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Supporting Information

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for

Engineering of *Thermus thermophilus* Cytochrome *c*552: Thermally Tolerant Artificial Peroxidase

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Experimental

General: All chemicals were purchased from Nacalai Tesque Co. and Sigma Co. and used without further purification. Deuteride buffers and D_2O_2 solution were prepared according to the methods previously reported.[1]

Site directed mutagenesis: Expression and purification of both wild type and variant cytochrome c_{552} were carried out according to the previous report.[2] The variants were constructed with a QuickChange site-directed mutagenesis kit (Stratagene Co.) by using oligonucleotides 5′-GTACCTCATCCTGGACCTTCTCTACGGCC-3′ (for replacement of Val49 with Asp) and 5′-GAAGTACAACGGCGTCGCGTCCTCCTTCG-C-3′ (for replacement of Met69 with Ala) and their complementary strands as mutation primers. The underlined sequences correspond to the amino acid residues of mutagenesis.

Measurements of peroxidase activity: A reaction mixture (3 mL) containing ferulic acid (200 μM) and a protein (0.02 μM for HRP, otherwise 0.2 μM) in MES-NaOH buffer (20 mM, pH 5.0) was preincubated at each reaction temperature (20 - 80 °C) for 10 min. To initiate reaction, H_2O_2 (500 mM in H_2O kept at 25 °C) was added to the solu-

tion at the final concentration of 1 mM. Since ferulic acid provides various oxidation products in the peroxidase reaction, observation of the reaction product is not applicable to the quantitative analysis by UV/Vis spectroscopy.[3] Therefore, the peroxidase activity was monitored with consumption of ferulic acid that shows absorption maximum at 310 nm at pH 5.0 (e_{310} = 15.6 mM^{-1} cm^{-1}).

Kinetic measurements for association of NH_2OH: The reactions of the cytochrome c_{552} variants (10 µM) with NH_2OH (500 - 1000 µM) were performed at 25 °C on a Unisoku stopped-flow apparatus RSP-1000 (Unisoku Co.) in 20 mM MES-NaOH buffer (pH 5.0). The kinetic traces at 398 nm that corresponds to the absorption maxima of the variants were used for determining pseudo first-order rates. The association rate constants were given by the slopes of plots of the observed rates versus NH_2OH concentration.

Reaction with cumenehydroperoxide: The reaction mixture containing 10 µM cytochrome c_{552} variants and 300 µM cumene hydroperoxide was incubated for 30 min at 25 °C and pH 5.0. Aliquots of the mixture were analyzed by HPLC (Shimadzu Shimpack VP-ODS column). The column was eluted with 50% water / 50% acetonitrile at flow rate of 0.5 mL/min and the effluent was monitored at 210 nm. Assignment of the components was based on the retention time of authentic samples.

Spectroscopy: UV/Vis spectra were recorded on MultiSpec 1500 UV/Vis spectrometer equipped with a temperature controller, 110-QS-10 (Shimadzu Co.). Proteins were dissolved into 20 mM MES-NaOH buffer (pH 5.0) at the final concentration of 10 µM. Extinction coefficients of soret bands in the ferric M69A and M69A/V49D variants were determined by pyridine-ferro-hemochrome method to be 161.7 and 146.7 mM^{-1} cm^{-1}, respectively. Temperature dependent CD spectra were recorded on J-720WN CD spectrometer with a temperature controller (Jasco Co.). A thin optical cell (1 mm path length) containing 200 µL protein solution (10 µM in 20 mM MES-NaOH buffer, pH 5.0) was used for measurements. The temperature was raised lineally from 20 to 95 ºC by the ratio of 1 °C/min. All data were corrected at 222 nm that corresponds to a maximum of a negative cotton effect by α-helices.

References

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