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Engineering of *Thermus thermophilus* Cytochrome c_{552}: Thermally Tolerant Artificial Peroxidase*

Hiroshi Nakajima,[a] Yusuke Ichikawa,[a] Yuh Satake,[a] Nobuyuki Takatani,[a] Soumen Kanti Manna,[b] Jitumani Rajbongshi,[b] Shyamalava Mazumdar,[b] and Yoshihito Watanabe*[c]

Application of peroxidases as catalysts for the oxidation of a wide spectrum of organic compounds has been a major focus in industrial sectors such as food processing, bioremediation, and biorefinement of oil[1] because they use hydrogen peroxide (H_2O_2), an environmentally low-load oxidant. The full-scale use of peroxidases is, however, restricted by poor thermal and environmental stability, as is the case with many commercially available enzymes. Therefore, peroxidases with improved thermal stabilities are highly desired. Their detection in thermophilic bacteria by screening techniques is a popular approaches to finding thermally tolerant enzymes, and several such peroxidases have been isolated and characterized.[2,3] Although these peroxidases show remarkable stabilities and enzymatic activities at elevated temperatures in their host strains or cell lysates, the properties of the purified enzymes are still not sufficient for industrial applications.[3] Some of the enzymes undergo rapid loss of activity in the presence of H_2O_2 over physiological levels. Partial compensation for the reduced thermostabilities and activities of the purified enzymes might be achievable by random mutagenesis achieved through evolutionary engineering.[4] However, there are difficulties in elucidating the obtained variations in chemical terms, which prevents further tuning to improve the desired properties.

Here we demonstrate that cytochrome c_{552} (Cyt c_{552}) from *Thermus thermophilus* HB8 can be transformed into a thermally stable artificial peroxidase by rational modification based on the molecular mechanisms of natural peroxidases. Cyt c_{552} is an electron transfer protein containing His15 and Met69 as heme axial ligands, and it has characteristically high stability against thermal denaturation.[5] In this study, two amino acid residues in Cyt c_{552} were chosen for mutagenesis in line with the peroxidase mechanism and three-dimensional structure of the protein (PDB ID: 1C52).[6] One was Met69, which was replaced with alanine in order to provide a reaction site immediately above the heme iron (M69A). Another was the mutagenesis of Val49 to aspartic acid (V49D), which would be expected to introduce general acid–base catalyst, a fundamental element of the peroxidase mechanism, in the heme cavity. Although weak peroxidase activity is a natural property of cytochrome *c*,[7] a variant bearing both mutations (M69A/V49D) exerts enhanced activity, which is observed neither for the wild-type nor for variants bearing only the M69A mutation.

Figure 1 shows the temperature dependence of CD spectra recorded at 222 nm for the Cyt c_{552} variants (M69A/V49D and M69A) together with the wild-type and the H64D myoglobin variant (Mb H64D), an engineered sperm whale myoglobin

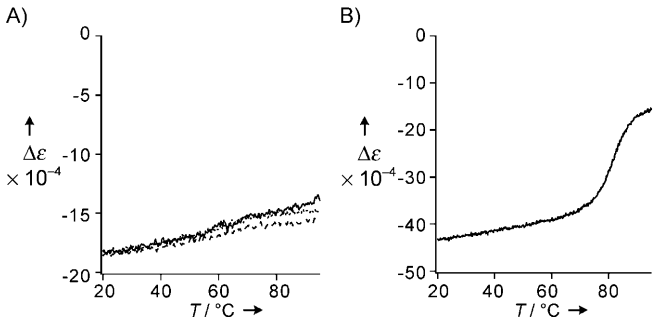

Figure 1. A) Temperature dependence of CD spectra recorded at 222 nm in a temperature range of 20 to 95 °C. ——: M69A/V49D. ····: M69A. -----: Cyt c_{552} wild-type. B) Mb M64D. Each protein was dissolved in MES/NaOH (20 mM, pH 5.0) buffer at a concentration of 10 μM.

that shows the highest enzymatic activity among artificial peroxidases.[8] Like the wild-type, the Cyt c_{552} variants showed gradual decreases in their ellipticities with rising temperature, while no obvious melting points were obtained throughout the measurement. This is in contrast to Mb H64D, which has an apparent melting point at about 82 °C. The mutagenesis applied in this study thus scarcely influences the conformational stability of Cyt c_{552}.

Although the Cyt c_{552} variants are stable with regard to thermal denaturation, the heme in M69A/V49D switches its coordination structure depending on temperature. Figure 2 shows the UV/Vis spectra of the Cyt c_{552} variants recorded every 10 °C between 20 and 90 °C. M69A shows Soret and small bands at 398 and 625 nm, respectively. Both bands are characteristic of pentacoordinated high-spin heme, consistently with the removal of the original sixth ligand, Met69. The spectrum of M69A is largely independent of the temperature change, except for slight hypochromism of the Soret band at higher temperatures. On the other hand, the spectral features of M69A/V49D change clearly above 70 °C: a red shift of the Soret band from 398 to 413 nm is seen, and the band at 625 nm disappears concomitantly with the emergence of a band at 533 nm, thus suggesting formation of a hexacoordi-

[a] *Prof. Dr. H. Nakajima, Dr. Y. Ichikawa, Dr. Y. Satake, Dr. N. Takatani*
Graduate School of Science, Department of Chemistry, Nagoya University
Nagoya 464-8602 (Japan)

[b] *Dr. S. K. Manna, J. Rajbongshi, Prof. Dr. S. Mazumdar*
Department of Chemical Sciences, Tata Institute of Fundamental Research
Homi Bhabha Road, Colaba, Mumbai 400005 (India)

[c] *Prof. Dr. Y. Watanabe*
Research Center of Materials Science, Nagoya University
Nagoya 464-8602 (Japan)
Fax: (+81) 52-789-3049
E-mail: yoshi@nucc.cc.nagoya-u.ac.jp

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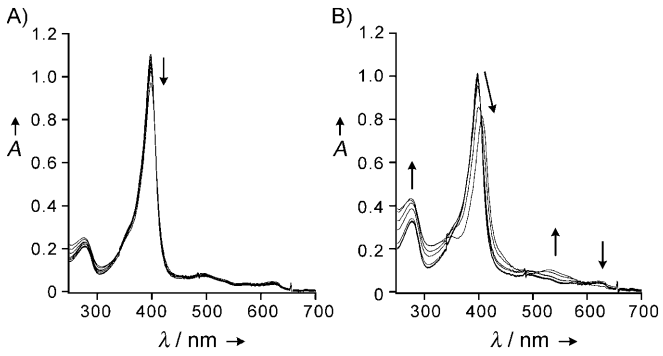

Figure 2. Temperature-dependent spectral changes in A) M69A and B) M69A/V49D ferric forms. Spectra were recorded every 10 °C between 20 and 90 °C in MES/NaOH buffer (20 mM, pH 5.0). Concentration of each variant is 10 μM. Arrows indicate direction of spectral changes with rising temperature.

nated heme. Despite this large change, the transformation between the penta- and hexacoordinated forms is largely reversible, and regeneration of the pentacoordinate heme takes place on reducing the temperature to 70 °C. Coordination switching hence appears to cause no critical perturbation leading the variants to denaturation.

EPR spectroscopy with M69A/V49D might provide additional evidence for the hexacoordinated heme and a clue as to the sixth ligand. However, quick reversal from the hexa- to pentacoordinated form with reducing temperature hampers the preparation of a frozen sample of the hexacoordinated form. Determination of the sixth ligand will be performed in a further study.

CD and UV/Vis spectroscopy revealed high thermal stabilities of the Cyt c_{552} variants relative to the wild-type. We then assayed the peroxidase activities of the variants at several temperatures. Figure 3 shows the temperature dependence of the peroxidase activities of the variants together with that of Mb H64D. The activity was evaluated on the basis of the initial reaction rate of one-electron oxidation of ferulic acid, a natural substrate for horseradish peroxidase (HRP).[9] Ferulic acid and its oxidation products show good stability under all reaction conditions employed in this study.

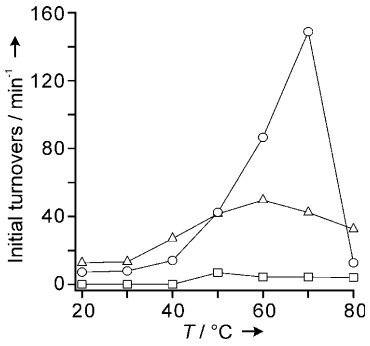

Figure 3. Temperature dependence of peroxidase activities evaluated by initial turnovers at several temperatures. Symbols represent M69A/V49D (○), M69A (□), and Mb H64D (△). Reaction conditions: enzyme variants (0.2 μM), H_2O_2 (1 mM), and ferulic acid (200 μM) in MES/NaOH buffer (20 mM, pH 5.0).

A reaction mixture containing ferulic acid and a variant in MES/NaOH buffer (20 mM, pH 5.0) was incubated at the indicated reaction temperature for 10 min prior to initiation of the reaction by addition of a small amount of concentrated H_2O_2 (500 mM, final concentration was adjusted to 1 mM) to the mixture. M69A/V49D showed higher enzymatic activities at higher temperatures, except at 80 °C. The rise in activity above 40 °C is remarkable. In contrast, Mb H64D showed a sluggish increase in activity, followed by a decrease after peaking at 60 °C. M69A/V49D thus surpasses Mb H64D in activity at 60 and 70 °C. This distinctive feature of M69A/V49D could be associated with the thermal stability of Cyt c_{552}. In Mb H64D, acceleration of each reaction step by heat would be subject to partial cancellation due to loss of catalytic activity through protein denaturation. The effects of denaturation become more prominent at higher temperatures, resulting in decreased activity. On the other hand, Cyt c_{552} would serve as a thermally stable scaffold to retain the catalytic activity of M69A/V49D. Consequently, the accelerating effect of heat is the predominant feature in the temperature dependence of overall peroxidase activity. The sudden drop in activity at 80 °C can be ascribed to the formation of the hexacoordinated heme described above. The uncharacterized sixth ligand fills the vacant site on the heme to prevent substrates from accessing the active reaction center. As a result, M69A/V49D loses its catalytic activity.

The initial activity assay also confirms the significantly low catalytic activity of M69A in relation to M69A/V49D. This is consistent with our previous study, in which M69A provided a suitable environment for facile observation of the hydrogen-peroxide-bound heme (Compound 0) that was hardly observable by normal spectroscopy at ambient temperature.[10] In contrast, Compound 0 produced in M69A can be detected by an ordinary EPR technique. This is due to the inability of M69A to produce a subsequent active intermediate, oxoferryl π cation radical, from Compound 0. These results with M69A thus confirm the essential role of the aspartic acid introduced at Val49 in the catalytic cycle. This point is discussed in detail below.

In addition to the higher initial reaction rates at higher temperatures, M69A/V49D shows acceptable performance as a thermally tolerant peroxidase in the durability of its enzymatic activity. Figure 4 shows representative time course results for consumption of ferulic acid observed at 70 °C in the presence of M69A/V49D, M69A, and Mb H64D as catalysts. In accordance with the initial reaction rates, M69A/V49D provides the highest consumption among the variants throughout the reaction period. Therefore, M69A/V49D is superior to Mb H64D in durability as a peroxidase at high temperatures. An apparent difference in the catalytic activity between M69A/V49D and M69A confirms that the Asp49 in M69A/V49D also exerts its function in a consecutive reaction. However, the high initial activity falls at 200 s and is followed by an inactive phase, as observed in the form of a plateau in the substrate consumption curve. A UV/Vis spectrum of the resultant M69A/V49D after 300 s revealed significant loss of heme from the protein. This indicates that, irrespective of the thermal stability of the protein itself, degradation of the heme, which is believed to an inactivation

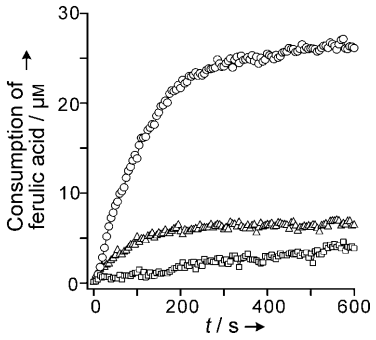

Figure 4. Time course of consumption of the substrate in the reaction at 70 °C up to 600 s. Symbols represent M69A/V49D (○), M69A (□), and Mb H64D (△). Reaction conditions: variants or hemin (0.2 μM), H_2O_2 (1 mM), and ferulic acid (200 μM) in MES/NaOH buffer (20 mM, pH 5.0).

process in peroxidase cycles, occurs during the catalytic reaction.[11] Thus, the peroxidase mechanism installed in Cyt c_{552} is resistant to the thermal disorder, which affords the high initial reaction rate and subsequent rapid consumption of substrate at high temperatures. Meanwhile, further improvements in the durability of the catalyst would require additional molecular design to suppress the degradation of the heme.

In order to examine the role of the introduced Asp49 in detail, kinetic isotope effects (KIEs) were determined by use of D_2O_2 in place of H_2O_2 in the catalytic reactions of M69A/V49D and M69A. M69A/V49D shows a KIE value of 3.1 (at 70 °C, pH/D 5.0), which is substantially smaller than the value for M69A (9.9 at 70 °C, pH/D 5.0) but similar to that of HRP for compound I[12] formation (1.6 at 25 °C, pH/D 7.0). The KIE value of M69A/V49D indicates that Asp49 is involved in the catalytic mechanism of the peroxidase cycle. For further understanding of the catalytic role of Asp49, we examined the ligation of NH_2OH to the variants as a model for H_2O_2. The pK_a value of NH_2OH (6.0) suggests that the majority is protonated to form NH_3^+OH under the reaction conditions of this study (pH 5.0). Association rate constants of NH_3^+OH to heme (k_{on}) at 25 °C were determined for M69A (1.48×10^3 M^{-1} s^{-1}) and M69A/V49D (10.0×10^3 M^{-1} s^{-1}). The seven-times higher k_{on} value for M69A/V49D could be ascribed to assistance of Asp49 as a carboxylate to pull off the proton from NH_3^+OH, accompanied by ligation of its deprotonated form to the heme iron. The base catalysis in the peroxidase reaction is essentially the same as this deprotonation process. It is thus reasonable to assume that Asp49 works as a base catalyst in the peroxidase reaction.

The possible acid function of Asp49 was also examined by product analysis in the reaction with cumene hydroperoxide. Heterolytic and homolytic cleavage of the hydroperoxide O–O bond affords cumyl alcohol and acetophenone as shown in Equation (1) and Equations (2) and (3), respectively.[8]

$$PorFe^{3+} + PhC(CH_3)_2OOH + Por^{\bullet+}Fe^{4+}{=}O + PhC(CH_3)_2OH \quad (1)$$

$$PorFe^{3+} + PhC(CH_3)_2OOH + PorFe^{4+}{=}O + PhC(CH_3)_2O^{\bullet} + H^+ \quad (2)$$

$$PhC(CH_3)_2O^{\bullet} + PhC(O)CH_3 + CH_3^{\bullet} \quad (3)$$

The acid catalyst would be expected to enhance heterolysis and therefore to raise the ratio of heterolysis over homolysis (cumyl alcohol/acetophenone). M69A/V49D showed a ratio of 2.8 at 25 °C and pH 5.0, 3.5 times higher than that observed for M69A (0.8) under the same conditions. It can therefore be concluded that Asp49 is also efficient as an acid catalyst in the reaction.

In this study we have demonstrated for the first time that Cyt c_{552} from *Thermus thermophilus* HB8 can be transformed into a thermostable peroxidase by molecular design modeled on the peroxidase mechanism. The prepared variant, M69A/V49D, retains thermostability comparable to that of the wild-type and exerts performance superior to that of Mb H64D, both in the initial reaction rate and in durability at high temperatures. Detailed inspection on the function of Asp49 in M69A/V49D revealed that the residue serves as a general acid–base catalyst, confirming the rationality of molecular design on Cyt c_{552}.

The initial reaction rate of HRP measured under these reaction conditions is 3.4×10^4 min^{-1} at 70 °C, about 230 times higher than that of M69A/V49D (1.5×10^2 min^{-1}, 70 °C) and still beyond the range of artificial peroxidases. In addition, heme degradation during the catalytic reaction is an obstacle to the durability. These problems require further refinement of Cyt c_{552} before practical application is possible. Nevertheless, we argue that rationally engineered Cyt c_{552} is a promising prototype as a thermally stable artificial peroxidase.

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