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Spectroscopic and binding studies of azide to type-2-copper-depleted ascorbate oxidase from zucchini

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Summary. Binding of azide to type-2-copper-depleted (T2D) zucchini ascorbate oxidase, containing reduced type-3 Cu centers, and met-T2D ascorbate oxidase, containing oxidized type-3 Cu centers, has been studied spectroscopically. In both cases titration with azide in 0.1 M phosphate pH 6.8 produces a broad near-ultraviolet band with maximum at 455 nm ($\Delta e \approx 2500\ M^{-1}\ cm^{-1}$, with respect to the met-T2D enzyme) and shoulder at 390 nm ($\Delta e \approx 1700\ M^{-1}\ cm^{-1}$), that are assigned to π(azide)→Cu(II) ligand-to-metal charge transfer (LMCT) transitions. This is accompanied by a reduction of absorbance at 330 nm in the met-T2D enzyme adduct ($\Delta e \approx -1400\ M^{-1}\ cm^{-1}$). A broad circular dichroic band of negative sign between 370–480 nm corresponds to the LMCT absorption band. Analysis of the titration data indicates that one azide ion binds independently to each of the binuclear T3 Cu couples with low affinity ($K = 50\ M^{-1}$). The ESR signal of the T1 Cu observed in frozen solutions of the T2D enzyme is also perturbed by the addition of azide. The analogies in the azide-binding characteristics between ascorbate oxidase and laccase are discussed.

Key words: Ascorbate oxidase – Copper proteins – Azide binding studies – Spectroscopic studies

Introduction

The enzyme ascorbate oxidase (L-ascorbate; O_2 oxidoreductase, EC 1.10.3.3) is a blue copper oxidase mostly found in plants, where it probably performs the aerobic oxidation of a variety of biological compounds (Dawson 1966; Kroneck et al. 1982). It contains eight copper atoms/molecule (M_r 140000); three type-1 (blue, T1), one type-2 (nonblue; T2) and two pairs of type-3 Cu (coupled binuclear, T3). The T1 Cu(II) dominates the visible absorption and electronspin resonance (ESR) spectra of the enzyme, while the optical features of T2 Cu(II) are weak and buried under the intense T1 Cu(II) absorption bands; though, it can be detected by ESR (Deinum et al. 1974; Marchesini and Kroneck 1979; Aikazyan and Nalbandyan 1979; Casella et al. 1984; Kawahara et al. 1985). The T3 Cu(II) is not detectable by ESR and probably occurs as pairs of strongly antiferromagnetically coupled Cu^{2+} ions; it contributes to the near-ultraviolet absorption of the enzyme and, in particular, it is mostly responsible for the prominent shoulder observed near 330 nm.

Redox studies on native (Sakurai et al. 1985, 1986) and type-2-copper-depleted (T2D) ascorbate oxidase from cucumber (Sakurai et al. 1987) have shown that the copper centers can be divided into two groups. The first of these groups constitutes the 'principal' active site of the enzyme; it comprises one T1, one T2 and a pair of T3 copper atoms and is thus similar to the copper set found in laccase, the smallest and best characterized member of the group of blue copper oxidases (Fee 1975; Reinhammar and Malmström 1981), while the other group consisting of two T1 and the second pair of T3 copper atoms forms a sub-route for electron transfer during the catalytic cycle. A recent report (Messerschmidt et al. 1989) on the preliminary X-ray structure analysis of two crystal forms of ascorbate oxidase from zucchini indicates the existence of laccase-like subunits requiring a stoichiometry of 2 T1, 2 T2 and 4 T3 Cu (instead of 3 T1, 1 T2 and 4 T3 Cu) atoms/dimeric protein molecule. A similar stoichiometry was obtained by ESR on a solution of the crystallized protein (Morpurgo et al. 1988). However, as we have shown separately (Casella et al., unpublished work), the crystallization process occurs with a modification of the native protein involving a loss of blue copper and concomitant increase of nonblue copper. A similar change in the ratio between T1 and T2 Cu is observed in aged ascorbate oxidase samples (Kroneck et al. 1982; Marchesini and Kroneck 1979; Avigliano et al. 1983; Casella et al. 1984). For both the crystallized and aged protein samples the process is accompanied by decrease in the activity of the enzyme. The X-ray struc-

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tural results are important, though, because they confirm the existence of trinuclear active sites in the copper oxidases, as originally proposed for laccase (Allendorf et al. 1985; Spira-Solomon et al. 1986; Kau et al. 1987).

We have recently found that a fraction of the T3 copper atoms are in the reduced state in native ascorbate oxidase from zucchini and the fully oxidized enzyme can be obtained by treatment with small amounts of hydrogen peroxide (Casella et al. 1988a). Further, azide binds with high affinity ($K > 5000\ M^{-1}$) to the enzyme when the T3 sites are reduced and with low affinity ($K \approx 100\ M^{-1}$) when these sites are oxidized. This behavior, and also the spectral features of the azide adducts, are similar to those exhibited by laccase (Allendorf et al. 1985; Spira-Solomon et al. 1986) so that structurally analogous active sites between the two enzymes seem possible. Since the mode of binding of azide involves both the T2 Cu and one of the T3 Cu pairs, a useful step toward a better definition of the active-site structure of ascorbate oxidase is represented by the investigation of the azide binding characteristics to the T2D derivative of the enzyme. This paper amplifies upon our preliminary report on the optical features of azide binding to T2D ascorbate oxidase from zucchini (Casella et al. 1989) and complements similar optical studies performed on the enzyme from cucumber (Sakurai et al. 1987) by reporting detailed spectroscopic and binding studies of the anion to the T2D form of zucchini ascorbate oxidase.

Experimental procedures

Materials

Ascorbate oxidase was purified from the green zucchini squash (*Cucurbita pepo*) according to the method of Marchesini and Kroneck (1979). The absorbance ratios of the present enzyme preparation were $A_{330}/A_{610} = 0.72$ and $A_{280}/A_{610} = 25.3$ (0.1 M phosphate, pH 6.8). The high purity of the enzyme was confirmed by polyacrylamide gel electrophoresis. Protein concentrations were determined by assuming $\varepsilon_{610} = 9800\ M^{-1}\ cm^{-1}$ and $M_r = 140\,000$. Sodium *N,N*-diethyldithiocarbamate was recrystallized from ethanol, carefully dried and stored under nitrogen at 4° C. All other reagents were of the highest grade commercially available. Buffers were prepared in water purified in a Milli-Q water purification system. Protein solutions were concentrated as needed using Sartorius collodion bags.

Type-2-copper depleted ascorbate oxidase was prepared according to the *N,N*-diethyldithiocarbamate method (Morpurgo et al. 1987). A 0.1 mM solution of ascorbate oxidase in 0.1 M phosphate, pH 6.0 was treated with 9 mol/mol of 1 mM sodium *N,N*-diethyldithiocarbamate in 0.1 M phosphate pH 6.0. The solution turned green and, by centrifugation at 20000 rpm for at least 4 h at 4° C, a brownish red precipitate could be removed. The resulting blue solution was extensively dialyzed against buffer at pH 6.0 to remove all traces of unreacted *N,N*-diethyldithiocarbamate.

Peroxide-treated T2D ascorbate oxidase was prepared by reacting the T2D protein with 20 mol/mol of hydrogen peroxide (0.01 M aqueous solution) in 0.1 M phosphate pH 6.8 at 4° C. Excess peroxide was immediately removed by dialysis against buffer containing 1 mM EDTA (disodium salt) and then against buffer alone.

Ligand titrations of the enzyme and data analysis

All spectroscopic measurements were made with protein dissolved in 0.1 M phosphate pH 6.8. Azide binding studies were performed by adding concentrated aqueous solutions of the anion to the protein solution in buffer, followed by incubation at 4° C ($\approx$ 48 h for the T2D enzyme and 15–40 h for the peroxide-treated T2D enzyme) before performing each spectroscopic measurement.

The equilibrium constant (K) for complex formation between the enzyme (E) and azide (L) was determined by using the Hill equation:

$$Y = K[L]^c/(1 + K[L]^c) \quad (1)$$

where Y is the fractional saturation of the enzyme with ligand bound, [L] the free ligand concentration, and c an empirical parameter measuring the degree of interaction between the sites, in the case of multiple binding (Antonini and Brunori 1971). Eq. (1) is usually written in the logarithmic form:

$$\log[Y/(1-Y)] = \log K + c \log[L] \quad (2)$$

A plot of $\log[Y/(1-Y)]$ versus $\log[L]$ should give a straight line with a slope of c and an intercept of $\log K$. In protein systems with a single binding site or with no interaction between the binding sites $c = 1$, while $c > 1$ indicates the presence of positive interactions between the sites.

In the case of multiple independent binding of ligand molecules by the protein, an evaluation of the number (n) of binding sites can be made only if it is possible to determine the average number ($\bar{v}$) of ligand molecules bound/molecule of protein ($\bar{v} = [L]_{bound}/[E]_{total}$; $\bar{v} = Yn$) through, for instance a Scatchard plot (Antonini and Brunori 1971). But this determination was impossible in the present case with the standard techniques available.

Physical measurements

Optical absorption spectra were recorded at room temperature using a Perkin-Elmer Lambda 5, a Philips PU 8700 spectrophotometer or an HP 8452 A diode array spectrophotometer.

Circular dichroic (CD) spectra were obtained on a Jasco J-500 C dichrograph. ESR spectra were measured in frozen solution at −150° C using a Varian E-109 spectrometer operating at X-band frequencies and a V-4000 variable-temperature-control apparatus. Total copper ion determinations were made by atomic absorption spectrometry. The amount of ESR-detectable Cu(II) was estimated by double integration of the spectra using Cu-EDTA as standard.

Results

Spectroscopic properties of T2D ascorbate oxidase

Removal of T2 copper from ascorbate oxidase was performed according to the diethyldithiocarbamate method (Morpurgo et al. 1987). We also prepared the T2D enzyme on a smaller scale following the EDTA method described for cucumber ascorbate oxidase (Sakurai et al. 1987) obtaining a protein with practically identical characteristics. Starting from 7.9 mol Cu/mol native ascorbate oxidase, the total amount of copper in the T2D enzyme preparation decreased to 6.7 mol/mol and the ESR-detectable Cu correspondingly decreased from 3.9 to 2.7 mol/mol. The electronic spectrum of T2D ascorbate oxidase shows a marked decrease in the ab-

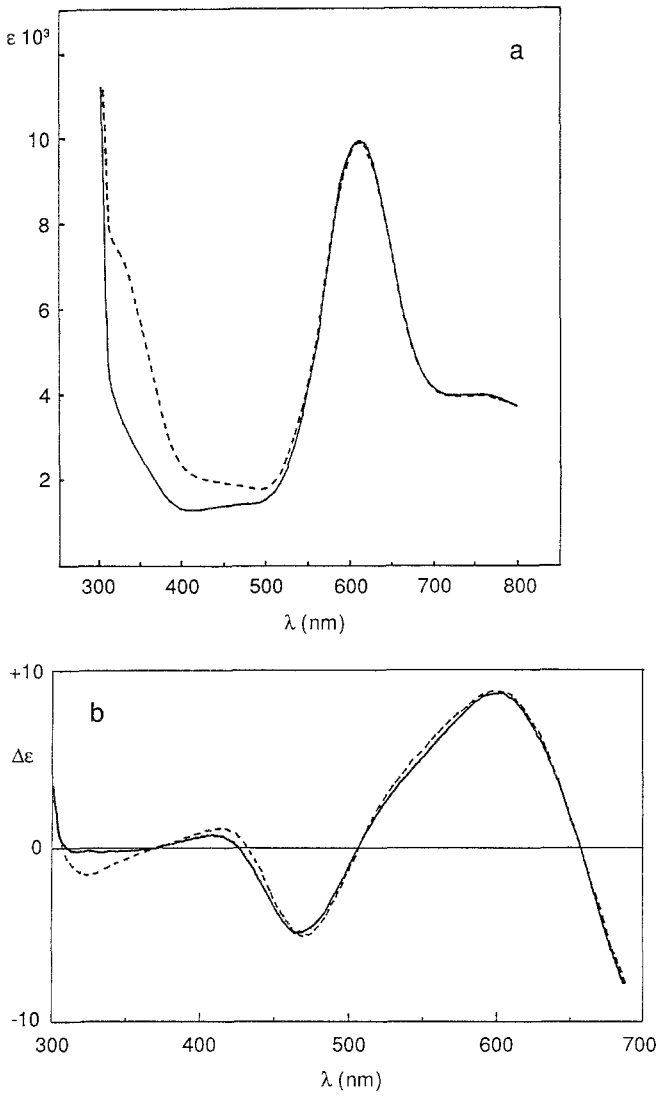

Fig. 1. Electronic (**a**) and CD (**b**) spectra of native (- - -) and T2D (——) ascorbate oxidase from zucchini

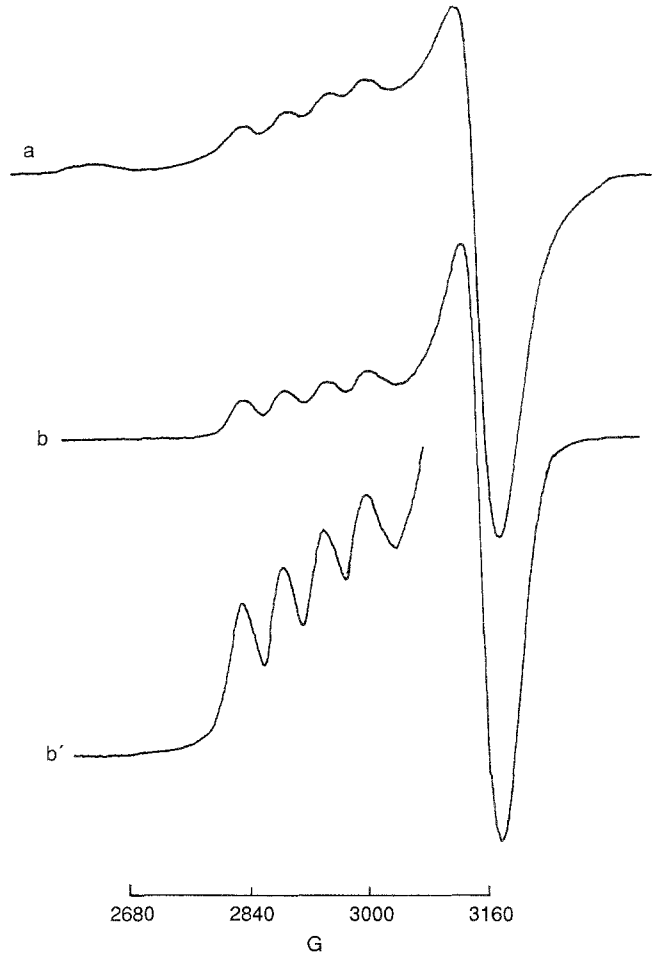

Fig. 2. ESR spectra of native (**a**) and T2D (**b**) ascorbate oxidase. Conditions: temperature −150° C; 8.98-GHz microwave frequency, 6.3-G modulation amplitude, 100-kHz modulation frequency, 5-mW microwave power. The low-field portion of the spectrum of the T2D enzyme (**b′**) is shown at a higher gain

sorbance at 330 nm (Fig. 1), the absorbance ratio A_{330}/A_{610} decreasing to 0.33. Comparable changes were observed previously in T2D enzyme preparations employing different methods (Sakurai et al. 1987; Avigliano et al. 1979; Kroneck and Jacob 1985). The ratio A_{280}/A_{610} of 24.5 shows that no loss of T1 Cu or degradation of the protein occurs with the *N,N*-diethyldithiocarbamate treatment.

The CD spectrum of T2D ascorbate oxidase shows that only some minor changes in the 300–500-nm region, with respect to the native protein, parallel to the decrease of absorbance at 330 nm (Fig. 1). No appreciable change occurs in the aromatic and far-ultraviolet regions; in particular, the intensity of the tryptophan CD peaks near 290 and 295 nm (Casella et al. 1984) does not seem to be affected.

The ESR spectrum of T2D ascorbate oxidase shows the complete loss of type-2 copper in the preparation (Fig. 2). The perpendicular component of the signal becomes narrower in the T2D enzyme (the peak-to-peak separation decreases from ≈65 G in the native to ≈55 G in the TD2 enzyme) and also the parallel component of the signal is better resolved, as if the three T1 Cu centers become more strictly equivalent. The following parameters can be calculated from the spectrum: $g_{\|}=2.220$, $A_{\|}=56$ G, and $g_{\perp}=2.055$.

Azide binding to T2D ascorbate oxidase

When T2D ascorbate oxidase is titrated spectrophotometrically with azide a broad band extending over 350–550 nm, with maximum at 455 nm ($\Delta\varepsilon\approx3000$ $M^{-1}\,cm^{-1}$) and shoulder near 390 nm ($\Delta\varepsilon\approx2300$ $M^{-1}\,cm^{-1}$), slowly develops (Fig. 3). Long incubation times are required to develop fully the optical features after each addition of the anion. Note that, contrary to the native enzyme, no decrease of absorbance at 330 nm occurs on binding of the anion. The changes observed in the CD spectrum of the T2D enzyme during this experiment are similar to those of the low-affin-

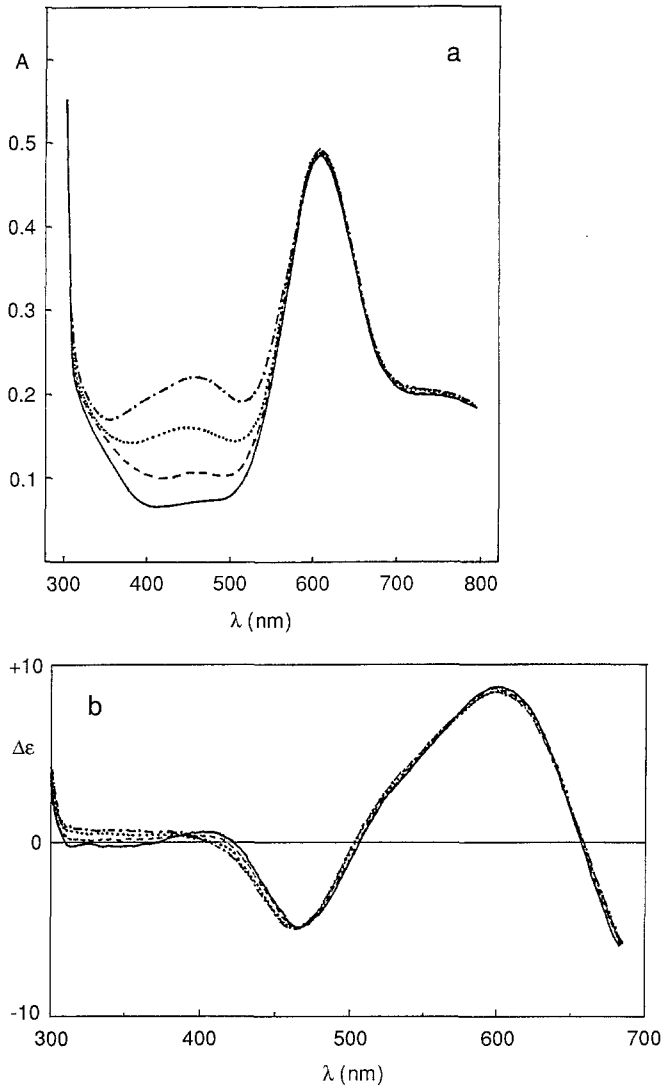

Fig. 3. Absorption (**a**) and CD (**b**) spectra of 52 μM T2D ascorbate oxidase before (——) and after the addition of 100 (– – –), 300 (· · · · ·), and 1000 (–·–) mol azide/mol protein

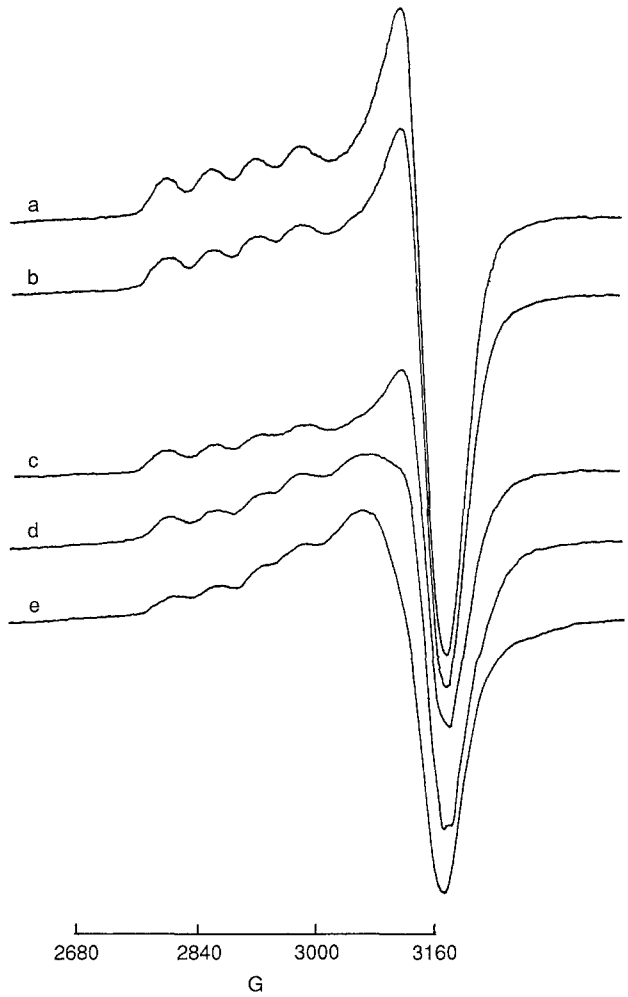

Fig. 4. ESR spectra of T2D ascorbate oxidase treated with azide. (**a**) T2D enzyme (0.3 mM); (**b**) with 5 mol azide/mol protein; (**c**) with 10 mol azide/mol protein; (**d**) with 50 mol azide/mol protein; (**e**) with 200 mol azide/mol protein. Conditions: temperature −150° C; 8.98-GHz microwave frequency, 6.3-G modulation amplitude, 100-kHz modulation frequency, 5-mW microwave power

ity azide binding to native ascorbate oxidase (Casella et al. 1988a) and consist of the progressive disappearance of the positive CD band near 420 nm, replaced by broad CD activity between 370–480 nm, and the replacement of the weak negative CD activity in the 300–350-nm range by ill-defined optical activity of opposite sign (Fig. 3). Binding of azide affects also the T1 copper ESR signal of T2D ascorbate oxidase, even though the optical features of the T1 Cu are negligibly affected, as is observed for the native protein (Fig. 4). In particular, both the parallel and perpendicular components of the signal are markedly broadened at high azide/protein ratios; possibly the T1 Cu atoms become nonequivalent in these conditions. However, compared to the native enzyme, larger amounts of the anion are required to perturb significantly the ESR spectrum. The overall intensity of the ESR signal does not change appreciably, within the experimental uncertainty of the measurements, on addition of azide.

Azide binding to peroxide-treated (met) T2D ascorbate oxidase

A met form of T2D ascorbate oxidase, containing oxidized T3 Cu centers, can be obtained by treatment with excess hydrogen peroxide. This treatment restores to a large extent the absorbance at 330 nm ($\Delta\varepsilon_{330} \approx 2500$ M^{-1} cm^{-1}, Fig. 5). The ratio A_{330}/A_{610} rises to 0.58, but this value is still lower than that of the native enzyme. Dialysis of excess peroxide produces no further appreciable change in the optical spectrum. Rather surprisingly, practically no change occurs in the CD spectrum of T2D ascorbate oxidase on peroxide treatment. Also the ESR spectrum is completely unaffected, but this is expected, since the optical features of the T1 Cu are unchanged.

When met-T2D ascorbate oxidase is titrated spectrophotometrically with azide, the growth of the same broad absorption band at 455 nm ($\Delta\varepsilon_{455} \approx 2500$

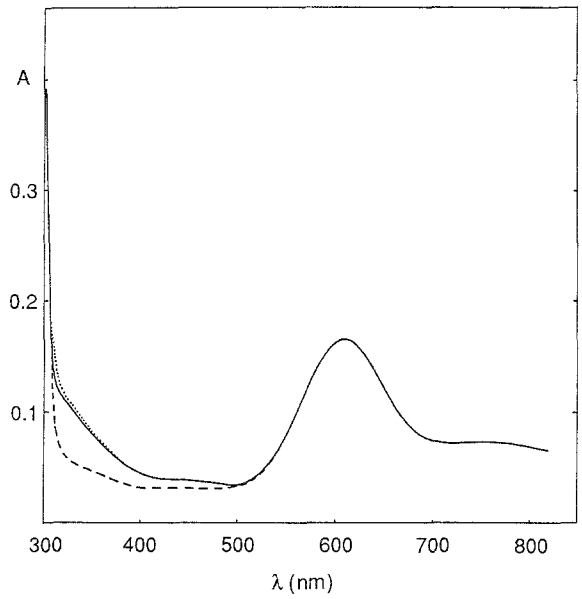

Fig. 5. Absorption spectra of T2D ascorbate oxidase (– – –), peroxide-treated T2D ascorbate oxidase (· · · · ·), and peroxide-treated, dialyzed T2D ascorbate oxidase (——)

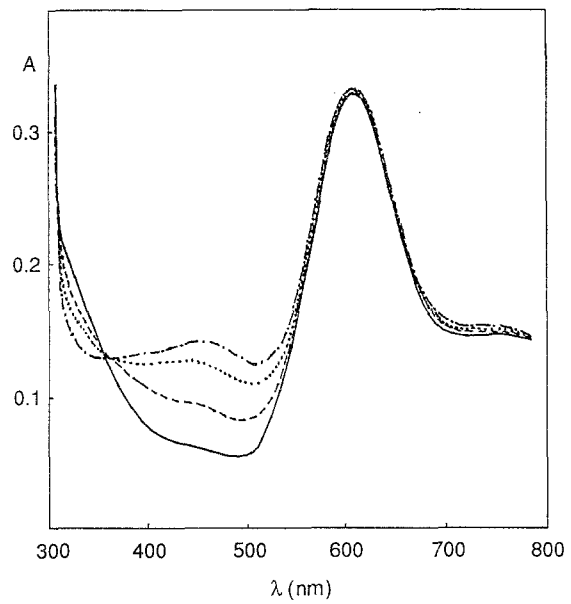

Fig. 6. Absorption spectra of 35 μM met-T2D ascorbate oxidase (——), and in the presence of 250 (– – –), 750 (· · · · ·) and 1200 (-·-) mol azide/mol protein

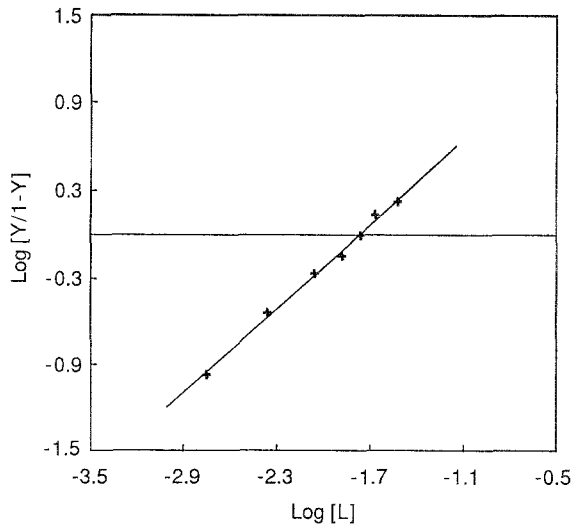

Fig. 7. Hill plot for azide binding to met-T2D ascorbate oxidase using the titration data at 455 nm

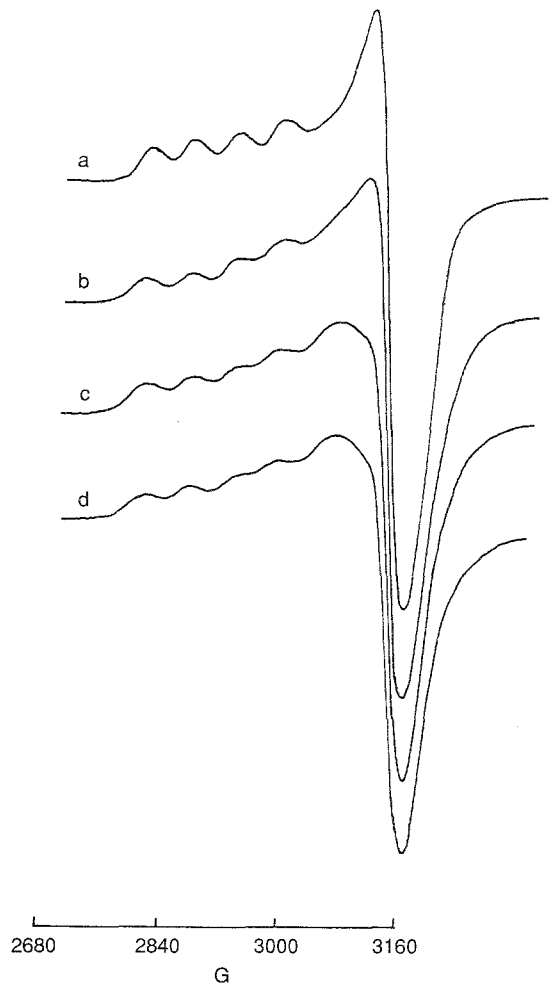

Fig. 8. ESR spectra of met-T2D ascorbate oxidase treated with azide. (**a**) Met-T2D enzyme (0.12 mM); (**b**) with 10 mol azide/mol protein; (**c**) with 50 mol azide/mol protein; (**d**) with 100 mol azide/mol protein. Conditions: temperature −150° C; 8.99-GHz microwave frequency, 6.3-G modulation amplitude, 100-kHz modulation frequency, 20-mW power

$M^{-1}\,cm^{-1}$), with shoulder near 390 nm ($\Delta\varepsilon_{390} \approx 1700$ $M^{-1}\,cm^{-1}$), observed for the T2D enzyme occurs (Fig. 6). But this is accompanied by a parallel reduction of the absorbance at 330 nm ($\Delta\varepsilon_{330} \approx -1400$ $M^{-1}\,cm^{-1}$), in contrast with the spectral data reported for the enzyme from cucumber (Sakurai et al. 1987). A comparable decrease of the absorbance at 330 nm was observed also on adding azide to H_2O_2-treated ascorbate oxidase

(Casella et al. 1988a). Analysis of the titration data at 455 nm using Eq. (2) yields an apparent intrinsic binding constant of 50 M^{-1} (correlation coefficient 0.99; Fig. 7). A Hill coefficient of $c = 1$ indicates the existence of either a single binding site or multiple, independent and noninteracting binding sites.

The CD spectral changes in the 300–450-nm region occurring on azide binding to met-T2D ascorbate oxidase are practically the same as those shown in Fig. 3b for binding to the T2D enzyme. Also the ESR spectral changes are similar, but the perturbations undergone by the T1 Cu signals are perhaps less pronounced (Fig. 8).

Discussion

Selective removal of type-2 copper from laccase and ascorbate oxidase is part of the strategy which has been followed to gain information on the active-site structure and reaction mechanism of these complex metalloproteins. For laccase, while there have been controversial reports on the oxidation state of the T3 Cu in the T2D enzyme (Reinhammar 1983a; LuBien et al. 1981; Morpurgo et al. 1980; Hanna et al. 1988; Frank and Pecht 1986), there is direct proof by X-ray absorption spectroscopy that bleaching of the 330-nm chromophore accompanying the T2-Cu depletion corresponds to reduction of the binuclear T3 Cu site (Kau et al. 1987). Similar results from X-ray absorption data have been reported recently for T2D cucumber ascorbate oxidase, although the amount of reduced T3 Cu could not be determined (Sakurai et al. 1988). Since even in native ascorbate oxidase (Casella et al. 1988a), as in laccase (Allendorf et al. 1985; Spira-Solomon et al. 1986), a fraction of the T3 Cu is stable to dioxygen in the reduced state, it is likely that T2-Cu depletion involves complete T3-Cu reduction. The reason for this difficulty of T3 Cu to remain in the oxidized state in the native copper oxidases is not known, but it is clearly related to some particular conformational state of the proteins, since on binding of the substrate the reaction with dioxygen becomes extremely fast (Kroneck et al. 1982; Reinhammar and Malmström 1981).

If we adopt the picture emerging from the preliminary X-ray structural data of ascorbate oxidase (Messerschmidt et al. 1989), showing a trinuclear cluster composed of one T2 Cu and a pair of T3 Cu atoms bound to a total of eight imidazole groups (three to each T3 Cu and two to the T2 Cu), it is possible to associate the bleaching of the 330-nm protein chromophore occurring upon T2-Cu depletion with the loss of LMCT features from both the T2 and T3 Cu(II) centers. In fact, the π(imidazole)→Cu(II) LMCT transition occurs in this range in the ultraviolet/visible and CD spectra of simple, tetragonal copper-imidazole complexes (including Cu-histidine) (Fawcett et al. 1980; Bernarducci et al. 1981; Casella and Gullotti 1983). In the cluster the optical contribution of each of these LMCT transitions ($\varepsilon \approx 200$–500 $M^{-1}\,cm^{-1}$) is clearly additive, while partial cancellation of the signed CD contributions ($|\Delta\varepsilon| \approx 0.05$–$0.25$ $M^{-1}\,cm^{-1}$) may occur according to the relative spatial orientations of the various chromophores. Hydroxide or alkoxide ions are additional potential ligands for the trinuclear Cu site that can contribute with LMCT transitions (Solomon et al. 1983) to the 330-nm protein chromophore, and also 'dimer' bands (Desjardins et al. 1987; Ross et al. 1989) may contribute to this near-ultraviolet region. Note that the residual absorption at 330 nm of the T2D enzyme does not necessarily imply incomplete T3-Cu reduction since the LMCT absorptions of Cu(I)-imidazole chromophores, with maximum near 290 nm (Sorrell and Borovik 1986; Casella et al. 1988b) tail into this region.

Treatment with hydrogen peroxide of T2D ascorbate oxidase yields the met-T2D enzyme form, containing oxidized T3 Cu centers. The possibility that excess peroxide can act as a ligand for the oxidized type-3 sites seems excluded by the relatively weak optical features at 330 nm, compared with the intense O_2^{2-}→Cu(II) LMCT transitions observed in oxyhemocyanin, oxytyrosinase and synthetic copper-dioxygen complexes (Solomon 1981; Tyeklar and Karlin 1989) and by the complete lack of CD features associated with the 330-nm chromophore in the met-T2D enzyme. Reoxidation of T3 Cu by peroxide does not lead to complete recovery of the absorbance at 330 nm present in native ascorbate oxidase, or rather the peroxide-treated native enzyme (Casella et al. 1988a) showing that T2 Cu is partly contributing to this protein chromophore. In practice, only the T3 Cu is responsible for the optical activity of the enzyme between 300–350 nm.

Azide binds to T3 Cu in T2D ascorbate oxidase, but the optical features slowly developed by the adduct are clearly those of Cu(II)-azide chromophores (Karlin et al. 1987; McKee et al. 1984; Agnus et al. 1984). In particular, the absorption bands at 390 and 455 nm can be assigned to π(azide)→Cu(II) LMCT transitions. The broad CD activity of negative sign developed between 370–480 nm, that obscures the original CD absorption of the T2D enzyme in the same range, can also be attributed to this chromophore. The same spectral features are in fact exhibited by the azide adduct formed by met-T2D ascorbate oxidase. Therefore, azide binding to T2D ascorbate oxidase induces a slow autoxidation process of T3 copper, which still remains ESR-undetectable and thus strongly antiferromagnetically coupled. Being a negative ion, azide is expected to stabilize the Cu(II) oxidation state and, in fact, azide binding to model complexes is found to lower the redox potential of the Cu(II)/Cu(I) couple (P. Zanello, unpublished observation). This behavior is different from that exhibited by T2D laccase, for which no new optical feature is produced by aerobic treatment with excess azide (LuBien et al. 1981), although the redox potential of the T3 Cu is higher in laccase (434 mV in *Rhus* laccase, 782 mV in *Polyporus* laccase) (Reinhammar 1983b) than in ascorbate oxidase (345 mV) (Kroneck et al. 1982). It is therefore likely that binding of azide to the reduced T3 centers in T2D laccase is insufficient to promote the

Table 1. Comparison of the binding characteristics of the azide adducts of various derivatives of laccase and ascorbate oxidase

Enzyme form	Laccase				Ascorbate oxidase			
	λ (nm)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	K (M^{-1})	ref.	λ (nm)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	K (M^{-1})	ref.
Native:								
high-affinity	410 br	630	10000	b	400 br	1000	5000	d
	500 br	500			500 br	1300		
low-affinity	400	1900	200	b	420	6000	100	d
	300	− 500[a]			330	−2000		
	450	900			455	2500		
Met-T2D	390 sh	1000	170	c	390 sh	1700	50	this
	330	−1000			330	−1400		work

br = broad, sh = shoulder

[a] Estimated from the spectra reported in Allendorf et al. (1985)

[b] Data from Spira-Solomon et al. (1986)

[c] Data from Spira-Solomon and Solomon (1987)

[d] Data from Casella et al. (1988a)

Cu(I) autoxidation. In addition, T2D ascorbate oxidase may preserve some possibility of electron transfer to O_2 through the sub-route provided by its 'secondary' active site (Sakurai et al. 1985).

When compared with binding studies of azide performed on native ascorbate oxidase (Sakurai et al. 1987; Casella et al. 1988a), the spectral features of the T3-Cu-azide adducts change when T2 Cu is removed. This confirms that T2 Cu is directly involved in the binding of the anion in the native enzyme, as it is in laccase (Spira-Solomon and Solomon 1987). The overall binding characteristics of the azide adducts of the various enzyme forms, summarized in Table 1, are also quite similar between ascorbate oxidase and laccase, so that structurally related active sites are a possibility. However, ascorbate oxidase contains two binuclear T3 Cu sites and it is likely that azide can bind to both of them. Although the binding experiments reported here do not directly prove the existence of two independent and noninteracting azide binding sites, the LMCT bands of the azide adducts of the various derivatives of ascorbate oxidase are systematically about twice as intense as those of the corresponding derivatives of laccase, for which a single azide molecule binds/protein molecule. The broad optical contribution of the T3-Cu-azide adduct at the 'secondary' site must be buried under the well-featured and intense LMCT band of the T2-Cu–T3-Cu-azide center in native ascorbate oxidase. It is surprising to find that the affinity of azide in the native oxidases, where it may be bridging between the T2 Cu and one of the T3 Cu centers, is about as low as that in the T2D enzymes, where it is apparently terminally bound to one atom of the T3 Cu couple (Spira-Solomon and Solomon 1987).

It is interesting to note that binding of azide ot met-T2D or native (Casella et al. 1988a) ascorbate oxidase, as well as to met-T2D (Spira-Solomon and Solomon 1987) or native (Spira-Solomon et al. 1986) laccase, produces a decrease in the absorbance at 330 nm. This observation is important because it indicates that either azide replaces one of the ligands in the binuclear T3 Cu unit or causes some marked conformational change at this site. Our binding studies of azide to model Cu(II) complexes (Casella et al. 1990) show that, for terminally bound azide, binding constants as low as those found here for ascorbate oxidase cannot be associated with Cu(II) centers carrying dipositive charge. Unless the protein backbone imposes some steric constraint to the coordinated azide so that its affinity for the T3 Cu(II) is lowered, it seems reasonable to assume that an anionic ligand is part of the coordination sphere of the T3 Cu(II) ion which binds N_3^-. This conclusion indirectly supports the view that some endogenous anionic ligand is present in the binuclear T3 Cu unit (Spira-Solomon and Solomon 1987).

A final comment is necessary on the remarkable ESR changes that we observe at the T1 Cu sites on addition of azide, even though these sites are not involved in the binding of the anion. The changes are clearly produced by freezing of the solutions, since at room temperature the optical and CD features of T1 Cu are only slightly affected. Similar behavior was noted previously on addition of azide to native ascorbate oxidase (Casella et al. 1988a). Freezing can induce local structure rearrangements at the T1 Cu sites but the dependence of the ESR changes on the azide concentration indicates that such changes are related to the binding of the ligand to the T3 Cu sites. At least part of the T1 Cu is probably located close to the T3 Cu site(s) and is sensitive to the variations occurring in the coordination sphere of the T3 Cu.

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