

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

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Hemocyanin is the oxygen carrier protein involved in the hemolymph of many mollusks and arthropods.^{1,2} It is a large multi-subunit protein whose structure varies significantly depending on the source of the protein. Arthropod hemocyanin subunits have $M \sim 75$ kDa and associate into oligomers of hexamers *in vivo*.^{1–3} On the other hand, mollusks' hemocyanin subunits contain seven or eight oxygen-binding sites, referred to as functional units, and have $M \sim 400$ kDa, which associate in multiples of 10 to yield cylindrical supramolecular assemblies.^{1,2,4}

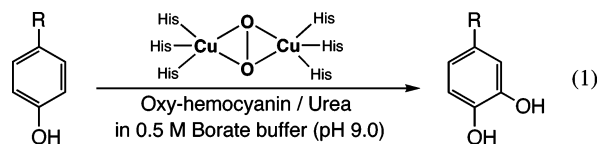
The X-ray crystallographic analyses of oxy-hemocyanins have clearly demonstrated the existence of a (μ - η^2 : η^2 -peroxo)dicopper(II) core structure in the active center.^{2,5,6} The side-on peroxo dinuclear copper(II) (Cu_2O_2) core is the common structural feature of the oxy forms of hemocyanin, tyrosinase, and catechol oxidase.^{5–9} Thus, the oxy forms of these copper proteins exhibit very similar spectroscopic features originated from the Cu_2O_2 unit, even though the overall structures of the proteins are quite different.^{5–9}

Despite having the same side-on peroxo dicopper(II) species, these copper proteins exhibit different chemical reactivity toward external substrates. Namely, tyrosinase catalyzes the *o*-hydroxylation of phenols to catechols (phenolase activity) as well as the oxidation of catechols to *o*-quinones (catecholase activity).^{7,8,10} On the other hand, catechol oxidase only exhibits the catecholase activity, but shows no monooxygenase activity.¹¹ In contrast to these dicopper enzymes, hemocyanin has essentially no redox reactivity toward external substrates, but exhibits only reversible dioxygen binding ability, thus acting as the oxygen carrier protein.

In this respect, recent studies on the enzymatic functions of arthropod hemocyanins are worthy to be noted. Beltrami and co-workers reported that arthropod hemocyanins exhibited the catecholase activity, and the catalytic activity increased by 1.5–5-fold when a certain amount of NaClO_4 was added into the solution.¹² The authors suggested that perchlorate ions induce a change in protein conformation, resulting in an increase of the enzymatic activity (V_{max}). Decker and co-workers demonstrated that tarantula hemocyanin got both the phenolase and catecholase activities after limited proteolysis with trypsin or chymotrypsin.^{13,14} In this case, the proteolytic cleavage removes an N-terminal fragment to open the entrance (phenylalanine-49) for the substrate incorporation. Söderhäll and co-workers recently reported a similar result using crayfish hemocyanin.¹⁵ On the other hand, horseshoe crab hemocyanin was demonstrated to get catecholase activity when it was allowed to interact with an antimicrobial peptide.¹⁶ In this case as well, the interaction may induce a conformational change of the protein to gain the catalytic activity. However, mechanistic details of the enzymatic functions of arthropod hemocyanins have yet to be addressed.

Molluscan hemocyanin isolated from *Octopus vulgaris* was also demonstrated to exhibit catecholase activity, for which a one-electron oxidation mechanism of catechol to the corresponding semiquinone (radical mechanism) was invoked.¹⁷ However, phenolase activity of molluscan hemocyanin has yet to be examined in detail so far.¹⁸

In this study, *Octopus vulgaris* hemocyanin has been found to exhibit efficient monooxygenase activity (phenolase activity) when it is treated with 8 M urea (eq 1). Mechanistic studies on the phenolase reaction have been performed to provide important insights into the chemical functions of (μ - η^2 : η^2 -peroxo)dicopper(II) species in the biological systems.



It was previously reported that the gigantic supramolecular assembly of *Octopus* hemocyanin was dissociated into the subunits consisting of seven or eight functional units when the supramolecular assembly of hemocyanin was treated with urea.¹⁹ Thus, in this study, we examined the effects of urea on the stability and redox reactivity of hemocyanin. In Figure S1 are shown the absorption spectra of isolated hemocyanin taken in a 0.5 M borate buffer solution (pH 9.0) containing 10% MeOH in the presence and absence of urea at 25 °C. The spectra exhibit an intense absorption band at 348 nm together with a relatively weak band at 578 nm, clearly indicating that hemocyanin was isolated as the oxy form containing the (μ - η^2 : η^2 -peroxo)dicopper(II) species.^{7,8} It should be noted that the spectrum of oxy-hemocyanin in the presence of high concentration of urea (8 M) (dotted line in Figure S1) is the same as that of oxy-hemocyanin in the absence of urea (solid line). This clearly indicates that the Cu_2O_2 core structure of oxy-hemocyanin remains intact even in the presence of the high concentration of urea. Moreover, oxy-hemocyanin was unexpectedly stable even in the absence of O_2 (under anaerobic conditions), and the stability of the peroxo species was hardly affected by the addition of urea. As clearly demonstrated in the inset of Figure S1, the decay rate of oxy-hemocyanin was very slow (after 10 min, $\sim 95\%$ of oxy-hemocyanin still remained under Ar atmosphere), and the decay rate in the presence of urea (dotted line) is nearly the same as that of oxy-hemocyanin in the absence of urea (solid line).

To our surprise, addition of *p*-cresol (4-methylphenol) (16 mM) to the anaerobic borate buffer solution of oxy-hemocyanin (0.17 mM) containing 8 M urea at 25 °C resulted in a rapid decrease of the absorption bands of the peroxo species, as shown in Figure 1A. From the final reaction mixture, the oxygenated product

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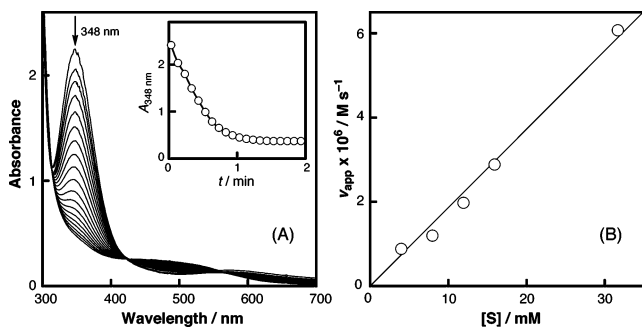


Figure 1. (A) Spectral change observed upon addition of *p*-cresol (16 mM) to *Octopus* hemocyanin (0.17 mM) in 0.5 M borate buffer (pH 9.0) containing 10% MeOH and 8 M urea at 25 °C under Ar. Inset: Time course of the absorption change at 348 nm. (B) Plot of v_{app} versus [*p*-cresol].

Table 1. Apparent First-Order Rate Constants for the Oxygenation of Phenols (ρ -RC₆H₄OH) by Oxy-Hemocyanin

| ρ -substituent (R) | k_{app} (s ⁻¹) |
|-------------------------|------------------------------|
| –OCH ₃ | 9.0×10^{-4} |
| –CH ₃ | 1.9×10^{-4} |
| –F | 3.5×10^{-5} |
| –Cl | 3.8×10^{-5} |
| –Br | 2.2×10^{-5} |
| –COOCH ₃ | 4.2×10^{-6} |
| –CN | 1.1×10^{-6} |

catechol (4-methyl-1,2-dihydroxybenzene) was obtained in a 74% yield based on the initial concentration of oxy-hemocyanin (the product yield of single-turnover reaction under anaerobic conditions). In the absence of urea, the spectral change was much slower, as shown in Figure S2. These results clearly indicate that the addition of urea causes not only the dissociation of the subunits from the supramolecular assembly but also a partial conformational change of the protein to open a space for the substrate binding as suggested previously.^{3,14} Nonetheless, the active site structure of the (μ - η^2 : η^2 -peroxo)dicopper(II) core of oxy-hemocyanin is maintained to react with the substrate. This is the first spectroscopic detection of the monooxygenation reaction of the (μ - η^2 : η^2 -peroxo)-dicopper(II) species in the biological systems.

From the initial slope of the time course of the absorption change shown in the inset of Figure 1A, the apparent rate constant (v_{app}) of 3.2×10^{-6} M s⁻¹ was determined. Then, the rate dependence on the substrate concentration was examined to obtain a linear correlation, as shown in Figure 1B. Thus, the apparent first-order rate constant, k_{app} , was determined to be 1.9×10^{-4} s⁻¹ from the slope. Similar spectral changes were obtained in the reactions with a series of phenol derivatives, and their rate constants, k_{app} , were determined similarly as listed in Table 1.

The linear dependence of v_{app} against the substrate concentration in the single-turnover reaction (Figure 1B) may indicate that the binding of the substrate to oxy-hemocyanin is weak. This is reasonable since hemocyanin is essentially an oxygen carrier protein, but not an enzyme, thus having no proper substrate-binding pocket.

To get insight into the oxygenation mechanism of phenols by oxy-hemocyanin, electronic effects of the phenol substituents (R) on the reaction rate were examined, as shown in Figure S3. The plot of $\log(k_{app})$ against the Hammett σ^+ gave a linear correlation, from which a Hammett ρ constant was obtained as -2.0 . It should be noted that the ρ value of the present reaction is very close to that of the phenolase reaction of mushroom tyrosinase ($\rho = -2.4$).²⁰ This result strongly suggests that the oxygenation of phenols by oxy-hemocyanin involves the same mechanism as the phenolase reaction of tyrosinase, that is, an electrophilic aromatic substitution

mechanism.^{20,21} Consistent with this mechanism, there was no kinetic deuterium isotope effect ($k_{app(H)}/k_{app(D)} = 1.0$) when deuterated substrate (*p*-ClC₆D₄OH) was employed (Figure S4).

The oxygenation of *p*-cresol (21 mM) by hemocyanin (2.2×10^{-3} mM) also proceeded *catalytically* when the reaction was carried out under *aerobic* conditions in the same borate buffer solution (pH 9.0) containing NH₂OH (7.0 mM) as an electron donor at 25 °C.²² The yield of catechol product was 77% based on the initial O₂ concentration (0.25 mM).

In summary, we have found that the oxygen carrier protein hemocyanin can get the monooxygenase activity when it is treated with a high concentration of urea (8 M). Oxy-hemocyanin is stable enough to be examined in the single-turnover reaction under anaerobic conditions. Thus, the oxygenation reaction of phenols by oxy-hemocyanin can be followed directly by using an ordinary UV-vis spectroscopic method (Figure 1). Preliminary kinetic studies on the single-turnover reaction have suggested that the reaction mechanism of the phenol–monooxygenation reaction by oxy-hemocyanin is the same as that of phenolase reaction of tyrosinase. Effects of urea on the stability and the reactivity of hemocyanin are now under investigation.

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Supporting Information Available: Details of the experimental procedures and the spectral and kinetic data (Figures S1–S5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- The reaction was carried out in a 0.5 M borate buffer in order to prevent over-oxidation of the primary oxygenation product catechols to the corresponding *o*-quinones. In this system, NH₂OH had to be used as an external electron donor for the reduction of met-hemocyanin to deoxy-hemocyanin (see ref 20).

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