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-4S]^{2+/1+} or [2Fe-2S]^{2+/1+} cluster and a novel type of hybrid cluster, [4Fe-2S-2O], the so-called hybrid cluster, and a cubane [4Fe-4S]^{2+/1+} cluster (3) or a dinuclear [2Fe-2S]^{2+/1+} cluster (4). HCPs are widespread among the three life domains, as genes encoding orthologs of HCP are observed in a wide range of distinct organisms such as enterobacteria, clostridia, Bacteroides, Cyanobacteria, Bacillus spp., 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-nitrosothiolactone. 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 over-producing 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_{2}O_{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...
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 ΔoxyR strains were grown aerobically or anaerobically in LB to an A_{600} of 0.3 and exposed to 3 mM H_{2}O_{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') 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_{2}O_{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 Ndel 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.

**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_{2}O_{2}-treated cells grown under aerobic or fermentative conditions (Fig. 1).

To investigate whether expression of hcp is induced by oxidative stress in an 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 (ATAGN₇CTATN₇ATAGN₇CTAT) (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
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 H2O2 in liquid cultures grown aerobically in LB followed by transfer of aliquots onto solid medium. The results (Fig. 2) showed that Δhcp exhibits lower viability (around 2 orders of magnitude) after treatment with H2O2 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 Δhcp 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 Δhcp 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 enzymic peroxidase activity determination, ascorbate, guaiacol, ABTS, NADH, and NADPH were used as substrates. Only with ascorbate was a measurable activity observed, with a Vmax of 0.2 μmol H2O2 min⁻¹ mg⁻¹ and a Km value for H2O2 of 0.3 mM for E. coli HCP, a Vmax of 0.04 μmol H2O2 min⁻¹ mg⁻¹ and a Km for H2O2 of 0.3 mM for D. desulfuricans HCP, at pH 7, and with Km for H2O2 calculated as described under “Experimental Procedures.” Although the Vmax values are much lower than those reported for heme-containing peroxidases, the Km values for H2O2 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 kcat of 9.9 min⁻¹ and 2.2 min⁻¹ and kcat/Km of 3.7 × 10⁴ M⁻¹ min⁻¹ and 7.9 × 10³ 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
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 H2O2, which was subsequently reduced; and enzyme reduced with sodium dithionite followed by treatment with hydrogen peroxide. The spectra revealed that H2O2 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 Δhcp 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 H2O2 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. 6a). 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. 6b), 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 completely discounted. The requirement for a reductant also explains why no reactivity is observed with...
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 obtained values 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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