Type III Coppers in an EPR Detectable Met Form of Multicopper Oxidases Afford an Identical EPR Signal with Type II Copper

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Abstract

The mixed metal derivatives of laccase, ascorbate oxidase and ceruloplasmin which contain mercury(II) in the type I copper binding site(s) and copper(II) in the type II and type III copper binding sites, have been prepared from the apoenzymes, Hg(II) and Cu(II). All copper(II)s were EPR detectable, giving a signal very similar to that of the intrinsic type II copper. Consequently, Cu(II)s in an EPR detectable met form appear to have a close structural resemblance with type II Cu(II).

Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) [1], ascorbate oxidase (L-ascorbate: oxygen oxidoreductase, EC 1.10.3.3) [2] and ceruloplasmin (Fe(II): oxygen oxidoreductase, EC 1.16.3.1) [3] are multicopper oxidases containing three distinct types of Cu centers. Numbers of type I (blue) Cu, type II (non-blue) Cu and type III (EPR undetectable) Cu in native laccase, ascorbate oxidase and ceruloplasmin are considered to be 1-1-2, 3-1-4 and 2-1-2, respectively. In spite of a variety of investigations on these enzymes, type I copper has prevented the characterization of the properties of type II and type III coppers by its strong masking effect. Metal substitution has been successfully achieved for many blue copper proteins [4–6], non-blue copper proteins [7, 8] and EPR undetectable binuclear copper-containing proteins [9, 10]. However, mercury(II) substitution [11, 12] and cobalt(II) substitution [13] have been carried out only for laccase among the three multicopper oxidases. Here we have performed mercury(II) substitution of the type I copper(s) in laccase, ascorbate oxidase and ceruloplasmin starting from apoenzymes, Hg(II) and Cu(II), coming to the conclusion that all copper(II)s incorporated into type II and type III copper sites are EPR detectable and give rise to an identical EPR signal. The structure of the type III copper(II) in an EPR detectable met form was shown to be very similar to that of type II copper(II).

Experimental

Laccase was prepared from latex of the Chinese lacqer tree supplied by Saito and Co. according to the method of Reinhammar [14]. A_{280}/A_{614} as a purity index was 16. Ascorbate oxidase, whose final A_{280}/A_{610} ratio was 24, was purified from cucumber peelings [15]. Human ceruloplasmin was purchased from Green Cross Corp. and bovine ceruloplasmin was isolated from bovine serum as described previously [16]. The absorption ratio, A_{280}/A_{610} was 24 for human ceruloplasmin and 23 for bovine ceruloplasmin. Apoenzymes were prepared by treating the reduced enzymes with KCN under N_2. The Hg(II) substituted derivatives at type I copper site(s) were obtained by reacting a stoichiometric amount of $HgCl_2$ with apolaccase and apoascorbate oxidase or excess $HgCl_2$ with apoceruloplasmin in 0.1 M, pH 7.0 acetate solution for 2 days. The resulting proteins were dialyzed against 0.1 M Tris buffer (pH 8.0) and then treated with a stoichiometric amount of $CuCl_2$ for 2 days. Otherwise, a large excess of Cu(I) was reacted with the Hg(II) derivatives under N_2 and extra copper was aerobically removed by dialysis against the Tris buffer solution.

Absorption and circular dichroic spectra were measured on a Hitachi U-3400 spectrometer and a Jasco J-500A spectropolarimeter, respectively. EPR spectra were obtained with a Jeol JES-FE1X X-band spectrometer at 77 K. The amount of EPR detectable Cu(II) was estimated by the double integration method using Cu–EDTA as a standard. The total amounts of copper in the enzyme derivatives were determined by atomic absorption spectroscopy with a Nippon-Jarrell Ash AA-1 spectrometer.

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Results and Discussion

EPR spectra of native multicopper oxidases and their derivatives are shown in Fig. 1. Apparently, the signal due to only one kind of species, which is very similar to that of the corresponding type II Cu in the native enzyme, is observable in the spectrum of each Hg derivative. EPR parameters of the type II Cu in the native enzyme and type II like-Cu in its derivative are $g_{\parallel} = 2.24$, $g_{\perp} = 2.05$, $|A_{\parallel}| = 0.020$ cm^{-1} for native laccase [17], $g_{\parallel} = 2.23$, $g_{\perp} = 2.05$, $|A_{\parallel}| = 0.020$ cm^{-1} for laccase derivative, $g_{\parallel} = 2.25$, $g_{\perp} = 2.05$, $|A_{\parallel}| = 0.020$ cm^{-1} for native ascorbate oxidase, $g_{\parallel} = 2.25$, $g_{\perp} = 2.05$, $|A_{\parallel}| = 0.019$ cm^{-1} for ascorbate oxidase derivative, $g_{\parallel} = 2.25$, $g_{\perp} = 2.05$, $|A_{\parallel}| = 0.019$ cm^{-1} for native ceruloplasmin [18], and $g_{\parallel} = 2.26$, $g_{\perp} = 2.05$, $|A_{\parallel}| = 0.018$ cm^{-1} for ceruloplasmin derivative. For nitric oxide- or ascorbate-treated enzymes, similar spectra bearing the characteristic of the only type II copper have been obtained [19–25].

The amount of EPR detectable Cu^{2+} in the derivatives of laccase, ascorbate oxidase and ceruloplasmin was 2.6, 4.7 and 1.8 per protein molecule, respectively. These values coincide with the total amounts of Cu which were determined by atomic absorption spectroscopy; 2.8, 4.7 and 1.9 for the derivatives of laccase, ascorbate oxidase and ceruloplasmin, respectively. The number of potential Cu binding sites subtracting that for the type I Cu site(s) is 3, 5 and 3 for the Hg derivatives of laccase, ascorbate oxidase and ceruloplasmin, respectively. Cu(II)s incorporated into enzyme derivatives are supposed to be accommodated by the type II and type III copper sites, affording a sole EPR signal. Supporting this, extra Cu(II)s beyond the number of the original Cu-binding sites were easily removed from excess Cu-treated Hg derivatives of laccase and ascorbate oxidase by dialysis against buffer solution. In the native enzyme type III Cu(II)s are EPR silent because of the strong antiferromagnetic coupling through a bridging group, presumably μ-oxo, hydroxo or another group [26, 27]. However, in the present derivatives, Cu(II)s possibly incorporated into type III Cu sites are in the EPR detectable met state, where no group bridges between a pair of Cu(II)s.

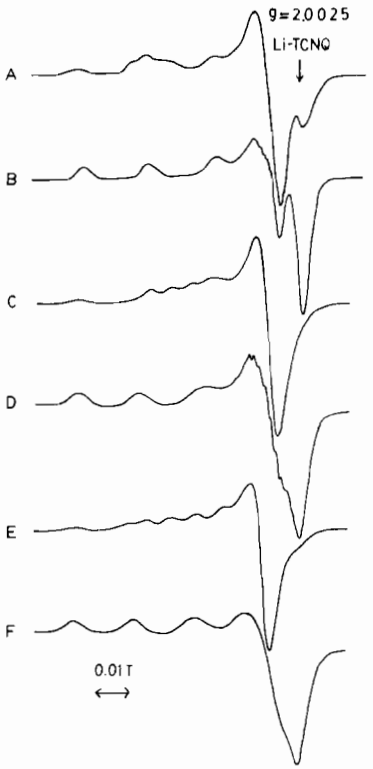


Fig. 1. EPR spectra of native enzymes (pH 6.0, phosphate buffer) and Hg derivatives (pH 8.0, Tris buffer): laccase (A), Hg laccase (B), ascorbate oxidase (C), Hg ascorbate oxidase (D), ceruloplasmin (E) and Hg ceruloplasmin (F).

In the ceruloplasmin derivative the total amount of Cu was not 3 but 1.9. This may have arisen from the fact that excess Hg(II) was used to prepare the Hg derivative. Since ceruloplasmin can bind some extra cuprous ions other than 5 Cu(II) ions, which occupy type I, II and III copper sites [3], excess Hg(II) was reacted with the apoenzyme in order to avoid the binding of coppers at these extra sites. Shortage of the amount of Cu(II)s incorporated into the Hg derivatives of ceruloplasmin may have been brought about by a portion of the Hg(II) ion occupying type II and/or type III copper site(s). However, the very low sensitivity of Hg for atomic absorption spectroscopy prevented the determination of the total amount of Hg incorporated into apoceruloplasmin.

Although Hg(II) has an especially high affinity for the type I copper site [11, 12], Hg(II) can also occupy type II and type III copper sites of multicopper oxidases. When excess Hg(II) or Ag(I) was reacted with native multicopper oxidases, only the type II Cu signal was left, since type I Cu(II) was displaced by Hg(II) or Ag(I) (data not shown). Inspection of the amounts of the residual Cu and the EPR detectable Cu^{2+} suggested that both of the type II and type III coppers are also displaced by Hg(II) or Ag(I) slowly.

The absorption and CD spectra of Hg derivatives are shown in Fig. 2. A weak optical band coming from the d–d transitions of the type II-like Cu(II)s (type II Cu and EPR detectable met type III Cu) was observed at around 600 nm. A similar absorption band has been reported for ascorbate-treated ceruloplasmin [23]. CD spectra of the present derivatives are composed of several bands, differing from the absorption spectra which show only one broad peak. The CD spectral feature over the 480–800 nm region of the ceruloplasmin derivative (Fig. 2C)

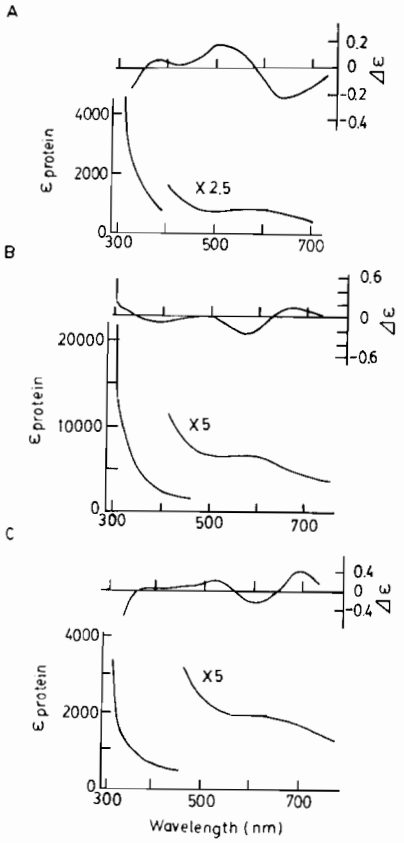
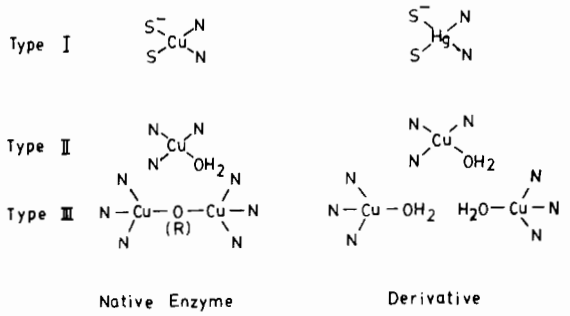


Fig. 2. Absorption and CD spectra of Hg derivatives of laccase (A), ascorbate oxidase (B) and ceruloplasmin (C).

is similar to that of ascorbate-treated ceruloplasmin [23], although the bands at 400 and 450 nm were not observed in our ceruloplasmin derivative. A rather similar feature was observed for the CD spectrum of the ascorbate oxidase derivative (Fig. 2B). In contrast, a very different feature was noticed for the CD spectrum of the laccase derivative (Fig. 2A).

McMillin and coworkers prepared a Hg derivative of laccase in which type III Cu(II)s are considered to be in the EPR undetectable form as in the native enzyme, although they did not give the absorption spectrum [11, 12]. On the other hand, according to our procedure using Cu(II) instead of Cu(I) [11, 12], the derivatives whose Cu(II)s are all EPR detectable are obtained. Nevertheless, the Cu(II) EPR signal of the laccase derivative given by McMillin and coworkers [11] appears to be identical with ours shown in Fig. 1. In addition, it is interesting that type III Cus in the EPR detectable met form give an identical EPR signal with that of type II Cu. Our recent Co(II) substitution study of laccase [13] indicated that the structure of the type III Cu site is similar to those of hemocyanin and tyrosinase. Both type II and type III Cu sites are revealed to be composed of the same ligand group set of 3NlO.

The present study suggests that the structures of type II and type III copper sites are similar, when a pair of type III Cus are not bridged by an intrinsic group. The structures of the metal binding sites of native enzyme and derivatives are figured as follows.



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