Supporting Information

For

Significant Enhancement of Monooxygenase Activity of Oxygen Carrier Protein Hemocyanin by Urea

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Experimental Section

Materials. All chemical reagents used in this study, except *p*-ClC_6D_4OH, are commercial products of the highest available purity and used as received. The deuterated phenol, *p*-Cl-C_6D_4OH, was prepared according to the reported procedure,[1] and its purity (more than 99%) was confirmed by ^{1}H-NMR and MS analyses using a JEOL FT-NMR Lambda 300WB and a JEOL JMS-700T Tandem MS-station mass spectrometer, respectively.

Purification of Octopus Hemocyanin. The hemolymph of octopus (*Octopus vulgaris*) was collected from living animals purchased at Fukahiro fish dealer at Kuromon market, Osaka City, Japan. The blood cells were removed by brief low-speed centrifugation for 20 min at 13,000 rpm (20,000 *g*). The clear supernatant was sedimented by centrifugation for 6 hours at 35,000 pm (120,000 *g*) using a Hitachi Preparative Ultracentrifuges Models CP 70MX. The sediment was resolved in a small volume of 0.1 M Tris-HCl buffer (pH 7.4), and sedimented again by centrifugation for 13 hours at 35,000 rpm (120,000 *g*). The sediment was then slowly resolved again in the same buffer, and stored at 4 °C in a chlomatochamber MC-8EF2.

Purity and Concentration-determination of Oxy-hemocyanin. The concentration of copper ion in a hemocyanin solution was first determined by atomic absorption spectroscopy. Then, the UV-vis spectrum of that solution was measured by a JASCO UV/VIS Spectrophotometer V-550 under oxygen-saturated conditions (Figure S5). The molar absorption coefficient at 578 nm [for (μ-η^2:η^2- peroxo)dicopper(II) form] was determined as 466 M^{-1} cm^{-1}. The degree of oxy-form of hemocyanin was evaluated from the absorbance ratio A_{345}/A_{280}, the value of 0.22 corresponding to 100% oxy-hemocyanin at pH 7.4 (in 0.1 M Tris-HCl buffer).

Reaction of Oxy-hemocyanin and Phenols under Anaerobic Conditions (Ar). Typically, a portion (200 μL) of a stock solution of oxy-hemocyanin (1.89 mM in 0.1 M Tris-HCl buffer, pH 7.4) was added to a 0.5 M borate buffer solution (2 mL, pH 9.0) containing 10 % methanol and 8 M urea placed in a UV cell at 25 °C. The UV cell was closed by a silicon-rubber cap, and Ar gas was flashed through a thin needle for 10 min in order to

remove O_2 from the cell. Then, the reaction was started by adding a methanol solution of the substrate (10 μL) at 25 °C. The time course of the reaction was followed by monitoring a decrease of the absorbance at 348 nm due to oxy-hemocyanin (Figure 1).

Product Analysis. The final reaction mixture was analyzed by using a HPLC system consisting of a Shimadzu LC-6A chromatographic pump, a Shimadzu UV-vis spectrophotometric detector, and an ODS column (Prodigi 250 mm x 4.6 mm, Phenomenex). The mobile phase was a mixture of acetonitrile/water (v/v = 65:35) containing 0.1 % trifluoroacetic acid, and the flow rate was 0.3 mL min^{-1}. The products were quantified by comparison against a known amount of internal standard using a calibration curve consisting of a plot of mole ratio (moles of product/moles of internal standard) versus area ratio (area of product/area of standard).

Figure S1

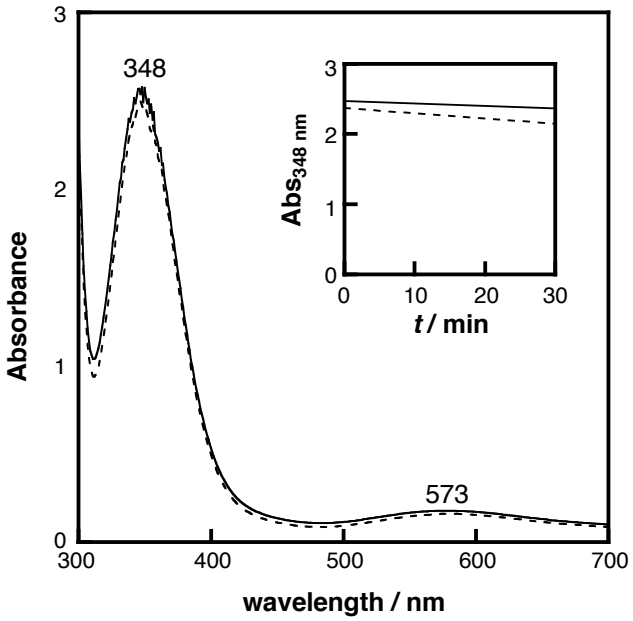

Figure S1. UV-vis spectra of octopus hemocyanin (0.17 mM) in a 0.5 M borate buffer (pH 9.0) containing 10 % MeOH in the absence (solid line) and the absence (dotted line) of urea (8 M) at 25 ˚C. Inset: Time course of the absorbance changes at 348 nm under anaerobic conditions (Ar).

Figure S2

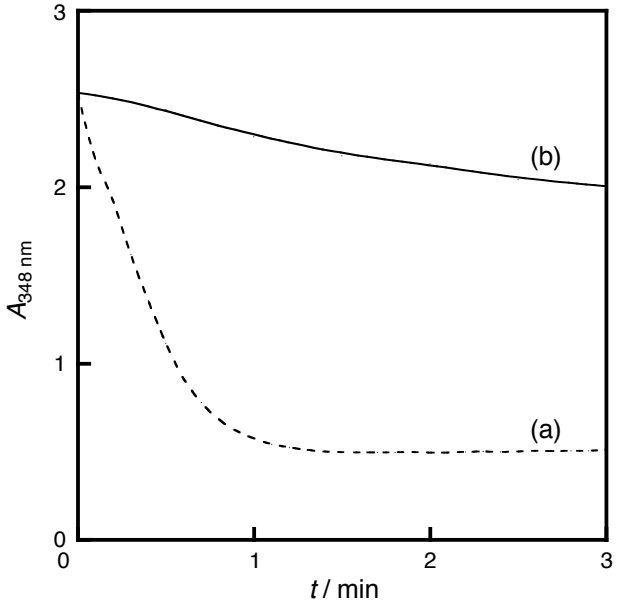

Figure S2. Time courses of the absorbance change at 348 nm for the reactions of oxy-hemocyanin (0.17 mM) and *p*-cresol (16 mM) (a) in the presence of 8 M urea and (b) in the absence of urea in 0.5 M borate buffer (pH 9.0) containing 10 % MeOH at 25 °C under anaerobic conditions.

Figure S3

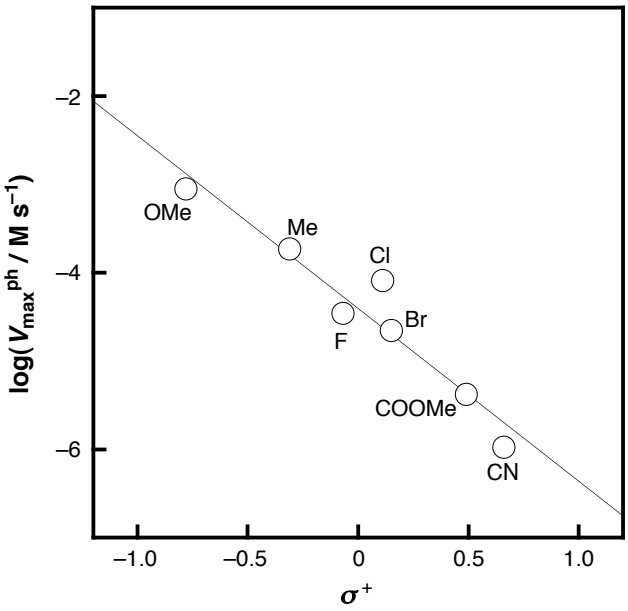

Figure S3. Hammett plot for the oxygenation of phenols by oxy-hemocyanin (single turnover reaction); data are taken from Table 1).

Figure S4

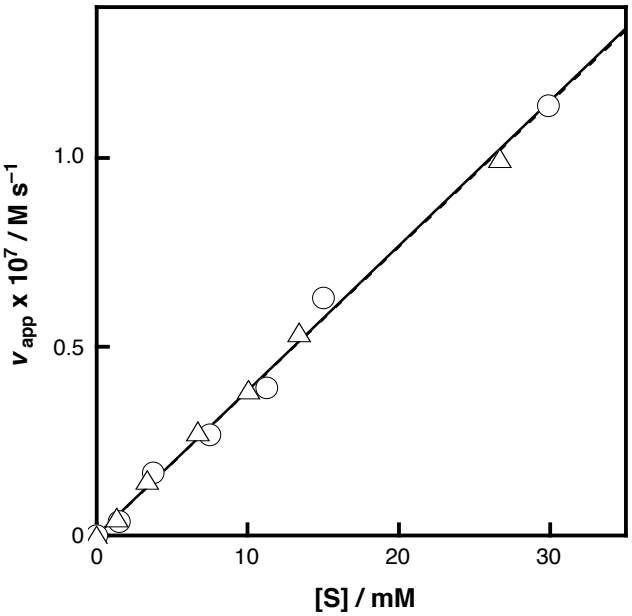

Figure S4. Plot of v_{app} against the substrate concentration for the reaction of *p*-ClC_6H_4OH (○) and of *p*-ClC_6D_4OH (△).

Figure S5

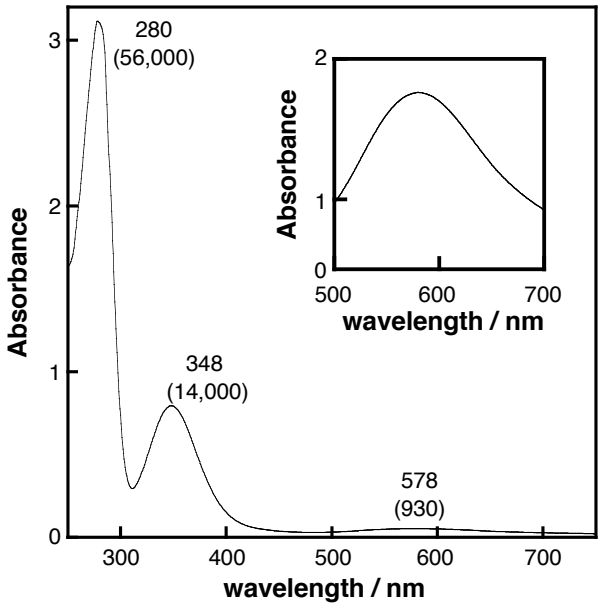

Figure S11. UV-vis spectrum of oxy-hemocyanin from *Octopus vulgaris* (5.5 x 10^{-5} M) taken in 0.1 M Tris-HCl buffer (pH 7.4) at 25 ˚C under O_2-saturated conditions. Inset: Magnified spectrum (1.9 x 10^{-3} M).

(1) Jallal, M. G.; Roger, A. S. *Tetrahedron Lett*. **1995**, *36,* 3893–3896.