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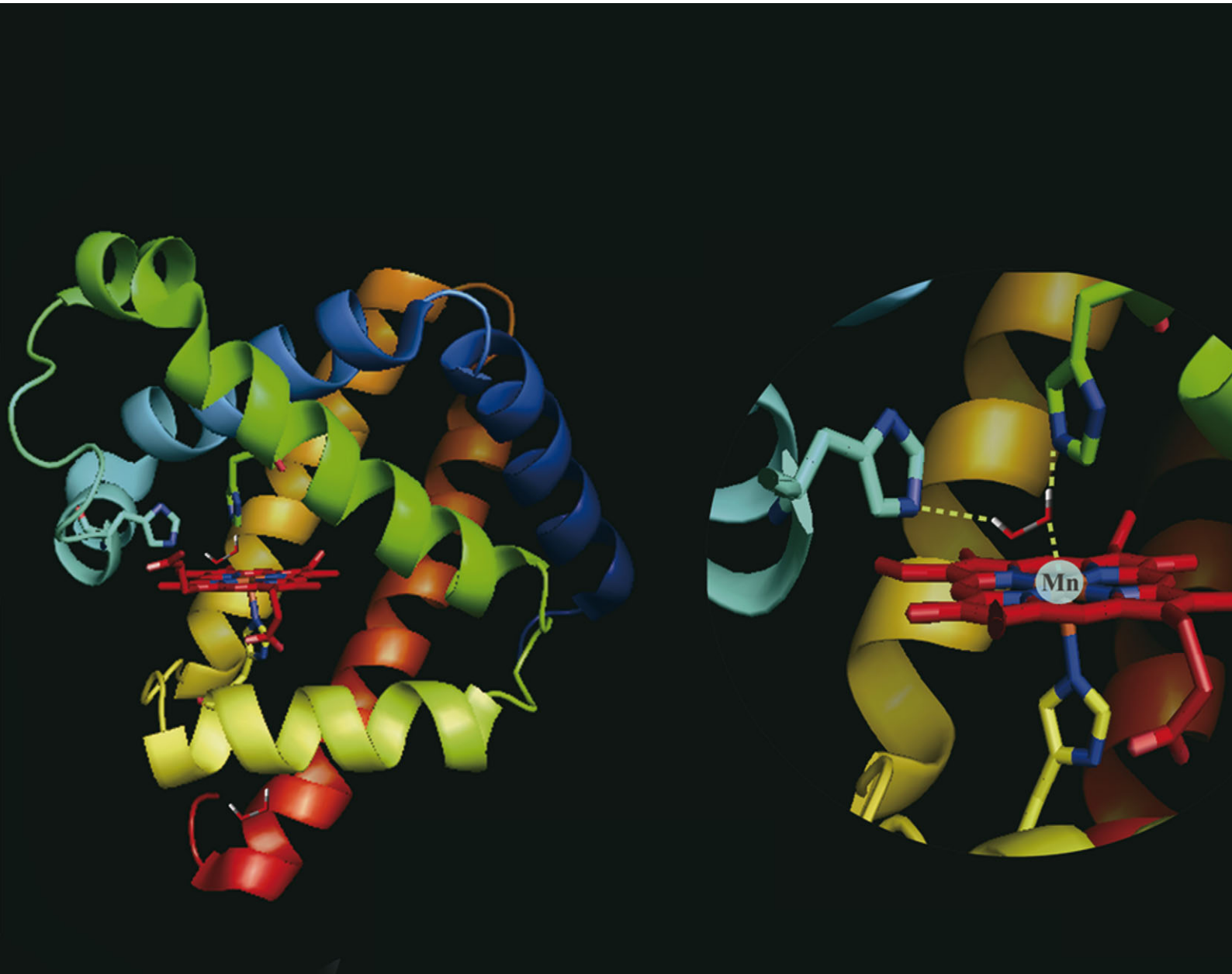

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Effect of distal histidines on hydrogen peroxide activation by manganese reconstituted myoglobin†

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Myoglobins provide an opportunity to investigate the effect of the secondary coordination sphere on the functionality and reactivity of non-native metal porphyrins inside well-defined protein scaffolds. In this work, we reconstituted myoglobin by the replacement of natural heme with manganese(III) protoporphyrin IX and firstly investigated the effect of distal histidine on the reaction of Mn^{III} porphyrin with H_2O_2 and one-electron oxidation of ABTS. We have prepared L29H, F43H, H64F, L29H/H64F, F43H/H64F, L29H/F43H and L29H/F43H/H64F mutants and reconstituted apo-myoglobins with manganese(III) protoporphyrin IX. Distal histidine at the 64 position plays an essential role in binding H_2O_2 through hydrogen bond formation, which facilitates the coordination of H_2O_2 to the Mn center. The second histidine at the 43 position is important in the cleavage of the O–O bond and to form the highly valent Mn(IV)-oxo intermediate. His29 has less efficiency to activate H_2O_2, because it is too far from the Mn center. The cooperative effect of dual distal histidines at positions 64 and 43 on the activation of H_2O_2 was observed and the F43H Mn^{III}Mb mutant exhibited 5-fold and 10-fold reaction rate increases in the activation of H_2O_2 and one-electron oxidation of ABTS *versus* wild-type Mn^{III}Mb. This is different from the distal histidine effect on the H_2O_2 activation by heme in Mb. This work will provide new insights to understand the fundamental chemistry of manganese in oxidation, and further construct biomimetic Mn models for peroxidase, inside or outside of protein scaffolds.

Introduction

Metalloproteins provide ''golden'' examples to illustrate the importance of the secondary coordination sphere, previously proposed by Alfred Werner in 1912, to the reactivity of metal centers.[1–7] To understand the secondary coordination sphere inside protein scaffolds, tremendous efforts have been taken to construct models by chemical synthesis as well as biosynthetic approaches.[2,8–13] In particular, the direct utilization of small, stable, easy-to-produce proteins with the characteristic scaffolds as ''ligands'' to confer the necessary structural features identified in native enzymes attracts considerable attention.[14,15] For example, myoglobins (Mb) have been used as protein scaffolds to mimic heme-containing metalloproteins by site-specific modulation of the proximal and secondary coordination spheres.[16–22] On the other side, apo-myoglobin (apo-Mb) provides an opportunity to investigate the functionality and reactivity of *non-native metal porphyrins* inside the well-defined protein scaffold. For the similar size, charge and configuration of metal prosthetic groups, manganese, cobalt and other metal protoporphyrin IXs have been employed as non-native metal cofactors through the replacement of the native heme.[23–32] Furthermore, expanding the scope of metal-substituted porphyrins to metal porphyrin-like complexes such as metal salens would enrich the realms of artificial metalloproteins together with the related catalysis. Thus, this approach not only provides new insights to understand the fundamental chemistry of metals in aqueous solution, but also advances the knowledge of their coordination chemistry in biomacromolecules.[33–39]

For the importance of manganese and its redox chemistry in biological systems such as photosystem II,[40,41] bacteria catalases,[42] and mitochondrial superoxide dismutase,[43] the chemistry of highly valent oxo-manganese(IV/V) have been extensively studied. Manganese reconstituted heme proteins such as horseradish peroxidase (Mn^{III}HRP),[44–46] cytochrome-*c* peroxidase (Mn^{III}C*c*P),[47,48] microperoxidase-8 (Mn^{III}MP-8),[27,49,50] and myoglobin (Mn^{III}Mb)[17,51,52] have been prepared and, through comparative studies with native peroxidases, showed much lower

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† Electronic supplementary information (ESI) available: UV-vis spectra of all Mn^{III}Mb mutants at pH 5.1. Spectra and kinetic data of reaction with H_2O_2 at pH 7.4 and 5.1. Spectra and kinetic data of ABTS oxidation at pH 7.4. ESI-MS spectra of all mutants. See DOI: 10.1039/c3mt20275e

reactivity toward hydrogen peroxide. The reactions between Mn^{III} protoporphyrin IX and H_2O_2 inside protein scaffolds provided valuable insights for the formation and reactivity of oxo-manganese intermediates in aqueous media. For example, the different ability to form peroxides on iron and manganese centers with H_2O_2 could be referred to different pK_as of Fe^{III}MP-8-OH_2 (9.2 ± 0.9) and Mn^{III}MP-8-OH_2 (11.2 ± 0.3),[50] for the similar pK_as of H_2O and H_2O_2 (14.0 *vs.* 12.0, respectively). Thus, pH plays an important role in the deprotonation/protonation of hydrogen peroxide, which is consequently converted to an active oxo-manganese intermediate. This may explain the lower reactivities of manganese reconstituted heme proteins than that of native heme proteins, which is in accordance with previous reports using water-soluble synthetic model porphyrins such as FeTDCPPS and MnTDCPPS.[53–55]

Despite extensive studies on the reactions of manganese porphyrins with H_2O_2, even performed within protein scaffolds, the influence of the secondary coordination sphere has been rarely considered. According to the studies of Wanatabe and coworkers, the distal heme environment affects the reactivity of native heme with H_2O_2 significantly.[19,21,22,56–58] The fact that distal histidine relocation mutants (H43F/H64L) increase the reactivity by 11-fold, relative to that of the wild-type Mb, encourages us to investigate the effect of distal ligands around manganese protoporphyrin on the reactivity as peroxidase.

Comparison of crystal structures of myoglobin (Mb) and manganese reconstituted myoglobin (Mn^{III}Mb) led us to propose that Mn protoporphyrin adopts a similar location and coordination sphere to that of native heme inside the Mb scaffold.[17,22,56] Thus, following Wanatabe's work, we chose L29H and F43H as the distal histidine with estimated iron–distal histidine distances of 6.6 and 5.4 Å, respectively.[56] It has been demonstrated that the distance for the F43H mutant is appropriate as a general acid/base. To examine the distal histidine relocation, we chose mutants L29H/H64F and F43H/H64F, for the similar size of phenyl ring to imidazole. To examine the accumulative effect of distal histidine, we chose L29H/F43H, in which three distal histidine are inside the Mb scaffold together with His64. The structures of sperm whale myoglobin (Mb) and the corresponding mutants are shown in Fig. 1. Thus, we investigated how the secondary coordination sphere, especially the hydrogen bond network, affects the reactivity of Mn center. Through reactions of Mn^{III}Mb with H_2O_2 (20 equiv.) in pH 7.4, we found that distal environment obviously influence the formation of oxo-manganese(IV) protoporphyrin. His64 is essential for the formation of Mn^{IV}=O, whereas it is of less importance to form an oxo-iron active intermediate for wild-type Mb. More importantly, the additional distal histidine and its location are important to accelerate the formation rates toward H_2O_2. The F43H Mn^{III}Mb mutant exhibits the highest reaction rate (*ca.* 5-fold higher than Mn^{III}Mb) among these mutants, whereas L29H decreases the reactivity compared with that of wild type Mn^{III}Mb. An ABTS assay for the peroxidase reactivity of these mutants demonstrated the same trend of distal histidine effect on the formation of the Mn^{IV}=O intermediate. Thus, in this work, constructing biomimetic Mn models inside the Mb scaffold provides new insights for the secondary coordination sphere effect on the oxidation reactivity of manganese porphyrins in aqueous media.

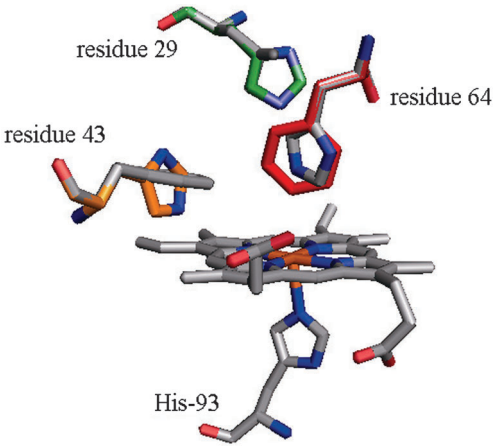


Fig. 1 Structures of the heme pocket of sperm whale myoglobin (gray). The residues mutated in our studies are also shown: His-29 (green), His-43 (orange), and Phe-64 (red). All N atoms were marked in blue. The PDB code for wild-type Mb is 1JP6. Structures of mutants were reproduced by PyMol.

Results

Spectroscopic features of mutated Mn^{III}Mb

Computer modelling was used to predict the location of distal histidines in sperm whale myoglobin that would position the Mn protoporphyrin IX cofactor in nearly the same location as the native heme within the protein pocket (PDB: 1JP6, Fig. 1). Residues selected from the model as potential distal points are Leu29, His64, Phe43. Construction, expression, and purification of myoglobin (Mb) with L29H, F43H, H64F, L29H/H64F, F43H/H64F, L29H/F43H and L29H/F43H/H64F mutants were carried out using procedures reported previously.[59] The residues at sites 29, 43, and 64 of these mutants were described in Table 1. The heme in those Mbs was removed by extraction with butanone.[36,60–62] To incorporate Mn protoporphyrin IX into apo-Mbs, 10 equiv. of Mn protoporphyrin in DMSO solution was added to 0.1 mM apo-Mb solution in 50 mM ammonia acetate buffer (pH = 5.1) at room temperature for 1–2 h. According to the previous reports, the evidence for Mn protoporphyrin incorporating apo-Mb was provided by UV/vis spectra of Mn

Table 1 Residues at site 29, 43, and 64 of wild-type Mb and different mutants

	Residues		
Mutant	Site 29	Site 43	Site 64
Wild-type	Leu	Phe	His
F43H	Leu	His	His
L29H	His	Phe	His
H64F	Leu	Phe	Phe
L29H/F43H	His	His	His
F43H/H64F	Leu	His	Phe
L29H/H64F	His	Phe	Phe
L29H/F43H/H64F	His	His	Phe

protoporphyrin displays two absorption bands at 280 and 376 nm with the ratio of 1 : 1.8.

Mn^{III}Mb and its mutants display characteristic "split" Soret band patterns at λ_{max} 376 and 460–480 nm and Q band at 560–590 nm (Fig. 2a and b). Interestingly, the Soret band at 460–480 nm is sensitive to H64X mutants. Wild-type Mn^{III}Mb exhibited an absorption maximum at 471 nm, whereas H64F Mn^{III}Mb showed a weaker one with a red shift to 486 nm (Fig. 2a). As reported in Fe^{III}Mb, His64 appears to stabilize the heme-bound water, and the loss of water ligation in H64L causes a shift from 408 nm to 393 nm for native heme.[56] In this work, F43H/H64F Mn^{III}Mb displays a "split" band at λ_{max} 471 and 483 nm (Fig. 2a) and L29H/H64F Mn^{III}Mb displays an absorption at 471 nm (Fig. 2b), blue shifted to that of H64F Mn^{III}Mb. The mutants F43H, L29H and L29H/F43H/H64F Mn^{III}Mbs, with two distal histidines, showed blue shifts of 466 and 468 mm, respectively, compared to that of wild-type Mn^{III}Mb (Fig. 2a and b). According to previous reports, the intensity of the second Soret band component at 486 nm is assigned to the distal-pocket uncoordinated water coordinating to the Mn^{III} ion. From our UV-vis spectra of these mutants, distal histidine plays an important role in controlling water binding to the Mn center and tuning the polarity of the distal environment.

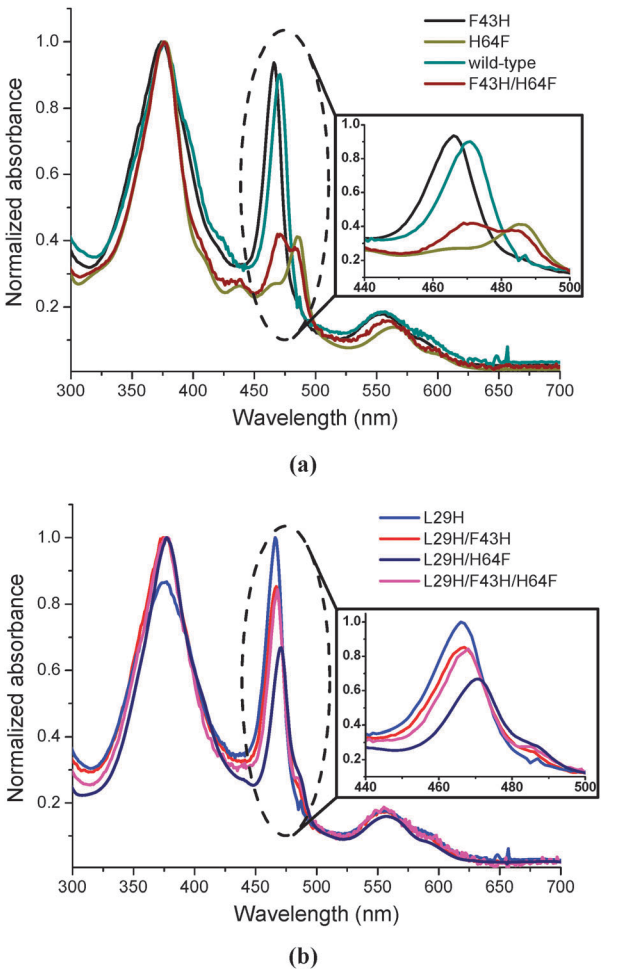


Fig. 2 Normalized absorption spectra of (a) wild-type, F43H, H64F, F43H/H64F Mn^{III}Mb and (b) L29H, L29H/F43H, L29H/H64F, L29H/F43H/H64F Mn^{III}Mb mutants in 50 mM potassium phosphate buffer (pH 7.4). Insets: enlarged spectra from 440 to 500 nm.

Reaction of Mn^{III}Mb mutants with H_2O_2

Since the reaction of wild-type Mn^{III}Mb with H_2O_2 proceeds much slower than that of Fe^{III}Mb, the kinetic studies for the formation of the oxo-Mn^{IV} species could be carried out by UV-vis spectrophotometer. Wild-type Mb reacted with 20 equiv. H_2O_2 to yield the Mn^{IV}=O species, with a decrease in absorption at 376 nm and 471 nm, and the appearance of a single Soret band at 411 nm. This is similar to Mn^{IV}=O hematoporphyrin IX in MP-8 and Mn^{III}HRP (401 and 412 nm, respectively),[44,50] indicating the formation of an Mn^{IV}=O species. The reaction also resulted in a nearly isosbestic conversion to Mn^{IV}=O and the kinetic trace obeyed pseudo first-order kinetics in the incubation with 20 equiv. H_2O_2, but not with 100 equiv. H_2O_2. In the presence of a large excess amount of H_2O_2 (>100 equiv.), the apparent deviation of the trace from single exponential curvature was observed, indicating that more complicated reactions occurred.

As the histidines in the Mb pocket are protonated at pH 5.1 and deprotonated at pH 7.4, the reaction rates of these Mb mutants were determined under pH 5.1 and 7.4. The rate constants k_{obs} were summarized in Table 2. The corresponding spectral changes were shown in Fig. 3 and Fig. S2–S9 (ESI†). From the reactions of Mn^{III}Mbs (pH 7.4), we found that histidine at residue 64 plays a critical role to activate H_2O_2. Removal of His64, H64F Mn^{III}Mb, replacing His64 with Phe, showed no reactivity. Even when the distal histidine was relocated to the positions of 43, or 29, or 43 and 29, the resulting Mn^{III}Mbs showed little reactivity. Interestingly, F43H Mn^{III}Mb, with two distal ligands (His43 and His 64), increases the reaction rate to 0.072 s^{-1}, *ca.* 5-fold than that of wild type Mn^{III}Mb. However, L29H Mn^{III}Mb (His29 and His 64) showed few absorption spectra changes upon the addition of H_2O_2. Increasing the number of distal histidines to 3, the mutant L29H/F43H Mn^{III}Mb exhibited a 2.4-fold higher reaction rate than that of wild-type Mn^{III}Mb. Thus, L29H would inhibit the formation of Mn^{IV}=O intermediate.

Table 2 Rate constants of different mutants with H_2O_2

	Formation of metal-oxo (k_{obs}/s^{-1})	
	Mn^{III}Mb[a]	
Mb	pH = 7.4	pH = 5.1
Wild-type	0.015	0.0078
L29H	N.D.[b]	N.D.[b]
F43H	0.072	N.D.[b]
H64F	N.D.[b]	N.D.[b]
L29H/F43H	0.036	N.D.[b]
L29H/H64F	N.D.[b]	N.D.[b]
F43H/H64F	N.D.[b]	N.D.[b]
L29H/F43H/H64F	N.D.[b]	N.D.[b]

[a] Reactions were performed with 29 μM mutants and 580 μM H_2O_2 in 50 mM potassium phosphate buffer at 20 °C. [b] Reaction rates were too slow to measure.

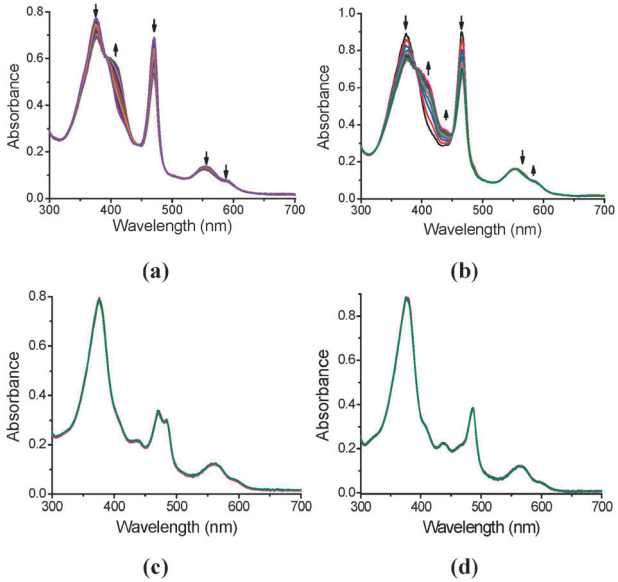


Fig. 3 Spectral changes of (a) wild-type $Mn^{III}Mb$, (b) F43H $Mn^{III}Mb$, (c) F43H/H64F $Mn^{III}Mb$ and (d) H64F $Mn^{III}Mb$ mixing with H_2O_2 at pH 7.4. The arrows in (a) and (b) indicate the directions of absorbance changes.

In addition, only wild type $Mn^{III}Mb$ produced an Mn^{IV}=O intermediate in pH 5.1 and the reaction rate decreased to 0.5 fold that in pH 7.4, as shown in Table 2.

To better illustrate the dual histidines at the 64 and 43 positions accelerating the formation of Mn^{IV}=O intermediate, we plotted the time course (k_{obs}), shown in Fig. 4, for the reactions of different mutated $Mn^{III}Mbs$ (29 μM) with H_2O_2 (580 μM) at pH 7.4, according to the changes of absorbance at 411 nm. This plot clearly showed that all mutants containing H64F didn't show H_2O_2 reactivity, and only F43H and L29H/F43H exhibited higher reactivity than that of wild-type $Mn^{III}Mb$.

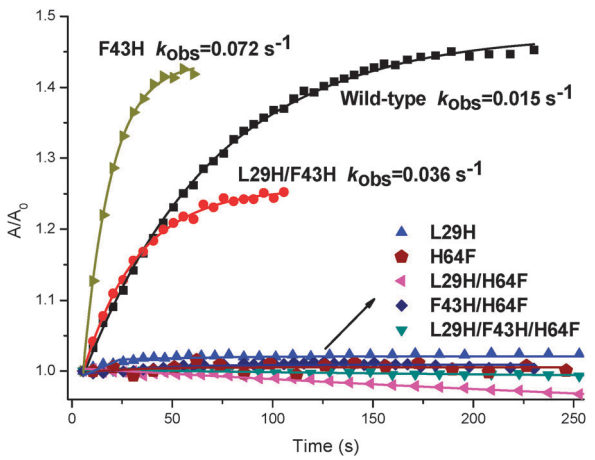


Fig. 4 Time courses of Mn^{IV}=O por formation in the reaction with H_2O_2 for different mutants. A/A_0 refers to ratio of the absorbance and initial absorbance at 411 nm. Reaction conditions: mutants, 29 μM; H_2O_2, 580 μM in 50 mM potassium phosphate buffer (pH 7.4) at 20 °C.

Table 3 Reaction rates (turnover/min) of different mutants with ABTS and H_2O_2 at pH 7.4

Mb	Rate (turnover min^{-1}) $Mn^{III}Mb^a$ pH = 7.4
Wild-type	0.023
L29H	0.0093
F43H	0.24
H64F	0.0012
L29H/F43H	0.15
L29H/H64F	0.0029
F43H/H64F	0.0017
L29H/F43H/H64F	0.018

[a] Reactions were performed with 29 μM mutants, 580 μM ABTS, and 580 μM H_2O_2 in 50 mM potassium phosphate buffer at 20 °C.

ABTS oxidation by $Mn^{III}Mb$ with H_2O_2

To examine the effect of distal histidine on the activity of $Mn^{III}Mb$ as peroxidases, we chose 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as a probe because it serves as a colorimetric indicator for hypervalent metal porphyrin[63–65] or Salen species.[66,67] The experiments for ABTS oxidation were carried out at 20 °C with the ratio of the [catalyst] : [H_2O_2] : [ABTS] being 29 μM : 580 μM : 580 μM (1 : 20 : 20). The reaction rates (TOF, turnover min^{-1}) were obtained by monitoring the change of absorption at 734 nm (ε = 1.5 × 10^4 M cm^{-1}). Table 3 summarizes the reaction rates of the $Mn^{III}Mb$ mutants. F43H $Mn^{III}Mb$ exhibited a ~10 folds higher TOF than that of wild-type $Mn^{III}Mb$ (0.023 min^{-1}). In contrast, the mutant L29H MnMb gave 0.5 fold lower TOF than wild-type $Mn^{III}Mb$. As expected, we could not find any significant activities for the $Mn^{III}Mb$ containing H64F mutant, except for L29H/F43H/H64F which exhibited comparable reactivity to wild type $Mn^{III}Mb$ for dual distal histidines. The changes in the TOF values correlate well with the reaction rates observed for the reactivities of $Mn^{III}Mb$ with H_2O_2, following the trend: F43H > L29H/F43H > wild-type > F43H/L29H/H64F > L29H > H64F, L29H/H64F, F43H/H64F.

Discussion

Previous studies for Mn substitution inside the protein scaffolds such as horseradish peroxidase ($Mn^{III}HRP$), cytochrome-*c* peroxidase ($Mn^{III}CcP$), microperoxidase-8 ($Mn^{III}MP$-8) and myoglobin ($Mn^{III}Mb$) showed the decrease of the reactivity toward H_2O_2 and the related peroxidase activity.[44,45,47–52] This might be interpreted on the basis of unfavorable H_2O_2 binding to the Mn center and the lower pK_a for H_2O_2 deprotonation to a less extent than the Fe center. Thus, increasing the pH of aqueous media becomes an important approach to increase the reactivity of Mn porphyrins, inside or outside of protein scaffolds. Although the effects of the location of distal histidine on the reactivity of native heme toward H_2O_2 inside myoglobin scaffold have been demonstrated by Watanabe and co-workers,[22,56] similar studies on the effect of the secondary coordination sphere on Mn reconstituted heme proteins have been seldom performed. Recently, Lu and coworkers reported the pH dependent

reactivity of an MnSalen cofactor, the analogue to Mn porphyrin, in myoglobin and demonstrated the effect of the distal ligand on binding, orienting and activating H_2O_2.[33] On the other hand, outside the protein scaffold, appending imidazole functional groups to the Salen or porphyrin ligand skeleton has been demonstrated to enhance their reactivity and selectivity toward oxidation. The imidazole functional groups are regarded as the distal or axial ligands to mimic the heme microenvironment in proteins.[2,68] Building on these initial successes, it is desirable to carry systematic studies for the effect of distal environment on Mn^{III} porphyrin at the defined locations inside myoglobin.

Effect of distal histidine on water coordinating to Mn center

Absorption spectra of Mn^{III}Mb mutants revealed the ability of distal His64 to stabilize a water molecule around the Mn center. Although the Mn coordination mode in Mn^{III}Mb is still in debate, the crystal structure of $Mn^{III}(H_2O)$Mb shows a mixture of a six-coordinate species (with axial water; 70% occupancy) and a five-coordinate species (without axial water; a non-bonded water refined to 30% in the distal pocket). Two conformations for the distal His64 residue refined to the corresponding 70% and 30% occupancies. Thus, this water is stabilized by hydrogen-bonding with the distal His64 residue. However, for the lower coordination ability of water to the Mn center, a five-coordinate species could also be observed, even though with a hydrogen bond forming between the distal His64 residue and water. Therefore, the mutant H64F MnMb showed the characteristic Soret band at 486 nm with low intensity, which might be assigned to be a five-coordinate Mn^{III} porphyrin.

Changing the location of the distal histidine from the 64 to 43 position, F43H/H64F Mn^{III}Mb showed a "split" of the second Soret band and an absorbance blue shift to 471 nm, indicating that the hydrophilicity of the distal environment increases. Another mutant L29H/H64F Mn^{III}Mb showed a blue shift of the second Soret band but with less intensity, compared with that of H64F Mn^{III}Mb, suggesting the distal histidine location resulted more hydrophilicity. This minor difference in the second Soret band may be explained by the crystal structures of L29H/H64L and F43H/H64L Mb, in which the distance between L29H and the water (2.67 Å) is shorter than that of F43H and water (2.75 Å).[22] Nevertheless, the distal histidine at 64 position could better stabilize a six-coordinate Mn por with an axial water than that at the 43 and 29 positions, which is different from the effect of the location of distal histidine residues on the heme-bound water in Mb mutants.

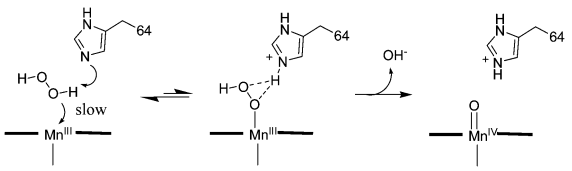
Interestingly, increasing distal histidine could facilitate a water binding Mn center and form a six-coordinate Mn porphyrin. From the absorption spectra of F43H, L29H, L29H/F43H and L29H/F43H/H64F Mb, we found a small blue shift (1–2 nm) of the second Soret band compared to the wild-type Mn^{III}Mb, suggesting the importance of hydrogen bond formation with distal His64 to water coordinating to the Mn center.

Effect of distal histidine on the reaction with H_2O_2 activation

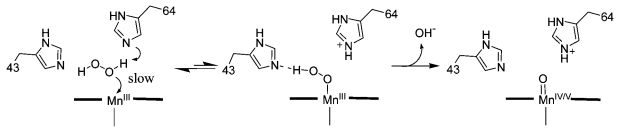
The reactivity of Mn por with H_2O_2 is largely dependent on the existence, location and number of the distal histidine. H64F Mn^{III}Mb with no distal histidine has no reactivity with H_2O_2. This is not surprising for low coordination ability of H_2O_2 to Mn center in the absence of His64, which was previously observed in MnSalen inside H64F Mb.[33] Changing the locations of distal histidine from the 64 to 29 or/and 43 positions does not show the reactivity toward H_2O_2. Thus, it is apparent that His64 in wild-type Mb plays an essential role to enhance the reaction with H_2O_2, as shown in Scheme 1.

The mutation of Phe43 to His43, F43H Mn^{III}Mb, results in 5-fold higher reactivity than wild-type Mn^{III}Mb; whereas L29H Mn^{III}Mb showed no reactivity. This indicates that an appropriate choice of the second distal histidine is important to the acceleration of the O–O bond cleavage of the Mn-bound peroxide. From the crystal structures of the ferric L29H/H64L, F43H/H64L Mb and wild-type Mb, we found that the distance of His29 from the metal ion center is *ca.* 6.6 Å, which is farther from the Mn porphyrin than that of His43 (5.7 Å) and His64 (4.3 Å). This indicates that His29 in the mutant facilitates the reaction less efficiently than His64 and His43 in wild-type and F43H Mb. For the F43H mutant, the distal His43 has a similar distance from the heme iron compared with peroxidases and could form a hydrogen bond with the H_2O_2 coordinated to the Mn center, which may enhance the O–O bond cleavage and showed a *ca.* 5-fold rate increase in the reaction with H_2O_2 *versus* wild-type Mn^{III}Mb. For the short distance to the Mn center, His64 makes a direct hydrogen bond with the Mn-bound H_2O_2, which stabilizes the coordination of H_2O_2. However, for the possible interaction of the distal histidine between both oxygen atoms of the Mn-bound peroxide, His64 does not facilitate the charge separation, as suggested for the role of the wild-type Mb.[56] Thus, as shown in Scheme 2, for Mn reconstituted Mb, His64 is critical to bind H_2O_2 and His43 facilitates the activation of H_2O_2 as a base to promote the O–O bond cleavage.

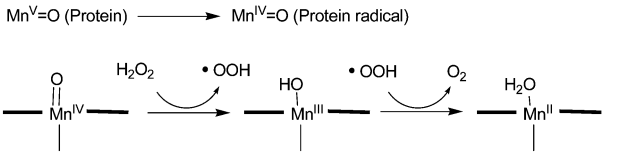
This cooperative effect of dual distal histidines had not been observed in the reaction between iron and H_2O_2 in native Mb and the mutated Mbs.[56] The difference between Fe^{III}Mb and Mn^{III}Mb was supposed due to the different coordination and deprotonation abilities of H_2O_2 coordinated to Fe^{III} and Mn^{III}. Harriman and co-workers have reported the first pK_a of coordinated water for Fe^{III} and Mn^{III} porphyrin complexes as 6.6 and 8.0, respectively.[69] In the protein scaffold, the same order has been reported by Veeger and coworkers.[50] The pK_a of metal-bound water for Mn^{III}MP-8 was 11.2, two units higher than that for Fe^{III}MP-8 (pK_a = 9.2). The higher pK_a for Mn^{III} indicated that H_2O deprotonated with more difficulty when it is coordinated



Scheme 1 The proposed mechanism of Mn^{IV}=O porphyrin formation and possible interactions between His64 and hydrogen peroxide in wild-type Mn^{III}Mb.



Scheme 2 The proposed mechanism of Mn^{IV}=O por formation and possible interactions between His-43/His-64 and hydrogen peroxide in F43H mutant.



Scheme 3 The proposed mechanism of Mn^{II} por formation from high valent Mn^{IV} or Mn^{V} species in the presence of large excess amount of H_2O_2.

to Mn^{III} than to Fe^{III}. Similar to H_2O, we proposed that the deprotonation of H_2O_2 is important to coordinate to Mn^{III}. It had been reported that the coordination of H_2O_2 to Mn^{III}Mb was less favorable than that to Fe^{III}Mb,[52] which supports our consideration.

Effect of distal histidine on peroxidase activity

The peroxidase activities of Mn^{III}Mb were measured for the 1-electron oxidation of ABTS, which roughly correlates with the reactivities of Mn^{III}Mbs with H_2O_2 (Tables 1 and 2). However, the 10-fold increase in F43H Mn^{III}Mb *versus* wild-type Mn^{III}Mb is greater than the 5-fold improvement in the reactivity of the F43H Mb with H_2O_2. Given the fast reaction between ABTS with high valent Mn^{IV} or Mn^{V} intermediates through one-electron oxidation in the presence of a large excess amount of the substrates, the rate-determining step for the one-electron oxidation is the formation of high valent Mn^{IV} or Mn^{V} intermediates. Thus, the difference between the reaction rates with H_2O_2 and one-electron oxidation of ABTS indicates the possibility of reactivity of H_2O_2 with high valent Mn^{IV} or Mn^{V} intermediates. Indeed, in the presence of a large excess amount of H_2O_2 (>100 equiv.), we observed the formation of Mn^{II} species in UV-vis spectra, which exhibit the absorption at 440 nm for the F43H mutant (seen in Fig. S1, ESI†). In accordance with the previous reports for Mn^{V}=O (ref. 70) and Fe^{IV}=O in protein scaffolds with excess H_2O_2,[71] we proposed the mechanism of Mn^{II} formation as described in Scheme 3.

Conclusions

In summary, we have prepared L29H, F43H, H64F, L29H/H64F, F43H/H64F, L29H/F43H and L29H/F43H/H64F mutants to clarify the effects of distal histidines to activate H_2O_2 by Mn protoporphyrin IX. The modulation of distal environment is achieved through hydrogen bonding interactions by the distal ligand through perturbation of H_2O_2 protonation/deprotonation equilibrium. Distal histidine at the 64 position plays an essential role to bind H_2O_2 through hydrogen bonding formation, which facilitates the coordination of H_2O_2 to Mn center. The second distal at the 43 position is important for cleavage of the O–O bond and forming the high valent Mn-oxo intermediate. This cooperative effect of dual distal histidines on the activation of H_2O_2 by Mn porphyrin inside protein scaffold is firstly observed. As a consequence, through tuning the distal histidine to bind and orient H_2O_2, the reactivity to activate H_2O_2 exhibits significant changes. The F43H Mn^{III}Mb mutant exhibited 5-fold and 10-fold increases in reaction rates in the activation of H_2O_2 and one-electron oxidation of ABTS *versus* wild-type Mn^{III}Mb. In our group, we will continue to investigate the activation of reactive oxygen species (ROS) by metal porphyrins through modulating the secondary coordination spheres of protein scaffolds, which would help us to understand the "trick" of nature to activate small molecules and further provide new insights to develop biomimetic catalysis.

Experimental section

General

Chemicals such as protoporphyrin IX, H_2O_2, and ABTS were purchased from Alfa Aesar and used without further purification. Manganese(III) protoporphyrin IX was prepared according to the reported method.[45,72] ESI-MS spectra were recorded with Bruker Apex IV FTMS spectrometer. UV-vis spectra were recorded with Agilent 8453 UV-Vis spectrometer.

Preparation of proteins

Mutations at desired locations were introduced *via* a polymerase chain reaction-based technique. Heme proteins were purified as described in previous reports.[33,59,73] The extraction of heme and reconstitution of manganese(III) protoporphyrin IX to apo-myoglobin were according to the literature.[36,51,60–62] All mutants were verified by ESI-MS.

Kinetic studies with H_2O_2

The reactions were initiated by 20 equiv. H_2O_2 in 50 mM potassium phosphate buffer, detected by UV-vis spectrometer. In the reaction, 580 μM H_2O_2 was added to 29 μM protein solution, the reaction temperature was 20 °C. The kinetic traces at 411 nm for Mn^{IV}=O Por were used for calculating the pseudo first-order rates k_{obs} by fitting to a single-exponential eqn (1):

$$y = y_0 + a(1 - e^{-x/t}) \quad (1)$$

where x is reaction time, and y is the absorbance at λ = 424 nm. $k_{obs} = -1/t$.

ABTS oxidation

Proteins were mixed with 20 equiv. ABTS in 50 mM potassium phosphate buffer. The reactions were initiated by adding 20 equiv. H_2O_2, monitored at 734 nm with a UV-vis spectrometer. Reaction conditions: 29 μM protein, 580 μM ABTS, and 580 μM H_2O_2 was mixed at 20 °C. The turnover numbers for ABTS were calculated at 734 nm (ε = 1.5 × 10^4 M cm^{-1} for $ABTS^{+\bullet}$).[74]

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