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Oxygen Binding to Tyrosinase from *Streptomyces antibioticus* Studied by Laser Flash Photolysis

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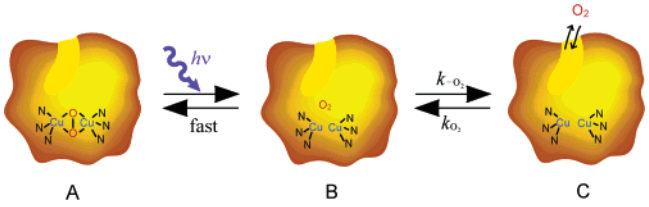
Tyrosinases (EC 1.14.18.1) are involved in the synthesis of melanin pigments in bacteria, plants, and mammals. They catalyze the *o*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity).[1] Tyrosinases possess a dinuclear copper active site similar to that of hemocyanin (Hc), which is an oxygen carrier protein in mollusks and arthropods, and to that of catechol oxidase.[2] Crystallographic studies have shown that Hc binds oxygen in a μ-η^2:η^2-peroxodicopper(II) binding mode,[3] while optical studies have shown that oxytyrosinase (Ty_{oxy}) and oxyhemocyanin exhibit similar absorption bands at about 345 and 600 nm.[4] They also exhibit similar resonance Raman bands at about 750 cm^{-1}, which are assigned to the peroxo stretching mode of the peroxodicopper site.[5] From these results, as confirmed by NMR spectroscopy,[6] the active site structure of tyrosinase (Ty) is thought to be similar to that of Hc. Model compound studies have shown that a μ-oxodicopper(III) species may be in equilibrium with the μ-η^2:η^2-peroxodicopper(II) species.[7] Binding of O_2 to a copper complex has been observed by photorelease of carbon monoxide from the CO complex in a mixture of CO and O_2.[8] Although Ty has attracted much interest because of its intriguing oxygen activation capability,[1] the information on the O_2-binding character of Ty is limited. Therefore, we have studied by flash photolysis how small molecules, such as carbon monoxide and *p*-nitrophenol (a substrate−analogue inhibitor[9]), may affect O_2 binding kinetics.

After photodissociation (Scheme 1, A→B), O_2 can stay in the active site pocket of the protein and perform geminate rebinding (Scheme 1, B→A), or leave the protein pocket, especially at higher temperature (Scheme 1, B→C). The amount of O_2 dissociation from

Scheme 1. Photodissociation of O_2 from Tyrosinase



the protein may depend on the accessibility of the active site and the protein flexibility. When the O_2 molecule leaves the copper site of Ty after flash photolysis and rebinds to it, the absorbance change can be observed in the microsecond to millisecond time scale. This behavior is well-known for heme proteins. For example, above 200 K, equilibrium fluctuations among conformational substates of myoglobin (Mb) open pathways for the ligand through the protein matrix.[10]

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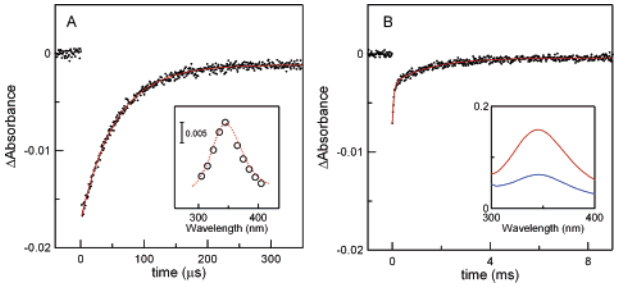

Figure 1. Flash-photolysis measurements of Ty_{oxy} by 355 nm pulse irradiation. Absorption changes at 345 nm under (A) 100% O_2 and (B) O_2:CO = 1:1 (v/v). (A) Single and (B) double exponential best-fitted curves are shown in red. Experimental conditions: Ty, 20 μM; O_2, (A) 1.54 mM and (B) 0.77 mM; 15 °C; 50 mM potassium phosphate buffer, pH 7.2; laser pulse, 30 mJ, 10 Hz. The traces are an average of (A) 128 and (B) 256 shots. (Inset) A: Overlap of wavelength dependence of the initial absorption changes (empty circles) with the difference absorption spectrum (red, oxy *minus* deoxy). B: Absorption spectra of Ty under 100% O_2 (red) and O_2:CO = 1:1 (v/v) (blue).

Purified met-tyrosinase was reduced with 1−10 mM hydroxylamine and dialyzed under anaerobic conditions.[11] The obtained reduced tyrosinase (deoxytyrosinase, Ty_{red}) was converted into Ty_{oxy} by aeration, and the solution was filtered before each measurement. The absorbance change of Ty_{oxy} at 345 nm under O_2 by 355 nm pulse irradiation is shown in Figure 1A. The absorption decreased immediately after the pulse and recovered on a microsecond time scale. The initial absorption decrease corresponds to dissociation of O_2 from the dicopper center, whereas the intensity recovery indicates rebinding of O_2 to the deoxy enzyme. Geminate recombination was not observed, although it may occur within the dead time of the instrument.[12] A first-order rate constant was obtained by fitting the absorbance change with an exponential curve ((2.0 ± 0.5) × 10^4 s^{-1}).[13,14] The O_2 binding rate constant (k_{O_2}) was then calculated as k_{O_2} = 13 ± 3 μM^{-1} s^{-1} by taking the oxygen concentration (1.54 mM at 15 °C) into account.[15] The largest absorbance change was observed at 345 nm, and the wavelength dependence of the initial intensity change in the 305−405 nm range showed a good correlation with the oxy 345 nm absorption band (Figure 1A, inset), which indicates that the absorption change is due to O_2 dissociation and rebinding. The O_2 rebinding kinetics did not change significantly on increasing the pH to 8.1, which suggests that there is no significant conformational change in Ty between pH 7.1 and 8.1.

To observe the effect of other molecules on O_2 binding, flash-photolysis experiments were performed with O_2 mixed with carbon monoxide (O_2:CO = 1:1 (v/v)) (Figure 1B). O_2 and CO binding to the dinuclear copper active site are mutually exclusive,[16] and CO caused formation of Ty_{oxy} to decrease to about 40% according to the absorption spectra (Figure 1B, inset).[17] Photodissociation of the

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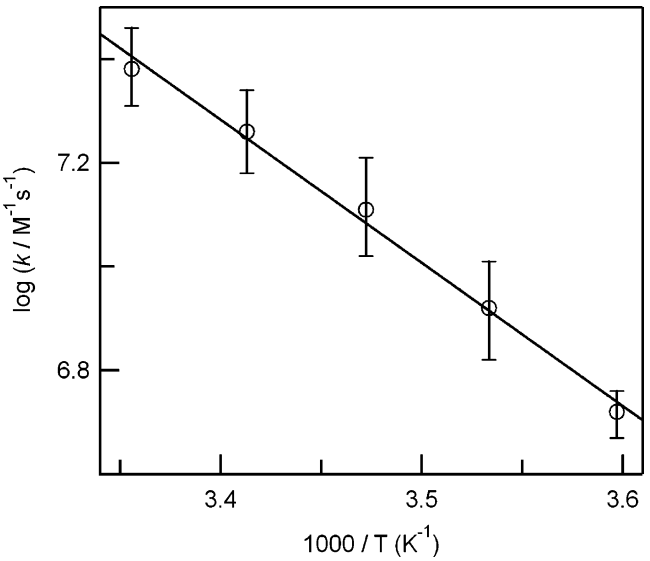

Figure 2. Arrhenius plot of oxygen binding rate constant of Ty_{oxy} obtained by 355 nm pulse irradiation at different temperatures (5−25 °C). Other experimental conditions are the same as those listed in Figure 1.

Table 1. Kinetic and Thermodynamic Parameters for Oxygen Binding to Tyrosinase and Hemocyanin

	k_{O_2} (μM^{-1} s^{-1})	k_{-O_2} (s^{-1})	K_D (μM)
Ty *Streptomyces antibioticus*	19[a]	310[b]	16.5
Ty *Agaricus bisporus*[22]	23	1100[b]	46.6
Hc *Panulirus interruptus*1[23]	31−154	150−470	2.0−9.1
Hc *Octopus vulgaris*[24]			90

[a] At 21 °C. [b] Calculated from k_{O_2} and K_D.

O_2 molecules from the active site by pulse irradiation also decreased to about 40%, whereas the recovery exhibited two phases (Figure 1B). The faster phase showed a rate similar to the binding rate observed in Figure 1A, and it is ascribed to O_2 binding to Ty_{red}. The O_2-rebinding rate constant of the slower phase amounted to 0.7 ± 0.1 ms^{-1}. This phase is attributed to Ty_{oxy} formation by dissociation of CO from the active site of the CO-bound species, the concentration of which increased transiently by O_2 photodissociation.[18] The rate constant of the slower phase was similar to that obtained by stopped-flow mixing of a Ty_{red} solution under CO atmosphere with O_2-saturated buffer (results not shown). The intensity of the initial absorption change by pulse irradiation decreased significantly when 100 μM *p*-nitrophenol was added, although Ty_{oxy} was generated as judged by the absorption spectrum. These results show that small molecules may occupy the active site and affect the O_2 binding kinetics of Ty.

The O_2-binding rate constant and the initial intensity change increased with temperature (Figure 2). The larger intensity changes for higher temperatures may correspond to an enhancement in protein fluctuations, which increase the dissociation of the O_2 molecule from the protein. It has been observed in flash-photolysis experiments on O_2 binding to Mb that the geminate fraction decreases by raising the temperature, which has been interpreted as enhancement of structural fluctuations at higher temperatures.[19] Using the reported O_2 dissociation constant for Ty from *Streptomyces antibioticus* ($K_D = 16.5$ μM) at 21 °C and estimating k_{O_2} at 21 °C from Figure 2,[20] the O_2 dissociation rate constant is calculated as 310 s^{-1} (Table 1). The activation enthalpy of the rate-limiting step of O_2 binding is calculated by the temperature dependence of k_{O_2} to be 12.8 ± 2.6 kcal/mol. It has been proposed that the rate-limiting step in CO binding to Mb involves some conformational change, and that conformational relaxation of Mb when going from the CO-bound state toward the deoxy state increases the barrier for CO binding.[21] The activation enthalpy for O_2 binding to Ty may also reflect a protein conformational change connected with binding of O_2 since the Cu−Cu distance has been shown to decrease from 4.6 to 3.6 Å by oxygenation for Hc.[3] The present study observes O_2 rebinding by flash photolysis and shows that the method would be a powerful tool to study the O_2 binding kinetics in Ty.

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(12) Geminate rebinding is difficult to observe due the difficulty in obtaining 355 nm picosecond pulses.
(13) Within experimental error, the rate varied linearly with O_2 concentration, although at less than 50% O_2 saturation, low S/N precluded reliable determination of the rate.
(14) Disappearance of the reduced species was also observed by monitoring the fluorescence change (Ty_{red} fluorescence is roughly halved upon binding of O_2).[9] It occurred with a similar rate as observed in Figure 1.
(15) Since $k_{obs} = k_{O_2}[O_2] + k_{-O_2}$, or $k_{obs}/k_{-O_2} = (1/K_D)[O_2] + 1$, it follows from the literature value of K_D and the value of k_{obs} that $k_{obs} \approx k_{O_2}[O_2] \gg k_{-O_2}$.
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(17) It follows that the constant for the equilibrium $Ty_{oxy} + CO \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ CO-bound Ty + O_2 is close to 1 and that $k_1 \approx k_2$.
(18) The ratio of the amplitude of the slower phase against that of the faster phase increased with the [CO]/[O_2] ratio. The rate constant of the slower phase was constant, in agreement with $k_1 \approx k_2$ (see ref 17).
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