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MODEL OF THE EXTRADIOL-CLEAVING MANGANESE(II) DIOXYGENASE PENICILLAMINE-MANGANESE(II) COMPLEX

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Summary: The structure of the green penicillamine(Pen)-Mn(II) complex prepared under air was determined from its electronic spectra, molar ratio, ESR spectra and oxygen consumptions at various pH values and by potentiometric titration. Pen bound with Mn(II) in a molar ratio of approximately 1:1 forming coordination bonds with a thiolate and an amino group, and the complex consumed about 1 mol of oxygen at pH 9-10. Oxygen binding to this complex was found to be reversible at room temperature. The oxygen adduct complex catalysed oxidative extradiol-cleavage of catechol at pH 7.0-7.5. The Pen-Mn(II)-O_2 complex seems to be a simple model of extradiol-cleaving manganese(II) dioxygenase, which was recently found in Bacillus brevis.

Oxidative cleavage of aromatic rings with incorporation of molecular dioxygen occurs widely in nature (1-3). In the reaction, known as the dioxygenase reaction, two types of enzyme are involved ; intradiol-cleaving enzymes and extradiol-cleaving enzymes. Well-characterized enzymes of this class have iron in their catalytic site and are usually labile (4-8). Besides those iron-containing enzymes, a unique manganese(II) containing extradiol-cleaving enzyme, 3,4-dihydroxyphenylacetate 2,3-dioxygenase, was recenlty purified from Bacillus brevis (9). Interestingly, this enzyme contains tightly bound manganese(II).

During investigations on the chemistry and biochemistry of manganese ion (10), we found that the reaction mode of the Pen-Mn(II) complex under air mimics that of the extradiol-cleaving dioxygenase which contains Mn(II). This paper reports studies on the Pen-Mn(II) complex as the first successful model of the enzyme.

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Materials and Methods

DL-Penicillamine was a product of Nakarai Chemicals, Kyoto. All other chemical reagents used were of reagent grade. Optical spectra were measured with a Hitachi 330 spectrometer. pH Titrations were carried out with a Radiometer TTT60 titrator, equipped with an REC61/REA160 titrigraph and TTA60 titrator assembly and an associated autoburrette ABU12. ESR spectra were recorded with a JES-ME3X spectrometer with 100KHz field modulation. As standards, DPPH powder and MgO powder doped with Mn(II) were used. Measurements were carried out at 293K in solution and 77K in the frozen state. Oxygen consumptions in solution were measured at 25°C with a biological oxygen monitor (Yellow Springs Instruments, Model 5331). Oxygen consumption was calculated assuming the presence of 250 μM oxygen in air-equilibrated buffer. Extradiol-cleaving activity was assayed by measuring the rate of formation of the yellow product, α-hydroxymuconic ε-semialdehyde, at 375 nm at 20°C in 0.2 M Tris-buffer, pH 7.4. The molar extinction coefficient of the product was taken as 33000 cm^{-1}M^{-1} (4).

Results and Discussion

When a mixture of Pen and manganese(II) chloride in a molar ratio of 2:1 in 0.2 M Tris-buffer (pH 7.4) was prepared under air at room temperature, a green color developed with absorption bands at 315 and 680 nm (Figure 1). On increasing the pH, no remarkable spectral changes in the absorption bands were observed at pH 7-10, but a hyperchromic effect on each band was seen at higher pH values. On bubbling inert gas through the solution, the color disappeared after 15 min, and on re-aeration the green color reappeared. This cycle could be repeated several times, suggesting that the green color was due to formation of a Pen-Mn(II)-dioxygen adduct complex. The Pen:Mn(II) binding ratio measured by the molar ratio method with monitoring at 680 nm was approximately 1:1 under air at three differ-

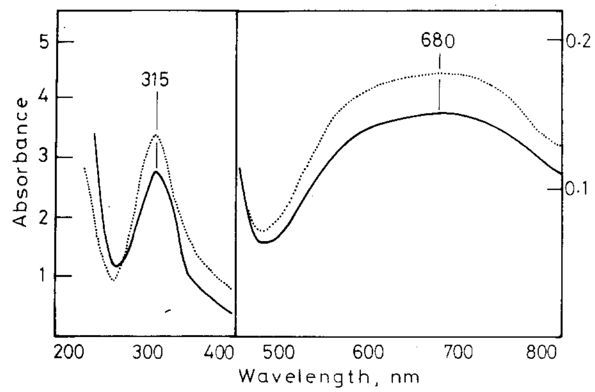

Figure 1. Electronic spectra of the green penicillamine-Mn(II) complex under air. pH: 7.4 (0.2M Tris-buffer), —— ; 10.0 (M/20 borate buffer), -----. The concentrations of Pen and $MnCl_2$, respectively, were 2 mM and 1 mM for the UV region and 10 mM and 5 mM for the visible region.

ent pH values (Figure 2). The dissociation of the two protons from Pen, observed by potentiometric titration of Pen-Mn(II) systems with mixtures in various ratios under nitrogen gas, indicating that at least two functional group, presumably thiolate and amino groups, could coordinate to Mn(II) and form a 2:1 Pen:Mn(II) complex. During titrations, the solution were colorless. The ESR spectra of the green Pen-Mn(II) complexes (1:1 and 8:1) prepared under air at pH 7.4 and 9.0 clearly showed the presence of manganese ion in its divalent form giving signals at g=2.0 and 5 at 77K (Figure 3). With Pen:Mn(II)=8:1 at pH 9.3 the hyperfine interval around g=2.0 was smaller (85.1 Gauss) than that (96.4 Gauss) with the same system at pH 7.4, indicating that in the alkaline pH region a relatively stable complex was formed. This result is consistent with the increased hyperchromic effect at higher pH values. The ESR spectral pattern reported for manganese(II) dioxygenase (9) suggests that the enzyme is similar to the Pen-Mn(II) (8:1) system at pH 9.3. Since the expected signal due to O_2^- was not observed under the conditions investigated, electron transfer from Mn(II) to O_2 for generation of O_2^- and Mn(III) seems unlikely to occur. Further work on this point is in progress. Oxygen consumption was investigated using an oxygen electrode. On initiating the reaction by addition of Mn(II) to a stable solution of Pen at three different pH values of 7.4, 9.3 and 10.0 at 25°C, rapid oxygen consumption was observed, the equilibrium being established within at least 2

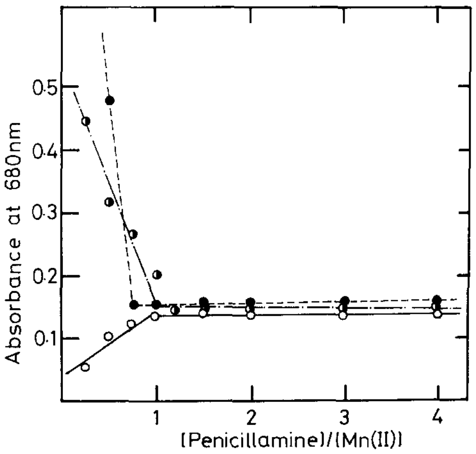

Figure 2. Effect of the molar ratio of the penicillamine-Mn(II) complex on the absorption under air. pH: 7.4,—○—: 9.3,--●--; 10.0,--◑--. The concentrations of Mn(II) were 5 mM, 10 mM and 5 mM at pH 7.4, 9.3 and 10.0, respectively.

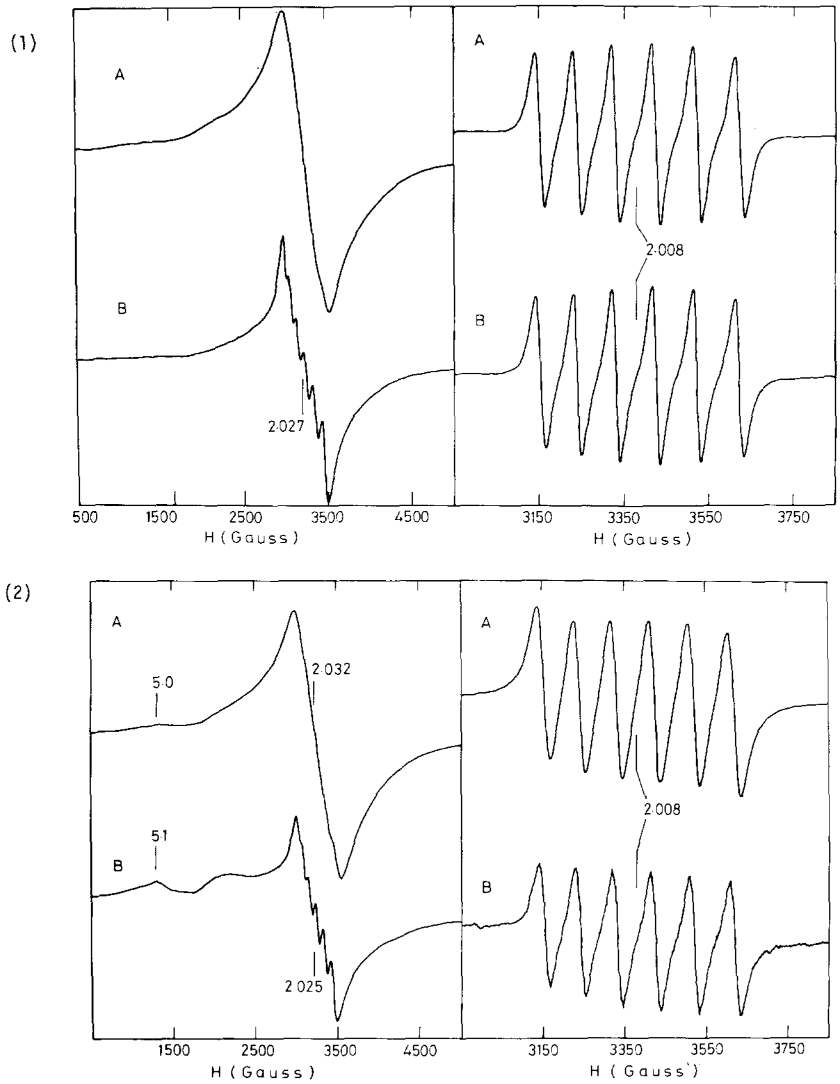

Figure 3. ESR spectra of green penicillamine-Mn(II) complexes. Complexes were prepared by the same procedure as for Figure 1 and the spectra were measured at 77K (left) and room temperature (right). pH= (1) 7.4; (2) 9.3. Mixing ratio of Pen:Mn(II) = (A) 1:1; (B) 8:1. The magnitudes of the hyperfine structural interval around g=2.0 were as follows: 1-A at 293K, 93.2 Gauss; 1-B at 77K, 96.4 Gauss; 1-B at 293K, 92.8 Gauss; 2-A at 293K, 93.6 Gauss; 2-B at 77K, 85.1 Gauss; 2-B at 293K, 93.2 Gauss. These values at 77K were obtained from the expanded ESR spectra.

min. Since it is well known that thiol compound are gradually auto-oxidized in neutral and alkaline pH regions, oxygen consumptions with the complex were calculated as the differences between the values for the Pen-Mn(II) complex and Pen alone under the same conditions[*2]. As seen in Figure 4, oxygen consumption,

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[*2] Mn(II) alone consumed very little oxygen under the same conditions.

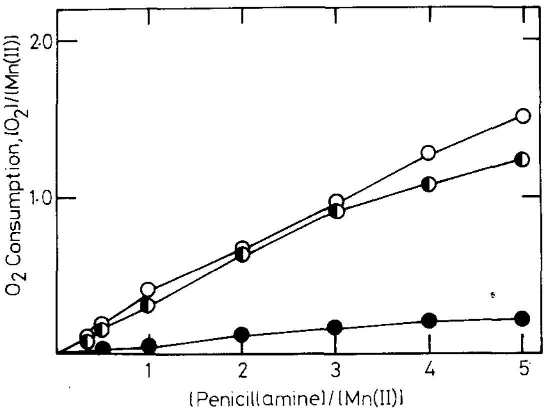
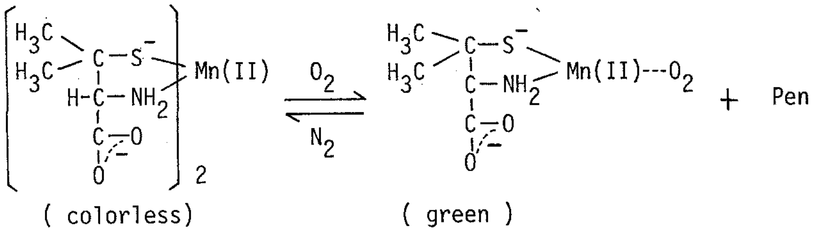


Figure 4. Oxygen consumptions by penicillamine-Mn(II) complexes at various pH values at 25°C. pH: 7.4 (0.2 M Tris-buffer), ● ; 9.3 (0.1 M borate buffer) ◐ ; 10.0 (M/20 borate buffer) ○ . The concentrations of $MnCl_2$ were 0.890 mM, 0.179 mM and 0.089 mM at pH 7.4, 9.3 and 10.0, respectively. The reaction was followed for at least 10 min and the data presented in the figure are consumption in 1 min.

expressed in terms of O_2 consumed per Mn(II), was enhanced in the presence of Pen and at elevated pH values. At neutral pH, Mn(II) was not fully oxygenated, but at pH 9-10, 1 mol of Mn(II) consumed about 1 mol of dioxygen. From these results, we tentatively concluded that the reaction of the Pen-Mn(II) complex under air were as follows :



Dioxygen binds so loosely to the Mn(II) center that it can be replaced by an inert gas such as nitrogen or argon.

An interesting feature of this complex is its activity for oxidative cleavage of an aromatic ring such as that of catechol. When a mixture of pyrocatechol and Pen-Mn(II) complex at pH 7.4 was prepared under air, a yellow color developed with an absorption maximun at 375 nm, bacause manganese(II) dioxygenase shows a similar spectral change in the presence of substrate (9). The Pen-Mn(II) complex formed this oxygenated product only in the system under air. Extradiol-cleaving activity was assayed by monitoring the rate of formation of the yellow product, α-hydroxymuconic ε-semialdehyde, at 375 nm at 20°C in 0.2 M Tris buff-

Table I. Catalytic extradiol-cleaving dioxygenation of pyrocatechol by penicillamine-Mn(II) complex[a]

Reaction time (min)	Amount of product (mM)	Conversion[b] (%)
45	0.0176	0.26
75	0.0864	1.30
105	0.0920	1.38

[a] Reaction conditions : Pen 1.33 mM, $MnCl_2$ 0.67 mM and pyrocatechol 6.67 mM in 0.2 M Tris-buffer, pH 7.4. The reaction mixture was stirred under air.
[b] Based on pyrocatechol.

er, pH 7.4, using pyrocatechol as substrate, as described in the literature (4, 9) (Table I). The reaction rate was increased by stirring the solution under air. The reaction showed an optimal pH of 7.0-7.5. At higher pH values, the intial stage of the reaction was faster but unidentified absorption bands appeared, probably due to formation of some other oxidation products. The mechanism of this reaction at higher pH is not fully understood. Complexes containing thiol compounds, such as cysteine and cysteine ethyl esters in place of Pen exhibited similar extradiol-cleaving activity on catechol[*3]. The Pen-Mn(II) complex also showed very slow reactions with a series of catechol analogs. In non-enzymatic dioxygenation of catechol with Cu(II) and O_2 leading to formation of cis,cis-muconic acid minomethyl ester (11), Groves proposed that an active metal species rather than active oxygen is involved in the ring cleavage reaction (12). Taking this suggestion into consideration, further studies on the relation between the structure of the complex and catalytic activity are in progress.

Thus the Pen-Mn(II)-O_2 complex having both Mn(II)-thiolate and amino coordination bindings and extradiol-cleaving dioxygenase-like activity may provide not only an insight into the coordination mode around the Mn(II) center of the enzyme but also a simple model for elucidating the mechanism of the catalytic reaction by the enzyme.

[*3] The hydroxyamino acid (serine or threonine)-Mn(II) complex did not show properties such as oxygen consumption and extradiol-cleaving dioxygenase activity under the same conditions.

Acknowledgments

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References

1. Nozaki,M. (1974) in : Molecular Mechanism of Oxygen Activation (ed. Hayaishi,O.) Academic Press, New York, 135-167.
2. Que,L.,Jr. (1980) Struc.Bond. 40, 39-72.
3. Wood,J.M. (1980) in : Metal Ion Activation of Dioxygen (ed. Spiro,T.G.) Wiley-Interscience Pub., New York, 163-180.
4. Nozaki,M., Kagamiyama,H. and Hayaishi,O. (1963) Biochem.Z. 338, 582-590.
5. Kita,H. (1965) J.Biochem. 58, 116-122.
6. Nozaki,M., Ono,K., Nakazawa,T., Kotani,S. and Hayaishi,O. (1968) J.Biol. Chem. 243, 2682-2690.
7. Ogasawara,N., Gander,J.E. and Henderson,L.M. (1966) J.Biol.Chem. 241, 613-619.
8. Dagley,S., Greary,P.J. and Wood,J.M. (1968) Biochem.J. 109, 559-568.
9. Que,L.,Jr., Widom,J. and Crawford,R.L. (1981) J.Biol.Chem. 256, 10941-10944.
10. Sakurai,H., Nishida,M. and Yoshimura,T. (1982) Inorg.Chim.Acta 66, L17-20.
11. Tsuji,J. and Takayanagi,H. (1974) J.Am.Chem.Soc. 96, 7349-7350, and Rogic,M.M. and Demmin,T.R. (1978) J.Am.Chem.Soc. 100, 5472-5487.
12. Groves,J.T. (1980) in : Metal Ion Activation of Dioxygen (ed. Spiro,T.G.) Wiley-Interscience Pub., New York, 125-162.