

Reassessment of the unusual ESR signal from type 3 copper of ascorbate oxidase reacted with hexacyanoferrate(II)

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Abstract

Anaerobic reactions of cucumber ascorbate oxidase and its type 2 copper-depleted derivatives with hexacyanoferrate(II) were investigated by absorption and ESR spectroscopies. An unusual ESR signal, which does not show a hyperfine structure in its g_{\parallel} region ($g_{\parallel}=2.28$, $g_{\perp}=2.08$), was developed by incubating the native enzyme with 120 equivalents of hexacyanoferrate(II). The unique ESR signal was not derived from the type 2 copper-depleted (T2D) derivative in which the type 3 coppers were in the reduced form, but it was derived from the T2D derivative in which the type 3 coppers were in the ESR silent met form. The ESR signal intensity and the microwave power saturation behavior suggested that the strange Cu^{2+} ESR signal comes from a sort of adduct of the type 3 copper with hexacyanoferrate(II).

Introduction

Ascorbate oxidase is a multicopper oxidase, together with laccase and ceruloplasmin, whose active sites are constructed by a type 1 copper and a trinuclear cluster composed of a type 2 copper and two type 3 coppers. Since ascorbate oxidase is a dimer, it contains eight copper ions (two type 1, two type 2 and four type 3 coppers) in the two active sites. The recent X-ray crystallographic study of ascorbate oxidase [1] showed how a dioxygen molecule is reduced to two water molecules by these enzymes.

Hexacyanoferrate(II) is concerned closely with multicopper oxidases. It is not only a good substrate but also a reagent for the selective removal of type 2 copper with the aid of chelating agents, dimethylglyoxime and ethylenediaminetetraacetic acid (EDTA) [2, 3]. We had discovered an unusual ESR signal in the course of the anaerobic reactions of ascorbate oxidase and its type 2 copper-depleted derivative (T2D) with hexacyanoferrate(II) [4]. Recently, we found a similar ESR signal ($g_{\parallel}=2.31$, $g_{\perp}=2.08$) for the reactions of native laccase and the H_2O_2 -treated T2D derivative [5]. These results on laccase urged us to reexamine the reactions of ascorbate oxidase and its derivatives with hexacyanoferrate(II) in detail.

Experimental

Cucumber ascorbate oxidase was purified from the crude enzyme supplied by Toyobo Co. according to the

method already reported [6]. The type 2 copper-depleted derivative was prepared according to the literature [3]. In order to oxidize the type 3 coppers, which had been reduced in the course of depleting the type 2 copper, the T2D derivative was treated with *c.* 20 fold excess of H_2O_2 and dialyzed. The anaerobic reaction of ascorbate oxidase with hexacyanoferrate(II) and the measurements were performed by using laboratory made quartz ware attached to a 5 mm ESR tube, 10 mm optical cell and three way stopcock. Hexacyanoferrate(II) and ascorbate were transferred into the quartz ware by using syringes under purified Ar or N_2 .

ESR spectra at 77 K were measured on a JEOL JES RE1X spectrometer and those at 4 K on a JEOL FE2XG spectrometer attached with a liquid helium transfer system. Cu-EDTA was used to estimate the ESR signal intensity by the usual double integration method. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used to calibrate the estimation error of the ESR signal intensities arising from the difference in tuning conditions. Absorption spectra were recorded at room temperature on a JASCO Ubest-50 spectrometer. Copper contents in the native enzyme and T2D derivative were determined by a Hitachi Z-6000 polarized Zeeman atomic absorption spectrometer.

Results and discussion

Native ascorbate oxidase was reacted with 120 equivalents of hexacyanoferrate(II) under N_2 or Ar and the

reaction processes were followed by absorption (Fig. 1) and ESR (Fig. 2) spectra. Soon after addition of hexacyanoferrate to ascorbate oxidase, the blue color of the solution decreased in intensity because the type 1 coppers were partly reduced. The reason why all type 1 coppers were not fully reduced as in the case of laccase is because the redox potential of the type 1 copper of ascorbate oxidase (370 mV) is lower than that of the tree laccase (394 mV) [7]. In the corresponding ESR spectrum (Fig. 2B), the signal due to the type 2 copper disappeared with reduction of the copper, although the type 1 copper signal was considerably reserved. (The ESR signal intensity decreased from 3.7 to 2.4 spins per protein molecule.) When the solution was allowed to stand at 4 °C for one day, its color gradually became green and the absorption band at 607 nm, which is characteristic of type 1 copper, was recovered nearly to its original intensity. In the corresponding ESR spectrum (Fig. 2C), the signal shape at around 3100 G was greatly changed when compared to that of the native enzyme. (The ESR spin intensity was 3.5 per the protein molecule at this stage.) Although every signal completely disappeared with dithionite, the addition of ascorbate ($\times c. 3$) to the sample afforded only the new ESR signal, which does not have a hyperfine structure at its g_{\parallel} region ($g_{\parallel} = 2.28$, $g_{\perp} = 2.08$) as shown in Fig. 2D. The signal intensity was 1.8 spins per protein molecule. The absorption spectrum (Fig. 1) exhibited a broad band between 400–500 nm, although its intensity was superficially stronger than the real value because the solution was slightly turbid at this stage. This unusual absorption band can be assigned to the Fe(II) to Cu(II) charge transfer (MMCT) band from laccase [5] and small molecule studies [8]. The behavior exerted by

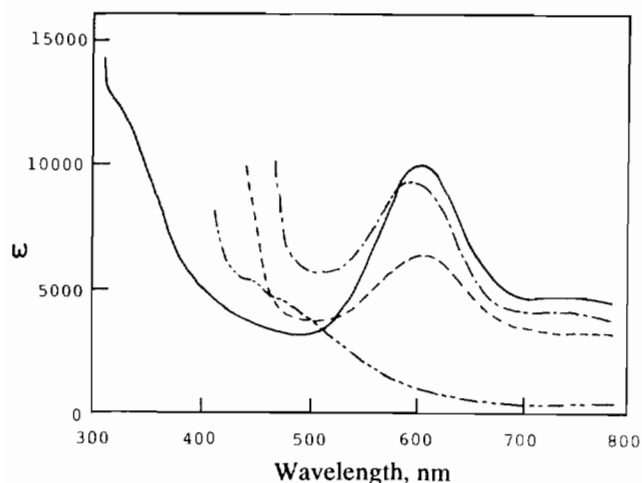


Fig. 1. Absorption spectra of ascorbate oxidase (—), hexacyanoferrate(II) ($\times 120$)-treated ascorbate oxidase (---) (10 min), (- - -) (1 day) and ascorbate-reduced $\text{Fe}^{\text{II}}(\text{CN})_6$ -ascorbate oxidase (.....). 50 μM enzyme in 0.1 M phosphate buffer (pH 6).

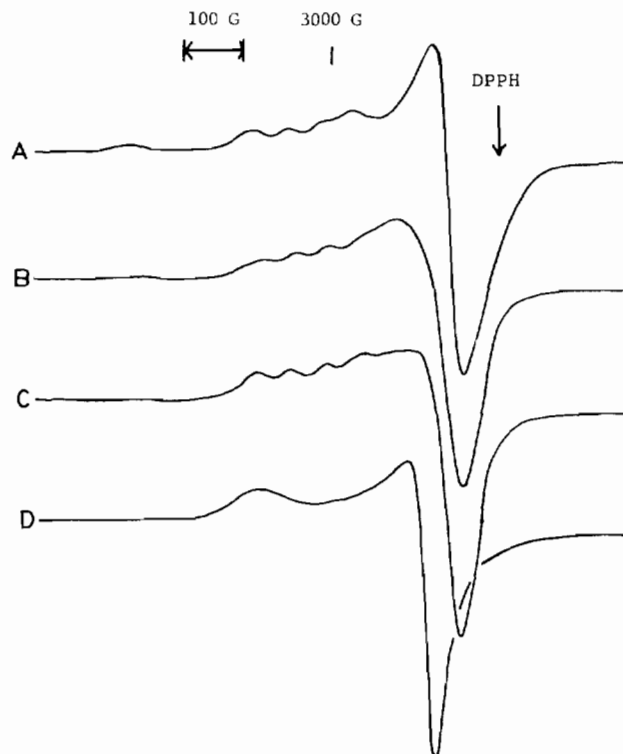


Fig. 2. The effect of hexacyanoferrate(II) on the ESR spectra of ascorbate oxidase. Native enzyme (A), after addition of $\text{Fe}^{\text{II}}(\text{CN})_6$ to (A), 2 min (B), 1 day (C), and ascorbate-reduced as (C) (D). The amounts of the ESR active spins are 3.7 (A), 2.4 (B), 3.5 (C), 1.8 (D), respectively. ESR conditions; frequency, 9.2 GHz, microwave power, 5 mW, modulation amplitude, 0.79 mT (100 kHz), temperature, 77 K.

ascorbate oxidase is not limited to this enzyme as shown for laccase in ref. 5. Since we could obtain similar results under vacuum, a fear of oxygen leakage will be eliminated at least after having started the reaction. The ESR signal in Fig. 2D disappeared when the reaction mixture was in contact with air. On the other hand, the control experiment using hexacyanoferrate(III) never gave the relevant ESR signal.

When hexacyanoferrate(II) was acted on by T2D ascorbate oxidase, we did not observe the new ESR signal (spectra omitted). The reason why we had observed a similar ESR signal in our previous paper for T2D ascorbate oxidase [5] might be that the native enzyme had been partly retained by standing the T2D derivative for several days [9] or a portion of the type 2 copper had not been depleted. In the present study, T2D derivatives were used soon after preparation. There has been discussion about the oxidation state of the type 3 coppers and the absorption feature of the T2D laccase and ascorbate oxidase at 330 nm [10–14]. However, the type 3 coppers in T2D laccase and ascorbate oxidase, which we have repeatedly prepared, seem to

be in the reduced form judging from XANES [11, 13] and titration studies [15].

The reduced type 3 copper in the T2D enzyme could be reoxidized by treatment with H_2O_2 . The band at 330 nm, which had disappeared in the course of the reaction to deplete the type 2 copper, developed again with H_2O_2 (spectra omitted) [15]. Figure 3 shows that the H_2O_2 -treated T2D ascorbate oxidase (T3Met) gives the new ESR signal (0.9 spins per protein molecule) by treatment with hexacyanoferrate(II). Therefore, the ESR signal appears to come from the oxidized type 3 copper, probably only in the absence of the type 2 copper. On the other hand, a broad MMCT band did not emerge, in contrast to the case of the native enzyme (spectrum omitted). We observed a very similar behavior in the reaction of the H_2O_2 -treated T2D laccase [5].

In order to obtain information about the new ESR signal, the microwave power saturation behavior was examined at 77 and 4 K. Figure 4 shows that the new ESR signal is scarcely saturated even at 4 K, although the half power saturation powers ($P_{1/2}$) of the type 1 and type 2 coppers in native enzyme are about 10 mW [16].

All results described above strongly suggest that the novel ESR signal arises from the oxidized type 3 copper which interacts with Fe(II) or another type 3 copper

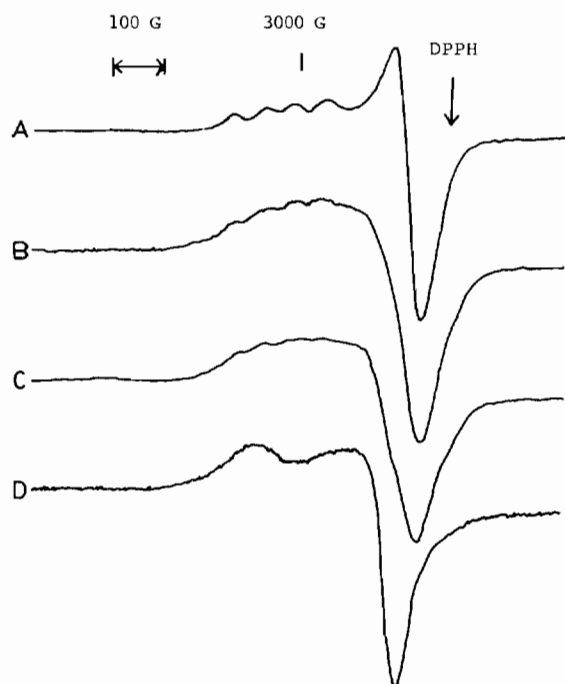


Fig. 3. The ESR spectra of H_2O_2 -treated T2D ascorbate oxidase reacted with hexacyanoferrate(II) ($\times 120$). T2D enzyme (A), after addition of $Fe^{II}(CN)_6$ to (A), 10 min (B), 1 day (C), and ascorbate-reduced as (C) (D). The amounts of the ESR active spins are 2.3 (A), 2.1(B), 3.0 (C), 0.9 (D), respectively. ESR conditions; frequency, 9.2 GHz, microwave power, 5 mW, modulation amplitude 0.79 mT (100 kHz), temperature, 77 K.

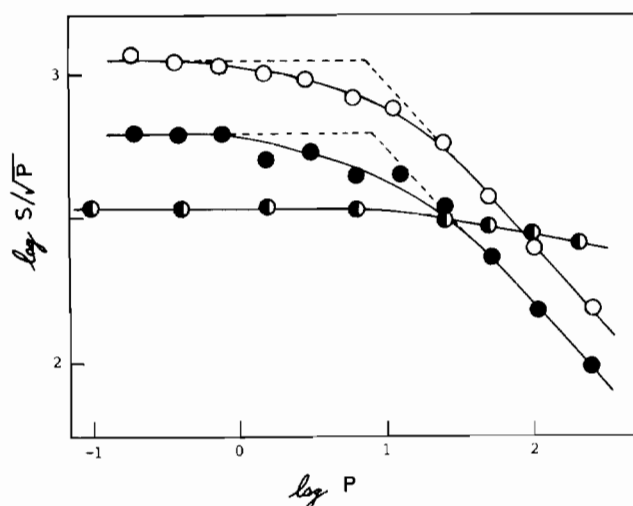


Fig. 4. The power saturation curves of the type 1 (○) and the type 2 (●) coppers of native ascorbate oxidase and the novel ESR signal (◐) of the ascorbate-reduced $Fe^{II}(CN)_6$ -ascorbate oxidase at 4 K. S and P denote signal intensity and microwave power (mW), respectively.

through a bridged CN^- ion. In line with this, the addition of free CN^- to the native and T3Met T2D derivative did not give the new ESR signal. Therefore, the CN^- bridged binuclear Cu(II) species will probably not be the origin of the relevant species. The lack of hyperfine structure might suggest that a spin exists on iron rather than on copper. According to the crystal structure analysis [1], ascorbate oxidase seems to have a crevice wide enough to accommodate a hexacyanoferrate(II) ion. The direct interaction of Cu(II) and hexacyanoferrate(II) has been suggested in superoxide dismutase [17]. Nevertheless, the species which gives rise to the MMCT band might be originated in the complex formed by the depleted-type 2 copper and hexacyanoferrate(II) [5], since the species in Fig. 3D did not give the corresponding MMCT band. Moreover, a portion of the type 3 copper was supposed to be in the reduced form judging from quantitative estimations of the absorption spectra and ESR signal intensities. (If the relevant ESR signal comes fully from all the type 3 coppers, the intensity should not be 1.8 (Fig. 2D) or 0.9 (Fig. 3D) but 4.) The present study shows that both ascorbate oxidase and laccase exhibit a similar behavior in anaerobic reactions with hexacyanoferrate(II) giving an unusual ESR signal. Further work on the ESR signal is in progress for both enzymes.

Acknowledgements

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