A Novel Versatility of Catalase I as a Dioxygenase for Indole-ring-opening Dioxygenation

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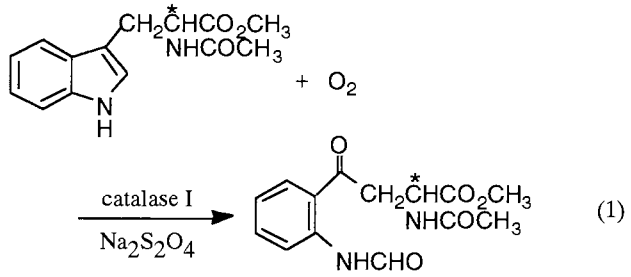
Catalase I (wild type) from *Bacillus stearothermophilus*, which was found to have catalase activity, catalyzed dioxygen-inserted indole-ring opening reaction of methyl *N*-acetyl L-tryptophanate as tryptophan 2, 3-dioxygenase (TDO) model in the presence of $Na_2S_2O_4$. Heme-reconstruction with protoporphyrin IX manganese(III) chloride (MnClPP) *via* annealing was examined and the reconstituted MnClPP-catalase I also revealed dioxygenolytic behaviour with higher TDO activity and higher selectivity than the catalase I (wild type).

Although organic synthesis with metalloenzymes seems of interest and significant in chemistry, it has hitherto been the subject of only limited investigation because of the low versatility of metalloenzymes (except such ones as cytochromes P-450). Recently, the asymmetric epoxidation of olefins by chloroperoxidase[1] or myoglobin,[2] and the sulfoxidation of thioethers by horseradish peroxidase[3] have been successfully performed with H_2O_2 as oxidants. In relation to heme-containing mono- or di-oxygenases, the oxygenase reactions by metalloenzymes with O_2 seem more attractive rather than those with previously activated oxygen sources such as H_2O_2, alkylhydroperoxides, iodosylbenzene, and ClO^-. This report first deals with the versatility of catalase I of *Bacillus stearothermophilus*[4] possessing a protoporphyrin IX iron(III) (hemin) in its subunit for the dioxygen-inserted indole-ring opening reaction of methyl *N*-acetyl L-tryptophanate as TDO model in 5 (or 10) vol% EtOH-phosphate buffer (pH 7.00) at an ambient temperature by using molecular dioxygen as an oxidant in the presence of $Na_2S_2O_4$ as shown in Eq. (1).[5] In our



(1)

previous work, we found that the TDO model reaction, addition of molecular oxygen to 3-methylindole to give ring cleavage, was catalyzed by Mn^{2+}, Mn^{3+}, Fe^{2+}, Fe^{3+}, Co^{2+}, and Co^{3+} complexes with tetraphenylporphyrin (TPP).[6] Among these complexes, Mn^{2+} and Mn^{3+}TPP complexes, having the highest activity for reaction of 3-methylindole in THF, were also observed to exhibit effective activity at 25 °C for oxygenation of methyl *N*-acetyl L-tryptophanate. In order to enhance the oxidation activity, a new strategy for heme-reconstruction with protoporphyrin IX manganese(III) chloride (MnClPP) *via* annealing is also examined.

Loprasert *et al.* purified a catalase I (wild type) from *Bacillus stearothermophilus*, which was found to have the catalase activity.[4] The apo-catalase I was prepared as follows: the hemin was extracted from catalase I (wild type) with 0.05% (v/v) 2 N HCl-acetone and 8 mol dm^{-3} of guanidine hydrochloride (pH 3.54), and then the apo-protein solution was diluted to 10 mmol dm^{-3} with guanidine hydrochloride. After dialysis of the apo-protein solution with 50 mmol dm^{-3} of potassium phosphate buffer (pH 7.00), the protein solution was heated on a steam-bath at 65 °C for 20 min, and followed by cooling to 0 - 5 °C in an ice-bath (a refolding method). Through the precipitation of the apo-protein with saturated ammonium sulfate and dialysis with 50 mmol dm^{-3} of potassium phosphate buffer, the concentrated apo-protein solution was obtained. On the other hand, after the hemin extraction from the holo-enzyme, following addition of MnClPP into the apo-protein-solution and heat treatment of the mixture at 65 °C for 20 min resulted effective incorporation into the heme crevice of apo-catalase I to yield reconstituted MnClPP-catalase I (a simultaneous refolding and reconstruction method). The catalase activity[7] of catalase I was found to be 3.05 x 10^5 min^{-1} (wild type) and 2.17 x 10^3 min^{-1} (reconstituted one), respectively. The decrease in absorbance at 240 nm (H_2O_2 concentration-change) was not observed in the mixed reaction system of MnClPP and apo-protein. This indicates that H_2O_2 degradation

Table 1. Dioxygenation of methyl *N*-acetyl L-tryptophanate with catalase I (wild type)[a]

Reaction system	Conv. / %	Yield[b] / %
hemin / $Na_2S_2O_4$ / O_2	6.1 ± 0.1	1.4 ± 0.2
apo-catalase I / $Na_2S_2O_4$ / O_2	6.3 ± 0.3	n.d.[c]
catalase I / $Na_2S_2O_4$ / O_2	32.0 ± 0.2	6.0 ± 0.6
catalase I / O_2	0	n.d.[c]
catalase I / $Na_2S_2O_4$ / N_2	3.4 ± 0.1	1.4 ± 0.1
$Na_2S_2O_4$ / O_2	3.4 ± 0.1	n.d.[c]

[a]The reaction was carried out in 10 vol% EtOH-phosphate buffer (pH 7.00) containing methyl *N*-acetyl L-tryptophanate (5.0 mmol dm^{-3}), catalase I (5.0 μmol dm^{-3}), and $Na_2S_2O_4$ (0 or 5.0 x 10^{-4} mol dm^{-3}) at 25 °C under O_2 (or N_2) for 1 day. The amounts of oxidation product (retention time of 8.1 min) and unreacted substrate (retention time of 11.8 min) were determined by high performance liquid chromatography (JASCO Finepack SIL C_{18}, UV 280 nm) eluted with 40 vol% MeOH-H_2O. [b]Yield of the oxidation product of methyl 2-acetamido-3-(2'-formamidobenzoyl)propanoate. The reaction forms other products such as *N*-acetyl L-tryptophan, (or methyl L-tryptophanate and L-tryptophan) in addition to the ring-opening dioxygenized one. The amounts of these by-products were negligibly small in the reaction, respectively. [c]Not detected.

did not occur. In this respect, MnClPP was incorporated effectively into the apo-protein by the simultaneous refolding and reconstruction method.

Catalase I (holo enzyme) exhibited the higher TDO activity than both the apo protein (without hemin) and the hemin *per se* (without the protein), as shown in Table 1. Addition of $Na_2S_2O_4$ as a reductant enhanced the catalytic activity for the dioxygenolysis. In the absence of $Na_2S_2O_4$, the dioxygenation did not occur. Since addition of $Na_2S_2O_4$ to the catalase I (wild type) solution changes the Soret band (406 nm) of

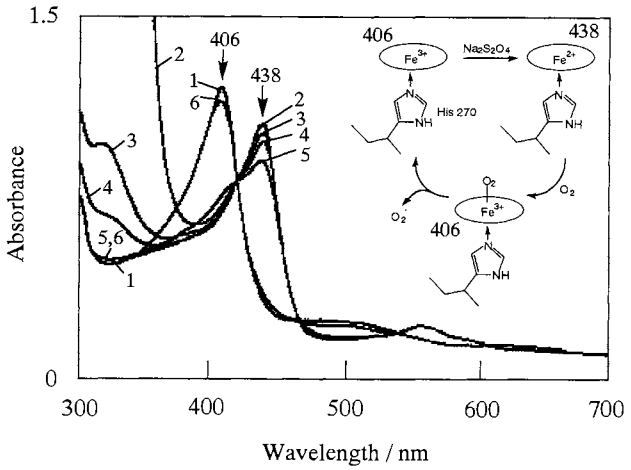


Figure 1. Electronic spectral changes of 10 μmol dm^{-3} catalase I (wild type) in 100 mmol dm^{-3} phosphate buffer caused by the addition of $Na_2S_2O_4$ (5.0 μmol): curve 1; catalase I (wild type) under Ar atmosphere. curve 2; after the $Na_2S_2O_4$ addition. curve 3-5; 2, 3, and 6 h after the exposure to air, respectively. curve 6; after the charge with O_2 for 10 min.

Table 2. Dioxygenation of methyl *N*-acetyl L-tryptophanate with various catalysts[a]

Catalyst	Conv. / %	Yield / %	Selectivity[b]
hemin	54.0 ± 0.6	23.0 ± 0.7	0.43
catalase I (wild type)	87.2 ± 0.5	34.2 ± 0.9	0.39
MnClPP	26.4 ± 0.2	22.0 ± 0.5	0.85
MnClPP / apo-catalase I[c]	69.2 ± 0.9	7.0 ± 0.8	0.10
MnClPP-catalase I	76.0 ± 0.6	39.0 ± 0.4	0.51

[a]The reaction was carried out in 5 vol% EtOH-phosphate buffer (pH 7.00) containing methyl *N*-acetyl L-tryptophanate (2.5 mmol dm^{-3}), catalyst (25 μmol dm^{-3}), and $Na_2S_2O_4$ (2.5 mmol dm^{-3}) at 30 °C under O_2 for 1 day. [b]The selectivity is the ratio of the yield/conversion values. [c]*In situ* prepared system.

protoporphyrin IX iron(III) in the enzyme to that (438 nm) of iron(II) one (Figure 1), the reaction of the reduced hemin and molecular dioxygen forms the Fe^{3+}-O_2^- complex which deprotonates the indole-ring at N-1 position of the substrate.

The reconstituted MnClPP-catalase I also revealed dioxygenolytic behavior with slightly higher TDO activity (1.1-fold) and higher selectivity (1.3-fold) than the catalase I (wild type), as shown in Table 2. The reconstituted MnClPP-catalase I exhibited the higher TDO activity (1.8-fold) than the MnClPP *per se* (without the protein). The evidently low TDO activity was observed in the case of *in situ* prepared reaction system of MnClPP and apo-protein. Thus, the reconstruction of heme-containing enzyme with MnClPP was found to be effective to increase TDO activity. The inclusion-efficiency of the manganese(III) porphyrin into the apo-enzyme was not 100%: *ca.* 74% of MnClPP was incorporated into the heme crevice of the apo-catalase I. The amount of the included manganese(III) porphyrin was estimated from the absorption spectroscopy measurement of the native enzyme and reconstituted MnClPP-catalase I at λ_{max} s = 280 nm (protein) and 406 (Fe^{3+} Soret band) or 464 nm (Mn^{3+} Soret band). In this respect, yield and the selectivity with correction based on the inclusion-efficiency in this reconstituted MnClPP-catalase I are 52.7% and 0.53, respectively. Further experiments to improve the inclusion-efficiency are now under progress.

References and Notes

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7 Enzyme assay was carried out as follows: Catalatic activity was measured spectrophotometrically at 30 °C in 100 mmol dm^{-3} potassium phosphate buffer (pH 7.00) containing 20 mmol dm^{-3} H_2O_2. The reaction was recorded as the decrease in absorbance at 240 nm (H_2O_2 concentration), and the reaction rate was calculated from the maximum slope of the trace using a molar coefficient of H_2O_2 of 43.6 mol^{-1} dm^3 cm^{-1} [S. Trakulnaleamsai, Ph. D. Dissertation, (Landscape around catalase I from *Bacillus stearothermophilus* and its application for protein engineering by artificial evolution), University of Osaka, Osaka, Japan, 1995, p. 4].