Isolation, purification and characterization of hemerythrin from *Methylococcus capsulatus* (Bath)

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ABSTRACT

Earlier work from our laboratory has indicated that a hemerythrin-like protein was over-produced together with the particulate methane monooxygenase (pMMO) when *Methylococcus capsulatus* (Bath) was grown under high copper concentrations. A homologue of hemerythrin had not previously been found in any prokaryote. To confirm its identity as a hemerythrin, we have isolated and purified this protein by ion-exchange, gel-filtration and hydrophobic interaction chromatography, and characterized it by mass spectrometry, UV-visible, CD, EPR and resonance Raman spectroscopy. On the basis of biophysical and multiple sequence alignment analysis, the protein isolated from *M. capsulatus* (Bath) is in accord with hemerythrins previously reported from higher organisms. Determination of the Fe content in conjunction with molecular-weight estimation and mass analysis indicates that the native hemerythrin in *M. capsulatus* (Bath) is a monomer with molecular mass 14.8 kDa, in contrast to hemerythrins from other eukaryotic organisms, where they typically exist as a tetramer or higher oligomers.

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1. Introduction

Recently we reported a quantitative proteomic analysis of the metabolic regulation by copper ions in *Methylococcus capsulatus* (Bath) (*M. capsulatus* (Bath)) [1]. Copper ions switch the oxidation of methane by the soluble methane monooxygenase (sMMO) to the particulate methane monooxygenase (pMMO) in this organism. Toward understanding the change in cellular metabolism related to this transcriptional and metabolic switch, we have undertaken genome sequencing and quantitative comparative analysis of the proteome in *M. capsulatus* (Bath) grown under different copper-to-biomass ratios by the cleavable isotope coded affinity tag (cICAT) technology. Of the 682 proteins identified, the expressions of 60 proteins were stimulated by at least two fold by Cu ions; 68 proteins were down-regulated by two fold or more. The 60 proteins over-produced included the methane and carbohydrate metabolic enzymes, while the 68 proteins suppressed were mainly responsible for cellular signaling processes, indicating a role of copper ions

in the expression of the genes associated with the metabolism of the organism downstream of methane oxidation. The study has also provided a complete map of the C₁ metabolic pathways in this methanotroph and clarified the interrelationships between them.

As part of this study, we also uncovered a hemerythrin in *M. capsulatus* (Bath) (McHr), which, like pMMO, was also over-produced in the cells. At a Cu concentration of 30 μ M in the growth medium, the expression of the hemerythrin was stimulated four fold relative to its level when the cells were grown in the absence of added Cu ions. It is a non-heme iron protein with a diiron center rather than a heme as the prosthetic group. In terms of function, the hemerythrin in *M. capsulatus* (Bath) most likely functions as an oxygen carrier to bolster the effective concentration of dioxygen at the aqueous-membrane interface of the cytoplasmic membrane for efficient consumption by the pMMO, particularly at the high levels of pMMO produced at 30 μ M Cu.

At the time of discovery of hemerythrin, heme-related proteins, such as myoglobin and hemoglobin, were already well known heme-based dioxygen carriers, whose structures and mechanisms have been extensively studied. Since then, the structure and mechanism of eukaryotic hemerythrin have also been well studied and reviewed [2]. While hemerythrin is thought to be present only in marine invertebrates in most of the current literature, several

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bacterial hemerythrin genes have recently been implicated by genomic sequencing [3]. The bacterial hemerythrin-domain located in the bacterial chemotaxis protein *Desulfovibrio vulgaris* (DcrH) was first identified and sequenced in 1994 (Swiss-Prot Q46583). This domain was further characterized in 2000 [4]. While this work represented the first attempt to clone and examine the properties of a bacterial hemerythrin, strictly speaking, the latter was only a fused domain in a much larger protein. Until our quantitative proteomics study [1], no native or bona fide bacterial hemerythrin has been identified, isolated and characterized. Interestingly, the hemerythrin that we have identified in *M. capsulatus* (Bath) was expressed at high levels in the copper-enriched environment. Accordingly, we have decided to isolate and purify the McHr after completion of the proteomics study. In comparison with known primary sequences from the marine invertebrate *Themiste dyscritum* (peanut worm) (Fig. 1), the diiron center chelating residues were all conserved despite only just 29% overall sequence identity. Since our preliminary account of the discovery of a bacterial hemerythrin from *M. capsulatus* (Bath), the cloning of this hemerythrin in *Escherichia coli* has also appeared [5]. However, the recombinant McHr was characterized in only one oxidation state, and the characterization was rather cursory. In particular, the iron content was not well determined due to the requirement to correctly incorporate iron atoms into the over-expressed diiron-containing protein clones in *E. coli*, and the difficulty in obtaining the extinction coefficient of the *holo* protein from the UV-visible spectra [5]. Accordingly, the identity and the physiological function of the putative hemerythrin have remained unsettled.

The active site of hemerythrin consists of a non-heme diiron center. According to the crystal structures of invertebrate hemerythrin [2,6], the two iron atoms are coordinated by a total of five histidines: three histidines are coordinated to Fe1 and the other two coordinated to Fe2 (Scheme 1). In addition, the binuclear center is bridged by two carboxylate side chains from Asp and Glu, and a μ -oxo bridge in the oxygen-bound form (oxyHr). In deoxygenated hemerythrin (deoxyHr), the two iron atoms are bridged by a hydroxide in addition to the two carboxylates (Scheme 1). Upon

Scheme 1. Three different states of hemerythrin: deoxy-hemerythrin, oxy-hemerythrin, and met-hemerythrin in invertebrates. Possible conversion of three oxidation forms is illustrated.

oxygenation, the proton is transferred to the bound dioxygen to form a hydroperoxo at Fe2. After long-time exposure to water, the diiron site will undergo auto-oxidation to produce methemerythrin (metHr), the oxidized state without bound dioxygen, as shown in Scheme 1.

In this study, we present the isolation and purification of McHr from cells of *M. capsulatus* (Bath) enriched in pMMO, as well as characterization of different states of the McHr via UV-visible, circular dichroism, resonance Raman, and electron paramagnetic resonance spectroscopy. A more complete study of the McHr is essential to verify its expression in *M. capsulatus* (Bath), and to define its function in the cells, particularly, in relationship with the pMMO. As a case in point, a report has just appeared describing

Fig. 1. Multiple sequence alignment of selected hemerythrin sequences from Swiss-Prot and TrEMBL databases. Q9REU3_DESVU: DcrH-Hr of *Desulfovibrio vulgaris* (PDB code 2AVKA), HEMT_THEDY: *Themiste dyscritum* hemerythrin (PDB code 1HMD), HEMTM_THEZO: *Themiste zostericola* myohemerythrin (PDB code 1A7D), Q60AX2: *M. capsulatus* (Bath) hemerythrin. The secondary structure of the DcrH-Hr initially assigned from its PDB file was also shown at the top of the first sequence. Note that the conserved McHr Leu114 should be labeled 115 here since the numbering system was constructed according to the secondary structure source DcrH-Hr. His22, His58, His77, His81, His117, Asp122 and Glu62 are the metal binding ligands.

⁵⁷Fe Mössbauer studies on cells of *M. capsulatus* (Bath) grown under high Cu and Fe concentrations. This work suggested that the pMMO enzyme itself might contain a non-heme diiron center just as in the sMMO [7]. Since the McHr contains a diiron center as well, the level of expression of the hemerythrin in *M. capsulatus* (Bath) must also be ascertained under different conditions of culturing the cells.

2. Experimental methods

2.1. Isolation and purification of hemerythrin

M. capsulatus (Bath) was cultured and grown at a final Cu concentration of 30 μ M in a Bioflo 3000 fermentor adapted with a hollow-fiber membrane bioreactor as previously described by Yu et al. [8]. The harvested cell paste was suspended in 25 mM PIPES buffer at pH 7.0 containing 1 mM DNase I and 1 mM benzamidine. Cells were broken by two to three passes through a French pressure cell at 20,000 p.s.i. Unbroken cells and cell debris were removed by centrifugation at 27,000g for 25 min at 4 °C. The supernatant was then ultra-centrifuged at 220,000g for 40 min at 4 °C to pellet the membrane fraction. After ultra-centrifugation, the supernatant was regarded as the cytosolic fraction.

The cytosolic fraction of cells grown at 30 μ M Cu was collected for the purification of proteins used in this study. All chromatographic processes were performed on an ÄKTA FPLC (GE Healthcare) and all gel media and empty columns were obtained from the same vendor. The crude extract was first passed through Hi-Prep 26/10 desalting column to exchange the buffer to 20 mM Tris–HCl pH 8.0, and then loaded on to a weak ion exchange gel column (DEAE-Sephacrose Fast Flow in XK 26/20); the flow-rate was set at 2 ml/min during sample loading and elution. Upon the elution of all unbound proteins, the salt gradient was increased to 50 mM NaCl (the base elution buffer was 1 M NaCl in 20 mM Tris–HCl, pH 8.0). These fractions contained methanol dehydrogenase and hemerythrin. After the absorbance of the eluent at 280 nm leveled off, the salt concentration was increased to a step-wise gradient of 10%, 20% and 50% to systematically collect proteins for further analysis.

The fractions from 0% to 5% gradient were concentrated by Amicon YM-10 Ultra-15 Centrifugal Filter Units (Millipore) to volumes less than 1 ml, and injected into a Sephacryl S100 HR gel-filtration C 16/100 column with 300 mM NaCl in 20 mM Tris–HCl buffer. The use of a lengthy column together with low flow rate (0.2 ml/min) ensured optimal resolution of the low molecular mass proteins. After collecting hemerythrin-containing fractions from the S100, 3.2 M ammonium sulfate solution in 50 mM Tris–HCl pH 8.0 was added 1:1 (v/v) directly to the sample in order to purify the proteins by hydrophobic chromatography (HiLoad 16/10 Phenyl Sepharose HP). The initial salt concentration was set at 1.6 M ammonium sulfate in 50 mM Tris buffer pH 8.0, followed by a 240 ml (12 column volumes) continuous gradient to 50 mM Tris buffer only. Hemerythrin was eluted around 0.7 M ammonium sulfate. All proteins purified were desalted by a HiTrap 5 ml desalting column against Tris–HCl pH 8.0 in order to eliminate the interference of ionic interactions. The hemerythrin was identified by SDS-PAGE and UV-visible spectrum recorded on a spectrophotometer (Model 8453, Hewlett Packard). SDS-PAGE was set-up according to Laemmli [9] with 16% (w/v) resolving gels and 4% stacking gels. The gel was stained with Coomassie brilliant blue G250 (Sigma).

2.2. Molecular mass calibration by Sephacryl S100 and mass spectra

A Gel-filtration LMW Calibration Kit (GE Healthcare) was used to determine the linear regression curve and to estimate the

molecular mass of the eluate. Five milligram of each component (bovine serum albumin, 67,000 Da; ovalbumin, 43,000 Da; chymotrypsinogen A, 25,000 Da, and ribonuclease A, 13,700 Da) were weighed and mixed in 2.5 ml of elution buffer. The separation conditions were the same as noted above.

Procedures for both in-gel and in-solution digestion of protein samples for mass spectrometry followed the protocols described by Kinter and Sherman [10]. Mass spectra were recorded on a Bruker MALDI-TOF (matrix assisted laser desorption/ionization time-of-flight) mass spectrometer equipped with a Scout source and delay extraction. The identity of the protein was established by reference to the NCBI non-redundant (nr) database using the MASCOT program. The purified protein was also desalted by Zip-Tip directly and the same MALDI-TOF facility was used to perform intact protein molecular mass analysis.

2.3. Iron content

Iron concentration was analyzed by polarized Zeeman atomic absorption spectrophotometer (Z8200, HITACHI). A 150 μ L of 1 mM hemerythrin was digested by 65% HNO₃ in a microwave accelerated reaction system (MARS5, CEM). The sample was diluted and subjected to atomic absorption spectroscopy. Iron dissolved in 2% HNO₃ was employed as standard. The protein concentration was determined by the Lowry method using bovine serum albumin (Sigma) as the standard.

2.4. Deoxy and various oxygenated forms of hemerythrin

The deoxyHr was obtained according to previous methods [4,11]. All experimental procedures were carried out in an anaerobic glove box (COX) at room temperature. The purified hemerythrin was dialyzed against five equivalents of sodium dithionite to the protein concentration for 6 h. This dialysis process was repeated at least twice. Subsequently, the deoxyHr was dialyzed against degassed Tris–HCl buffer (pH 8.0) to remove excess sodium dithionite for 15 h. Dithionite-free buffer was displaced every 5 h. The deoxyHr was confirmed by UV-visible spectrum.

To form the oxyHr, the deoxyHr was directly exposed to air. The transparent deoxyHr gradually turned into the red–purple color. The complete formation of oxyHr was determined by its absorbance at 500 nm. Upon continued exposure of the sample to air, however, the protein was converted to metHr, and the solution returned to a yellow color. The azide derivative (azideHr) was prepared by addition of 50 equivalents of sodium azide to the metHr sample and stirred for about 12 h at ambient temperature.

2.5. Spectroscopy methods

The UV-visible spectra of hemerythrin were monitored by a HP 8453 UV-visible spectrometer. Circular dichroism spectra were recorded on a JASCO spectropolarimeter (J-720 JASCO, Tokyo) between 200 nm and 300 nm. All electronic spectroscopy was carried out at room temperature. The life time ($t_{1/2}$) of auto-oxidation was measured in a Hewlett Packard 8453 by observing the change at 500 nm. All processes were monitored at ambient temperature.

EPR spectra of the various hemerythrin samples were recorded at X-band (9.60 GHz; modulation frequency 100 kHz; and modulation amplitude 5 G) on a Bruker E580 spectrometer equipped with a Bruker dual-mode ER 4116DM cavity. The different forms of hemerythrin (2.5 mM) were loaded separately into EPR tubes, and then quickly frozen by immersing into a cold isopropanol bath chilled by liquid nitrogen. During EPR experiments, the sample temperature was maintained at 4 K by using an Oxford

Instruments continuous liquid helium cryostat equipped with a turbo pump to lower the vapor pressure of the liquid helium.

Resonance Raman spectroscopy was obtained on a home-built instrument. The laser source was an Ar⁺ laser (Coherent INNOVA 90) operating at 514 nm to excite the sample at ambient temperature. Spectra were collected by a 0.85 m double spectrometer (Spex 1403) equipped with a liquid N₂ cooled CCD detector (SI-502). Rayleigh scattering was blocked by a holographic notch filter and Raman scattering were collected in a ~30°-scattering geometry from the excitation of the solution sample. Throughout this study, the spectroscopy data were dealt with in Origin 7.

2.6. Bioinformatic analysis

Genomic sequencing of *M. capsulatus* (Bath) was performed by the National High-Throughput Genome Sequencing Center at the National Yang-Ming University [1]. The entire shotgun sequencing is not finished; however, we have obtained both the 4× and 8× coverage contigs. EMBOSS 2.8.0 [12], NCBI BLAST 2.2.6 [13], and HMMER 2.3.2 [14] were installed on FreeBSD 6.2 RELEASE with one Intel® Pentium® 4 processor operating in the batch mode. Open reading frames (ORFs) were predicted and translated into protein sequences by the GETORF function of the EMBOSS, and their homologues protein names were searched against the Swiss-Prot, TrEMBL, and the NCBI non-redundant databases with the expectation value of 1E-05 as the criterion. Multiple sequence alignment analysis was done by CLUSTALW [15,16]. The tertiary structure modeling was performed by SWISS-MODEL service online (<http://swissmodel.expasy.org/>) [17] and secondary structure was predicted by PSIPRED [18].

3. Results

3.1. Hemerythrin purification

The desalted cytosolic fraction of *M. capsulatus* (Bath), grown and harvested in a growth medium containing 30 μM Cu, was passed through a DEAE-Sepharose Fast Flow column. The hemerythrin was identified in the 0–50 mM NaCl fraction by SDS-PAGE, followed by in-gel trypsin digestion and MALDI-TOF. The desalting

and pH adjusting step was important since the hemerythrin only bound to the DEAE-Sepharose Fast Flow under this condition; otherwise it would be eluted in the unbound fraction together with some high-abundant low molecular mass proteins, increasing the difficulty of purification (Fig. 2, lanes 6–8). These fractions were further purified by Sephacryl S100, and the hemerythrin-containing fractions were collected (Fig. 2, lanes 2–5). Finally hydrophobic interaction chromatography was used to separate impurities and heme proteins that interfere with the UV-visible spectra from the hemerythrin, and a near pure protein band could be identified by SDS-PAGE (Fig. 2, lane 1). After concentrating and buffer exchange, in-solution trypsin digestion was performed directly for protein peptide mass fingerprinting analysis. The results showed that the only match was gi|53805087 in the NCBI non-redundant database [13], corresponding to McHr with 90% sequence coverage. The intact protein molecular mass was also measured by MALDI-TOF and the determination gave a value of 14887.28 Da (Fig. 3). This result is in excellent agreement with the mass of the intact protein (metHr) obtained by adding the mass of the diiron oxo core and the associated hydroxyl group (145 Da) to that of the McHr polypeptide calculated from the NCBI database (14714.65 Da).

The molecular mass of the native hemerythrin could also be estimated by linear regression analysis of protein bands on a calibrated Sephacryl S100 HR column. In this manner, the molecular mass of the hemerythrin eluted was determined to be 16.3 kDa. Together with the intact mass mentioned earlier, the result is in reasonable correspondence with the molecular mass calculated from the protein sequence database (14.71 kDa). The retention time of the final purified protein was almost identical to that determined by gel-filtration before the treatment with ammonium sulfate. Thus, we have obtained no evidence for polymeric forms of McHr. We assume that the native McHr is a monomer. Thus, it is evident that the hemerythrin has been successfully purified from *M. capsulatus* (Bath).

3.2. UV-visible spectra

The UV-visible spectra of the purified deoxyHr, oxyHr and metHr (10^{−5} M) from *M. capsulatus* are shown in Fig. 4. Basically, the absorption features observed for the three different forms of

Fig. 2. Purification of Hemerythrin. This electrophoretogram shows the sequence of purification: lane 1: Final elution from Phenyl Sepharose HP; lanes 2–5: Sephacryl S-100 HR fractions 36–39; lanes 6–8: Fractions from DEAE-Sepharose Fast Flow 0–50 mM NaCl gradient with increasing retention time. Molecular mass maker (Mr): 97 kDa, 66 kDa, 45 kDa, 30 kDa, 20 kDa and 14.4 kDa.

Fig. 3. Molecular-weight validation. The hemerythrin purified by phenyl sepharose chromatography was subjected to intact protein molecular mass determination by MALDI-TOF. The data demonstrate that the protein mass is 14887.28 Da, which corresponds well with the molecular mass of the monomer.

Fig. 4. UV-visible spectra of hemerythrin (100 μ M of each) from *M. capsulatus* (Bath) in 50 mM Tris-HCl buffer (pH 8.0) at room temperature. Solid line represents the met form; dashed line, the oxy form; and dotted line, the deoxy form. The inset depicts the UV-visible spectrum of 140 μ M hemerythrin in 50 mM Tris-HCl (pH 8.0) after adding 50 equivalents of sodium azide to the protein solution.

hemerythrin are very similar to those previously reported for hemerythrin from marine invertebrates. MetHr exhibited two major absorption peaks at 327 nm ($4431 \text{ M}^{-1} \text{ cm}^{-1}$) and 376 nm ($3715 \text{ M}^{-1} \text{ cm}^{-1}$), and one weaker feature near 492 nm, all of which could be assigned to ligand-to-metal charge transfer (LMCT) transitions from the μ -oxo to Fe(III), according to previous studies of hemerythrins in invertebrates [4,6,11]. There should be no absorption peaks in this region of the spectrum for deoxyHr. The small absorptions observed could be attributed to traces of oxyHr or metHr formed from oxidation of the deoxyHr by air diffused into the cuvette during transfer of the sample from the anaerobic glove box to the aerobic spectrophotometer. These absorptions increased in intensity within minutes after recording of the deoxyHr spectrum and as soon as the lid of the cuvette was removed to expose

the solution to air. The sharp absorption peak at 328 nm ($4296 \text{ M}^{-1} \text{ cm}^{-1}$) and the broad shoulder peak near 371 nm ($3677 \text{ M}^{-1} \text{ cm}^{-1}$) in oxyHr were assigned to LMCT transitions from oxo to Fe(III) [4,6,11]. Perhaps, more diagnostic was the broad peak near 500 nm ($1416 \text{ M}^{-1} \text{ cm}^{-1}$), which became more prominent. This peak is a LMCT transition from the hydroperoxo to Fe(III), and thus constitutes major evidence for oxyHr [4,6,11].

3.3. Auto-oxidation

The rate of auto-oxidation of metHr of *M. capsulatus* (Bath) ($t_{1/2} \sim 50$ min) was much faster than that of hemerythrin from marine invertebrates ($t_{1/2} \sim 20$ h) [11], and was closer to the rate recently reported for DcrH-Hr, the hemerythrin-like domain of chemotaxis protein from *Desulfovibrio vulgaris* ($t_{1/2} < 1$ min) [4,19]. In mutagenesis experiments, Kurtz found that the conserved Leu104 (Leu114 in McHr, Fig. 1) played an important role in controlling the auto-oxidation in the marine invertebrate hemerythrin. It was suggested that the hydrophobicity of this amino acid limited the entry of the solvent to reach the diiron core [11]. However, from sequence alignment of McHr and myohemerythrin of *Themiste dyscritum* as well as to DcrH-Hr (Fig. 1), we noted that the WLVNHI domain which included Leu114 was still conserved. In a recent crystallographic structural analysis of the hemerythrin-like domain of chemotaxis protein from *D. vulgaris* [19], Kurtz and Chan concluded that DcrH-Hr contained a putative “substrate tunnel” which was not found in the marine invertebrate Hrs. This substrate tunnel would facilitate access of the solvent to the diiron core in DcrH-Hr. Presumably a similar substrate tunnel is implicated in McHr, since the similarity between McHr and DcrH-Hr is 35% (data not shown), and all iron-binding residues are conserved (Fig. 1).

3.4. Secondary and tertiary structure

Molecular modeling of the McHr based on the structure of DcrH-Hr (PDB code: 2AVKA) by Swiss-Model revealed a four-helix bundle structure, in which the amino acids 21–37, 41–68, 74–96, 100–124 are superimposed on the helix structure of the DcrH-Hr.

Fig. 5. Circular dichroism spectrum of met-hemerythrin (7.6 μ M) in 20 mM Tris-HCl (pH 8.0) from *M. capsulatus* (Bath). Two major negative peaks appeared at 208 and 222 nm, respectively, supporting the predicted four-bundle α -helix structure.

Note that in Fig. 1 the iron binding domains are conserved in all the aligned α -helical sequences.

Secondary structure prediction by the “garnier” application in EMBOSS also supported amino acids 1–11, 17–37, 46–104 as α -helical domains, which covered 68.7% of the total length. Another secondary prediction tool PSIPRED implicated amino acids 8–36, 41–69, 75–97, and 104–116 as α -helical structures, totaling 65.6% of the length. These predicted four-helix bundle structural models are consistent with the CD spectrum of McHr, which showed two major negative peaks at 208 and 222 nm, characteristic of α -helices (Fig. 5). Taken together, these data indicate that the structure of *M. capsulatus* (Bath) hemerythrin is similar to that of DcrH-Hr.

BLAST search of McHr was also performed against all hemerythrin homologues of known structures. It is very interesting that polymeric hemerythrins are closer to McHr than myohemerythrins, which exist as monomers. In all the entries (Fig. 1) only DcrH-Hr is of bacterial origin, and it shows the highest similarity. Further analysis of McHr to Swiss-Prot and TrEMBL databases also revealed that the overall identity of McHr was higher to eukaryotic hemerythrins than to myohemerythrins (data not shown).

3.5. The diiron core

Atomic absorption spectroscopy yielded two iron atoms per protein. From structural modeling, the conserved amino acids His22, His58, His77, His81, His117, Asp122 and Glu62 offered the best residues for chelating the two irons. Resonance Raman spectroscopy confirmed the existence of a μ -oxo-bridged diiron core (Fe–O–Fe). As shown in Fig. 6, a Raman band corresponding to the symmetric stretch of Fe–O–Fe was observed at 503 cm^{-1} , similar to the band previously assigned to this vibration in marine invertebrates. There was no apparent asymmetric peak near 750 cm^{-1} because its intensity would be much weaker than the symmetric mode. In comparison with earlier work [4,11,20], the observed frequency suggests that the Fe–O–Fe angle in McHr is about 130 degree. Thus, we surmise that McHr also consists of a diiron oxo core occluded by a four-helix bundle.

According to previous studies, the two irons in hemerythrin are antiferromagnetically coupled in metHr, oxyHr, and deoxyHr, due to the presence of a bridging μ -oxo between them. Thus, only the semi-met hemerythrin is expected to yield EPR signals in the $g \sim 2$ region. A non-integer Kramer doublet can exist only in the semi-met Hr, where antiferromagnetic coupling between the Fe(II) and Fe(III) ions yields a $S = 1/2$ ground state. Thus, surprisingly, EPR

Fig. 6. Resonance Raman spectrum of met-hemerythrin from *M. capsulatus* (Bath). The sample concentration was 2.5 mM in 20 mM Tris-HCl (pH 8.0). A 200 mW Ar^+ laser operating at 514.5 nm was used to excite the metHr at ambient temperature.

Fig. 7. 4 K EPR spectra of hemerythrin from *M. capsulatus* (Bath). The solid spectrum was recorded for freshly purified metMcHr. Other spectra were obtained from samples of oxy-hemerythrin (dashed line) and deoxy-hemerythrin (dotted line). The signals observed near $g \sim 2$ were readily assigned to the presence of small amounts of semi-met Hr in these samples (see text). In the deoxy-McHr sample, the small signal was attributed to traces of non-fully reduced hemerythrin. The signal at $g \sim 4.24$ in the spectrum of oxy-McHr was from a high-spin Fe(III) contaminant. Microwave frequency, 9.63 GHz; modulation frequency, 100 kHz; modulation amplitude, 5 G; power, 0.2 mW.

signals were observed at $g \sim 1.86$ in our metHr and oxyHr in Fig. 7. However, these signals are characteristic of the partially reduced semi-met Hr from one-electron reduction of the met-hemerythrin. The sample of metHr as isolated from *M. capsulatus* (Bath) yielded an EPR spectrum with g at 1.90, 1.84 and 1.50, which was similar (the g -values are somewhat more isotropic here) to the EPR observed for the semi-met Hr in the case of the marine invertebrate hemerythrins [21–23]. EPR spin-counting indicated that the semi-met form accounted for no more than 22% of the as-isolated protein sample. With this extent of partial reduction, it would not be possible to ascertain the presence of the semi-met form in the UV-visible spectrum of the as-isolated metHr sample.

When the EPR was recorded for deoxyHr, a small signal was also observed near $g \sim 1.86$ (Fig. 7). Again, this signal could be attributed to small amounts of the semi-met Hr in the sample due to incomplete reduction of the metHr at the outset. Also, it was diffi-

cult to maintain totally anaerobic conditions during the handling of the samples, and the metHr could be formed by the rapid reoxidation of deoxyHr with dioxygen to form oxyHr, and the subsequent auto-oxidation of the oxyHr. Spin count of the EPR signal at $g \sim 1.86$ provided an estimate of ca. 3.6% of total protein concentration in the semi-met form.

A small high-spin Fe(III) was also detected at $g \sim 4.27$ in Fig. 7. The intensity of this signal is ca. 8.9% of the intensity of the semi-met signal at $g \sim 1.86$, and would account for only 0.03% of the iron in the sample.

4. Discussion

Hemerythrin is an iron-containing dioxygen carrier typically found in marine organisms, but the bacterial fusion-domain homologues are also known [3]. Recently, we reported the identification of a bona fide bacterial hemerythrin from *M. capsulatus* (Bath). In this study, we have successfully isolated and purified this hemerythrin. UV-visible, CD, resonance Raman, and EPR demonstrated that the structure of the protein, including the diiron core, was identical to those of hemerythrins from other sources. The iron content and auto-oxidation rate of McHr have also been measured. Thus, the McHr should function as an oxygen carrier in *M. capsulatus* (Bath), just like hemerythrins from marine invertebrates. Unlike eukaryotic hemerythrins, which are typically multimeric, however, molecular mass determination during and after the purification indicated that McHr existed as a monomer in vitro. We were also able to confirm that the McHr is over-expressed in the parent organism under the regulation of copper ions, in accord with our earlier proteomic analysis [1]. This is the first native bacterial hemerythrin isolated and characterized from prokaryotes.

Although the X-ray crystallographic analysis of McHr is still pending, the crystal structure of the hemerythrin-domain of the DcrH-Hr, the only reference bacterial hemerythrin-like protein to date, is already available [19]. Given the similarity in the sequences between McHr and DcrH-Hr, we expect the tertiary folds of the two proteins to be similar.

According to the multiple sequence alignment in Fig. 1, there are two gaps located between the helix bundles in the eukaryotic hemerythrins, which do not appear in McHr. These sequence gaps are also not found in DcrH-Hr. The intervening amino acids in DcrH-Hr are largely α -helical in the protein structure, in effect extending the length of the helices forming the helix bundles, although the packing of the helices is somewhat disordered in the extended loop region. Interestingly, similar modeling of McHr based on DcrH-Hr as the template revealed similar lengths of the helices in the four-helix bundle. In fact, the α -helices matched well, and all the metal chelating residues fitted in with almost identical spatial orientation (data not shown). Karlsen et al. [5] had earlier predicted on the basis of PSIPRED that the α -helices of McHr are longer. At the time, these authors compared the length of McHr to *Thermotoga zostericola* myohemerythrin (PDB code 1A7D) as the structure of DcrH-Hr had not been solved then. Thus, compared to Hr in marine invertebrates, the helix bundles in the prokaryotic hemerythrins are apparently longer. This might be the origin of the larger substrate tunnel found in DcrH-Hr. Thus, a similar substrate tunnel might exist in *M. capsulatus* (Bath) hemerythrin, which would account for the faster rate of auto-oxidation observed for McHr than in marine invertebrate hemerythrins.

Recently French et al. have reviewed all single- and multi-domain hemerythrin-like proteins available in public genomic databases, and they have postulated a list of diverse physiological functions for these proteins [3]. Oxygen transport is the most common suggestion to account for the existence of a “stand-alone”

hemerythrin in the light of marine worms. The physiological environment of methanotrophic bacteria is distinct. When *M. capsulatus* (Bath) was cultured and grown in $30 \mu\text{M}$ Cu, the pMMO in the plasma membrane is over-expressed by at least 10-fold, and the production of hemerythrin was elevated by 4-fold [1]. The McHr was also detected in both the cytosolic and membrane fractions of the cell. With the pMMO in the plasma membrane accounting for 80–90% of the membrane proteins [8], the organism needs to come up with a method to increase the effective intracellular O_2 concentration to facilitate turnover of the pMMO. It would be unreasonable to have even a small fraction of the pMMO idle in the membrane given the energetic cost to over-produce it. Since the level of expression of the hemerythrin parallels the overproduction of pMMO under these conditions, we surmise that hemerythrin functions as an oxygen carrier and its function in the bacterium is to enhance the effective dioxygen concentration in the cytoplasm or at the membrane–aqueous interface of the cytoplasmic membrane so that the dioxygen within the cell body could be readily available for consumption by the pMMO. We stress that the pMMO is immobilized in the membranes, so the dioxygen must be delivered to the membranes either by diffusion in the free form or associated with a diffusing carrier such as hemerythrin. As it is not possible to increase the concentration of the free dioxygen beyond the solubility limit, the only way is to increase the effective dioxygen concentration using a water-soluble dioxygen carrier that is capable of delivering the dioxygen to the membrane. Consistent with this scenario, the McHr is found not only in the cytosolic proteome but it is also associated with the plasma membranes.

In the cellular environment, the consumption of O_2 by pMMO must be significantly faster than the rate of auto-oxidation of the oxy-McHr, based on the turnover frequency of the pMMO. In addition, the cell is sufficiently reducing so that the steady state concentration of the met-McHr cannot be high. Thus, there is a major difference in the importance of the auto-oxidation in the cellular milieu versus a protein solution in buffer. Auto-oxidation of hemerythrin is primarily an artifact of studying hemerythrin in buffer.

5. Abbreviations

C ₁	one-carbon
<i>M. capsulatus</i> (Bath)	<i>Methylococcus capsulatus</i> (Bath)
pMMO	membrane-bound or particulate methane monooxygenase
sMMO	soluble methane monooxygenase
Hr	hemerythrin
McHr	<i>M. capsulatus</i> hemerythrin
deoxyHr	deoxygenated hemerythrin
oxyHr	oxygenated hemerythrin
metHr	methemerythrin
DcrH-Hr	the bacterial hemerythrin-domain located in the bacterial chemotaxis protein <i>Desulfovibrio vulgaris</i>
MALDI-TOF MS	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
cIcAT	cleavable isotope coded affinity tag
LMCT	ligand-to-metal charge transfer
BLAST	basic local alignment search tool
ORFs	open reading frames
PDB	protein data base

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