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Reduction of Ascorbate Oxidase with Hexacyanoferrate(II)

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Ascorbate oxidase (AOase) (EC 1.10.3.3) is known as a blue, copper-containing protein, having eight atoms of copper per molecule weighing 1.3–1.4 × 10^5 [1–3]. It catalyzes the oxidation of L-ascorbate to dehydroascorbate accompanied by the reduction of the oxygen molecule to water. According to EPR studies of the native and modified enzymes, the eight copper atoms consist of three type 1-, one type 2-, and four (two pairs of) type 3-copper ions [4, 5].

We have recently isolated the AOase from cucumber peelings, and studied the reduction of copper ions using hexacyanoferrate(II) in order to shed light on the electron transfer mechanism of the copper ions at the active site of the enzyme. It has been disclosed that the electron transfer from type 1 Cu to type 2 Cu which is forbidden in laccase [6] may occur in AOase.

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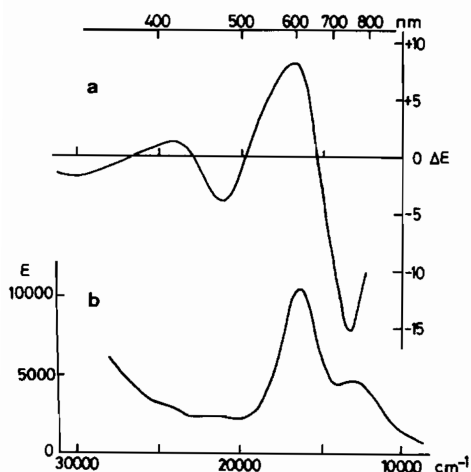

Fig. 1. CD (A) and electronic (B) spectra of AOase isolated from cucumber peelings at room temperature (0.1 *M* phosphate buffer, at pH 7.2). The ϵ and $\Delta\epsilon$ are expressed per mole of AOase. The CD spectrum was obtained with a JASCO-500A spectropolarimeter.

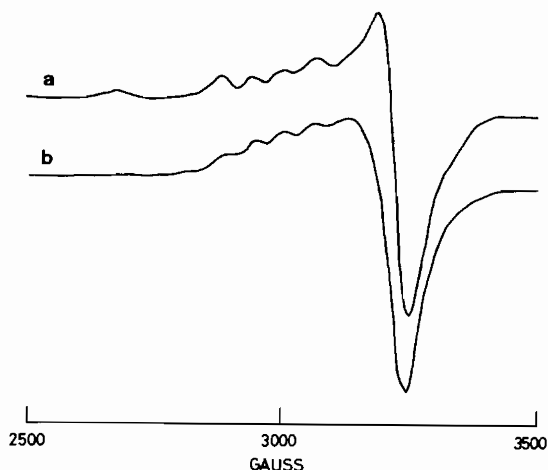

Fig. 2. EPR spectra of native (a) and hexacyanoferrate(II)-treated (b) AOase at 77 K (0.1 *M* phosphate buffer, at pH 6.0). Conditions of spectrum b: Cu conc. in AOase, 2.4 × 10^{-4} *M*; $K_4[Fe(CN)_6]$ conc., 2.9 × 10^{-2} *M*; The period between the mixing of AOase and $[Fe(CN)_6]^{4-}$ under nitrogen atmosphere and EPR measurement (JEOL JES-FE1X spectrometer) was about 3 hr.

The AOase was obtained from cucumber peelings as previously described [7]. The final absorption of A_{280}/A_{610} was 25, being almost equal to the reported value [8]. The purity of the protein was confirmed by polyacrylamide gel electrophoresis.

Figure 1 represents the electronic and CD spectra of the native AOase isolated from cucumber peelings at room temperature.

The blue color of AOase stems from the strong absorption band at 610 nm, of which the extinction coefficient is estimated to be 10,400 M^{-1} cm^{-1} as to protein. The absorption spectrum is nearly identical with those reported previously for cucumber [8] and squash [4, 5, 9] AOases. The CD spectrum also bears a strong resemblance to that of squash AOase [9]. These spectra exhibit typical absorption bands of blue copper proteins such as plastocyanins and azurins except for the negative CD band at around 320 nm, which was observed for multicopper oxidases as laccase [6].

It is known that there are two kinds of EPR-detectable coppers (type 1 Cu and type 2 Cu) in AOase as reported by many investigators [4, 5, 8]. The EPR spectrum for the cucumber AOase at 77 K is illustrated in Fig. 2a. The EPR parameters obtained from the signal due to type 1 Cu in the figure are $g_{\parallel}$ = 2.23 and $A_{\parallel}$ = 63 G. The latter value is somewhat greater than that for the squash AOase ($A_{\parallel}$ = 54–60 G) [5]. Figure 2b indicates the EPR spectrum of AOase treated with a 120-fold excess of hexacyanoferrate(II) under nitrogen atmosphere. A comparison of Figs. 2a and 2b reveals that a hyper-

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Fig. 3. Plot of reciprocal velocity of O_2-uptake against reciprocal hexacyanoferrate(II) concentration. 10 μl of AOase (3×10^{-5} *M*) was injected into 2 ml of 0.1 *M* phosphate buffer (pH 6.0) containing potassium hexacyanoferrate(II) at 30.8 °C (IRICA oxygen monitor Model PO-701).

Fig. 4. EPR spectrum of t2d-AOase at 77 K (0.1 *M* phosphate buffer, at pH 6.0).

fine peak at around 2675 G (Fig. 2a) arising from the type 2 Cu disappeared unequivocally with the reduction (Fig. 2b). Further, a somewhat altered type 1 Cu signal ($g_{\parallel}$ = 2.23 and $A_{\parallel}$ = 59 G) which is clearly different from that of the original type 1 Cu appears with the reduction in the region of 2800–3400 G. Double integration of the spectrum of the same type 1 Cu appears with the reduction in the region of 2800–3400 G. Double integration of the spectrum of the same type 1 Cu was carried out by use of *Pseudomonas denitrificans* azurin [10] as a standard. The EPR-detectable Cu content in AOase was thus estimated to be 54% of total copper concentration. On the other hand, the intensity of the absorption band at 610 nm was unchanged after anaerobic reduction by hexacyanoferrate(II) at 20 °C. These findings suggest that the redox potential of hexacyanoferrate(II) (+425 mV *vs.* NHE) [11] lies between that of type 1 Cu in squash AOase (E_o' = +344 mV) has by the following sequence: type 2 Cu > $[Fe^{II}(CN)_6]^{4-}$ > type 1 Cu. In fact, the redox potential of type 1 Cu in squash AOase (E' = +344 mV) has already been reported to be lower than that of hexacyanoferrate(II) [3]. Secondly the number of EPR-active copper atoms can be estimated to be 4.3 g atoms out of 8 g atoms of copper in one mole of the enzyme. These may consist of 3 g atoms of type 1 Cu and 1.3 g atoms of type 3 Cu origin as was reported for the EPR-detectable type 3 Cu in 2-mercaptoethanol treated tyrosinase [12]. A superposition of the signal for type 1 Cu and that for the originally type 3 Cu might give rise to the new signal as observed in Fig. 2b, which is distinctly different from the signal of the native AOase (Fig 2a).

The treatment of native AOase with hexacyanoferrate(II) under aerobic conditions did not produce the above new type 1 Cu signal in the EPR spectrum but only the signal of native AOase. However, the mixture of AOase and hexacyanoferrate(II) was observed to consume molecular oxygen using an oxygen monitor equipped with a Clark-type electrode. Figure 3 depicts the Lineweaver–Burk plots of the reaction of hexacyanoferrate(II) with AOase at 30.8 °C. According to the plots the Michaelis constant (K_m) and the maximum velocity (V_{max}) of the overall reaction were evaluated as 5.2×10^{-3} *M* and 29 molO_2/min per mol of enzyme, respectively. The reaction rate of hexacyanoferrate(II) corresponds to *ca.* 1/5 of that of ascorbic acid [8]. These facts may be elucidated by taking electron-transfer from type 2 Cu to type 3 Cu into consideration.

In order to interpret the role of type 2 Cu in the hexacyanoferrate(II) reaction, we prepared type 2 Cu-depleted AOase (t2d-AOase) [13]. The EPR spectrum of t2d-AOase reveals a typical signal for type 1 Cu, displaying the parameters of $g_{\parallel}$ = 2.22, $g_{\perp}$ = 2.06, and $A_{\parallel}$ = 63 G, as illustrated in Fig. 4. The EPR spectrum is of course devoid of the hyperfine peak for type 2 Cu near 2675 G. The removal of type 2 Cu is accompanied by a decrease in the total copper content from 8 to 5.7 g atoms per mol of the enzyme, and lowers the ascorbate [13] or hexacyanoferrate(II)-oxidizing activity to 3% of that of native AOase. It may be considered that the reduction of type 2 Cu in native AOase with hexacyanoferrate(II) causes the consumption of O_2 in the oxidation of hexacyanoferrate(II).

In the light of the above findings, we propose the following scheme for the electron transfer mechanism in AOase:

ascorbate → dehydroascorbate; type 1 Cu $\xrightarrow{e^-}$ type 2 Cu $\xrightarrow{e^-}$ type 3 Cu; O_2 → H_2O; $[Fe^{II}(CN)_6]^{4-}$ → $[Fe^{III}(CN)_6]^{3-}$

Since the redox potential of type 2 Cu is higher than that of type 1 Cu, electrons must be transferred from type 1 Cu to type 2 Cu and then to type 3 Cu for which the redox potential is the highest among the three types of copper. The coupled binuclear copper-(I) ions (type 3 Cu) may catalyze the reduction of molecular oxygen as described in the mechanisms of laccase [6, 14] and tyrosinase [15]. In the oxidation of hexacyanoferrate(II) in the presence of O_2, the type 2 copper(I) ion which has accepted an electron from $[Fe^{II}(CN)_6]^{4-}$ may transfer it to the type 3 Cu site to produce H_2O. Although the uptake of O_2 by AOase occurs in the presence of O_2 (Fig. 3), the reduction of a part of type 3 Cu as well as type 2 Cu was also observed in the absence of O_2 as confirmed by the EPR-measurement (Fig. 2b). In the case of ascorbate the reduction of type 1 Cu with ascorbate might result in the reduction of O_2 at the type 3 Cu site *via* an electron transfer through type 2 Cu. Thus the type 2 Cu is considered to behave as a mediator in the electron transfer chain. This assumption is supported by the fact that the removal of the type 2 Cu from AOase inhibited the reduction of O_2 in the ascorbate and hexacyanoferrate-oxidizing reactions.

Of special interest is the fact that the type 1 Cu exhibits a higher redox potential than type 2 Cu in laccase [16, 17]. For this reason a different mechanism involving the direct electron transfers from both the type 1- and type 2- to type 3-Cu has been proposed for laccase [6, 14]. The type 2 Cu in laccase has also been shown to contribute to the bond-breaking reduction of the peroxide intermediate bound to type 3 Cu.

Acknowledgements

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