A Hemerythrin-like Domain in a Bacterial Chemotaxis Protein†

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ABSTRACT: Hemerythrin (Hr) is an O_2-carrying protein found in some marine invertebrates. A conserved sequence motif in all Hrs provides five histidine and two carboxylate ligands to an oxo-/hydroxo-bridged diiron active site, as well as a hydrophobic O_2 binding pocket. Database searches located a previously unrecognized Hr-like sequence motif at the 3′ end of the gene, *dcrH*, from the anaerobic sulfate-reducing bacterium, *Desulfovibrio (D.) vulgaris* (Hildenborough). This gene encodes a putative methyl-accepting chemotaxis protein, DcrH. We have established by immunoblotting that a full-length DcrH, including the Hr-like domain, is expressed in *D. vulgaris* (Hildenborough). The C-terminal domain of DcrH, when expressed separately in recombinant form in *Escherichia coli*, was found to fold into a stable protein, DcrH-Hr. The UV−vis absorption and resonance Raman spectra of DcrH-Hr, and of its azide adduct, provide clear evidence for an oxo-bridged diiron(III) site very similar to that found in Hr. Based on UV−vis absorption spectra, exposure of the reduced (colorless, presumably diferrous) DcrH-Hr to air resulted in formation of an O_2 adduct also very similar to that of Hr. Unlike that of Hr, the O_2 adduct of DcrH-Hr autoxidized within a few minutes at room temperature. The O_2 binding pocket of DcrH-Hr appears to be larger than that of Hr. Given the air-sensitive nature of *D. vulgaris* and the putative chemotactic function of DcrH, one possible role for the Hr-like domain of DcrH is O_2-sensing. DcrH-Hr is the first characterized example of a Hr-like protein from any microorganism.

Up to now the non-heme iron O_2-carrying proteins hemerythrin and myohemerythrin [hereafter referred to generically as hemerythrin (Hr)[1]] have been found only in a few phyla of marine invertebrates. A set of characteristic features defining Hr includes a four-helix bundle protein fold surrounding an oxo-/hydroxo-bridged diiron site, as shown in Figure 1 (*1*). Fe2, shown in Figure 1, reversibly binds O_2, as well as small anions such as azide, in a bent, end-on fashion. A conserved amino acid sequence motif in Hr (shown in Figure 2) furnishes five terminal histidine and two bridging carboxylate ligands to the diiron site. This sequence motif is distinct from that which furnishes ligands to the diiron sites in microbial O_2-activating enzymes, such as methane monooxygenase and aerobic ribonucleotide reductase (*2*).

Hr has not, heretofore, been reported in microorganisms. However, during searches of protein and nucleotide sequence databases, we discovered a previously unrecognized Hr-like sequence motif at the C-terminus of the putative protein, DcrH, from the anaerobic, sulfate-reducing bacterium *Desulfovibrio (D.) vulgaris* (Hildenborough) (*5*). An alignment of the C-terminal end of the DcrH amino acid sequence with that of Hr is shown in Figure 2. The 959-residue DcrH is the hypothetical product of the *dcrH* gene, 1 of at least 12 *Desulfovibrio c*hemoreceptor (*dcr)* genes identified by Voordouw and co-workers in the genome of *D. vulgaris* (Hildenborough) (*11*). The chemoreceptor attribution was based primarily on the presence of "excitation" and "methylation" amino acid sequence motifs that are homolo-

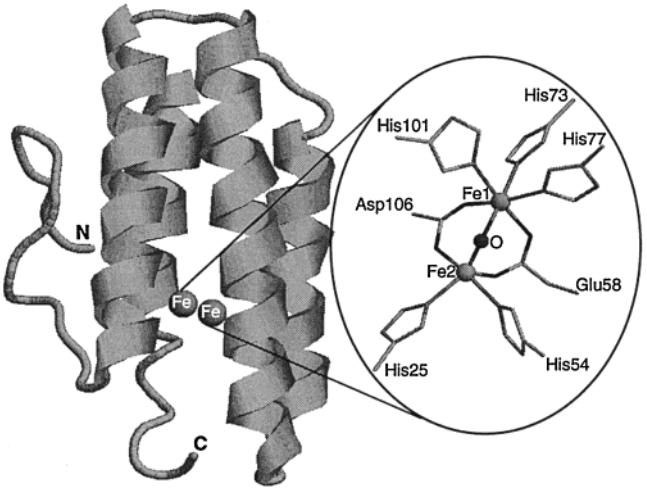

FIGURE 1: Ribbon diagram of the four-helix bundle structure of the *T. dyscritum* metHr subunit and a wireframe representation of its constituent diiron site (*3*). The drawings were generated using RASMOL (*4*) and coordinates from 2HMQ in the Protein Databank.

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[1] Abbreviations: Hr, hemerythrin; myoHr, myohemerythrin; PCR, polymerase chain reaction; DcrH, *Desulfovibrio* chemoreceptor protein H; DcrH-Hr, Hr-like domain of DcrH; CDcrH, putative cytoplasmic domain of DcrH; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetate; Tricine, *N*-tris(hydroxymethyl)methylglycine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LB/amp, Luria−Bertani medium containing 100 μg/mL ampicillin; Bis-Tris-HCl, [bis(2-hydroxyethyl)amino]tris-(hydroxymethyl)methane hydrochloride; MWCO, molecular weight cutoff; ORF, open reading frame.

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824 G..DADVLVKWSEDLANLP......S.IDTQHKRLVDYINDLYRAARRRD 864
    |    |  | |  |    |       | || :|| |  : | ::  |   |
  1 GFPIPDPYV.W..D....PSFRTFYSIIDDEHKTL..F.NGIFHLA..ID 38
              W                  D  H  L
                .          .          .          .
865 MDKAREVFDALKNYAVEHFGYEERLF.A.DYA.YPEATRH.KEIHRRFV 909
     | |  . : |:    .||  |: |  |  |  |  |    | || |  |:
 39 .DNADNLGE.LRRCTGKHFLNEQVLMQASQYQFYDE...HKKE.HEGFI 81
                   HF  E                 H    H  F
                .          .          .          .
910 ETVL.KWEKQLAAGDPEVVMTTLRGLVDWLVNHIMKE.DKKYEAYLRERGVS 959
      |  | |    ||   |    :    ||||||  |  | ||   :
 82 H.ALDNW.K....GD...V....KWAKSWLVNHI.KTIDFKYKGKI..... 113
                              WL  HI    D  Y
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FIGURE 2: Amino acid sequence alignment using the GAP algorithm (*6*) of *D. vulgaris* (Hildenborough) DcrH residues 824−959 [Genbank accession number AAB50497, (*5*) (upper)] with *P. gouldii* Hr [accession number P02244, (*7*−*9*) (lower)]. Symbols between the sequences indicate identical (|) and similar (:) residues, respectively. Letters below the Hr sequence indicate those residues common to both sequences *and* which are also conserved in the 10 known Hr sequences (*1, 10*). Boldface letters indicate residues furnishing iron ligands in Hr. Letters in shaded background indicate Hr residues known to reside in helical regions (*1*).

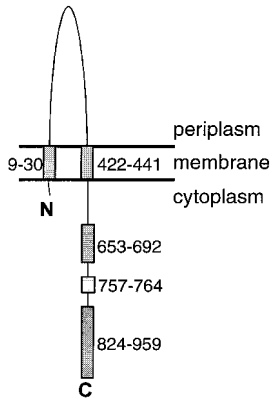

FIGURE 3: Schematic diagram of membrane topology proposed for *D. vulgaris* (Hildenborough) DcrH based on hydrophathy analysis and sequence homologies to other bacterial chemoreceptors (*5*). Shaded boxes indicate putative membrane-spanning (residues 9−30 and 422−431), excitation (653−692), and methylation (757−764) regions. The C-terminal box (824−959) indicates the Hr-like region.

gous to proteins of known methyl-accepting chemotaxis proteins in enteric bacteria. Thus, the *dcr* gene family was proposed to comprise a set of inner-membrane-spanning proteins used by *D. vulgaris* to sense and respond to specific molecules in or physical states of its environment. These chemotaxis proteins typically contain a periplasmic N-terminal sensing domain, a C-terminal cytoplasmic transmitting domain, and a trans-membrane domain linking the former two. Transduction from the sensing to transmitting domains triggers a phosphorylation/methylation cascade which results ultimately in a change in frequency of flagellar motor reversal, causing the bacteria to swim either up or down a concentration gradient of the environmental stimulus (*12, 13*).

DcrA is currently the only characterized member of the *D. vulgaris* chemoreceptor family. Its membrane-spanning topology has been established (*14*), and the protein has been shown to contain a heme group (*15*). The heme group was proposed to be a *c*-type and to be covalently attached to the periplasmic domain at a CXXCH sequence motif. DcrA was, therefore, proposed to function as an O_2 or redox sensor, a presumably essential function since *D. vulgaris* cannot survive prolonged exposure to air. A *dcrA*-deletion mutant of *D. vulgaris* was shown to be more aerotolerant than was the wild-type, although no difference in anaerotaxis (i.e., the tendency to swim away from a liquid−air interface) compared to the wild-type was noted (*16*).

The previously proposed membrane topology of DcrH, shown in Figure 3 (*5*), would place the putative Hr-like domain on the cytoplasmic side of the inner membrane. However, no reports of detection, isolation, or expression of the putative protein product of the *D. vulgaris dcrH* gene have appeared up to now. No amino acid sequence homology between DcrH and the periplasmic sensing domain of DcrA was noted previously (*5*), and our own database searches (*17, 18*) have not found significant homologies between the putative periplasmic domain of DcrH and any other proteins of known function. In this paper, we show that DcrH is indeed expressed in *D. vulgaris*, and that the C-terminal domain of DcrH has properties characteristic of Hr.

EXPERIMENTAL PROCEDURES

Materials and General Methods. General molecular biology procedures followed those described in *Current Protocols in Molecular Biology* (*19*). Restriction enzymes were obtained from either Boehringer Mannheim or Promega, Inc. PCR products were purified using Wizard PCR purification kits (Promega, Inc.). Nucleotide sequences were determined at the University of Georgia Molecular Genetics Instrumentation Facility. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Growth conditions for *D. vulgaris* (Hildenborough) and for isolation of its genomic DNA followed previously described procedures (*20*). *P. gouldii* myoHr isoform I was obtained as previously described (*21, 22*).

Cloning and Overexpression of the dcrH Hr Domain. The *D. vulgaris* genomic DNA was subjected to restriction digestion by *Hin*dIII, *Nde*I, and *Xba*I. This restriction-digested DNA was used as template for PCR amplification. Nucleotide sequences of the PCR primers were as follows: DVHR5, 5′-ACTGGCCcatatgGGTGACGC-3′; DVHR3, 5′-CGggatccTCAGGAGACT-CCGCGTTC-3′. These two primers have *Nde*I and *Bam*HI restriction sites, respectively, indicated in lower case letters. The sequences following the start and stop codons (underlined) in DVHR5 and DVHR3, respectively, duplicate the 5′ and complementary 3′ ends of the *dcrH* nucleotide sequence encoding the putative Hr-like domain of *dcrH* (nucleotides 2769−3179 in GenBank accession number U30319) (*5*). The PCR was carried out with PCR buffer and *Pfu* DNA polymerase, both from Stratagene, Inc., and using Stratagene's recommended mixtures and amounts. The PCR thermocycling parameters used were the following: denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2 min, for a total of 30 cycles in a Thermolyne, Inc., thermocycler. The PCR product was purified and ligated into plasmid pT7-7 (*23*) after *Nde*I/*Bam*HI double digestion of the PCR product/pT7-7 mixture. The ligation mixture was transformed into *E. coli* JM109. One plasmid from this transformation containing an *Nde*I/*Bam*HI insert of the appropriate length was purified using a tip-100 column (Qiagen, Inc.). Nucleotide sequences of both forward and reverse strands of the insert in this plasmid, pDVHR, were obtained. The nucleotide sequence determined for the insert was identical to that of nucleotides 2769−3179 published for *dcrH* (*5*) with the exception of nucleotides 2902−2905, which were found to have the sequence 5′-CGAG-3′ rather

than the reported 5′-GCAC-3′. The nucleotide sequence encoding the amino acid sequence of DcrH-Hr listed in Figure 2 has been deposited in the GenBank database, accession number AF210632 (*24*). Plasmid pDVHR was transformed into *E. coli* BL21(DE3) (*25*). Using analogous procedures, the *dcrH* Hr-domain PCR product was ligated into the *Nde*I/*Bam*HI sites of the vector pET-16b (Novagen, Inc.), which adds an extra 20 residues, including a 10×His-Tag, to the N-terminus of the overexpressed protein. The resulting plasmid, pETDVHR, was purified and transformed into *E. coli* BL21(DE3).

Induction of DcrH-Hr overexpression was accomplished as follows. *E. coli* BL21(DE3)[pDVHR] was inoculated into several 50 mL batches of LB/amp and grown overnight in a 37 °C/250 rpm incubator/shaker. These 50 mL cultures were then used to inoculate 1 L batches of LB/amp, and incubation was continued at the same temperature and shaker speed. When the 1 L cultures reached an OD_{600} of ∼1.0 (ca. 2 h), isopropyl-*β*-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. Incubation was continued at 37 °C/250 rpm for ∼3 h until the OD_{600} reached ∼2.1. The cells were harvested by centrifugation at 5000*g* for 5 min. Tricine SDS−PAGE analysis (12% gels) (*26*) using Bio-Rad molecular mass standards showed that both supernatant and pellet from the lysed cells contained an overexpressed protein running at a molecular mass of ∼16 kDa, as expected for DcrH-Hr. Upon induction of *E. coli* BL21(DE3)[pETDcrH-Hr], as described for DcrH-Hr, a protein of ∼18 kDa appeared to be overexpressed from SDS−PAGE analysis, as expected for the His-tagged dcrH-Hr.

Purification of and Iron Incorporation into DcrH-Hr. *E. coli* cells were lysed by sonication in an ice−water bath. Centrifugations and concentrations/redilutions were carried out at 4 °C, the latter in Amicon cells under argon pressure using YM-3 (3500 MWCO) membranes. All other steps were carried out at room temperature. Fifty millimolar Tris + 200 mM KCl (pH 8.0) was used as the buffer throughout. The cells (25−30 g) from six combined l L cultures of induced *E. coli* BL21(DE3)[pDVHR] were resuspended in 200 mL of buffer and lysed. After centrifugation of the lysed-cell suspension at 30000*g* for 30 min, the pellet was resuspended by gentle stirring into 20 mL of 6 M guanidine hydrochloride in buffer under an argon atmosphere. The suspension was stirred for 1 h, and then centrifuged at 30000*g* for 30 min. The supernatant was transferred to a 500 mL Schlenk-type flask. The flask was connected to a vacuum manifold and subjected to three evacuation/argon-flushing cycles. One hundred microliters of 2-mercaptoethanol was added to the flask via a gastight syringe, and the mixture was stirred for 15 min. One milliliter of 0.14 M ferrous ammonium sulfate solution (freshly prepared in buffer under argon) was then added via a gastight syringe into the mixture with stirring. The mixture was allowed to equilibrate for 15−30 min, and anaerobic buffer was then added dropwise through a pressure-equalizing dropping funnel with magnetic stirring. This dropwise addition was carried out over the course of several hours to overnight in multiple preparations to reach a final volume of 200−250 mL. The resulting suspension was centrifuged for 30 min at 30000*g*. The clear supernatant, which at this stage was usually yellow−green, was concentrated to a volume of 5 mL. Excess guanidine hydrochloride was removed by repeated concentrations/dilutions. Final purification was achieved by gel filtration on a Superdex-75 16/50 FPLC column (Pharmacia LKB) equilibrated with buffer and elution at a flow rate of 0.5 mL/min. At this stage, the DcrH-Hr was homogeneous as analyzed by Tricine SDS−PAGE. The yield of DcrH-Hr purified in this fashion was ∼15 mg/L of *E. coli* culture using an extinction coefficient at 322 nm of 6500 M^{-1} cm^{-1} (vide infra).

The 10×His-Tagged DcrH-Hr was purified as follows. Combined cells from four 1 L cultures of induced *E. coli* BL21(DE3)[pETDVHR] were resuspended in 100 mL of binding buffer [5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9)], lysed by sonication, and centrifuged at 30000*g* for 30 min, yielding a yellow supernatant. The supernatant was concentrated to 20 mL, and then passed over a 10 mL column containing approximately 2.5 mL of Ni-chelated His·Bind resin (Novagen, Inc.). After the column was washed with 15 mL of washing buffer [60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9)], the His-tagged DcrH-Hr was eluted as a yellow−green band with 5 mL of elution buffer [1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9)]. The eluate was subjected to several concentrations/redilutions with imidazole-free buffer [50 mM Tris, 200 mM KCl (pH 8.0)]. The His-tagged DcrH-Hr was homogeneous, as analyzed by Tricine SDS−PAGE. The yield of purified His-tagged DcrH-Hr was estimated to be 10 mg/L of culture.

Cloning and Overexpression of the Entire DcrH Protein and of the Putative Cytoplasmic Domain. The region of *dcrH* encoding the putative cytoplasmic domain of DcrH was PCR-amplified using the restriction-digested *D. vulgaris* (Hildenborough) genomic DNA as template and primers CDcrH5 (5′-CGCATCcatatgCGGCTGGTGG-3′, with the *Nde*I restriction site indicated in lower case letters and the start codon underlined) and DVHR3 (vide supra). The PCR thermal cycling conditions used were a "hot-start" at 97 °C for 5 min followed by 30 cycles, each consisting of 97 °C for 1 min, 67 °C for 1 min, 72 °C for 3 min. The ∼1.6 kb PCR product was excised from a 1.2% agarose gel and purified. After *Nde*I/*Bam*HI double digestion, the purified PCR product was ligated into both pET-16b and pT7-7. The resulting plasmids, pETCDcrH and pCDcrH, respectively, were verified to contain nucleotide sequences duplicating that encoding the putative cytoplasmic domain of DcrH (cf. Figure 3), which we named CDcrH (consisting of residues 442−959 in GenBank sequence U30319 except for the differences noted above for pDVHR). These plasmids were transformed into *E. coli* BL21(DE3).

The *dcrH* gene, encoding the entire DcrH protein (residues 1−959), was also cloned from *D. vulgaris* (Hildenborough) genomic DNA using primer-pairs DCRH5 and DVHR3. The nucleotide sequence of DCRH5 is 5′-GAGGAACcatatgA-CACTCAAGCGCAA-3′ with an *Nde*I restriction site indicated by lower case letters and the start codon by an underline. PCR thermal cycling conditions were a "hot-star" at 97 °C for 5 min followed by 30 cycles, each consisting of 97 °C for 1 min, 66 °C for 1 min, and 72 °C for 6 min. The 3.0 kb PCR product was isolated, purified, and ligated into pET-16b as described for pETCDcrH. The resulting plasmid, pETDcrH, was transformed into *E. coli* BL21(DE3). Overexpression and purification of both His-tagged CDcrH from pETCDcrH and His-tagged DcrH from pETDcrH were conducted as described above for His-tagged DcrH-Hr.

Preparation of Polyclonal Antibodies against DcrH-Hr and Western Blotting. Antisera were raised in rabbits against

purified DcrH-Hr at the Animal Resource Facility of the University of Georgia. A total of 600 μg of purified DcrH-Hr in 1 mL of 20 mM potassium phosphate (pH 7.0) was used. A first boost injection was given to the rabbits on day 21 after the initial injection of the DcrH-Hr (day 1). Three subsequent boost injections were given at 3 or 4 week intervals. Serum (120 mL from two rabbits) obtained 10 days after the fourth injection was collected. The antisera were precipitated by 50% ammonium sulfate, and centrifuged at 30000*g* for 30 min. The pellet was redissolved in 50 mM Tris and 200 mM KCl (pH 8.0), and passed through a 5 mL Sephadex G-25 column. The eluted antisera (5 mL) were transferred to dialysis tubing (3500 MWCO) and dialyzed against 2 L of buffer [50 mM Tris and 200 mM KCl (pH 8.0)] for >48 h at 4 °C. The dialyzed antisera were centrifuged at 30000*g* for 15 min and divided into 1 mL aliquots, which were stored at −20 °C.

The immunoblotting and immunodetection of DcrH from *D. vulgaris* and of recombinant DcrH proteins from *E. coli* followed standard procedures (*27*). Tricine SDS−PAGE (10% gel) was carried out on DcrH protein samples or *D. vulgaris* cell extracts. The electrophoresed samples/extracts were transferred from the gel to a nylon membrane using a tank transfer system (both from Bio-Rad, Inc.). Upon completion of the transfer procedure, the membrane was incubated at room temperature for 30 min in a minimal volume (10 mL) of blocking buffer [100 mM Tris, 0.9% NaCl (pH 7.5), plus 10% w/v nonfat dry milk]. The primary antibody (anti-DcrH-Hr) was added to a 1:1000 dilution, and the membrane was incubated for 1 h at room temperature with gentle agitation. The membrane was then washed several times with TBS buffer [100 mM Tris and 0.9% NaCl (pH 7.5)], and incubated for 1 h at room temperature with a secondary antibody alkaline phosphatase−anti-(rabbit-IgG) (Boehringer Mannheim, Inc.) in blocking buffer. The membrane was washed again with TBS buffer (3 × 15 mL) and stained with chromogenic visualization solution (BCIP/NBT) (Boehringer Mannheim, Inc.).

Characterization of DcrH-Hr. Unless otherwise noted, DcrH-Hr solutions were in 50 mM Tris and 200 mM KCl (pH 8.0), and all procedures were carried out at room temperature (~24 °C). The molecular weight of DcrH-Hr in solution was determined by passage over a Superdex 10/30 column (PharmaciaLKB) equilibrated with buffer after calibration with carbonic anhydrase (M_r 29 000), cytochrome *c* (M_r 12 400), and aprotinin (M_r 65 000). The molecular weight of DcrH-Hr in water-diluted samples (0.15 μM) was also determined by electrospray ionization mass spectrometry using a Sciex API-1 quadrupole mass spectrometer in the Mass Spectrometry Facility at the University of Georgia. The DcrH-Hr sample was diluted to 48:48:4 vol % acetonitrile/water/acetic acid before introduction into the mass spectrometer via loop injection. Protein concentrations were estimated with a Protein Assay kit (Bio-Rad, Inc.) using bovine gamma globulin and bovine serum albumin as standards. The as-isolated DcrH-Hr was reduced under an argon atmosphere by addition of a 5-fold molar excess of sodium dithionite at room temperature. The solution gradually became colorless over the course of several hours. The excess sodium dithionite was removed by repeated anaerobic dialyses against dithionite-free buffer in an anaerobic Coy chamber. Reaction of the reduced DcrH with O_2 was achieved by manually bubbling air through the solution of the reduced protein. Azide binding to as-isolated DcrH-Hr was achieved by addition of sodium azide to a final concentration of 50 mM. For phenol binding to as-isolated DcrH-Hr, a drop of water-saturated phenol was added to an Eppendorf tube containing 1 mL of DcrH-Hr, and the tube was centrifuged to remove any precipitation. Catalase activity of DcrH-Hr was assayed as described by Lück (*28*) in 50 mM phosphate (pH 7.0). Phosphatase activity was assayed using *p*-nitrophenyl phosphate as substrate. The assay solution contained 15 mM *p*-nitrophenyl phosphate, 50 mM Bis-Tris-HCl (pH 6.0), 10 mM sodium ascorbate, 20 μM ferrous ammonium sulfate (*29*). To start the reaction, DcrH-Hr was added from a stock solution, such that its concentration in the assay mixture was in the micromolar range, and, after mixing, the absorbance change at 410 nm was monitored continuously for 5−60 min. Superoxide dismutase (SOD) activity of micromolar concentrations of DcrH-Hr was assayed using a standard method (*30*).

Spectrometers and Spectral Conditions. All absorbance readings and UV−vis absorption spectra were obtained in 1 cm path length semi-micro quartz cuvettes on a Shimadzu UV/2101PC spectrophotometer. Resonance Raman spectra were obtained at the Oregon Graduate Institute of Science & Technology on 1−2 mM samples of DcrH-Hr and its azide adduct. Spectra were collected on a McPherson 2061 spectrograph with an 1800-groove grating and a Princeton Instruments liquid N_2 cooled (LN100PB) CCD detector. Rayleigh scattering was attenuated with a Kaiser Optical holographic super-notch filter. The excitation source was provided by a Coherent Innova 90-6 Ar laser operating at 488.0 nm with incident power at the sample ~20 mW. Spectra were collected in a 90°-scattering geometry from solution samples contained in glass capillary tubes in contact with a copper-finger immersed in an ice−water bath.

RESULTS

The PCR products resulting from our amplification of the *D. vulgaris* (Hildenborough) *dcrH* gene contained differences from the published nucleotide sequence at three positions (*5*). These base changes resulted in changes at two adjacent amino acid residues in the translated DcrH sequence; namely, residues 868 and 869 of the sequence in Genbank accession number AAB50497 were changed from G and T to A and R, respectively. These changes have been incorporated into the sequence listed in Figure 2, which then represents the amino acid sequence of the overexpressed protein, DcrH-Hr, resulting from our PCR amplification of *dcrH*. These changes do not appear to be the result of errors during PCR amplification because exactly the same changes were found in nucleotide sequences of products from two independent PCR amplifications, one of the Hr-like domain and the other of the putative cytoplasmic domain of *dcrH*. Both PCR amplifications used *D. vulgaris* (Hildenborough) genomic DNA as template, but different N-terminal primers.

Overexpressed DcrH-Hr was found almost totally in the lysed-cell pellet of *E. coli* BL21(DE3)[pDVHR]. This crude DcrH-Hr contained little or no iron, a situation we have encountered previously when overexpressing other small heterologous diiron proteins in *E. coli* (*31*). Following a procedure previously developed in our laboratory (*31*), the

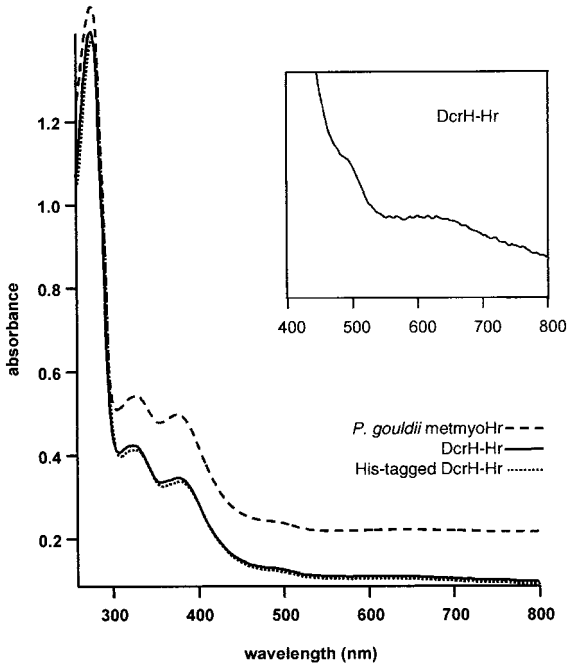

FIGURE 4: UV−vis absorption spectra of as-isolated DcrH-Hr, His-tagged DcrH-Hr, and *P. gouldii* metmyoHr. All proteins are in 50 mM Tris-HCl, 200 mM KCl (pH 8.0). The metmyoHr absorption spectrum is offset vertically for clarity. Inset: expanded spectrum showing lower intensity absorption features of DcrH-Hr.

pelleted DcrH-Hr was solubilized with guanidine hydrochloride, and constituted with iron. Somewhat smaller quantities of the "His-tagged" DcrH could be purified from the soluble portion of the lysed *E. coli* BL21(DE3)[pETDVHR] cells by metal-chelate affinity chromatography. This purified His-tagged DcrH-Hr ran as a single band at a molecular weight of ∼18 000 on Tricine SDS−PAGE, compared to a calculated molecular weight of 18 651 for the DcrH-Hr sequence listed in Figure 2 plus the 21-residue N-terminal His-tag sequence: GHHHHHHHHHHSSGHIEGRHM. The calculated molecular weight of 16 130 based on the DcrH-Hr sequence listed in Figure 2 (i.e., without an N-terminal Met residue) agreed exactly with the molecular weight of the non-His-tagged DcrH-Hr determined by electrospray ionization mass spectrometry (data not shown). Another peak at 16 245 of low intensity in the mass spectrum is at a mass consistent with the incorporation of two irons. The DcrH-Hr sample used for mass spectrometry presumably lost most of its iron either in the solvent (48:48:4 vol % acetonitrile/water/acetic acid) used for mass spectrometry or during electrospray ionization. Calibrated gel filtration chromatography of DcrH-Hr gave M_r ∼16 000 (data not shown). Thus, DcrH-Hr is a monomer in 50 mM Tris and 200 mM KCl (pH 8.0). DcrH-Hr tested negative for phosphatase, catalase, and SOD activities. These negative results are not surprising, since Hr and myoHr show none of these activities (Kurtz, D. M., Farmer, C. S., and Zhong, J., unpublished results).

As shown in Figure 4, the resolubilized, iron-constituted DcrH-Hr and the "His-tagged" DcrH-Hr have essentially identical UV−vis absorption spectra. Furthermore, these spectra closely resemble that of the met [i.e., diiron(III) oxidation state] form of myoHr from *P. gouldii* (*21*). The absorption spectrum of as-purified DcrH-Hr shows maxima at 322 nm ($\epsilon \sim 6500$ M^{-1} cm^{-1}) and 380 nm ($\epsilon \sim 4700$ M^{-1} cm^{-1}) and weaker features at ∼480 and ∼620 nm

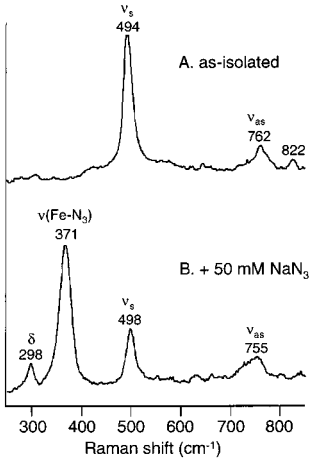

FIGURE 5: Resonance Raman spectra of 1−2 mM DcrH-Hr (A) as-isolated and (B) as-isolated + 50 mM NaN_3. Laser excitation wavelength was 488 nm. Samples were in 50 mM Tris and 200 mM KCl (pH 8.0) and were kept at ice temperature. Peaks are labeled according to their frequencies and their assigned Fe−O−Fe vibrational modes, except for the Fe−N_3 peak in (B).

(Figure 4, inset). The extinction coefficients were calculated from the estimated protein concentrations and absorbances, and are very similar to those of analogous absorption bands of metmyoHr. This pattern of spectral features and absorption intensities, including the minor, lower-energy features, is characteristic of ligand-to-metal-charge-transfer transitions observed for the $Fe^{III}-O^{2-}-Fe^{III}$ unit in other proteins and in synthetic complexes with nitrogen and oxygen ligands (*32*, *33*). The near-coincidence with the metmyoHr absorption spectrum in Figure 4 shows that both DcrH-Hr and the His-tagged DcrH-Hr contain essentially their full expected complement of diiron sites, i.e., one diiron site per protein molecule. The spectrum of the His-tagged DcrH-Hr, which was purified from the soluble fraction of the *E. coli* cell lysate by a one-column procedure, proves that an oxo-bridged diiron site can be incorporated into DcrH-Hr in vivo, and that the 20-residue His-tag has no influence on the absorption spectrum. All of the remaining results discussed below were obtained on DcrH-Hr that had been solubilized from the pellet of *E. coli* BL21(DE3)[pDVHR], i.e., the protein without the His-tag.

The structure of the diiron(III) site in DcrH-Hr was further probed by resonance Raman spectroscopy. This technique selectively enhances the intensities of those Raman-active vibrational modes that are coupled to an absorbing chromophore, and has been used extensively as a selective probe of the oxo-bridged diiron(III) unit in proteins (*32*). As-isolated DcrH-Hr gives the resonance Raman spectrum shown in Figure 5. The two major peaks have frequencies and relative intensities closely resembling those assigned to symmetric and asymmetric Fe−O−Fe vibrational modes of metHr, which is known to have a dicarboxylate-bridged $Fe^{III}-O^{2-}-Fe^{III}$ unit (cf. Figure 1). Table 1 compares assignments of the resonance Raman vibrational frequencies for the oxo-bridged diiron(III) sites of DcrH-Hr and various forms of Hr. The $Fe^{III}-O^{2-}-Fe^{III}$ vibrational frequencies are known to be highly sensitive to the Fe−O−Fe angle (*32*, *34*). The close correspondence of the Raman frequencies of the DcrH-Hr and metHr diiron sites provides strong evidence for additional carboxylate bridges, which close the Fe−O−Fe angle compared to oxo-bridged

Table 1: Resonance Raman Vibrational Frequencies for *D. vulgaris* (Hildenborough DcrH-Hr and *P. gouldii* Hr

protein	ν_s(Fe−O−Fe)	ν_{as}(Fe−O−Fe)	δ(Fe−O−Fe)	ν(Fe−N_3)
DcrH-Hr[a]				
as-isolated	494	762	no[c]	–
as-isolated + 50 mM N_3^-	498	755	298	371
P. gouldii Hr[b]				
met	510	∼750	n.o.[c]	–
met(N_3)	507	768	292	375
met(OH)-*trans*	506	780	n.o.[c]	
met(OH)-*cis*	492	n.o.[c]	n.o.[c]	–
oxyHr	486	∼753	n.o.[c]	–

[a] From this work. [b] From Shiemke et al. (*35*). [c] Not observed.

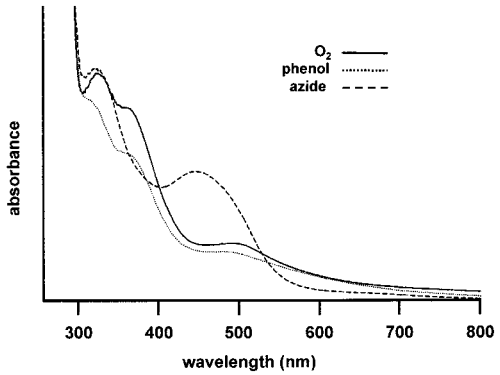

FIGURE 6: UV−vis absorption spectra of the O_2 adduct of reduced DcrH-Hr, and azide and phenol adducts of as-isolated DcrH-Hr in 50 mM Tris, 200 mM KCl (pH 8.0).

diiron(III) complexes with no supporting bridges. Similarly, the resonance Raman spectrum of the azide adduct of DcrH-Hr closely resembles that of the azide adduct of metHr, even showing the Fe−O−Fe bending mode at 298 cm^{-1}. From correlations of the symmetric and asymmetric Fe−O−Fe stretching frequencies with compounds of known structure, the frequencies for DcrH-Hr and its azide adduct indicate an Fe−O−Fe angle of ∼130° (*32*, *34*). The Fe−O−Fe frequencies of DcrH-Hr and its azide adduct are somewhat closer to those of oxyHr and met(OH)-*cis*Hr than to that of metHr. The oxo bridges in both oxy- and met(OH)-*cis*Hrs are hydrogen-bonded, whereas that of metHr is not (*35*). However, we found no D_2O effects on the Raman frequencies for DcrH-Hr.

Figure 6 shows the UV−vis absorption spectra of the azide and phenol adducts of as-purified DcrH-Hr and also the O_2 adduct of reduced DcrH-Hr. As-purified DcrH-Hr can be reduced by sodium dithionite under anaerobic conditions to a colorless, presumably diferrous form. Bubbling of air through a dithionite-free solution of reduced DcrH-Hr resulted in changes from colorless to transient pink−rose to yellow within approximately 1 min, indicating formation of an O_2 adduct followed by rapid autoxidation. The absorption spectrum shown in Figure 6, attributed to the O_2 adduct of DcrH-Hr, was obtained as quickly as possible after the procedure described above, and undoubtedly contains a significant proportion of the autoxidized [i.e., diiron(III)] spectrum. Nevertheless, when compared to spectra of oxyHr (*35*, *36*), the prominent feature at 500 nm in the DcrH-Hr spectrum ($\epsilon \sim 2200$ M^{-1} cm^{-1} per diiron center for oxyHr) is indicative of an O_2 adduct. The addition of excess azide to as-isolated DcrH-Hr gave a UV−vis absorption spectrum (cf. Figure 6) with distinct peaks at 450 nm ($\epsilon \sim 4000$ M^{-1} cm^{-1}) and ∼320 nm. These features are very similar to those of the azide adduct of metHr [broad absorption at 446 nm (3700 M^{-1} cm^{-1}) and peak at 326 nm (6800 M^{-1} cm^{-1}) (*35*, *36*)]. These absorption spectra are consistent with the resonance Raman spectra of DcrH-Hr plus excess azide discussed above, i.e., with the formation of an azide adduct. As judged spectrophotometrically, the room-temperature reaction between excess azide and DcrH-Hr is complete within several minutes while the corresponding reaction of excess azide with metHr or metmyoHr takes several hours (*37*). This observation, coupled with the relatively rapid autoxidation noted above, suggested to us that the exogenous ligand binding pocket in DcrH-Hr is somewhat larger and/or more flexible than that in Hr. To test this possibility, we added excess phenol to both DcrH-Hr and *P. gouldii* metmyoHr. We observed no change in the absorption spectrum of metmyoHr upon addition of phenol, whereas the DcrH-Hr absorption spectrum changed rapidly upon addition of phenol (cf. Figure 6), giving a visible absorption feature maximizing at 500 nm, which can be assigned to the ligand-to-metal charge-transfer transition of a phenolate−Fe^{III} adduct (*38*). The spectrum of the DcrhH-Hr + phenol solution also exhibits absorption maxima at ∼320 and ∼375 nm, indicating that the $Fe^{III}-O^{2-}-Fe^{III}$ unit is retained in the phenol adduct. This result supports the hypothesis that DcrH-Hr has a larger exogenous ligand binding pocket than does Hr.

The rapid autoxidation of reduced DcrH-Hr upon exposure to air may indicate that other domains of the DcrH protein are needed to stabilize the O_2 adduct. Therefore, the putative cytoplasmic domain of DcrH (CDcrH) was cloned and overexpressed in *E. coli*. The starting amino acid residue for CDcrH was chosen to be Arg442, based on the predicted membrane topology as shown in Figure 3. The purified recombinant His-tagged CDcrH ran at ∼60 kDa on SDS−PAGE, consistent with the calculated molecular mass from the predicted amino acid sequence plus the His-tag (59 458). However, the vast majority of this CDcrH was expressed as an inclusion body, and all our attempts to solubilize and incorporate iron into CDcrH were unsuccessful. A small amount of "His-tagged" CDcrH was purified from the supernatant of lysed BL21(DE3)[pETCdcrH] cells using metal-chelate affinity chromatography, but the amount was insufficient to carry out any structural, chemical, or spectroscopic characterizations.

The complete *dcrH* gene was also cloned into vector pET-16b and transformed into *E. coli* BL21(DE3). The overexpression of the His-tagged full-length DcrH in *E. coli* BL21(DE3)[pETDcrH] was relatively poor and essentially undetectable by Tricine SDS−PAGE of the whole cells. The supernatant upon cell lysis was passed over a Ni-chelate affinity column (Novagen, Inc.), and a colorless eluate in the presence of 1 M imidazole was collected. This eluate contained a minimal amount of "His-tagged" recombinant DcrH that could be detected by immunoblotting using antibody against DcrH-Hr (anti-DcrH-Hr) (Figure 7, lanes 1 and 2). The major band in lane 1 and the only band visible in lane 2 were assumed to have a molecular weight of 107 118, the calculated molecular weight of the recombinant His-tagged DcrH. This His-tagged protein was then used as a standard in Western-blot probing for DcrH in *D. vulgaris* (Hildenborough) whole cells. The Western-blot in Figure 7,

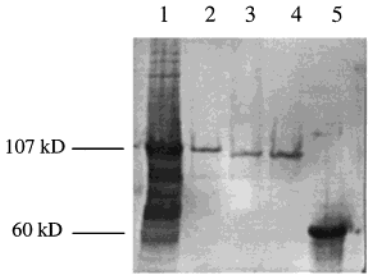

FIGURE 7: Western-blot probe of DcrH using anti-DcrH-Hr after blotting from a 10% Tricine SDS−PAGE gel. Lane 1, whole cells of *E. coli* BL21(DE3)[pETDcrH] after IPTG induction; lane 2, purified His-tagged DcrH from the induction in lane 1; lanes 3 and 4, whole cells from two independent cultures of *D. vulgaris* (Hildenborough); lane 5, whole cells of *E. coli* BL21(DE3)-[pETCDcrH].

lanes 3 and 4, revealed that a slightly smaller protein that binds anti-DcrH-Hr is indeed expressed in *D. vulgaris*. This result is consistent with the slightly smaller calculated molecular weight, 104 728, of full-length DcrH without a His-tag. For size comparison, the His-tagged CDcrH was run in lane 5 of the Western blot. No other protein band was reproducibly detected in Western blots of *D. vulgaris* (Hildenborough) cell extracts probed with anti-DcrH-Hr antibody.

DISCUSSION

The existence of the *Desulfovibrio* chemoreceptor protein, DcrH, had, heretofore, only been inferred from the sequence of a gene named *dcrH*. The immunoblotting experiments in Figure 7 using anti-DcrH-Hr antibody prove that a protein containing a DcrH-Hr-like domain is indeed expressed in *D. vulgaris* (Hildenborough), and that the size of this protein matches that inferred from the nucleotide sequence of the *dcrH* gene. We have further demonstrated that the C-terminal domain of DcrH can fold independently in vitro into a stable protein, DcrH-Hr. The UV−vis absorption and resonance Raman spectra of DcrH-Hr and its azide adduct clearly demonstrate the presence of an oxo-bridged diiron(III) site whose structure is very similar to that in Hr; e.g., the diiron site probably also contains bridging carboxylate ligands. The ligands to the diiron site in Hr are contributed from residues in each of the four helices (cf. Figures 1 and 2), thereby effectively cross-linking the protein and stabilizing the four-helix bundle (*39*). Given the conservation of all five of these ligand residues in DcrH (cf. Figure 2), the formation of a structurally very similar diiron site in DcrH-Hr constitutes strong evidence for a four-helix bundle structure in the C-terminal Hr-like domain of DcrH. A predominantly helical secondary structure is predicted for DcrH-Hr using various prediction programs.

An additional striking correlation is that all of the residues lining the O_2 binding pocket of Hr are conserved in the DcrH Hr-like domain. The O_2 binding pocket of Hr is diagrammed in Figure 8. All of the nonligand residues shown in this diagram have a side-chain atom within 4 Å of the bound O_2 (*1*), and a comparison with the amino acid sequence alignment in Figure 2 shows that all of these nonligand pocket residues are conserved in DcrH. The conservation of both iron−ligand and O_2-binding-pocket residues strongly suggests that the biological function of the diiron site in DcrH is similar to that in Hr, and most likely involves interaction

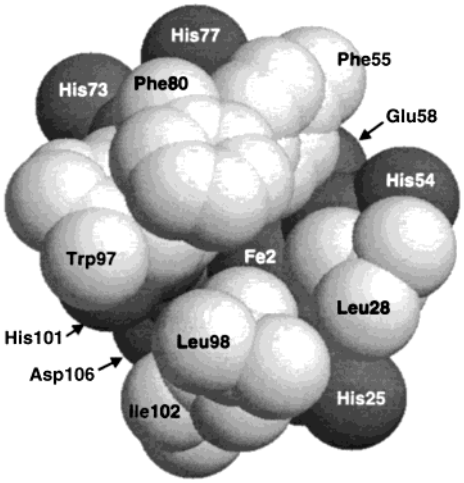

FIGURE 8: Space-filling depiction of residues lining the O_2 binding pocket in a subunit of *T. dyscritum* oxyHr viewed approximately parallel to the long axis of the four-helix bundle. The drawing was generated using RASMOL (*4*) and coordinates from 2HMO in the Protein Databank (*46*). Residues are labeled at their β-carbons. Iron−ligand residues are in darker shade, and residues lining the O_2 binding pocket are in lighter shade. Bound O_2 atoms would have obscured Fe2 and were omitted for clarity. The Fe1−Fe2 axis is oriented approximately diagonally from upper left to lower right.

with O_2. The dithionite-reduced, presumably diferrous DcrH-Hr formed an O_2 adduct upon exposure to air that was very reminiscent of oxyHr. However, unlike oxyHr, the DcrH-Hr O_2 adduct autoxidized within minutes at room temperature. No activity of DcrH-Hr with either superoxide or peroxide was observed, which is also the case for Hr. DcrH-Hr appears to have a larger or more flexible ligand-binding pocket than does Hr or myoHr. This inference is based upon its rapid autoxidation, which is presumably due to increased solvent access (*40*), its relatively rapid binding of azide, and its ability to form an Fe^{III}−phenol adduct, unlike Hr or myoHr. Since we were unsuccessful in obtaining either the full-length DcrH or its putative cytoplasmic domain in forms or quantities sufficient for detailed characterization, we could not investigate the possibility that the O_2 adduct of DcrH-Hr would be stabilized by additional interactions with other regions of the protein. However, the transient stability of the O_2 adduct of DcrH-Hr is not necessarily inconsistent with an O_2-sensing function. The very reducing intracellular environment of *D. vulgaris* is likely to provide a rereduction pathway for the autoxidized DcrH in vivo, and the rate of this rereduction could exceed that of reduction of O_2 by the same intracellular reducing components. Assuming that the autoxidation reaction of DcrH-Hr is analogous to the (much slower) autoxidation of Hr, hydrogen peroxide would be the product of O_2 reduction by DcrH (*40*). Thus, a rationale for the more open binding pocket in DcrH-Hr is that it facilitates rapid autoxidation, and that the O_2-sensing mechanism of DcrH involves production of hydrogen peroxide.

The structures of cytoplasmic domains of bacterial chemoreceptors have proven notoriously difficult to characterize, a phenomenon attributed to their highly dynamic nature (*13*). Very recently, the first X-ray crystal structure of a cytoplasmic domain of a bacterial methyl-accepting chemoreceptor protein was reported (*41*). This domain, from the *E. coli* serine chemoreceptor, crystallized as a supercoiled dimer of helical coiled-coils. Other lines of evidence suggest that this dimeric coiled-coil structure is the generally functional one

for cytoplasmic domains of bacterial methyl-accepting chemotaxis proteins (*13*). A distinct *intrasubunit* four-helix bundle, as would be expected for the Hr-like domain of DcrH, was not apparent in the crystal structure of the *E. coli* serine chemoreceptor cytoplasmic domain.

Given the air-sensitive nature of *D. vulgaris* and the putative chemotactic function of DcrH, an obvious candidate function for the DcrH Hr-like domain is O_2 sensing, such as in the anaerotaxis noted earlier (*16*). The sensor in DcrA, the other *Desulfovibrio* chemoreceptor so far characterized, is proposed to be a *c*-type heme attached to the periplasmic domain. *c*-type hemes are not known to bind O_2; it seems more likely that DcrA would instead sense redox potential changes (*15*). Both the proposed function of DcrA and its sensor location are, thus, distinct from those proposed for DcrH. Other bacterial chemoreceptor proteins that are known to be involved in O_2 sensing include FixL, whose sensing domain contains a *b*-type heme (*42*), Aer, and ArcB. The latter two proteins are proposed to sense O_2 indirectly by responding to intracellular redox potential changes (*43*). The redox sensor in Aer appears to be a bound flavin; the molecular sensor in ArcB is not known. The sensing domains in the latter three chemoreceptors all appear to be cytoplasmic (*43*). Thus, a cytoplasmic localization for the Hr-like domain of DcrH (cf. Figure 3) does not a priori rule out an O_2-sensing function. None of FixL, Aer, or ArcB has any apparent sequence homology to DcrH. Conversely, no other bacterial chemoreceptor protein has been reported to have a Hr-like domain.

In fact, DcrH-Hr is the first reported characterization of a Hr-like protein from any microorganism. Of the published microbial genome sequences, those of *Methanococcus (M.) jannaschii* (*44*), a methanogenic archaeon, and *Aquifex (A.) aeolicus* (*45*), a hydrogen-oxidizing bacteria, also have ORFs encoding Hr-like amino acid sequences (accession numbers AAB50497 and AAC07584, respectively). The proteins encoded by these latter ORFs have not yet been reported, but, unlike DcrH, their amino acid sequences appear to consist of essentially only Hr-like domains with no obvious membrane-associating motifs. Both *M. jannaschii* and *A. aeolicus* are hyperthermophiles; the former is classified as a strict anaerobe and the latter as a microaerophile (i.e., it is poisoned by air but can survive and respire under sub-atmospheric partial pressures of O_2). Therefore, O_2 sensing or, perhaps, detoxification are possible functions for these other putative Hr-like proteins. Our database searches failed to detect Hr-like ORFs in the genomes of the yeast *Saccharomyces cerevisiae* or the nematode *Caenorhabditis elegans* (or in any other eukaryotic nucleotide sequences deposited in the NCBI Genbank as of November, 1999). Thus, any evolutionary linkage between Hr-like proteins in microbes and marine invertebrates remains mysterious. Nevertheless, given the three very different microorganisms so far found to contain Hr-like ORFs, we suspect that additional occurrences of this motif await discovery.

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