FT-IR Spectra of the Azide-Type 3 Copper in Laccase and Ascorbate Oxidase

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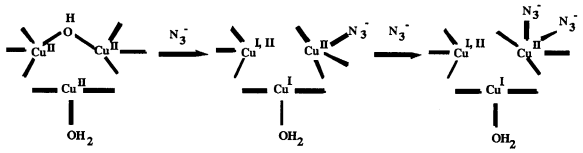
The azide-treated laccase and ascorbate oxidase gave the asymmetric stretching bands (υ_a) at 2056 and 2054 cm^{-1} in the FT-IR spectra, respectively.

Azide has been utilized as an effective inhibitor in studying the structure and function of the metal binding sites of metalloproteins and metalloenzymes. When azide is acted on multicopper oxidases, it binds to the trinucelar center comprised of a type 2 copper (non-blue copper) and a pair of type 3 coppers (EPR non-detectable coppers).[1] Although there had been controversial conclusions about the binding mode of azide,[2] the X-ray crystal structure analysis of the azide-ascorbate oxidase[3] and the spectroscopic and magnetic studies of laccase and ascorbate oxidase[4] unequivocally showed that azide exclusively binds to one of type 3 coppers, making the azide-bound type 3 copper to be EPR detectable. Therefore, in order to obtain further information about the unique trinucelar center in multicopper oxidases we measured the FT-IR spectra of the azide-treated multicopper oxidases, laccase from lacquer latex and ascorbate oxidase from cucumber peeling for the first time.



Addition of increasing amount of azide on laccase gave a very broad band at around 490 nm and a band at 405 nm due to the charge transfers from azide to Cu(II) (figures not shown).[2,4] The former band has been assigned to coming from the "high affinity azide" and the latter from the "low affinity azide". From the biphasic absorption changes (Figure 1) the two binding constants, K_1 = 80000 M^{-1} and K_2 = 70 M^{-1} were obtained (Since the band at 490 nm is very broad, its slope prevails on the absorption at 405 nm). In line with this, the binding constants at 77 K, K_1 = 400 M^{-1} and K_2 = 150 M^{-1} were obtained from the EPR signals derived from the $1N_3^-$-type 3 copper and $2N_3^-$-type 3 copper, respectively, which become EPR detectable in harmony with the appearance of the charge transfer bands (data not shown).[4] Although the binding constants at room temperature and 77 K do not coincide because of the possible conformational change of the protein structure, the fact that the changes associated with the action of azide on laccase are biphasic is the same. Similarly, we obtained K_1 = 2300 M^{-1} and K_2 = 300 M^{-1} for the biphasic binding of azide to ascorbate oxidase from the absorption changes at room temperature.

While the appearance of the CT bands coming from azide -Cu(II) has been repeatedly reported,[2,4] no vibrational spectrum for the azide ion(s) bound to copper(II) has been available. Therefore, we measured the azide-treated laccase and ascorbate oxidase using a horizontal ATR attachment (ZnSe crystal) by

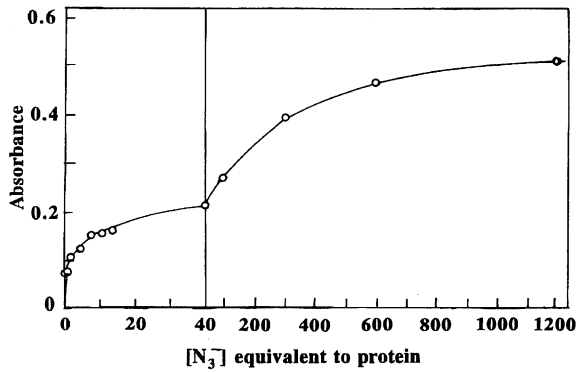


Figure 1. Absorption change at 405 nm with addition of the increasing amount of sodium azide on laccase. Conditions: protein concentration, 1 x 10^{-4} mol dm^{-3}; pH 6.0; 0.2 mol dm^{-3} phosphate buffer.

averaging 1024 scans. In Figure 2 are shown the difference spectra derived from azide acted on laccase and ascorbate oxidase. Soon after addition of the two to five fold excess of azide on laccase and ascorbate oxidase only a band due to free azide was observed at 2046 cm^{-1} (υ_a) (υ_s was not observed because of its low intensity). However, after the incubation for an hour an extra band appeared at 2056 cm^{-1} for laccase and 2054 cm^{-1} for ascorbate oxidase. These bands are apparently derived from the high affinity azide. In line with this, the resonance Raman excitation into the azide absorption bands of laccase has been reported to give a stretch at 2050 cm^{-1},[5] almost coinciding with the present FT-IR data. Further, when a large excess of azide was acted on laccase and ascorbate oxidase, no band coming from the low affinity azide was resolvable at around 2050 cm^{-1} because of the possible masking effect due to a large amount of free azide. Nevertheless, after incubation for more than an hour, a band appeared at 2148 and 2146 cm^{-1} for laccase and ascorbate oxidase, respectively. Since these bands appeared at the high wavenumber region as coming form an azide anion, they might be derived from the decomposition of azide (the similar band was also observed when a large excess of azide was acted on superoxide dismutase). If they come from the low affinity azide, their electronic state might be profoundly different (higher triple bond character) from that of the high affinity azide.

According to the small molecule studies, the mononuclear Cu(II) complexes, in which one or two azide ions coordinate in the end-on fashion, show the tendency to give the vibrational band at a higher energy region (< 2100 cm^{-1}) compared to free azide anion.[6] On the other hand, binuclear Cu(II) complexes, in which one or two azide ions bridge between the two Cu(II) ions (1,1- and 1,3-bridging mode), have usually given the vibrational band at ca. 2060 - 2020 cm^{-1}.[7] This fact also supports that azide ion coordinates to the type 3 copper in multicopper oxidases with

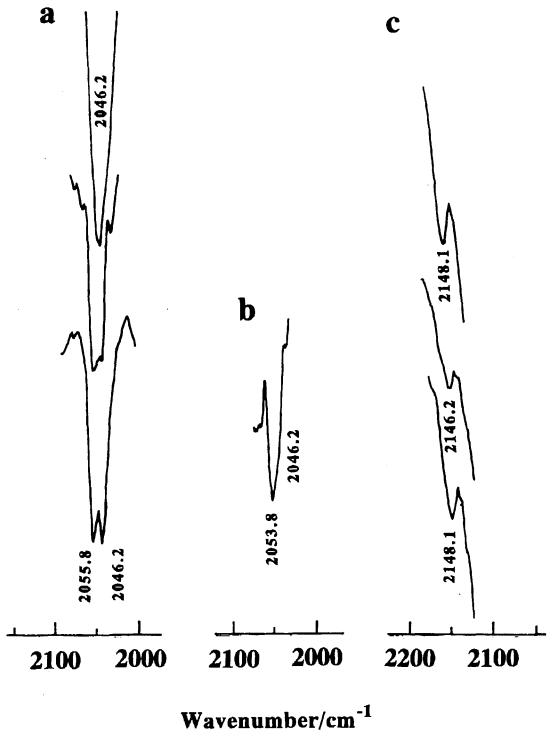


Figure 2. Difference FT-IR spectra of (a) azide(x5)-treated laccase measured soon after addition of azide, measured after 3 h incubation, and measured after 24 h incubation (from top to bottom), (b) azide(x5)-treated ascorbate oxidase measured after 1 h incubation, and (c) a large excess of azide(x300)-treated laccase, ascorbate oxidase, and superoxide dismutase (from top to bottom). Protein concentrations were 1 x 10^{-3} mol dm^{-3}, 6 x 10^{-4} mol dm^{-3}, and 9 x 10^{-4} mol dm^{-3} for laccase, ascorbate oxidase, and superoxide dismutase, respectively.

the end-on fashion.

As a conclusion, azide stepwisely binds to the trinuclear center in multicopper oxidases, giving the charge transfer bands and a vibrational band and also making the azide-bound type 3 copper to be EPR detectable.[4]

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