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Peroxidase activity enhancement of myoglobin by two cooperative distal histidines and a channel to the heme pocket

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ABSTRACT

To reveal the structure-function relationship of heme proteins, and to provide clues for creating artificial heme proteins with improved functions, we here use myoglobin (Mb) as a model protein, and report that its peroxidase activity can be enhanced by construction of two distal histidines and a channel to the heme pocket. It showed that in addition to a single distal histidine with a suitable distance to the heme iron (Phe43 to His43 mutation), a second distal histidine (Leu29 to His29 mutation) can work cooperatively to increase the turnover number, mimicking the role of well-known His-Arg pair in native peroxidases. Moreover, a channel created to the heme pocket by removal of the native His64 gate (His64 to Ala64 mutation) was shown to facilitate the binding of substrate, resulting in enhanced catalytic efficiency for the triple mutant L29H/F43H/H64A Mb, which is beyond the addition of both double mutants, L29H/H64A Mb and F43H/H64A Mb. These results provide valuable information for elucidating the structure-function relationship of heme proteins. In addition, this study provides clues for design of artificial heme proteins, and the strategy of creating a channel to the heme active center is expected be extended to design of other artificial enzymes with improved catalytic performance.

1. Introduction

Heme proteins perform a large array of biological functions, including electron-transfer, oxygen delivery, catalysis and signaling [1–11]. More fantastically, the same heme protein may exhibit diverse functions in different situations. For example, an electron-transfer protein, cytochrome *c*, shows enhanced peroxidase activity upon interacting with membrane, due to conformational change in the heme active site [2,12,13]. Moreover, the oxygen carrier myoglobin (Mb) (Fig. 1A) [14], was found to exhibit other functions such as peroxidase activity by activation of H_2O_2 [15,16], and nitrite reductase (NIR) activity by reduction of nitrite to nitric oxide [17–19]. Therefore, it is of significance to reveal the structure and diverse functions relationship of heme proteins, which will not only deepen our understanding of how natural proteins work in biological systems, but also provide us clues for creating artificial heme proteins with improved functions.

To address the structure and peroxidase activity relationship of Mb, Watanabe and co-workers found that a single distal histidine in the heme pocket may regulate the peroxidase activity, and a suitable distance of the distal histidine to the heme iron is crucial for the activation of H_2O_2 [15]. In previous study, we showed that introduction of a second distal histidine at position 29 (Leu29 to His29 mutation, L29H Mb, Fig. 1B [20]) can slightly enhance the peroxidase activity of Mb [21]. To address the structure and NIR activity relationship of Mb, we recently found that a single distal histidine (Phe43 to His43 mutation, F43H Mb, Fig. 1C) with a suitable distance to the heme iron, together with mutation of His64 to Ala64, significantly enhances the NIR activity of Mb [22]. The X-ray structure of F43H/H64A Mb (Fig. 1D) showed that by mutating the distal His64 gate to Ala, a water channel to the heme pocket was formed, which plays crucial roles in enhancing the NIR activity of Mb. Meanwhile, whether a channel to the heme pocket is also crucial for the peroxidase activity of Mb remains elusive, although creation of a substrate binding domain is helpful for enhancing the reactivity [16]. Moreover, since a His-Arg pair is found in native peroxidases such as cytochrome *c* peroxidase (CcP) [23] and horseradish peroxidase (HRP) [24], it is not well-understood whether double or

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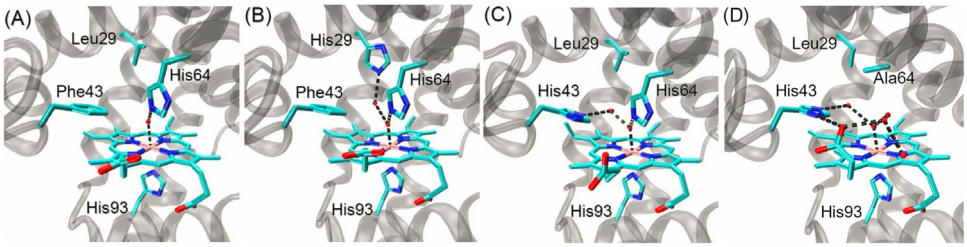

Fig. 1. Crystal structure of the met form of WT Mb (PDB entry code 1JP6), L29H Mb (B, PDB code 4IT8), F43H Mb (C, PDB code 4PQC), and F43H/H64A Mb (D, PDB code 5HLQ), showing the hydrogen-bonding network in the heme pocket.

multiple distal histidines in heme proteins can play a similar role to that of the distal His-Arg pair in native peroxidases.

To address these important issues, we evaluated and compared the peroxidase activities of wild-type (WT) Mb and a series of Mb mutants, including distal histidine mutants (L29H Mb, F43H Mb, L29H/F43H Mb) and channel mutants (L29H/H64A Mb, F43H/H64A Mb, L29H/F43H/H64A Mb). Although we initially constructed these Mb mutants for probing the structure and NIR activity relationship of heme proteins [20,25], we found that these mutants are excellent protein models for probing the structure and peroxidase activity relationship of heme proteins as well. As shown in this study, we found that the peroxidase activity of Mb was enhanced by two cooperative distal histidines and a channel to the heme pocket by removal of the His64 gate, which provides clues for design of functional heme proteins.

2. Materials and methods

2.1. Protein preparation

WT sperm whale Mb was expressed using the Mb gene of pMbt7-7 and purified using the procedure described previously [26]. The L29H Mb, F43H Mb, L29H/F43H Mb, L29H/H64A Mb, F43H/H64A Mb and L29H/F43H/H64A Mb gene were constructed using the QuickChange Site Directed Mutagenesis Kit (Stratagene). The mutations were confirmed by DNA sequencing assay. The proteins were prepared as described in previous study [25]. L29H Mb, F43H Mb, L29H/F43H Mb, and F43H/H64A Mb were expressed in BL21(DE3) and purified using the same procedure for WT Mb [26]. Meanwhile, both L29H/H64A Mb and L29H/F43H/H64A Mb were expressed in inclusion bodies, which were purified using a denaturation-refolding procedure, as described previously [27].

2.2. Peroxidase activity assay

The peroxidase activities of WT Mb, its distal histidine mutants (L29H Mb, F43H Mb, L29H/F43H Mb, L29H/H64A Mb), and channel mutants (L29H/H64A Mb, F43H/H64A Mb and L29H/F43H/H64A Mb), were evaluated on a dual mixing stopped-flow spectrophotometer (SF-61DX2 Hi-Tech KinetAsystTM), using guaiacol and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as two representative substrates and H_2O_2 as the oxidant, respectively. Typically, one syringe contains 2 mM protein (in 100 mM potassium phosphate buffer, pH 7.0) in presence of (0.1–6–mM) guaiacol or (0.03–0.9–mM) ABTS, and the second syringe contains 200 mM H_2O_2, as determined with $\varepsilon_{240nm} = 39.4\ M^{-1}\text{–}cm^{-1}$. The reaction was stated with mixing of equal volume of solutions from the both syringes. The reaction using guaiacol as a substrate was followed by monitoring the change in absorbance of the product at 470 nm, as proposed to be a dimeric form of guaiacol (3,3′-dimethoxy-4,4′-biphenylquinone) [28]. The reaction using ABTS as a substrate was followed by monitoring the formation the $ABTS^{+\cdot}$ cation radical at 660 nm. The initial rate was calculated based on the initial linear changes using an extinction coefficient of $\varepsilon_{470\ nm} = 26.6\ mM^{-1} \times cm^{-1}$ [29], and $\varepsilon_{660nm} = 14.0\ mM^{-1} \times cm^{-1}$ [30], respectively. The curve of initial rates *versus* substrate concentrations was fitted to the Michaelis-Menten equation: $v/[protein] = k_{cat}[substrate]/(K_m + [substrate])$.

3. Results

3.1. Peroxidase activity assay with guaiacol as a substrate

To determine the peroxidase activity of the distal histidine mutants and channel mutants of Mb, we first selected guaiacol as a typical substrate. The initial rate of guaiacol oxidation was measured spectroscopically by monitoring the formation of colored product at 470 nm. The steady-state reactions at various concentrations of guaiacol were then performed. As shown in Fig. 2, the kinetic data of these mutants can be well-fitted to the Michaelis-Menten equation, and the obtained kinetic parameters, k_{cat} (the turnover number) and K_m (the Michaelis constant) are listed in Table 1. The results showed that both L29H Mb and L29H/H64A Mb have similar profiles to that of WT Mb (Fig. 2A), and L29H Mb has an overall catalytic efficiency (k_{cat}/K_m) ~2-fold compared to WT Mb (Table 1). In case of F43H Mb (Fig. 2B), it has ~2.3-fold higher k_{cat} value and slightly decreased K_m value, resulting in ~2.9-fold higher catalytic efficiency. For the channel mutant of Mb, when the His64 gate was removed, the catalytic efficiency was further enhanced for F43H/H64A Mb (~9-fold higher), due to both increase of k_{cat} (~5.3-fold higher) and decrease of K_m (~1.6-fold lower) compared to WT Mb (Table 1). These results agree with that of F43H/H64L Mb in previous study (~11-fold higher) [15], and suggest that a single distal histidine with a suitable distance to the heme iron is important for enhancing the peroxidase activity. It should be noted that in previous study by Sato et al. [31], a larger K_m value (54 ± 15 mM) was determined for WT Mb than in this study (3.53 ± 0.05 mM), and thus a significantly decreased K_m value (0.98 ± 0.30 mM) was observed for H64A Mb. These results were obtained in different experimental conditions such as in 20 mM sodium malonate buffer (pH 6.0), suggesting that the protonation state of distal His64 plays a key role in substrate binding. With the distal His64 removed, other distal histidine in the channel mutants may also fine-tune the substrate binding by hydrogen-bonding interactions, resulting in slightly increased K_m values (Table 1) compared to H64A Mb.

More interestingly, L29H/F43H/H64A Mb was found to exhibit ~12-fold higher k_{cat} value and ~2.2-fold lower K_m compared to that of WT Mb, resulting in ~28-fold higher catalytic efficiency (Fig. 2C). The enhancement is beyond the addition of two double mutants, L29H/H64A Mb (~1.6-fold) and F43H/H64A Mb (~8.9-fold), which suggests that the double distal His29 and His43 work cooperatively in activation of H_2O_2 and in oxidizing guaiacol efficiently. Noted that in case of L29H/F43H Mb with three distal histidines, it showed similar catalytic efficiency to that of L29H/H64A Mb and WT Mb (Fig. 1C). This is likely due to the fact that the three distal histidines form a stable hydrogen-bonding network in the heme distal pocket