized in terms of the three-dimensional x-ray structures that have been determined for the oxidized as-isolated form (27) and dithionite fully reduced form (15). Thus, the substrate will bind to the reduced form of HCP in the vicinity of position *Y* (Fig. 6*a*). The ability of Asn-307 and Lys-489 in the vicinity of *Y* to form hydrogen bonds may assist this interaction. The addition of two electrons transferred to the hybrid cluster center via the cubane cluster will then cause the peroxide moiety to divide into two hydroxide units, which then bridge Fe6 and Fe8 and Fe7 and Fe8, respectively (Fig. 6*b*), as the enzyme transforms into its oxidized form. The addition of a reductant will then enable the HCP to return to the fully reduced state with the release of the two hydroxide moieties as two molecules of water through the *X* position. In the case of a putative alkyl peroxide substrate, the alkyl moiety will be released during this process into the large hydrophobic cavity adjacent to the hybrid cluster prior to final release from the enzyme. The presence of the two bridging oxygen moieties considerably enhances the putative function of HCP as a peroxidase, although other functions such as a hydroxylamine reductase cannot be completed discounted. The requirement for a reductant also explains why no reactivity is observed with

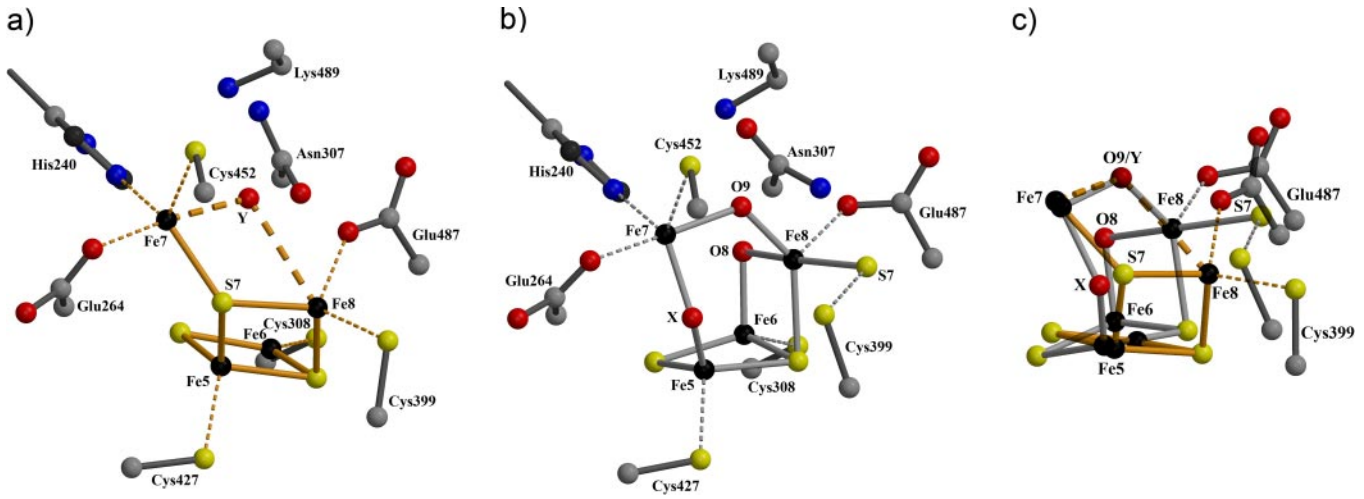

FIGURE 6. **Scheme of the hybrid cluster in *D. desulfuricans* HCP.** *a*, the reduced enzyme (Protein Data Bank code 1OA0) with no bridging oxygen moieties. *Y* represents the putative substrate binding site. The distance from *Y* to residue Lys-489 (NZ) is 2.88 Å and from *Y* to residue Asn-307 (OD1) is 3.07 Å. *b*, the as-isolated oxidized enzyme (Protein Data Bank code 1GNL) with bridging oxygen moieties (OH^-) O-8 and O-9 between Fe6 and Fe8 and between Fe7 and Fe8, respectively. The nature of *X* is not known, but it probably represents the exit pathway of water molecules. The distance from the oxygen O-9 to the residue Lys-489 (NZ) is 2.99 Å and from O-8 to residue Asn-307 (ND2) is 3.02 Å. *c*, superposition of the two forms emphasizing the large movements of both Fe8 (~2 Å) and Ser-7 (~4 Å) during the transformation from the reduced to the oxidized form and vice versa.

ABST or guaiacol, which have very high redox potentials of +700 and +800 mV, respectively, compared with the reduction potentials of ascorbate (30 mV), *E. coli* HCP (with −35 mV for the [2Fe-2S] center and +385, +85, and −50 mV for the hybrid cluster (4)), and *D. desulfuricans* HCP (+285, −5, and −165 mV for the hybrid cluster (28)). In the absence of the structures of the intermediate redox states of the hybrid cluster (not yet determined), it is not possible to know at which state the μ-oxo-bridges are broken. Also, the function of the extracanonical cluster can be understood to act as an electron donor to the hybrid cluster, being easily accessible to an external electron donor and therefore generating the reduced ready state. Lacking the physiological electron donor, assays could not be performed at the optimal conditions, and therefore the activity values obtained are only indicative. However, because of the low redox potential of the cytosol of these organisms (approximately −200 mV), it is expected that upon reoxidation by hydrogen peroxide the enzyme will be rapidly re-reduced to the catalytically active state. Also, because of the redox potentials determined for HCPs, ascorbate may reduce the hybrid cluster to an intermediate redox state. The absence of reactivity with NADH or NADPH can be understood as due to the lack of appropriate binding sites in HCP for these substrates. It should be stressed, however, that it is possible that HCPs may still have a type of reactivity, as yet not clarified, with hydrogen peroxide.

In conclusion, it has been shown that HCP clearly confers protection to *E. coli* toward hydrogen peroxide and is regulated by the peroxide response regulator, OxyR. This is a much clearer role for HCP, which is spread among anaerobic Bacteria, Archaea, and protozoa, than for unrelated metabolisms, namely those not involved in the nitrogen cycle. In addition, this study provides another example of the multiple roles of iron-sulfur proteins, in particular in relation to oxygen and oxidative stress protection.

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