

## X-ray Absorption Study on the Type II Copper-depleted Cucumber Ascorbate Oxidase

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Ascorbate oxidase (EC 1.10.3.3) is a multicopper oxidase, as are ceruloplasmin and laccase. There are three type I Cu(II) ions, one type II Cu(II), and two pairs of type III Cu(II) ions in the native enzyme. Selective depletion of a type II copper has been successfully performed for laccase and ascorbate oxidase as a strategy to shed light on the active site and reaction mechanism of these enzymes. However, there has been controversial discussion about the oxidation state of the type III coppers in the type II copper-depleted laccase. Reinhammar and Oda [1] reported that a prominent shoulder band at 330 nm was intact even after the type II copper was taken away from native laccase. In contrast, other groups [2–4] showed the band disappears or is greatly reduced in intensity because of reduction of the type III coppers. Although a similar discrepancy has not arisen with ascorbate oxidase, our recent studies about type II copper-depleted cucumber ascorbate oxidase [5, 6] indirectly suggested that type III coppers are reduced during the process to deplete type II copper selectively. Here we show direct evidence for the reduction of type III coppers from X-ray absorption edge spectroscopy.

Figure 1 shows the K-edge spectra of the type II copper-depleted cucumber ascorbate oxidase (a) and its H<sub>2</sub>O<sub>2</sub> (X28)-treated derivative (b). A very weak pre-edge peak due to the 1s → 3d transition, which is characteristic of Cu(II) species [7], is observed at 8980 eV. The absorption edge spectra of Cu(I) complexes afford a useful feature in recognizing the presence of Cu(I) species from a distinct transition (1s → 4s) at approximately 8984 eV with a strength about half that of the principal peak [8]. Accordingly, the low energy shoulder at about 8984 eV in Fig. 1(a) comes from the Cu(I) species. The reduction of the type I Cu(II) ions did not occur even after irradiation by strong X-rays. Consequently, the cupric species recognized in Fig. 1(a) are type I Cu ions and the cuprous species must be type III

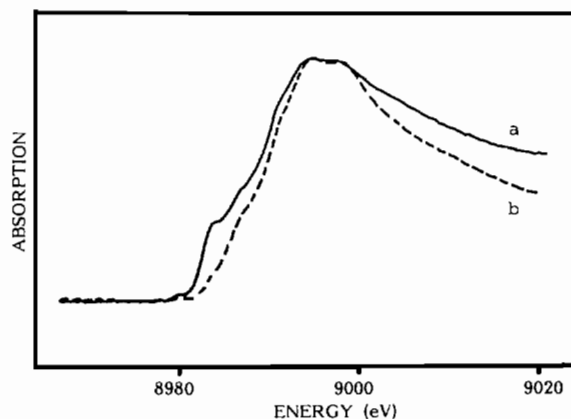


Fig. 1. Normalized Cu edge spectra for type II copper-depleted cucumber ascorbate oxidase (a) and its H<sub>2</sub>O<sub>2</sub> (X28)-treated derivative (b). Measurements were carried out on the storage ring of the Photon Factory at KEK, running at 2.5 GeV with a beam current of 230–260 mA (1.0 mM protein concentration; 0.1 M, pH 6.0, phosphate buffer; 0 °C).

Cu ions. It is concluded that type III coppers are reduced during the process to deplete type II copper, as has been suggested in our previous papers [5, 6]. In addition, according to a model study [8], three-coordinate Cu(I) complexes exhibit a second feature on the higher energy side of the 8984 eV peak [8]. Therefore the cuprous species present in Fig. 1(a) are also assumed to be three-coordinate. This is in accordance with the X-ray structure analysis that each copper ion in the possible deoxy form of hemocyanin is coordinated with three histidine imidazole groups [9]. In contrast, only cupric species were observed in the K-edge spectrum of the type II Cu-depleted ascorbate oxidase treated with H<sub>2</sub>O<sub>2</sub> (X28) (Fig. 1(b)) because type III Cu(I) ions were reoxidized.

The present X-ray absorption spectra unequivocally indicate, even though qualitatively, that type III coppers are reduced during the procedure to deplete type II copper and are stable in the cuprous state in air in the absence of type II copper. In addition, the concomitant disappearance of the band at 330 nm on selective removal of the type II copper and the reappearance of it after treatment of the enzyme derivative with H<sub>2</sub>O<sub>2</sub> (spectra not shown) indicate that the absorption band at 330 nm reflects directly the oxidation state of the coupled binuclear site.

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