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Peroxidase-like activity of L29H myoglobin with two cooperative distal histidines on electrode using O_2 as an oxidant

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ABSTRACT

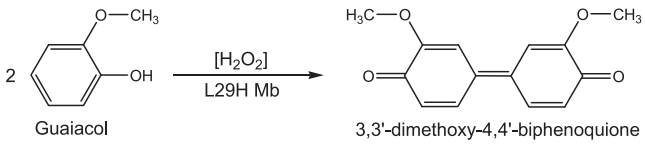
We recently showed that by introduction of a second distal histidine (Leu29 to His29 mutation) in the heme pocket of sperm whale myoglobin (Mb), L29H Mb mutant exhibits an enhanced peroxidase activity compared to wild type (WT) Mb using H_2O_2 as an oxidant. In this study, we further showed that when L29H Mb was immobilized in didecyldimethylammonium bromide (DDAB) on glassy carbon electrode (GCE), it can efficiently reduce O_2 in aerobic buffer solution to H_2O_2, followed by electrode-driven oxidation of guaiacol, a common peroxidase substrate, which is more effective (~3.6-fold at pH = 7.0) than that of WT Mb with a single distal His64. Moreover, the peroxidase-like activity of immobilized L29H Mb was found to be affected by pH values, i.e., the slope of the plot of reduction peak vs. guaiacol concentration was increased from $(0.83 \pm 0.04) \times 10^{-3}$ μA/mM at pH = 8.0 to $(3.08 \pm 0.07) \times 10^{-3}$ μA/mM at pH = 6.0, which suggests that the distal histidines play a key role in reductive activation of O_2 by providing protons. Since there is no native peroxidase with two distal histidines and native peroxidase usually uses H_2O_2 as an oxidant, this study is thus instructive for creating functional enzymes beyond those in nature.

1. Introduction

Myoglobin (Mb) is a well-known heme protein with a function of oxygen storage and delivery [1]. The heme group in Mb is five-coordinated with a proximal histidine (His93), where a distal histidine (His64) can stabilize O_2 binding by forming a hydrogen bond [2]. Moreover, other biological functions, such as peroxidase and nitrite reductase activities, have also been discovered for Mb, which has been drawn much attention recently [3–8]. Meanwhile, the relationship of the structure and multiple functions for Mb is still not fully understood.

Being much smaller and easier to express in high yields, Mb has also been widely used as a scaffold to engineer functional proteins to mimic native enzymes, such as cytochrome *c* oxidase (CcO), nitric oxide reductase (NOR), and nitrite reductase (NIR) [9–18]. Furthermore, by genetic and/or chemical modifications, Mb can also be engineered to exhibit novel functions beyond native enzymes [3,4,9,10,19]. For example, Matsuo et al. showed that an "ultra-native" biocatalyst can be constructed by the combination of a point mutation in Mb and precise design of an appropriate chemically modified heme cofactor [19].

Recently, we have designed a mutant of Mb with two distal histidines, L29H Mb, by mutating a distal Leu29 to His29 and retaining the native distal His64 [20], and resolved its X-ray crystal structure (Fig. 1) [8]. With a second distal histidine, L29H Mb exhibited a ~3.7-fold increased peroxidase activity compared to wild type (WT) Mb, using hydrogen peroxide (H_2O_2) as an oxidant for guaiacol oxidation (Eq. (1)) [20,21]. This new enzyme with two cooperative distal histidines has not been found in native peroxidase, the latter usually has a functional distal histidine–arginine pair [22,23].



(1)

On the other hand, although Mb is not primarily an electron carrier, it exhibits excellent electron transfer properties when immobilized on gold electrodes through modification of the electrode surface by cysteine [24] or modification of the heme group [25], etc. As investigated by Huang and co-workers [26], Mb also influences the electrochemical behavior of other heme proteins such as cytochrome b_5 by forming a protein–protein complex. Moreover, when imbedded in ordered liquid crystal surfactant films, such as

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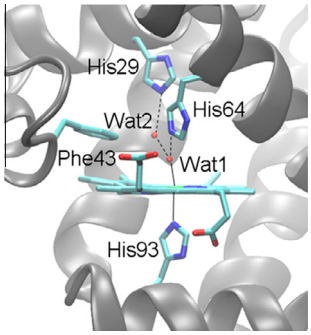

Fig. 1. Crystal structure of L29H Mb (PDB code 4IT8 [8]). The heme distal pocket shows the hydrogen-bonding network (dashed lines) and the coordination bonds (solid lines).

didecyldimethylammonium bromide (DDAB) film with a multiple bilayer structure, on graphite electrodes [27–32] or other chemical modified electrodes [33–39], Mb exhibits excellent electron transfer properties, which provides good models for reactions of the membrane-bound enzyme such as cytochrome P450 that dehalogenates organohalides by reductions and oxidations in living systems [27–32]. In addition, spectroelectrochemistry has also been successfully used to measure the redox potential of Mb as well as its mutants in solution in the presence of organic mediators [15,40,41].

Inspired by these progresses, we are interested in evaluating whether Mb and its L29H Mb mutant could exhibit peroxidase-like activity on electrode using molecular oxygen (O_2), instead of H_2O_2, as an oxidant. It is interesting to explore the ability of a deigned peroxidase to use O_2, as O_2 is normally used by cytochrome P450s for oxidation of organic substances [42], whereas the optimal substrate of peroxidase is H_2O_2 [43]. As shown herein, L29H Mb immobilized in DDAB film on electrode can reduce O_2 in aerobic buffer solution to H_2O_2, followed by electrode-driven oxidation of substrate guaiacol, which is more effective than that of WT Mb with a single distal His64.

2. Materials and methods

2.1. Materials

Plasmid pMbt7-7 containing WT sperm whale Mb gene was provided by Prof. S.G. Sligar and Prof. Y. Lu at University of Illinois at Urbana–Champaign (UIUC). *Escherichia coli.* BL21(DE3) cells were purchased from Sangon Biotech, China. QuickChange Site Directed Mutagenesis Kit was product of Strategene, USA. DEAE and Sephacryl S-100 columns were products of GE Healthcare, USA. Glassy carbon electrode (GCE) and saturated Ag/AgCl electrode were purchased from CorrTest Co., China. DDAB (80%, hydrosol solution) was purchased from Alddin-Reagent Co., China. Guaiacol, KH_2PO_4, KCl, and other chemical were analytic grade.

2.2. Protein preparation

L29H Mb gene was constructed using the QuickChange Site Directed Mutagenesis Kit, which was confirmed by DNA sequencing. WT Mb and L29H Mb mutant were expressed in *E. coli.* BL21(DE3) cells, and were purified by both DEAE and Sephacryl S-100 columns, using a procedure described previously [8,11,20]. The fractions eluted from Sephacryl S-100 column with R/Z (A_{409nm}/A_{280nm}) value greater than 4.8 were collected. The purity and identity were confirmed by SDS-PAGE and electrospray ionized MS (WT Mb, observed: 17 332 ± 1 Da; calculated: 17 334 Da; L29H Mb, observed: 17 353 ± 1 Da; calculated: 17 355 Da).

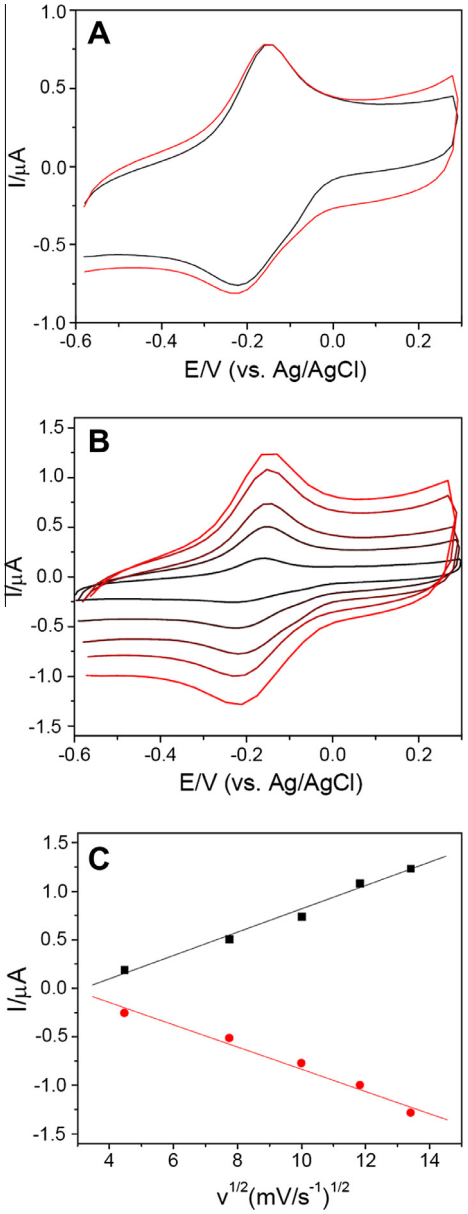

Fig. 2. Redox behavior of L29H Mb. (A) Cyclic voltammograms of L29H Mb–DDAB/GCE (dashed line) and WT Mb–DDAB/GCE (solid line) in N_2-degassed 100 mM KH_2PO_4, 50 mM KCl (pH 7.0) with a scan rate of 100 m V s^{-1}. (B) Cyclic voltammograms of L29H Mb–DDAB/GCE in condition of (A) at different scan rates (20, 60, 100, 140 and 180 m V s^{-1}). (C) The linear dependence of anodic (■) and cathodic (●) peak current vs. scan rate$^{1/2}$.

2.3. Protein-DDAB films preparation

L29H Mb/DDAB and WT Mb/DDAB films were prepared using a similar method as reported previously [27–31]. A 10-μL portion of 50 mM DDAB solution was cast onto the surface of a GCE and dried at 4 °C overnight, then 10-μL portion of 0.2 mM L29H Mb or WT Mb was cast on the ordered DDAB film and dried for a minimum of 12 h at 4 °C before use. The protein concentration was calculated using an extinction coefficient of ε_{409nm} = 157 mM^{-1} cm^{-1} for WT Mb [11] and ε_{409nm} = 155 mM^{-1} cm^{-1} for L29H Mb [8,20], respectively.