Visible and Magnetic Circular Dichroism Studies on Cobalt(II)-substituted *Rhus vernicifera* Laccase

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Abstract

The visible and magnetic circular dichroism (MCD) spectra of Co(II) derivatives of *Rhus vernicifera* laccase are reported. Anaerobic incorporation of 1 g-atom of Co(II) into apolaccase gave bands at 528 (ϵ = 248), 558 (254) and 589 nm (shoulder) attributable to d–d transitions. The MCD spectrum in the corresponding region is similar to that of Co(II)-substituted hemocyanin, indicating that the Co(II) ion incorporated into apolaccase is tetrahedral. On increasing the amount of Co(II) ion acting on the apolaccase, both the intensities of the absorption and the MCD spectra increased, and 2 g-atoms of tetrahedral Co(II) ion were introduced into the apolaccase. Very similar absorption and MCD spectra were obtained when laccase whose type I copper site was occupied by Hg(II) and both type II and type III copper sites were vacant (TlHg apolaccase) was treated with Co(II); this clearly supports the hypothesis that Co(II) cannot be incorporated into a type I copper site but may possibly be incorporated into a type III copper site. A tetrahedral Co(II) ion was also introduced into a type II copper site of type II copper-depleted (T2D) laccase, although its MCD bands were shifted *ca.* 20 nm to the longer wavelength region from the MCD bands due to tetrahedral Co(II) ion incorporated into type III copper site(s). The present study demonstrates that a tetrahedral Co(II) ion is introduced into type II or type III copper site(s) of laccase.

Introduction

Laccase (EC 1.10.3.2), which oxidizes *o*- and *p*-diphenol derivatives, phenylenediamine, Fe(II) and ascorbate by molecular oxygen, has been purified from latex of the lacquer tree [1] and many different fungi like *Polyporus versicolor* [2]. Laccase contains four copper ions per protein molecule and is a multicopper oxidase, together with ceruloplasmin and ascorbate oxidase. The characteristic strong band at 614 nm (ϵ = 5700) and the EPR signal with the unusually small hyperfine splitting constant ($|A_z|$ = 0.0043 cm^{-1}) comes from type I copper [3]. Type II copper gives an EPR signal with the usual magnitude of hyperfine splitting ($|A_z|$ = 0.0206 cm^{-1}) at its Z-component as for the cupric ion. Type III coppers exhibit a prominent shoulder at around 330 nm (ϵ = 4600–6200) but are EPR-undetectable because of the strong antiferromagnetic coupling between a pair of Cu(II) ions.

As one of the mild modification methods for the preparation of metalloproteins, Co(II) substitution has been successfully performed for copper proteins such as plastocyanin, azurin, stellacyanin [4, 5], plantacyanin [6], nitrite reductase [7] superoxide dismutase [8], amine oxidase [9], hemocyanin [10] and tyrosinase [11], affording structural information about the active sites of these proteins. However, all these copper proteins contain only type I, II or III copper(s) and Co(II) substitution has never been achieved for multicopper oxidase containing all types of coppers. In agreement with this, Larrabee and Spiro [12] reported that Co(II) could be substituted in the type I copper site of laccase. But it is revealed in the present study that type I copper cannot be substituted by a Co(II) ion. Here we show the absorption and MCD spectra of *Rhus vernicifera* laccase whose type II or type III copper site(s) was substituted by Co(II). The structures of the Co(II) binding sites are discussed based on the absorption and MCD spectra.

Experimental

Laccase was isolated from high quality latex of *Rhus vernicifera* produced in China and imported by Saito and Co. Osaka, Japan. Acetone powder was prepared carefully under the direction of Professor Nakamura of this university. Purification of laccase was performed according to Reinhammar [13] with a minor modification. The purity of the isolated laccase was checked by electrophoresis. A_{280}/A_{614} was found to be 16, as has been reported in the literature [13]. Apolaccase was prepared by removing all coppers from laccase with KCN under N_2. The

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amount of residual Cu was below the detection limit of atomic absorption spectroscopy. Selective depletion of type II copper from laccase was performed according to the method of Graziani *et al.* [14]. The amounts of total Cu and EPR-detectable Cu^{2+} were 2.8 and 1.0, respectively, and no type II Cu^{2+} EPR signal was observed at a high gain (spectrum not shown). Apolaccase with Hg(II) incorporated at its type I copper site (TlHg apolaccase) was prepared by treating apolaccase with a slight excess of Hg(II) ion, as according to Morie-Bebel *et al.* [15, 16]. Co(II)-substitution in apolaccase, T2D laccase and TlHg apolaccase was performed by using a Thunberg cuvette under purified N_2. Incubation of Co(II) (99.999%) was usually continued for 2 days. Tris-HCl buffer (pH 8.0, 0.1 M) was used throughout the experiments.

Absorption spectra were measured on a Hitachi U-3400 spectrometer, and circular dichroism (CD) and MCD spectra on a JASCO J-500A spectropolimeter attached to a DP-500N data processor. An electromagnet (1.31 T) was used for measurements of MCD spectra. All these spectra were obtained at room temperature. EPR spectra were measured on a JEOL FE-1X X-band spectrometer at 77 K. The amount of EPR-detectable Cu^{2+} ion was estimated using Cu-EDTA as standard. The total content of copper in native laccase and the modified laccase was determined by using a Nippon Jarrell-Ash AA-1 atomic absorption spectrometer.

Results and Discussion

Into apolaccase was introduced 1 g-atom of Co(II) ion and the resulting absorption and MCD spectra are shown in Fig. 1. Three bands are noticeable at 528 (ϵ = 248), 558 (254) and 589 nm (sh). The strong intensities of the d–d bands exclude the octahedral

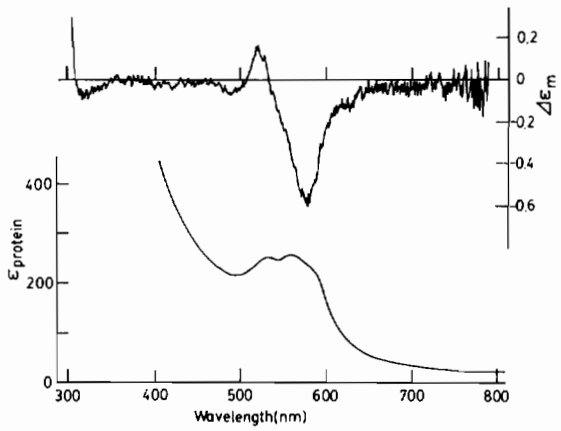

Fig. 1. Absorption and MCD (1.31 T) spectra of Co apolaccase (protein concentration, 143 μM; pH 8.0, 0.1 M Tris-HCl buffer).

structure as the stereochemistry of the Co(II) ion [17]. The whole absorption feature is very similar to those of Co(II)-substituted hemocyanin [10] and Co(II)-substituted tyrosinase [11] which have only type III copper sites originally. The concomitantly shown MCD spectrum gives a negative band at 580 nm and a smaller positive band at 529 nm, which are characteristic of a high-spin tetrahedral Co(II) ion, as shown by model studies. (The transition involved has been explained as $^4A_2(F) \rightarrow {}^4T_1(P)$ [18].) A small negative band at *ca.* 500 nm originates from extra octahedral Co(II) ion, although it is not easy to estimate its content. The MCD spectral features of the tetrahedral Co(II) ion in laccase are very similar to that of tetrahedral Co(II) ion introduced into hemocyanin [10]. On the other hand, the intensities of the MCD bands are slightly stronger than those of Co(II)-substituted zinc metalloenzymes, thermolysin and carboxypeptidase A containing tetrahedral Co(II) [18]. No transition coming from S^-(Cys) → Co(II) was observed in the near-UV region; this differs from absorption and MCD spectra of Co(II)-substituted blue copper proteins like plastocyanin, azurin, stellacyanin [4, 5], plantacyanin [6] and nitrite reductase [7]. Larrabee and Spiro [12] also found a similar absorption spectrum for excess Co(II)-treated apolaccase, and they proposed that Co(II) was incorporated into a type I copper site. However, all Co(II)-substituted blue copper proteins give highly split d–d bands in the region 500 to 700 nm, since the structure of the Co(II) ion in these proteins is highly distorted tetrahedral or trigonal bipyramidal. Compared to these spectra, the d–d bands of the present Co(II)-substituted laccase (*ca.* 500–600 nm) are not split as widely as the literature values [12]. This is because the Co(II) in laccase has a higher symmetry, almost tetrahedral. Larrabee and Spiro [12] observed the band at 305 nm and assigned it to S^-(Cys) → Co(II). But two bands have been observed between 330–400 nm for all Co(II)-substituted blue copper proteins [4–7]. In the present Co(II) derivative, the relevant bands were never observed. Accordingly, Co(II) ion does not seem to be introduced into a type I copper site. It is unlikely that Co(II) ion introduced into a type I Cu site is lacking in the S^-(Cys) → Co(II) transitions (330–400 nm). A type III Cu site is the most probable binding site for the Co(II) ion (*vide infra*).

A similar Co(II) substitution was successfully performed for laccase, whose type I copper site had been substituted by Hg(II) and both type II and type III copper sites were vacant (TlHg apolaccase). Since the type I copper site had been occupied by a Hg(II) ion which specially favors soft ligand groups like cysteine and methionine, an exogenous Co(II) ion could not expel Hg(II) ion from a type I copper site. Both the absorption and MCD spectra of the resulting Co(II) derivative (Fig. 2) are apparently very similar to those

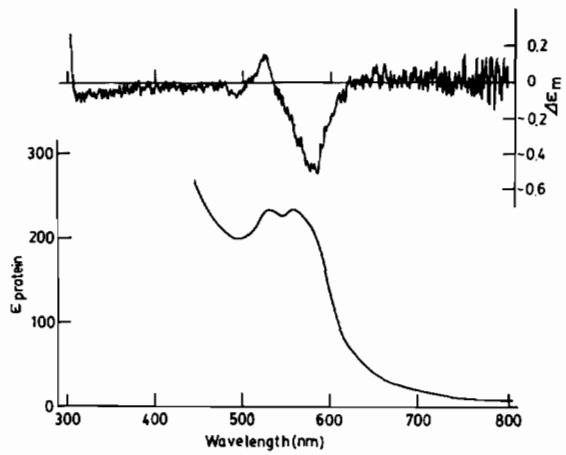

Fig. 2. Absorption and MCD (1.31 T) spectra of TlHg Co apolaccase (protein concentration, 119 μM; pH 8.0, 0.1 M Tris-HCl buffer).

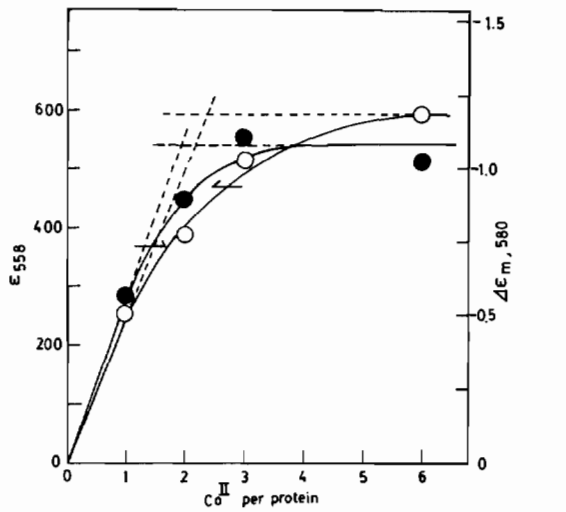

Fig. 3. Saturation–absorption and MCD curve for Co(II)-treated apolaccase. Intensity of absorption at 558 nm (○) and MCD signal at 580 nm (●) are plotted against the amount of Co(II) in the laccase molecule.

found in Fig. 1. Therefore, Co(II) ion is considered to be incorporated into the same site, *i.e.* a type III copper site both in apolaccase and TlHg apolaccase. In addition, it appears that the presence and absence of a metal ion at a type I copper site does not affect the character of Co(II) at a type III copper site. This fact supports the idea that type I copper sites and type III copper sites are separated by a certain distance [19].

The action of increasing the amount of Co(II) ion on apolaccase gave stronger bands due to tetrahedral Co(II) species in the absorption and MCD spectra (Fig. 3). The intensities of the absorption at 558 nm and the negative MCD band at 580 nm were plotted against the amount of Co(II) ion incorporated into apolaccase. Extrapolation of the linear regions at both ends of the curves shows that 2.4 or 2.0 tetrahedral Co(II) ions are incorporated into apolaccase. The former value might be slightly overestimated, since the absorption intensity at 558 nm continued to increase gradually because of the increase in the amount of octahedral Co(II) ion which contributes to the absorption around 500 nm. On the other hand, the latter value obtained using MCD spectral intensity reflects the amount of the incorporated tetrahedral Co(II) ion directly. This fact strongly suggests that two tetrahedral Co(II) ions are incorporated into two type III Cu sites of apolaccase. Incubation of apolaccase with Co(II) ion was usually carried out for 2 days. The presence of octahedral Co(II) species might arise from an insufficient incubation time. However, equilibrium conditions were ascertained to be retained within 2 days from a prolonged incubation of Co(II) ion for 7 days. Therefore, it appears that more than *ca.* three Co(II) ions are required to incorporate two tetrahedral Co(II) ions into apolaccase, according to our method, probably because the binding constant of the Co(II) ion is relatively small. Furthermore, two tetrahedral Co(II) ions introduced into type III copper sites appear to contribute equally both to the absorption and MCD spectra.

When T2D laccase was treated with a large excess of Co(II) ion for 2 days and dialyzed to buffer solution, 0.7 Co(II) ion was introduced into T2D laccase. The total copper content was 2.9 and the EPR-detectable amount of Cu^{2+} (only type I copper was observed) was 1.0. A decrease in the total Cu ion from the protein molecule was not observed, although the blue color of T2D laccase was partly bleached because reduction of type I copper by excess Co(II) proceeded to a certain extent (Fig. 4). However, the decrease in the blue color was reversed, though not fully, by treating Co(II)-treated T2D laccase with ferricyanide. The characteristic MCD bands due to a tetrahedral Co(II) ion at 600 nm and a very broad negative MCD band due to type I copper at around 670 nm [20] are superimposed on the MCD spectrum. The band attributable to type I copper disappeared on addition of dithionite to Co(II)-treated T2D laccase and the MCD band due to the tetrahedral Co(II) ion was left (spectrum not shown). The negative band is present at 600 nm, a 20 nm longer wavelength than that found for Co(II) apolaccase. All these data strongly support the idea that the Co(II) ion is introduced into a type II copper site in T2D laccase. However, excess Co(II) was still not able to completely introduce 1 g-atom of Co(II) into a type II copper site. This is probably because the binding ability of Co(II) ion at a type II Cu site is weaker than that at a type III Cu site. It should be noted that Co(II) ion can be introduced into a type II copper site only when type III copper sites are occupied by the intrinsic Cu(II) ions. A structurally

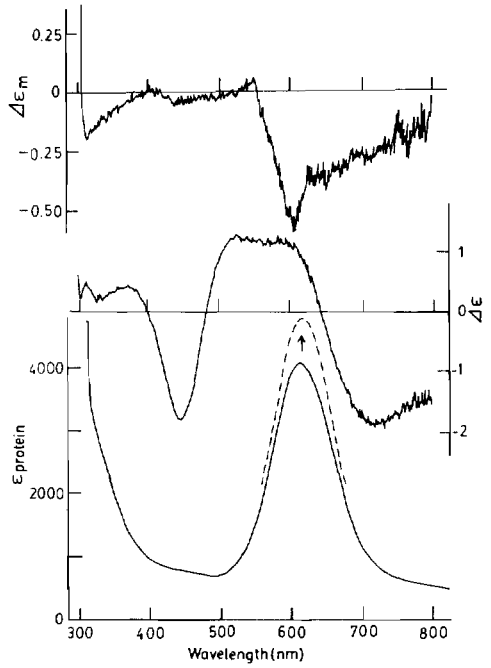

Fig. 4. Absorption, CD and MCD (1.31 T) spectra of Co(II)-treated T2D laccase (protein concentration, 260 μM; total copper content, 772 μM; total cobalt content, 180 μM; pH 8.0, 0.1 M Tris-HCl buffer). The dotted line indicates the absorption spectrum after $Fe(CN)_6^-$ treatment.

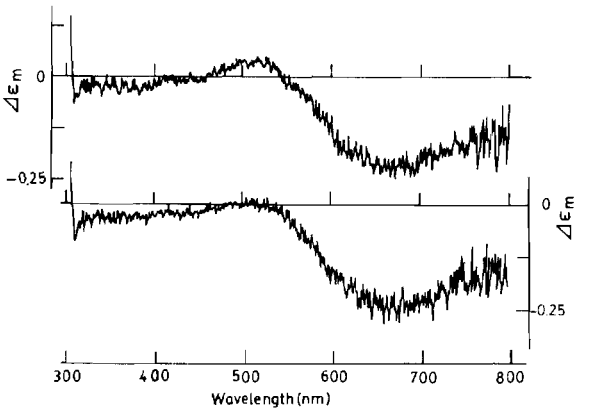

Fig. 5. MCD (1.31 T) spectra of native laccase (upper spectrum) and 1 g-atom of Co(II)-treated native laccase (lower spectrum).

close relation between type II and type III Cu sites [19, 21] will delicately affect the binding ability of exogenous Co(II) ion. The fact that Co(II) does not have a specific binding site besides type II and type III copper sites is supported by the fact that no tetrahedral Co(II) was noticeable in the MCD spectrum of Co(II)-treated laccase under N_2 (Fig. 5).

One of the significant conclusions of the present study is that Co(II) is found to be incorporated into type II or type III copper site(s) of laccase. It is surprising that Co(II) cannot be introduced into a type I copper site in spite of the successful Co(II) substitutions for many blue copper proteins. Type I copper may be buried relatively deep inside the laccase molecule compared to simple blue copper proteins. However, this does not seem to coincide with the suggestion by Mims *et al.* [22] that the type I copper of laccase is directly accessible to solvent from deuterium modification pattern in the electron spin-echo envelopes. Alternatively, the type I copper site may not have such strong binding ability for Co(II) as that of simple blue copper proteins because of a delicate difference in local structure, although all these type I or blue coppers have very similar properties to each other and are reasonably supposed to have similar structure around the metal binding sites. It seems to be difficult to explain such a peculiar property of the type I Cu site of laccase at the present stage. According to our unpublished results, ascorbate oxidase and ceruloplasmin also cannot incorporate Co(II) ion at their type 1 copper sites. Nevertheless, valuable information that both type III coppers of laccase and tyrosinase or hemocyanin have a structural resemblance has been obtained. The present Co(II)-substitution study permits us to suppose that three imidazole nitrogens coordinate to each type III copper of laccase. This is in line with the result of an ENDOR study for half-met type III coppers of laccase [23]. Co(II)-substitution in place of type II copper is only limited for superoxide dismutase [8] and amine oxidase [9] and the structure of the Co(II) has been revealed to be tetrahedral or trigonal bipyramidal for the former and to be tetrahedral for the latter. Although the original type II copper site is tetragonal, it is not necessarily surprising that Co(II) incorporated into a type II copper site is also tetrahedral in the case of laccase. The deviation of 20 nm in the MCD spectra for the Co(II) ion incorporated into type II and type III copper sites suggests that the ligand-field strengths afforded by coordinating groups (probably three histidine imidazole groups and a water or a hydroxide molecule) are not identical but are similar both for type II and type III copper sites.

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