



The bacteriohemerythrin from *Methylococcus capsulatus* (Bath): Crystal structures reveal that Leu114 regulates a water tunnel



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ABSTRACT

The bacteriohemerythrin (McHr) from *Methylococcus capsulatus* (Bath) is an oxygen carrier that serves as a transporter to deliver O₂ from the cytosol of the bacterial cell body to the particulate methane monooxygenase residing in the intracytoplasmic membranes for methane oxidation. Here we report X-ray protein crystal structures of the recombinant wild type (WT) McHr and its L114A, L114Y and L114F mutants. The structure of the WT reveals a possible water tunnel in the McHr that might be linked to its faster autoxidation relative to hemerythrin in marine invertebrates. With Leu114 positioned at the end of this putative water tunnel, the hydrophobic side chain of this residue seems to play a prominent role in controlling the access of the water molecule required for autoxidation. This hypothesis is examined by comparing the autoxidation rates of the WT McHr with those of the L114A, L114Y and L114F mutants. The biochemical data are correlated with structural insights derived from the analysis of the putative water tunnels in the various McHr proteins provided by the X-ray structures.

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1. Introduction

Classical hemerythrin (Hr) are molecular oxygen carriers in certain marine invertebrates, such as sipunculids, brachiopods, priapulids, and annelids [1–4]. These proteins are found to exist in different oligomeric forms (α_n), including monomers (myohemerythrin (myoHr)), dimers, trimers, tetramers and octamers, each α monomer sharing a highly conserved four-helix bundle structure and a non-heme di-iron center as the O₂ binding site [5,6]. Hr are not normally found in prokaryotes, though a Hr-like domain has been identified in the bacterial chemotaxis protein *Desulfovibrio vulgaris* chemoreceptor (DcrH-Hr) and has been structurally characterized [7–10]. Recently, Kao and co-workers have also reported a bacteriohemerythrin from *Methylococcus capsulatus* (Bath) (McHr), a monomeric form of hemerythrin co-expressed in this methanotroph when the bacterium is grown under high copper to biomass conditions to overproduce the particulate

methane monooxygenase (pMMO) [11–13]. From the effects of the McHr on the activity of the pMMO, it has been proposed that McHr acts as a shuttle to transport dioxygen from the cytoplasm of the cell to the intracytoplasmic membranes for delivery of the O₂ to the pMMO enzyme for methane oxidation [14].

McHr has been cloned and over-expressed in *Escherichia coli* and the recombinant protein has been studied by various biophysical methods [13]. The protein has also been purified from *M. capsulatus* (Bath) cells for biophysical/biochemical characterization [11]. However, a crystal structure of the McHr has not been reported for comparison with the known structures of Hr from *Phascolopsis gouldii* [15], *Themiste dyscritum* [16], *Themiste pyroides* [17], and DcrH-Hr [7–9].

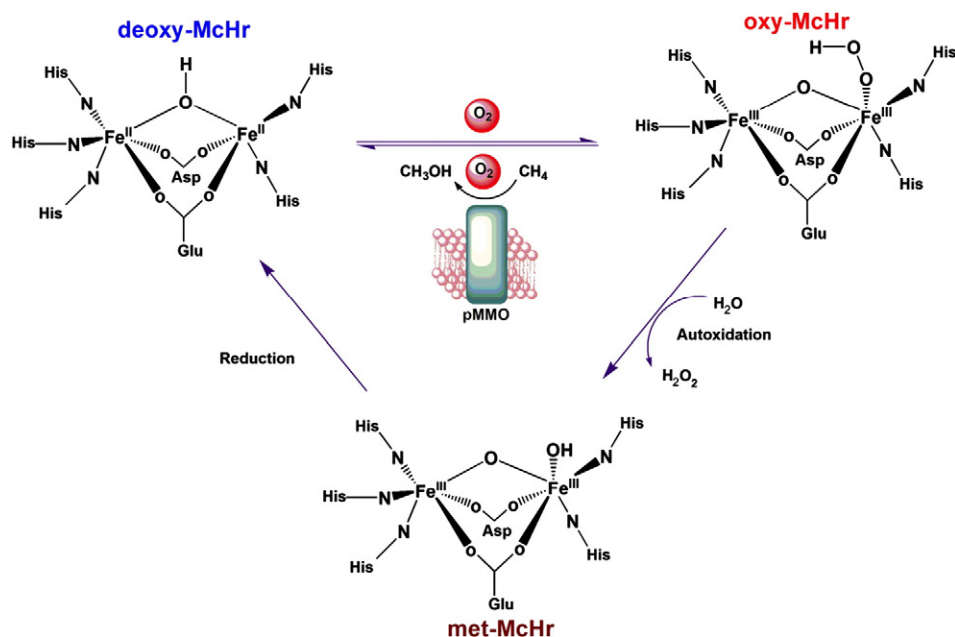
McHr delivers O₂ to the pMMO for the controlled oxidation of methanol into methanol by the enzyme [11,12,14]. However, upon prolonged exposure to O₂ and water, oxy-McHr also undergoes autoxidation to form met-McHr and hydrogen peroxide [5,18], as with Hr from other species (Scheme 1). The rate of autoxidation varies from minutes to hours depending on the species of Hr. Mutagenesis studies of specific residues located in the vicinity of the di-iron center in Hr from the marine invertebrate *P. gouldii*, myohemerythrin (myoHr) and the bacterial DcrH-Hr have shed some structural insights into this issue [7–10, 15–17]. Kurtz and co-workers have discovered that the conserved Leu104 in myoHr (Leu98 in Hr, Leu115 in DcrH-Hr, and Leu114 in

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Scheme 1. The various states of hemerythrin from *M. capsulatus* (Bath). Shown are the oxidation states of the two iron atoms in the di-iron center together with the ligand environments in the deoxy-, oxy- and met-forms. The formation of oxy-McHr from doxy-McHr and the autoxidation of oxy-McHr to give met-McHr are also shown.

McHr) plays a critical role in limiting the entry of water molecules to reach the di-iron core [19]. Apparently, the hydrophobicity of the Leu104 residue controls the autoxidation and regulates the dioxygen binding properties of myoHr [15]. Subsequently, a water tunnel was identified in the crystal structure of DcrH-Hr, which was not found in Hr from marine invertebrates [7], and it was suggested that this water tunnel was the cause of the rapid autoxidation observed for DcrH-Hr. The autoxidation of McHr is also rapid, although not as fast as in the case of DcrH-Hr. Based on sequence alignment of McHr, DcrH-Hr, Hr and myoHr (Fig. 1) [20,21], Leu114 is part of a conserved WLVNHI alpha-helical motif forming the putative water tunnel. However, we surmise that the packing of this helix in the protein structure might affect the accessibility of solvent to the di-iron center [11].

In this study, we report ultra-high-resolution X-ray structures (~1.0 Å) on the recombinant wide-type (WT) McHr as well as on the L114A, L114Y and L114F McHr mutants. The WT McHr crystal structure demonstrates the existence of the putative water tunnel in McHr similar to DcrH-Hr, and we find that Leu114 indeed plays a prominent role at the end of the water tunnel controlling access of water to the di-iron core.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents used in this study were of molecular biology grade or higher and were obtained from Sigma-Aldrich. Restriction enzymes were purchased from New England BioLabs.

2.2. Molecular cloning, site-directed mutagenesis and protein expression of the WT and recombinant McHr mutants

The cDNA encoding the McHr from *M. capsulatus* (Bath) was amplified by PCR and inserted into the tobacco etch virus (TEV) protease recognition site in the modified pGEX-4T-1 expression vector (GE Healthcare Inc.). The pET21a McHr WT was used as the template during the PCR experiments. The construct contained the glutathione S transferase (GST) fusion tag. The L114A, L114F and L114Y mutants of McHr were constructed using the QuickChange® site-directed mutagenesis kit according to

the manufacturer's protocol (Stratagene Inc.). The primers are shown in Supplementary Data Table A.1. The vector constructed was transformed into *E. coli* BL21 (DE3) cells (Novagen Inc.). A 5-liter vessel fermenter (Major Science Inc.) was used in the cell culture experiments. The bacteria were grown in Luria-Bertani (LB) medium in the presence of ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL) at 37 °C until the cell density reached an OD₆₀₀ of 0.6–0.8. The cultures were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. The cells were harvested by centrifugation at 6000 rpm for 30 min at 4 °C followed by snap freezing in liquid nitrogen and stored at –80 °C.

2.3. Purification of the recombinant McHr proteins

Frozen bacterial pellets were re-suspended in the lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0), and the cells were lysed at 20,000 psi in a French Press. The cell lysate was then subjected to centrifugation at 27,000 ×g for 40 min followed by ultracentrifugation at 220,000 ×g for 1 h at 4 °C to remove the cell debris and the membrane fraction from the lysis solution. Subsequently, we collected the supernatant solution after further purification by passing the lysis solution through a 0.22-µm filter.

To obtain the purified recombinant McHr protein, the crude protein was incubated with glutathione agarose bead (Thermo Inc.) for 2 h in a 4 °C cold room. After washing the column with the lysis buffer, TEV protease was added into the column for 1 h at room temperature to remove the GST tag. The recombinant McHr protein was then eluted with elution buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). Finally, the eluted samples were concentrated and injected into a Superdex 75 column (GE Healthcare Inc.) with 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, and the purified McHr was evaluated by using SDS-PAGE and UV-visible (UV-vis) spectroscopy.

2.4. Preparation of deoxy-, oxy- and met-forms of the recombinant McHr

The deoxy-form of the recombinant McHr was prepared by sodium dithionite treatment. The experimental procedures were carried out in an anaerobic glove box (type A vinyl anaerobic chamber, Coy Lab. Products Inc.) at room temperature. The purified recombinant McHr was dialyzed against ten equivalents of sodium dithionite to the protein

Table 1
Statistics of X-ray data and structure refinement.

Protein	McHr-WT	L114A	L114Y	L114F
PDB ID	4XPX	4XQ1	4XPY	4XPW
Data collection				
Space group	P6	P6	P6	P6
Cell dimensions (Å)				
<i>a</i> , <i>b</i> , <i>c</i>	83.43, 83.43, 30.89	83.88, 83.88, 31.18	83.11, 83.11, 31.01	83.28, 83.28, 30.96
Resolution (Å)	24.08–1.03	27.45–1.40	23.49–1.13	24.84–1.17
<i>R</i> _{merge} (%)	5.8(43.8)	8.1(54.1)	5.5(22.4)	6.5(42.6)
Completeness (%)	99.7(99.8)	96.7(98.9)	97.2(99.6)	95.0(99.5)
Redundancy	4.5(13.2)	11.6(12.2)	4.4(10.4)	4.2(10.5)
Refinement				
Resolution (Å)	24.08–1.03	27.45–1.40	23.49–1.13	24.84–1.17
No. of reflections	57,799	22,962	43,683	39,403
<i>R</i> _{work} / <i>R</i> _{free} (%)	15.7/16.6	15.6/18.6	15.1/16.9	15.2/16.5
No. of atom				
Protein	1025	1030	1037	1036
Fe/O	2/2	2/1	2/2	2/2
Water	156	197	175	147
Average <i>B</i> -factor (Å ²)	13.0	14.3	12.4	12.4
rmsd				
Bond length (Å)	0.028	0.028	0.029	0.027
Bond angle (°)	2.204	2.293	2.226	2.286

Values in parentheses are for the highest-resolution shell.

3. Results

3.1. Preparation of the recombinant WT MChr and its variants

We have cloned and over-expressed the WT MChr and its L114A, L114Y and L114F mutants in *E. coli* as a fusion protein with the GST affinity tag in order to harvest sufficient quantities of the highly purified proteins for single-crystal X-ray diffraction and biochemical experiments. In each case, the recombinant MChr protein is isolated from the host *E. coli* cells following the standard procedures of French Press, ultracentrifugation and column chromatography. The cytoplasm of the cells is loaded onto a glutathione agarose open column pre-equilibrated with the lysis buffer. The bound protein is cleaved by TEV protease on the column and then eluted out by the lysis buffer. The highly purified recombinant MChr-containing fractions are collected by Äkta FPLC by size-exclusion chromatography using a superdex75 size-exclusion column. SDS-PAGE analysis shows that the recombinant WT, L114Y, L114F and L114A MChr proteins appeared as a highly purified polypeptide at 14.8 kDa (Fig. A.1).

3.2. UV-vis spectra

The UV-vis spectra of the purified WT MChr, L114F, and L114Y and L114A recombinants are summarized in Fig. 2a–c.

The UV-vis spectra of the purified deoxy-, oxy- and met-forms of the WT MChr (Fig. 2c) are identical to those previously reported for the hemerythrin purified from *M. capsulatus* (Bath). No UV-vis absorption features are discernible for the deoxy-form of the WT MChr. Upon exposure to the air, it is rapidly converted to the oxy-form with its characteristic μ -oxo to Fe(III) and -OOH to Fe(III) ligand-to-metal charge transfer bands at 328 nm and 371 nm, respectively. In the presence of air, the oxy-MChr is eventually converted to the met-form, as evidenced by slight shifts of the ligand-to-metal charge transfer (LMCT) bands to 327 nm and 376 nm, respectively. The autoxidation reaction half-time ($t_{1/2}$) is determined to be around 1 h, consistent with our previous report [11].

As with the WT MChr, no LMCT absorptions are observed for the purified deoxy forms of the L114F and L114Y MChr proteins. Upon exposure to air, both mutants are converted from the deoxy- to the oxy-forms just as fast as for the WT MChr. Again, the met-forms are obtained by continuous exposure of the oxy-MChr to air, but the autoxidation $t_{1/2}$ increases

to 12 h and 20 h for the L114F and L114Y mutants, respectively. Fig. 2a and b shows the UV-vis spectra for the oxy-forms of these two mutants. In the case of the L114F MChr, LMCT bands are observed for the oxy-form at 320 nm and 371 nm, and for the met-form at 316 nm and 380 nm (Fig. 2a). The oxy- and met-L114Y MChr are purple in color, with a distinctive feature at 550 nm in the UV-vis spectrum due to the phenolate to Fe(III) charge transfer from the tyrosine residue, in addition to the charge transfer bands from the μ -oxo to Fe(III) in the region 337 to 330 nm (Fig. 2b).

Unexpectedly, only weak UV-vis absorptions are elicited for the as-isolated L114A MChr even after a 15-min purge with pure O₂ (Fig. 2c).

3.3. Metal analysis and near-edge X-ray absorption spectroscopy

Metal-ion analysis by atomic absorption spectroscopy shows that the recombinant WT and the three Leu114 MChr mutants contain 1.9–2.2 iron atoms per 14.8-kDa polypeptide. Near-edge X-ray absorption spectroscopy (Fig. 3) shows that the oxidation states of the iron atoms in the purified recombinant WT, L114F, L114Y and L114A MChr are mixed valence as isolated, similar to the ferrous ferric oxide (Fe₃O₄, Fe(II, III, III)), indicating that the WT and mutants MChr exist in the stable semi-met form in air as purified. It is interesting to note that, although one of the two irons apparently exists as Fe(III) in the L114A MChr as purified, no LMCT bands arising from μ -oxo- or oxo-charge-transfer to this Fe(III) are observed in the UV-vis spectrum. As described later, the crystal structure of this mutant reveals that a molecule of sodium nitrate is lodged near the non-heme di-iron site precluding dioxygen binding.

3.4. Crystal structures of the recombinant WT and mutant met-MChr: the water tunnel

Crystal structures of the DcrH-Hr protein have been previously reported by Chan et al. and Hayashi et al. [7,8]. Evidence for a putative water tunnel was provided in the structure of Chan et al. [7], and these workers suggested that rotation of the side chain of Leu115 at the end of the tunnel would open a channel to allow rapid access of a water molecule to the oxygenated non-heme di-iron center, facilitating the facile autoxidation ($t_{1/2} < 1$ min) required for detection of the dioxygen bound [7,19].

In this study, we have determined the protein structures for the WT met-MChr and its L114Y, L114F and L114A mutants at ultra-high

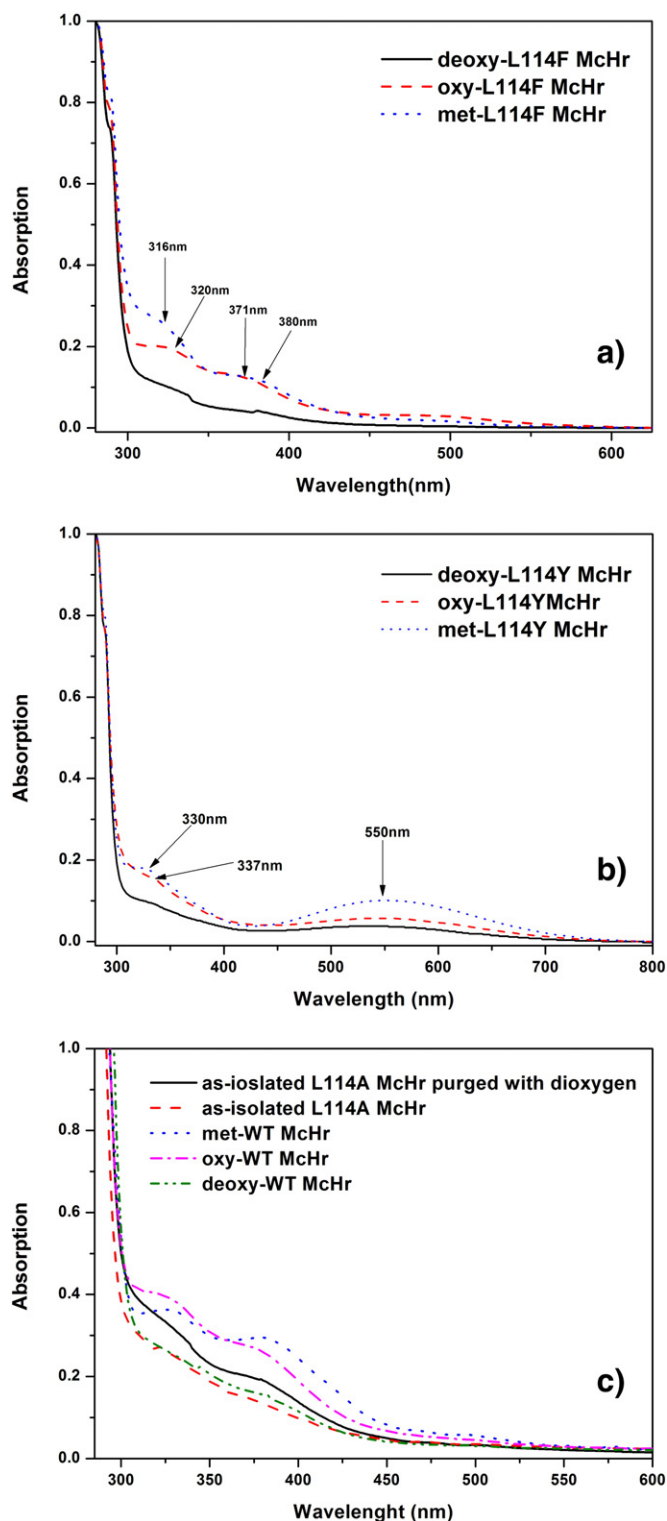


Fig. 2. (a) The UV-vis absorption spectra of the recombinant L114F McHr mutant in various iron oxidation states. The 300–400 nm absorption features are the characteristic μ -oxo to Fe(III) and -OOH to Fe(III) ligand-to-metal charge transfer bands. (b) The UV-vis absorption spectra of the recombinant L114Y McHr mutant in various iron oxidation states. The 300–400 nm absorption features arise from the characteristic μ -oxo to Fe(III) and -OOH to Fe(III) ligand-to-metal charge transfer bands associated with the oxygenated di-iron center. The 550 nm peak is assigned to the phenolate (114Y) to Fe(III) LMCT charge transfer absorption. (c) The isolated L114A McHr mutant exhibits no apparent UV-vis absorption. After a 15-min pure oxygen purge, a weak LMCT absorption is observed.

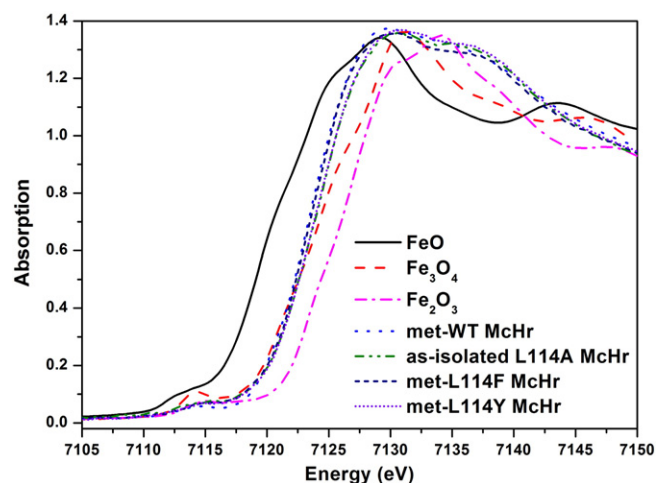


Fig. 3. The Fe K-edge X-ray absorption spectra of the purified recombinant met-WT, met-L114Y, met-L114F and as-isolated L114A McHr proteins together with various iron oxidation standards. Black line: FeO, Fe(II); red line: Fe₃O₄, Fe(II)(III)(III); blue line: Fe₂O₃, Fe(III)(III). Details of the X-ray absorption experiments are given in the text.

resolution (1.0–1.4 Å) with space group P6 (Table 1). Their overall structures are similar upon superimposition, with rmsd of ~0.11 Å for the C α backbone between the structures of the WT met-McHr and the three mutants. These ultra-high resolution structures allow us to correlate

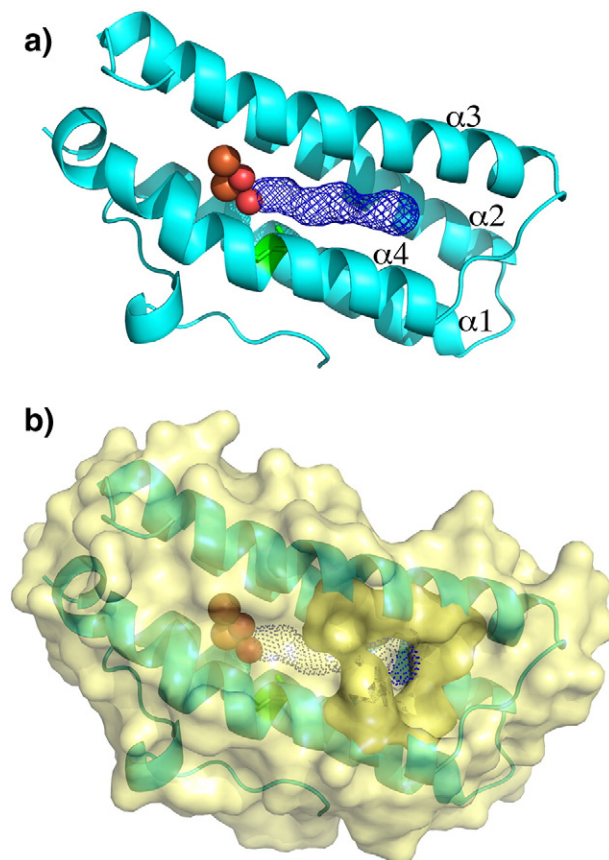


Fig. 4. (a) The protein crystal structure of the WT McHr comprises a four-helix bundle (light blue), a non-heme di-iron center (orange) and a water tunnel (deep blue mesh) oriented parallel with the long axis of the four-helix bundle. The oxygen molecule bound to the non-heme di-iron center and Leu114 is highlighted in red and green respectively. (b) Based on CAVER 3.0 transport pathway analysis, the putative water tunnel (deep blue dots) is shown to penetrate from the protein surface to the core of the di-iron center.

the autoxidation rates observed for the various McHr species with the water tunnel environments (Figs. 4 and 5). According to the protein X-ray crystallographic analysis, the WT met-McHr structure consists of a four-helix bundle ($\alpha 1$: 15–37, $\alpha 2$: 41–69, $\alpha 3$: 74–97 and $\alpha 4$: 103–129) with the non-heme di-iron center coordinated by His22, His58, His77, His81, His117, Asp122 and Glu62 (Fig. 5a).

Based on transport pathway analysis by CAVER 3.0 [26], we have located a putative water tunnel in the McHr oriented parallel to the long axis of the four-helix bundle. This water tunnel is lined by the side chains of the hydrophobic residues His22, Ile25, Val29, Leu32, Leu48, Leu51, Ile52, Val55, His58, Phe59, Glu62, His81, Leu84, Val87, Leu91, Phe109, Val110, Trp113, Leu114, His117, Ile118, and Asp122, and is terminated by the hydrophilic amino acids Cys88, Gln92, Thr106, and Thr107 adjacent to protein surface between helix $\alpha 3$ and $\alpha 4$ (Fig. 4a).

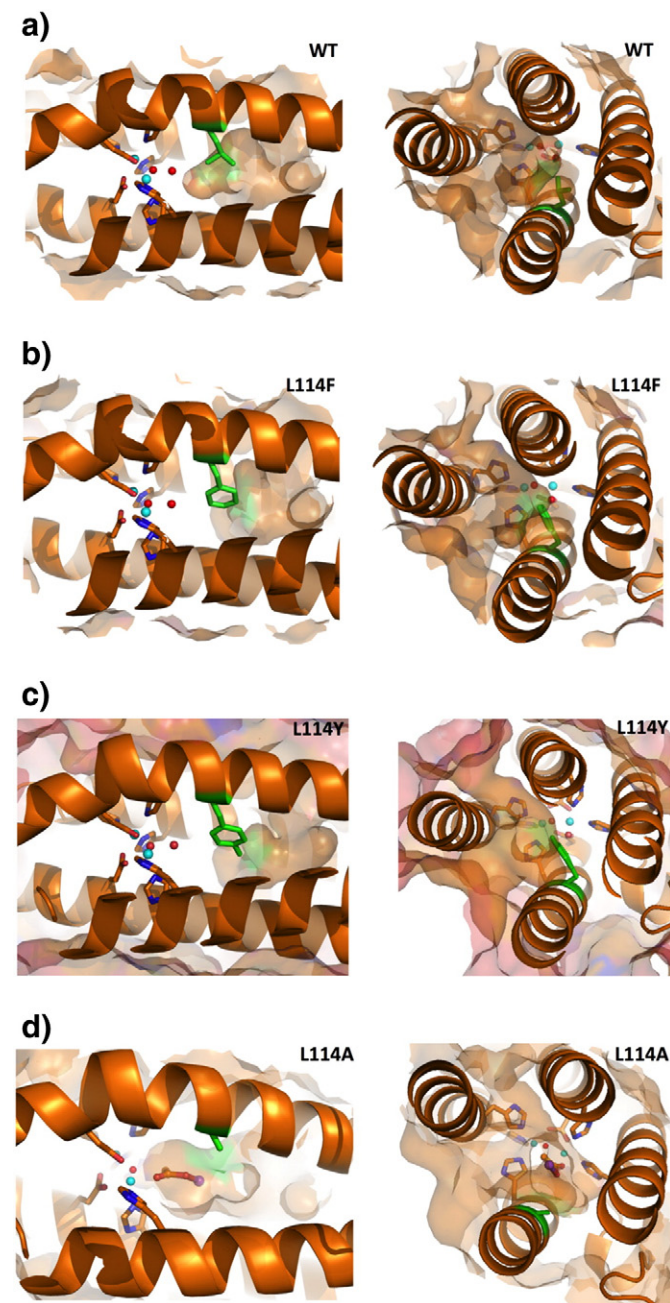


Fig. 5. The ultra-high-resolution X-ray structures of the recombinant WT met-McHr and three leucine 114 mutants: (a) WT; (b) L114F; (c) L114Y; and (d) L114A. Iron: sphere in blue; oxygen: sphere in red; amino acid mutated: stick in green; sodium acetate: stick in red and ball in purple.

According to our earlier study, the McHr autoxidation rate ($t_{1/2}$) is about 50 min, which is much faster than that observed for Hr from a marine invertebrate ($t_{1/2} = 20$ h) [27–29]. In the L114F McHr mutant, the relatively larger phenyl side chain of phenylalanine (Phe) has replaced the branched aliphatic isobutyl side chain of the leucine (L114) in McHr. This substitution should result in steric effects in the opening and closing of the putative water tunnel (Fig. 5b). Consistent with this structural expectation, the autoxidation rate ($t_{1/2}$) measured for this mutant McHr is 20 h, which is similar to the marine invertebrates, and considerably slower than that in the WT McHr.

In the case of the recombinant L114Y McHr mutant, the side chain from tyrosine (Tyr) is expected to exhibit both steric and polar effects. The phenyl group here is similar in size to that in the L114F mutant, but the polar hydroxyl group of the tyrosine side chain will influence the affinity of water and its movement in the putative water tunnel (Fig. 5c). As expected, the $t_{1/2}$ of L114Y is 12 h, thus the autoxidation is relatively slower than in the case of the WT McHr, but significantly faster than for the L114F McHr mutant. Interestingly, the high-resolution X-ray protein structure of this L114Y mutant reveals that the mutated residue Tyr exhibits a double conformation in the vicinity of the active site, with the conformation of the side chain directed either toward the di-iron center or toward the putative water tunnel (Fig. 6).

In the L114A McHr mutant, the significantly smaller aliphatic methyl group has replaced the isobutyl side chain of Leu114 in McHr. Any steric effects associated with the opening and closing of the putative water channel should be diminished (Fig. 5d). Thus we would expect more facile solvent access to the di-iron site here. However, to our surprise, our biochemical experiments indicate that the as-isolated L114A mutant has lost the ability to form the oxy-McHr derivative. According to the crystal structure of the purified L114A McHr, a sodium acetate or sodium nitrate molecule is lodged near the non-heme di-iron site precluding dioxygen binding, and the putative water tunnel is completely open (Fig. 7). We cannot distinguish between an acetate anion from a nitrate. The $F_o - F_c$ map can be fitted with either sodium acetate or sodium nitrate. The fitting results are shown in Supplementary Data Fig. A.2. Structure refinement gives a similar good density fitting with R_{work} and R_{free} values of 15.61 and 18.41% for NO_3^- and 15.49 and 18.28% for CH_3COO^- as the anion. We assume that the L114A McHr mutant has received the sodium acetate or sodium nitrate from the inorganic salts in the LB medium used to culture the host *E. coli* cells, or from the ammonium acetate/sodium nitrate in the HEPES buffer used in the crystallization of the protein.

3.5. Crystal structure of the WT met-McHr: comparison with the met-forms of DcrH-Hr and the invertebrate Hrs

The crystal structure of the WT met-McHr displays the same μ -oxo bridged di-iron metal center previously reported for the DcrH-Hr and met-Hrs from invertebrates. A close view of the non-heme di-iron site

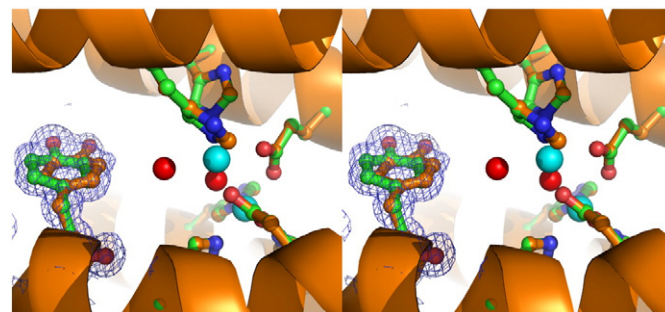


Fig. 6. The high-resolution X-ray protein structure of the met-McHr L114Y mutant reveals a double conformation of the mutated Tyr114 side chain with clear electron density (blue mesh) seen in the vicinity of the active site, with one conformation directed either toward the di-iron center (brown) and the other toward the putative water tunnel (green) shown in a stereo view.

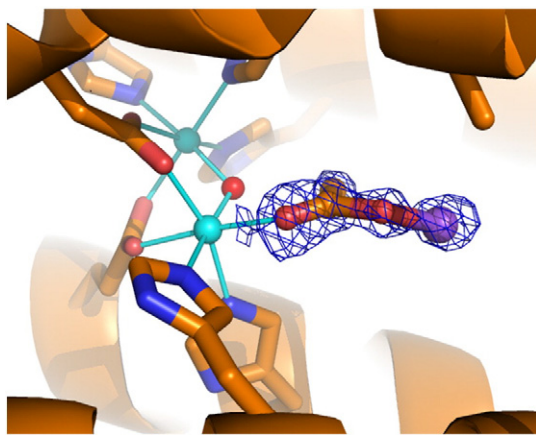


Fig. 7. A putative molecule of sodium acetate (blue mesh) is shown lodged near the non-heme di-iron site, precluding the binding of dioxygen to the di-iron center in the purified L114A met-McHr. The sodium cation is shown in purple. The omitted $2F_o - F_c$ electron density map is contoured at 2.3σ .

is shown in Fig. 8, where we compare the geometries and bond distances of the two Fe atoms with the surrounding residues and O atoms between the different species of hemerythrins. The coordination environments and the iron–ligand bond distances of the μ -oxo bridged di-iron center in McHr are analogous to those found in previous met-Hr

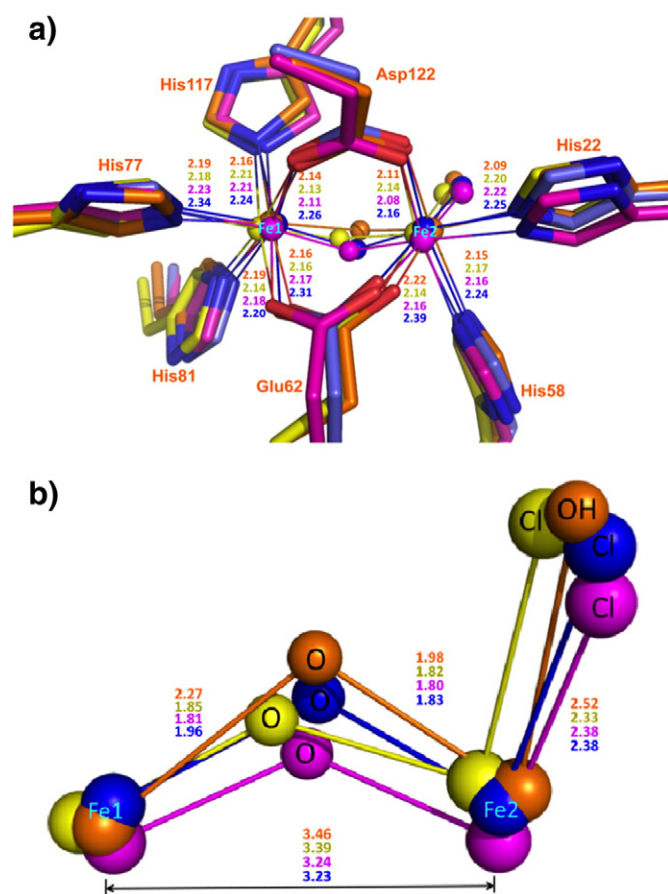


Fig. 8. Superimposed structures of the di-iron site in met-McHr (orange), met-DcrH-Hr from *D. vulgaris* (PDB: 2AWY; yellow), met-myoHr from *T. zostericola* (PDB: 1A7D; magenta) and met-Hr from *P. gouldii* (PDB: 114Y; blue). (a) The geometries and bond distances of the Fe atoms with their associated amino acid residues; and (b) zoom-in views of the Fe–O–Fe linkage at the non-heme di-iron site in the various met-Hrs.

structures (Fig. 8a). Fe1 is six-coordinated, with the ligands His77, His81, His117, Asp122 and Glu62. A hydroxide and the protein ligands His22, His58, Asp122 and Glu62 are associated with Fe2. The Fe1–Fe2 distance (3.46 Å) in WT met-McHr (Fig. 8b) is similar to that in DcrH-Hr (3.39 Å) [7] but it is significantly longer than those found in met-Hrs (3.23 Å and 3.24 Å) from invertebrates [15].

4. Discussion

It is conceivable that the different putative functions of various classes of hemerythrins are reflected in their autoxidation behaviors. A hemerythrin from a marine invertebrate is assumed to possess a dioxygen storage function, employing the non-heme di-iron center to bind O_2 within its vascular system for delivery to tissues when and where needed. Consistent with this function, the autoxidation of the oxy-Hr is extremely slow, with a half-life $t_{1/2}$ of ~ 20 h before it is autoxidized to the met-form [27–29]. In contrast, the autoxidation $t_{1/2}$ of oxy-DcrH-Hr, the hemerythrin-like domain in the fusion protein from *D. vulgaris*, is less than 1 min [10]. In the X-ray crystal structure of this DcrH-Hr, Chan and coworkers have previously identified a water tunnel that might facilitate the rapid autoxidation of the oxy-DcrH-Hr [7]. In addition, they have proposed that this rapid autoxidation is consistent with the function of this hemerythrin-like fusion domain as the dioxygen sensor in the chemotaxis receptor, which utilizes a redox-dependent conformational change to transduce a sensory signal to the neighboring methylation domain. The sensory mechanism could involve simple rotation of the side chain of Leu98 in the tunnel to allow for facile solvent access to the di-iron site.

The autoxidation $t_{1/2}$ of McHr is around ~ 50 min, which is much faster than that of hemerythrin from marine invertebrates, but significantly longer relative to that of DcrH-Hr. In the present study, we have mutated the L114 in McHr to F, Y and A. We find a 12- to 20-fold increase in the autoxidation $t_{1/2}$ of the L114F and L114Y McHr mutants relative to the WT McHr, rendering the autoxidation more akin to the behavior observed for the hemerythrins from marine animals. The observed longer time of autoxidation observed for the L114 McHr mutants suggests that this leucine plays the role of a water valve in the water tunnel of McHr. We surmise that Leu114 is serving as a hydrophobic barrier for passage of a water molecule from the water tunnel to the non-heme di-iron center. From the WT McHr high-resolution protein crystal structure, the diameter of the water tunnel is approximately 3.0–4.5 Å. This size of the water tunnel is wide enough to allow one water molecule to access the di-iron center of the McHr via passage across the Leu114 with the assistance of the hydrophobic repulsive force. There are no water molecules observed inside the water tunnel. The autoxidation rates that we have observed with the L114F and L114Y McHr mutants are consistent with this picture.

Evidently, hemerythrins can participate in dioxygen delivery, storage, or sensing in different organisms without distinct structural changes in the non-heme di-iron center in the various species. Indeed, the main difference between the bacterial Hrs and invertebrate Hrs is the water tunnel in the structural fold of the bacterial Hrs. The water tunnel is not found in invertebrate Hrs [15–17]. In the case of DcrH-Hr and McHr, where there is structural evidence for the water tunnel, the details of the water tunnel are also different (Fig. 9). In DcrH-Hr, the water tunnel passes from the left to right across the center of the protein surface from between $\alpha 2$ and $\alpha 3$ to between $\alpha 1$ and $\alpha 4$ [7,8]. The length of the putative tunnel between the metal center and the protein surface is also relatively short, which should lead to the observed fast rate of autoxidation. Unlike DcrH-Hr, the water tunnel in McHr penetrates just to the di-iron center without running through the two ends of the protein surface. The length of the tunnel is also relatively longer in McHr relative to DcrH-Hr.

In light of the above discussion, the oxy-form of invertebrate Hrs should be stable. Without a water tunnel, they would not be prone to the autoxidation reaction. Thus, a water tunnel is not required for a

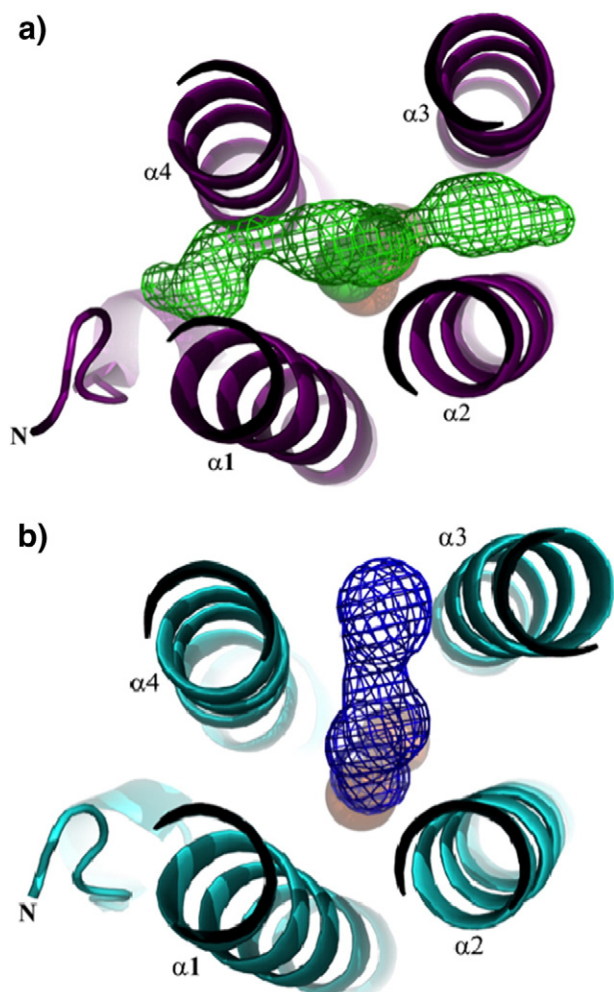


Fig. 9. Comparison of the putative water tunnel between DcrH-Hr and McHr. (a) Met-DcrH-Hr from *D. vulgaris* (PDB: 3AGT; purple), water tunnel is highlighted in green mesh. (b) Met-McHr from *M. capsulatus* (Bath) (light blue), water tunnel is shown in deep blue mesh.

hemerythrin that is functioning merely as storage for dioxygen. All hemerythrin shared the same highly conserved non-heme di-iron center for dioxygen binding. Due to its small size and nonpolar properties, a dioxygen molecule is able to gain access to the non-heme di-iron core of the protein by taking advantage of the micropores in the protein structure. The on-off rates of dioxygen binding to the non-heme di-iron center are rapid regardless of species, and whether or not there is a water tunnel in the hemerythrin. For example, in the case of DcrH-Hr, the k_{on} is $5.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and k_{off} is $\sim 2 \times 10^3 \text{ s}^{-1}$ [30], orders of magnitude faster than the autoxidation rate constant even for this hemerythrin.

Finally, we address the issue of why autoxidation might be built into the design of the McHr. McHr is expressed in the cells of *M. capsulatus* (Bath) when this methanotroph is cultured and grown under high copper to biomass conditions to over-produce the pMMO [12]. At sufficiently high levels of pMMO in the intracytoplasmic membranes of the cell, the turnover of the pMMO becomes limited by the O_2 at the membrane interface, and McHr acts as a carrier to transport O_2 from the cytosol to the pMMO for the turnover of the enzyme [14]. Upon association with the pMMO, the O_2 would be rapidly released and transferred from the oxy-McHr to the pMMO. However, we surmise that when the O_2 is not consumed by the pMMO for methane oxidation for a prolonged period, for example, when there is no hydrocarbon substrate available to the pMMO, a mechanism is built to abort the oxy-McHr by autoxidation. Once converted to the met-form, the McHr can

be dissociated from the pMMO and released from the membrane interface to the cytosol for re-reduction and eventual re-oxidation for another cycle of dioxygen transport and delivery. The autoxidation rate of McHr ($t_{1/2} \sim 50 \text{ min}$) is consistent with this function of the hemerythrin.

Thus, by protein design, nature has evolved various hemerythrin for various functions, demonstrating once again that biochemical unity underlies biological diversity.

Abbreviations

McHr	bacteriohemerythrin in <i>Methylococcus capsulatus</i> (Bath)
pMMO	particulate methane monooxygenase
<i>M. capsulatus</i> (Bath)	<i>Methylococcus capsulatus</i> (Bath)
LB medium	Luria–Bertani medium
TEV protease	tobacco etch virus protease
FPLC	fast protein liquid chromatography
LMCT	ligand-to-metal charge transfer

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jinorgbio.2015.04.001>.

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Appendix A. Supplementary data:

Supplementary material 1:

The bacteriohemerythrin from *Methylococcus capsulatus* (Bath): Crystal structures reveal that Leu114 regulates a water tunnel

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Figure Legends

Fig. A.1. SDS-PAGE analysis of the purified recombinant WT and McHr mutant proteins. Each lane contains 10 μg of the purified McHr proteins (WT, L114F, L114Y, and L114A) in 13.3% SDS-PAGE and is stained by coomassie blue staining to ensure protein purity. The molecular weight markers are indicated on the left column (kDa).

Fig. A.2. F_o-F_c maps showing the extra electron density (blue) in the water tunnel near the non-heme di-iron site in the L114A McHr mutant and fits of the extra electron density with a **(a)** sodium nitrate or **(b)** sodium acetate molecule. The structure refinement gives similar good density fitting with R_{work} and R_{free} values of 15.61 and 18.41% for Na nitrate **(a)** and 15.49 and 18.28% for Na acetate **(b)**, respectively.

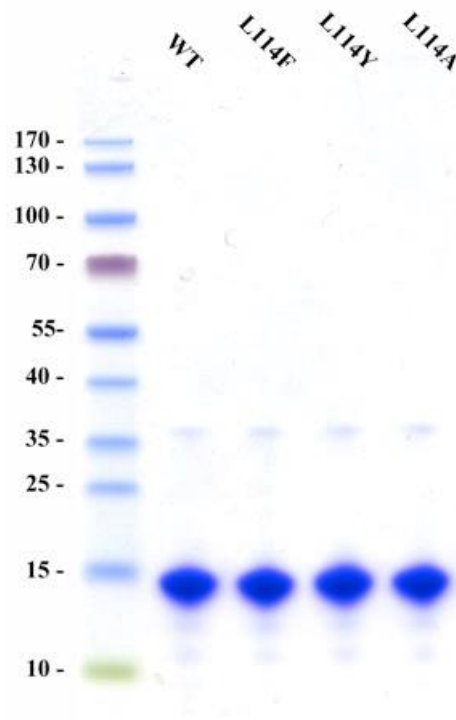


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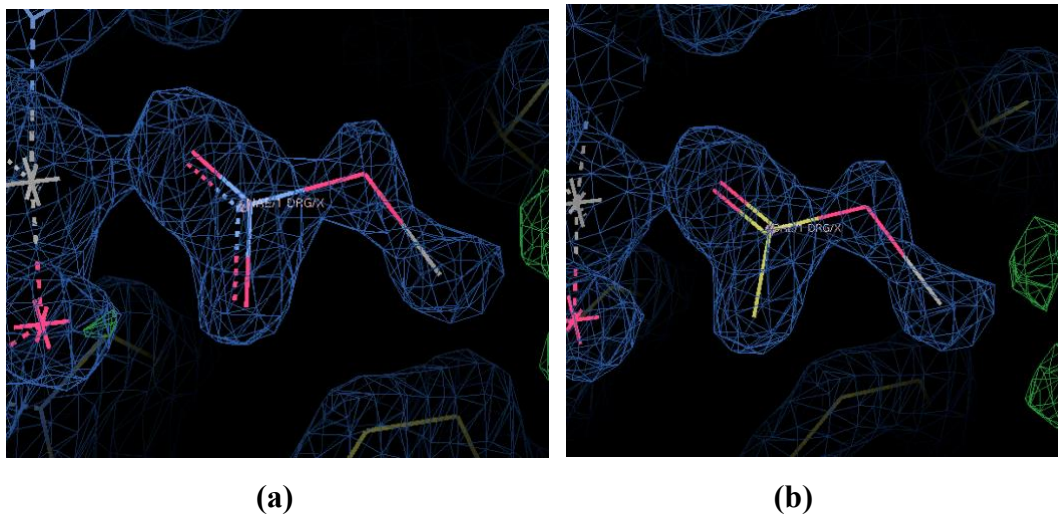


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Table. A.1. The primers used in the production of the L114A, L114F and L114Y mutants of McHr.

Primer	Sequence (5' - 3')
L114A F primer	5'-GGGTATGTGGTTGACCGCCCAGTCCCGTACGAAG-3'
L114A R primer	5'-CTTCGTACGGGACTGGGCGGTCAACCACATACCC-3'
L114F F primer	5'-GGGTATGTGGTTGACGAACCAGTCCCGTACGAAGC-3'
L114F R prime	5'-GCTTCGTACGGGACTGGTTCGTCAACCACATACCC-3'
L114I F primer	5'-GGGTATGTGGTTGACGATCCAGTCCCGTACGAAG-3'
L114I R primer	5'-CTTCGTACGGGACTGGATCGTCAACCACATACCC-3'
L114Y F primer	5'-GGGTATGTGGTTGACGTACCAGTCCCGTACGAAGC-3'
L114Y R primer	5'-GCTTCGTACGGGACTGGTACGTCAACCACATACCC-3'