Cytochrome *c* Peroxidase-Like Activity of Metmyoglobin Modified with Diethylenetriaminepentaacetic Acid

Keiichi Tsukahara* and Kimiko Kiguchi
Department of Chemistry, Faculty of Science, Nara Women's University, Nara 630-8506

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The oxidation of the reduced form of cytochrome *c* by hydrogen peroxide was catalyzed through a diprotein complex by metmyoglobin modified with diethylenetriaminepentaacetic acid, whose activity was higher than that of native metmyoglobin.

Cytochrome *c* peroxidase (CcP) is a heme enzyme which catalyzes an oxidation of the reduced form of cytochrome *c* (cyt *c*(II)) by hydrogen peroxide. The active site of CcP has iron(III) protoporphyrinIX which coordinates His imidazole and a water molecule, as well as that in metmyoglobin (metMb).[1] Therefore, metMb also shows the CcP activity, but the reaction is much slower than that of CcP resulting in cross-linking and heme degradation.[2] The myoglobin heme pocket differs from CcP in the following ways; myoglobin lacks the distal His-Arg pair essential for efficient peroxide cleavage and the strong proximal H-bonding network involving His175, Asp235, and Trp191 in CcP is missing.[1] A recent mutation technique has demonstrated the possibility of the conversion of metMb into a peroxygenase by replacement of the amino acid residue(s) of the heme environment.[3] Moreover, CcP binds cyt *c* to form a diprotein complex,[4] followed by a rapid electron transfer, while metMb cannot bind cyt *c*. We have recently reported that zinc-substituted myoglobin, whose lysine residue is modified with diethylenetriaminepentaacetic acid (DTPA), binds cyt *c*(III) followed by an *intra*complex photoinduced electron-transfer quenching.[5] In this work we report that the formation of a diprotein complex of metMbDTPA with cyt *c*(II) accelerates the oxidation of cyt *c*(II) by H_2O_2.

Horse heart metMb was treated with five-fold of DTPA dianhydride in a 0.1 M Tris/HCl buffer at pH 8.6 (M = mol dm^{-3}). After purifying the modified myoglobin by a column chromatography, one of the isomers of the singly modified metMbDTPA (F-4)[5,6] was subjected to kinetic measurements. The solution of purified cyt *c*(III) was reduced by sodium ascorbate in excess, which was then removed by dialyzing with a 0.01 M phosphate buffer under a nitrogen atmosphere. The solution containing metMbDTPA and cyt *c*(II) was mixed with the solution of H_2O_2 and then the reaction was followed at 549 nm spectrophotometrically. The initial rate method was applied to determine the kinetic parameters. The experimental conditions are as follows: $[\text{metMbDTPA}]_0 = (0—5.0) \times 10^{-6}$ M, $[\text{cyt } c(\text{II})]_0 = (0—5.0) \times 10^{-5}$ M,

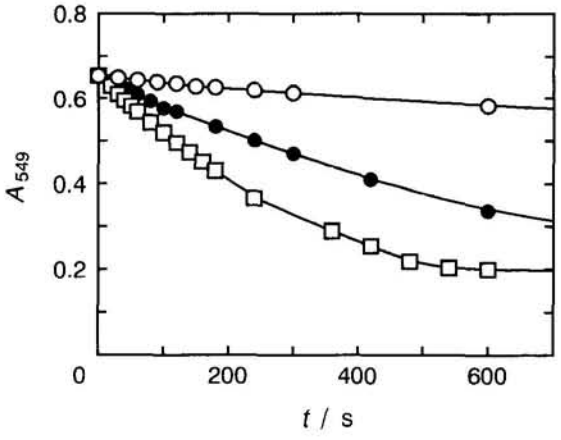


Figure 1. Decay of cyt *c*(II) in the oxidation of cyt *c*(II) (2.5×10^{-5} M) by hydrogen peroxide (5.0×10^{-5} M) monitored at 549 nm in the absence of metMb (○) and in the presence of 1.0×10^{-6} M native metMb (●) and 1.0×10^{-6} M metMbDTPA (□) at 25 °C, pH 7.0 (0.01 M phosphate buffer), and I = 0.02 M.

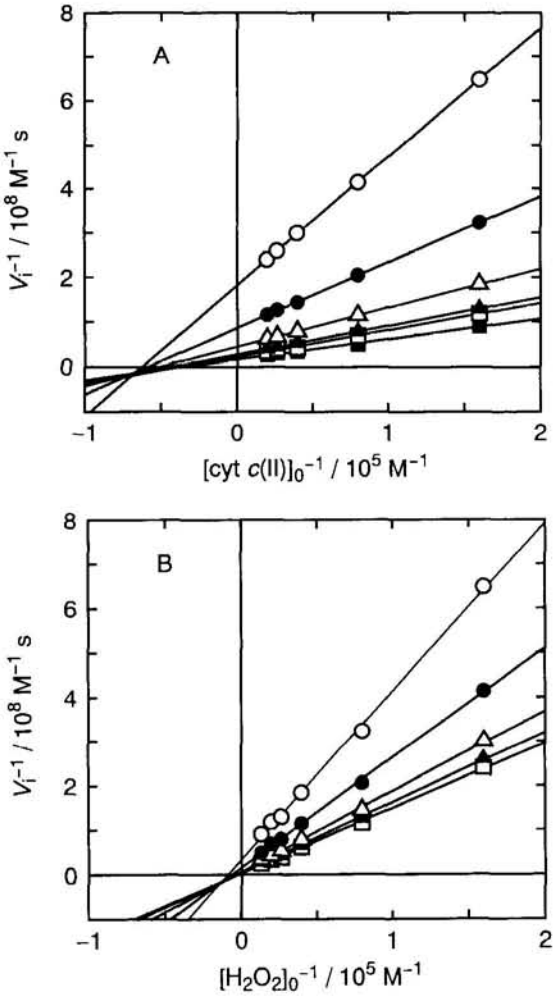
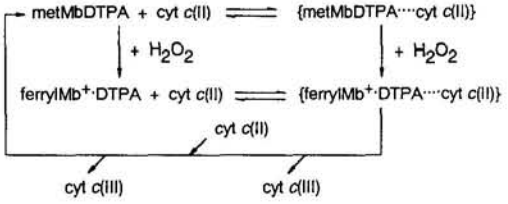


Figure 2. Double reciprocal plots of the initial rate (V_i) against the concentrations of cyt *c*(II) (A) and H_2O_2 (B) for the oxidation of cyt *c*(II) by H_2O_2 catalyzed by metMbDTPA (1.0×10^{-6} M) at 25 °C, pH 7.0 (0.01 M phosphate buffer), and I = 0.02 M. The concentrations of the reactants are 6.3×10^{-6} M (○), 1.3×10^{-5} M (●), 2.5×10^{-5} M (△), 3.8×10^{-5} M (▲), 5.0×10^{-5} M (□), and 7.5×10^{-5} M (■), respectively.

$[H_2O_2]_0$ = (0.6 — 5.0) × 10^{-5} M at 25 °C, pH 7.0 (a 0.01 M phosphate buffer), and an ionic strength (I) of 0.02 M.

Although the reaction of cyt c(II) with H_2O_2 was very slow, metMbDTPA catalyzed the oxidation of cyt c(II) by H_2O_2 and the rate of reaction was faster than that for native metMb (Figure 1). On the basis of a 2:1 stoichiometry, the initial rate (V_i) was determined from the initial slope of the change in absorbance vs. t ($-\Delta A/\Delta t$) divided by $2\Delta\varepsilon l$, where $\Delta\varepsilon$ and l are the difference in molar absorption coefficients between cyt c(II) and cyt c(III) at a given wavelength and the optical cell length, respectively. The V_i was saturated with increasing the initial concentrations of H_2O_2 ($[H_2O_2]_0$) for both native metMb and metMbDTPA, indicating that both myoglobins form the complex with H_2O_2. The V_i was also saturated with increasing the initial concentrations of cyt c(II) ([cyt c(II)]$_0$) for metMbDTPA, although for native metMb the linear dependence of V_i on [cyt c(II)]$_0$ was observed, indicating the formation of a diprotein complex of metMbDTPA, not for native metMb, with cyt c(II). Figure 2 shows Lineweaver-Burk plots for two substrates at constant concentrations of H_2O_2 (A) and cyt c(II) (B), respectively. An intersection was observed at a left-hand side of the V_i^{-1} axis for both plots. Moreover, H_2O_2 interacts with both metMb and metMbDTPA to form ferrylMb in the presence or absence of cyt c(II). The rate of the formation of ferrylMb and ferrylMbDTPA was found to be comparable to that of the catalyzed reaction: 1.5 × 10^2 M^{-1} s^{-1} for native metMb and 9.4 × 10 M^{-1} s^{-1} for metMbDTPA. FerrylMbDTPA containing a protein radical (ferrylMb$^+$·DTPA), which was produced by the reaction of metMbDTPA with H_2O_2 and by removing excess H_2O_2 by catalase, was found to react with cyt c(II) 20-fold faster than ferrylMb$^+$· derived from native metMb. Therefore, the following mechanism might account for the present experimental results:[7-9]



The following equation is derived from the above mechanism,

$$1/V_i = 1/V_{max} + K_S^{H_2O_2}K_M^{cyt}/V_{max}K_S^{cyt}[H_2O_2]_0 + K_M^{cyt}/V_{max}[cyt c(II)]_0 + K_S^{H_2O_2}K_M^{cyt}/V_{max}[H_2O_2]_0[cyt c(II)]_0 \quad (1)$$

At the constant concentrations of H_2O_2 and cyt c (II), Eqs. 2 and 3 are derived, respectively,

$$1/V_i = (1+K_S^{H_2O_2}K_M^{cyt}/K_S^{cyt}[H_2O_2]_0)/V_{max} + K_M^{cyt}(1+K_S^{H_2O_2}/[H_2O_2]_0)/V_{max}[cyt c(II)]_0 \quad (2)$$

$$1/V_i = (1+K_M^{cyt}/[cyt c(II)]_0)/V_{max} + K_S^{H_2O_2}K_M^{cyt}(1+K_S^{cyt}/[cyt c(II)]_0)/V_{max}K_S^{cyt}[H_2O_2]_0 \quad (3)$$

Here, V_{max} is a maximum rate at the saturated concentrations of both substrates, K_S^{cyt} and $K_S^{H_2O_2}$ are the dissociation constants of the complexes of metMbDTPA with cyt c(II) and H_2O_2, respectively, and K_M^{cyt} is the concentration of cyt c(II) required at a half of V_{max} when the saturated concentration of H_2O_2 is present. From the slopes and intercepts of the plots in Figure 2, we obtained $K_S^{H_2O_2}$ = (7.7 ± 1.0) × 10^{-5} M, K_S^{cyt} = (1.5 ± 0.2) × 10^{-5} M, and V_{max} = (5.0 ± 0.5) × 10^{-7} M s^{-1} for 1.0 × 10^{-6} M metMbDTPA and $K_S^{H_2O_2}$ = (8.6 ± 1.0) × 10^{-5} M, $1/K_S^{cyt} \approx 0$, and V_{max} = (1.0 ± 0.1) × 10^{-7} M s^{-1} for native metMb under the same experimental conditions. The V_{max} was also dependent on the concentrations of metMb. Therefore, we can conclude that higher activity of metMbDTPA compared with native metMb arises from its larger V_{max} and high affinity for cyt c(II), although the affinity for H_2O_2 is similar to each other.

In conclusion, metMbDTPA, which is an acidic protein derived from native metMb, shows CcP-like activity for the oxidation of cyt c(II) by H_2O_2 through the formation of a diprotein complex. We are now determining the modified site of metMb with DTPA to elucidate the relationship between the enzyme-mimic activity of metMbDTPA and the binding site of a diprotein complex of myoglobin with cyt c(II).

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References and Notes

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6 Analysis of the unmodified Lys residues by using sodium 2,4,6-trinitrobenzene-1-sulfonate revealed that one of the Lys residues of metMb is modified with DTPA. Moreover, SDS-polyacrylamide gel electrophoresis of metMbDTPA showed only one band.
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8 One-electron reduced ferrylMbDTPA (compound II) might react with the second cyt c(II) with similar affinity and faster rate.
9 The oxidation of cyt c(II) by H_2O_2 also occurred slowly and the initial rate was corrected by subtracting the initial rate of the reaction of cyt c(II) with H_2O_2.