Spectral Study of Ascorbate Oxidase*

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Received July 7, 1983

The electronic, CD and EPR spectra of ascorbate oxidase isolated from the green zucchini squash (Cucurbita pepo medullosa) *in 0.1* M *phosphate buffer (pH 6.8) have been investigated. The visible absorption bands are clearly resolved in the CD spectrum, where the extrema occur at 735, 610, 550, 475 and 330 nm, while weak additional CD activity possibly occurs near 420 nm. The near-UV spectrum is dominated by the absorption of the aromatic amino acid residues centered at 280 nm, while resolved CD bands occur at 296, 291, 283, 265 and 240 nm. In the far-UV region the protein CD spectrum reflects its secondary structure: a single negative maximum at 218 nm suggests a predominant antiparallel β conformation for ascorbate oxidase. The frozen solution EPR spectrum of the protein has been fitted according to a new computer simulation procedure. The following parameters were obtained: for the type 1 copper* $g_z = 2.222$, $g_x = 2.032$, $g_y = 2.056$, $A_z = 59$ G, $A_x = 11$ G, *and* $A_y = 5$ G; *for the type 2 copper* $g_\parallel = 2.240$, $g_\perp = 2.057$, $A_\parallel = 179$ G *and* $A_\perp = 1$ G. *Of the eight copper atoms present in the protein four are EPR-detectable: three of type 1 and one of type 2, as shown by computer simulation of the EPR spectrum. Ascorbate oxidase is a rather unstable protein when purified and it is sensitive to a number of environmental factors. Aging of the protein leads to a decrease in the ratio between the type 1 and type 2 coppers. A new species formed at the early stages of the aging process, that has been spectrally characterized, suggests that the loss of the type 1 copper is preceded by a change in the symmetry of the original type 1 site from pseudotetrahedral to pseudotetragonal.*

*Presented at the 1st International Conference on Bioinorganic Chemistry, Florence, Italy, June 13–17, 1983.

Introduction

Ascorbate oxidase (L-ascorbate:O_2 oxidoreductase, EC 1.10.3.3) is the largest, most complex and least well defined member of the group of enzymes known as the blue copper oxidases [1]. The protein contains the three types of biological copper, according to the Malmström classification [2], in the stoichiometry of three type 1, one type 2 and four type 3 copper atoms per molecule (M_r = 140000). The ratio between type 1 and type 2 copper was established by EPR measurements [3, 4], while the type 3 coppers are EPR nondetectable. The type 2 center can be selectively removed by anaerobic dialysis against EDTA-DMG [5], though the resulting protein (t 2d enzyme) shows a marked decrease in the original activity. The molecular properties and catalytic activity of ascorbate oxidase have been recently reviewed [6, 7]. The spectroscopic properties of the blue copper sites have also been recently investigated [8], though the only available CD data in the UV region refer to an impure preparation of the protein [9]. We wish to report here the results of our spectral study of ascorbate oxidase from green zucchini squash. This includes an EPR and a CD investigation, extended to the near-UV and far-UV regions, of the protein and an attempt to elucidate the spectral changes observed upon storage of the protein.

Experimental

Ascorbate oxidase was extracted from the green zucchini squash *Cucurbita pepo medullosa)* and purified according to the most recently published procedure [4]. Protein concentration was determined assuming $\epsilon_{610} = 9700\ M^{-1}\ cm^{-1}$ in phosphate

0020-1693/84/$3.00

buffer (0.1 *M*, pH 6.8) and M_r = 140000, while the ratio A_{330}/A_{610} for our preparation was 0.90. This value remained unchanged after preparative gel electrophoresis on polyacrylamide gel under the conditions described previously [4]. A copper content of 8.2 ± 0.2 atoms per mole of protein was determined by flame atomic absorption using a copper standard in the same buffer solution.

Absorption spectra were measured with a Perkin Elmer Lambda 5 or Beckman DK-2A spectrophotometer. CD spectra were recorded on a Jobin Yvonne Mark III instrument, calibrated with a solution of isoandrosterone in dioxane ($\Delta\epsilon$ = +3.31 at 304 nm). All recordings were made at 22 °C with protein in 0.1 *M* phosphate buffer solution (pH 6.8), using cylindrical quartz cells of optical path varying between 0.01 and 1 cm and protein concentrations appropriate for readings in the range 200–800 nm. The CD data in the visible and near-UV regions are expressed in terms of differential molar extinction coefficient $\Delta\epsilon$ (M^{-1} cm^{-1}), while those in the far-UV region are expressed as mean residue ellipticities $[\theta]$, in deg cm^2 $dmol^{-1}$, using a mean residue weight of 129 (calculated on the basis of M_r = 140000 and 1085 amino acid residues per enzyme molecule [4]).

The EPR spectra were recorded on a Varian E-109 spectrometer operating at X-band frequencies. Solutions of ascorbate oxidase ~0.5 m*M*, as used for the visible absorption and CD spectra, were placed in quartz EPR tubes and frozen in liquid nitrogen. Spectra were routinely measured at −140 °C by employing a V-4000 variable temperature control apparatus. The concentration of EPR-detectable copper was calculated against a copper standard on digitalized spectra, according to the method of Vänngård [10]. The amount of EPR-detectable copper in frozen solution was 49.5% of total copper, in agreement with similar preparations of ascorbate oxidase [3, 4]. The protein EPR spectra were fitted according to the following computer simulation procedure. We minimized the sum of errors:

$$Z = \sum_i w_i [G^{exp}(H_i) - G^{th}(H_i)]^2$$

where G(H) is the Lorentzian line-shape function sampled at 220 discrete points of the field and w_i are weighting factors chosen to have an even distribution of the sum of residuals over the whole spectral region. The theoretical line-shape was considered as a sum of the type: $G^{th} = \alpha_1 G_1{}^{th}(g_z, g_x, g_y, A_z, A_x, A_y) + \alpha_2 G_2{}^{th}(g_\parallel, g_\perp, A_\parallel, A_\perp)$ where α_1 and α_2 are the molar fractions and $G_1{}^{th}$ and $G_2{}^{th}$ the theoretical spectra of the type 1 and type 2 copper species, respectively. The lowest accuracy (±2%) in the parameter determination is that relative to α_1 and α_2, due to a rather smooth variation of the Z value with respect to these parameters. The line-shape function was computed using a modified version of the SIM 14 QCPE program [11].

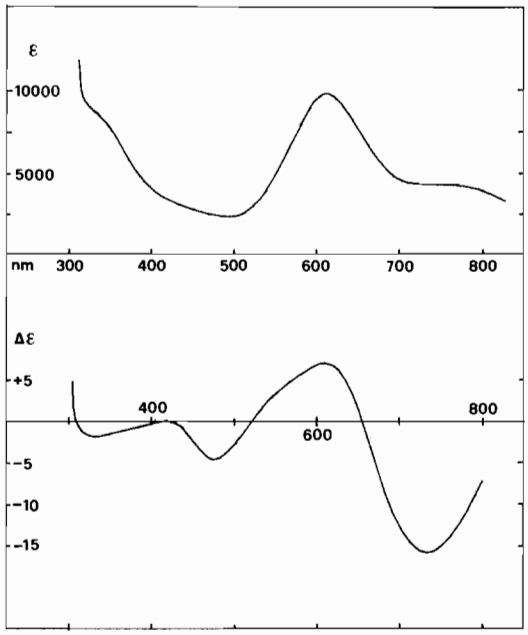

Fig. 1. Visible absorption and CD spectra of ascorbate oxidase (0.1 *M* phosphate buffer solution, pH 6.8) at room temperature.

Results and Discussion

The visible absorption and CD spectra of ascorbate oxidase are dominated by bands related to the blue copper chromophores (Fig. 1). Except for the shoulder at 330 nm, corresponding to negative CD activity and usually attributed to type 3 copper [12], all the visible bands that are well resolved in the CD spectra have been assigned to charge transfer transitions from the ligands to type 1 coppers [8]. The enzyme from our preparation exhibits visible CD extrema at 735 ($\Delta\epsilon$ = −15.85), 610 (+6.97), 550sh (+3.70), 475 (−4.85) and 330 nm (−2.08). Additional very weak positive CD activity possibly occurs near 420 nm, though this is actually often indistinguishable from zero. While the magnitude of the Cotton effects within these visible CD bands is similar to that reported by Gray [8], the location of the extrema occurs at slightly different wavelengths. The CD features of ascorbate oxidase above *ca.* 400 nm resemble those of ceruloplasmin [13–15], tree laccase [16, 17], and the other blue copper proteins [18], suggesting closely related asymmetric environments for the type 1 copper sites in these proteins, while the visible CD spectrum of fungal laccase is significantly different [16].

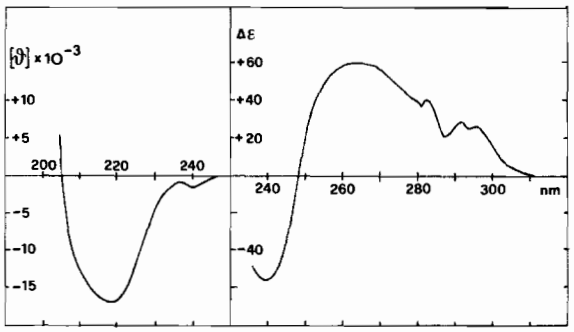

Fig. 2. Near-UV and far-UV CD spectra of ascorbate oxidase (0.1 *M* phosphate buffer solution, pH 6.8) at room temperature. In the far-UV region the ordinate represents the mean residue ellipticity (deg cm^2 $dmol^{-1}$).

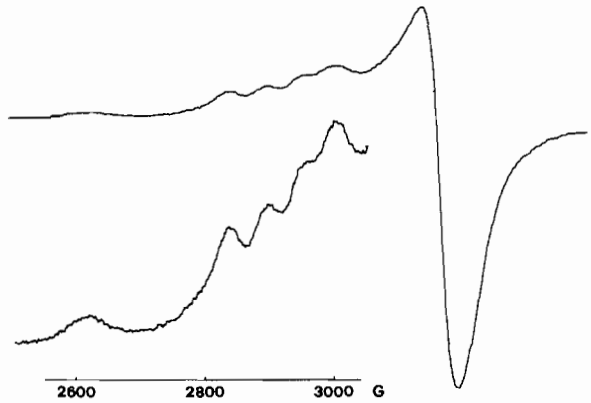

Fig. 3. Frozen solution EPR spectrum of ascorbate oxidase (0.1 *M* phosphate buffer, pH 6.8). Conditions: temperature −140 °C; modulation frequency 100 kHz; modulation amplitude 3.2 G; microwave power 10 mW; microwave frequency 9.075 GHz. The low-field part of the spectrum is also shown at a higher gain.

Comparison of the CD spectra of the various proteins below *ca.* 400 nm, however, reveals several differences and there appears to be no simple feature relating the chromophores involved.

In the near-UV region the absorption and CD spectra of ascorbate oxidase are mainly contributed by the various aromatic amino acid residues (40–45 tryptophan, 42–43 tyrosine and 46–47 phenylalanine, according to the most recently published amino acid composition [4]) and by the disulfide bonds of cystine residues [19], while minor contributions may also arise from higher energy charge transfer transitions from ligands (*e.g.* amino, carboxylate or amide groups) to type 2 or type 3 copper [20, 21]. The near-UV absorption spectrum comprises an intense, broad band centered at 280 nm ($\epsilon \sim$ 250,000) with shoulders near 290 and 260 nm, while several positive near-UV maxima are resolved in the CD spectrum at 296 ($\Delta\epsilon$ = +24.6), 291 (+28.4), 283 (+39.7) and 265 nm (+60.5, broad) as shown in Fig. 2 (unresolved shoulders also occur near 280 and 270 nm), while additional negative CD activity near 240 nm appears as a shoulder on the protein CD band at higher energy. The partially overlapping peaks at 296 and 291 nm most probably originate from tryptophan residues, since they occur at longer wavelengths than generally observed for tyrosine residues [22], while the higher intensity of the CD peak at 283 nm indicates that this is contributed by both tryptophan and tyrosine residues. The broad CD band extending from 280 to 250 nm represents unresolved contributions from several aromatic side chains and disulfide bonds. The presence of two peaks of comparable intensity above 290 nm, attributed to tryptophan residues suggests that these may be grouped roughly into two nonequivalent classes. In fact, the rotatory strength both at 291 and 296 nm can be considered to result from roughly additive contributions by half of the ~40 tryptophan residues of the protein ($\Delta\epsilon \sim 1.4$ for each single tryptophan residue [23]). However, the CD contribution above 290 nm by each single tryptophan residue can approach $\Delta\epsilon \sim 2$ by tightly packing or immobilizing the residue into a highly specific and rigid conformation [24], as shown by the CD spectra of azurin [25] and other proteins [26]; therefore CD measurements of ascorbate oxidase at low temperatures will probably be required to firmly establish this important point. The negative CD shoulder near 240 nm can possibly be assigned to transitions within the disulfide chromophores [27], though it may also originate from charge transfer transitions involving copper(II) ions [20].

The far-UV CD spectrum of ascorbate oxidase is shown in Fig. 2. The CD activity in this region is essentially due to the peptide groups of the protein, while minor contributions may arise from the aromatic residues and also from ligand to copper(II) charge transfer transitions [20], *e.g.* imidazole → Cu(II) [28]; The shape of the CD curve, with a single negative maximum at 218 nm, similar to that observed for the β form of poly-L-lysine [29, 30], indicates that a significant portion of the amino acid residues of ascorbate oxidase is present in the β conformation. Also, the absence of a peak or shoulder near 208 nm suggests that the fraction of amino acid residues with α-helical conformation is probably negligible. These qualitative features of the CD spectrum of ascorbate oxidase are very similar to those reported for ceruloplasmin [14].

The frozen solution EPR spectrum of ascorbate oxidase is shown in Fig. 3. The best fit of the EPR spectrum was obtained with the following set of parameters:

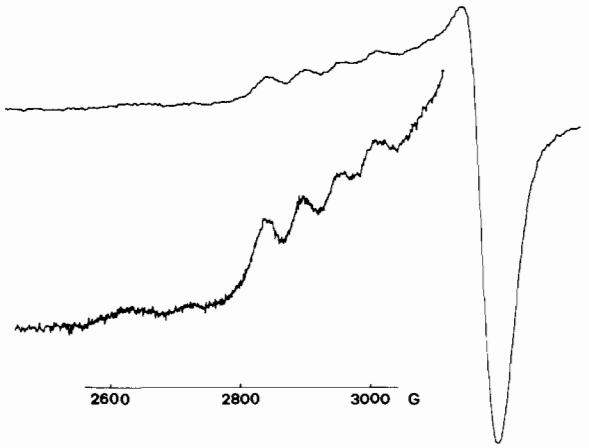

Fig. 4. Frozen solution EPR spectrum of ascorbate oxidase (0.1 *M* phosphate buffer, pH 6.8) after storage at room temperature for approximately two days. Conditions: temperature −140 °C; modulation frequency 100 kHz; modulation amplitude 3.2 G; microwave power 10 mW; microwave frequency 9.074 GHz. The low-field part of the spectrum is also shown at a higher gain.

$$\text{type 1}\begin{cases} g_z = 2.222, g_x = 2.032, & g_y = 2.056 \\ A_z = 59\ \text{G}, & A_x = 11\ \text{G}, \quad A_y = 5\ \text{G} \end{cases}$$

$$\text{type 2}\begin{cases} g_{\parallel} = 2.240, g_{\perp} = 2.057 \\ A_{\parallel} = 179\ \text{G}, A_{\perp} = 1\ \text{G} \end{cases}$$

The molar fractions of type 1 and type 2 copper were estimated as 0.75 ± 0.02 and 0.25 ± 0.02, respectively. We note that the accuracy of our simulation procedure in evaluating these parameters is certainly higher than that of the method usually followed based on integrations of the first, low-field, hyperfine line of type 2 copper [10], since this method completely neglects contributions of type 1 coppers to the type 2 hyperfine line that are actually non-negligible. Our EPR data, therefore, provide further convincing evidence for the stoichiometry of 3:1 between type 1 and type 2 coppers and rule out the possibility that ascorbate oxidase contains two identical 'laccase-like' subunits, each containing four copper atoms [31]. The *g* and *A* values obtained for the type 1 and type 2 sites, however, are slightly different from those reported earlier for similar preparations of the protein [3, 4].

Ascorbate oxidase, unlike tree and fungal laccase [2], but partly like ceruloplasmin [32], is a rather unstable protein when purified. It is also sensitive to a number of factors such as pH, temperature, ionic strength of the buffer solution, presence of external metal ions *etc.* [7]. Aging of the protein is accompanied by progressive loss of type 1 copper, as shown for instance by the decrease in intensity of the 'blue'

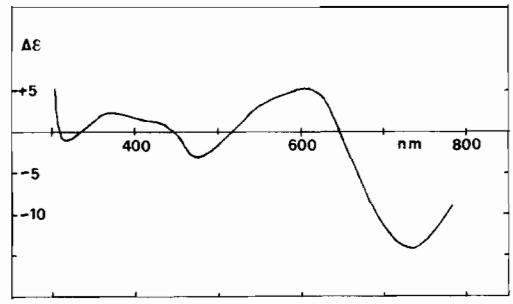

Fig. 5. Visible CD spectrum of ascorbate oxidase (0.1 *M* phosphate buffer, pH 6.8) after storage for approximately two days at room temperature.

band at 610 nm and the CD bands in the visible spectrum. This process also occurs slowly during storage of the protein at 4 °C [3] and eventually leads to a marked decrease in the ratio between type 1 and type 2 copper, as is clearly shown by the EPR spectra of aged preparations of the protein. Similar results are obtained upon lyophilization of the pure enzyme [4, 7]. Spectra recorded at earlier stages of the aging process, however, reveal new interesting features. Figure 4 shows the frozen solution EPR spectrum of ascorbate oxidase after storage at room temperature for approximately two days (compare with the EPR spectrum in Fig. 3). A new feature near 2730 G is clearly resolved between the $m_I = -3/2$ hyperfine line of the type 2 copper and the type 1 copper signal. At the same stage of the process the CD spectrum of the protein shows the appearance of new positive CD activity near 370 nm, accompanied by an overall decrease in intensity of the CD bands above 400 nm, particularly those at 610 and 735 nm (Fig. 5). The increase in CD activity near 370 nm corresponds to an increase in intensity of the broad absorption band between 300 and 400 nm. Since the 610-nm band also decreases in intensity, the result is an overall increase in the ratio A_{330}/A_{610} (from 0.90 to 1.20 in the present case). It is possible to relate the new EPR signal and the new CD band if we assume that the EPR feature near 2730 G in Fig. 4 represents the low-field, $m_I = -3/2$, hyperfine line of the EPR signal of the new copper species. The position of this hyperfine line is at higher field than the corresponding $m_I = -3/2$ line of the type 2 copper, but it must be related to a copper center of nearly tetragonal symmetry, since the EPR spectrum clearly shows the absence of any closely spaced $m_I = -½$ line that would characterize a copper site of tetrahedral or lower symmetry, such as that of the type 1 copper. The $m_I = -3/2$ line of the new copper center, therefore, suggests a rather low $g_{\parallel}$ value for this species, most likely in the range between 2.15 and 2.20, requiring equatorial coordination of at least one sulfur donor

atom to copper(II) [33]. The new copper species, therefore, may simply result from distortion of the original pseudotetrahedral type 1 site toward a pseudotetragonal symmetry, since an increase of $A_{\parallel}$ and a decrease of $g_{\parallel}$ are expected for such a transition [34]. Further support for this view comes from the CD data, since the positive CD activity observed near 370 nm occurs in the typical range for $S(\sigma) \rightarrow$ Cu(II) charge transfer transitions in tetragonal complexes containing equatorial sulfur donors [35]. It can be concluded, therefore, that the release of type 1 copper by the protein is preceded by a change in the original type 1 site symmetry from pseudotetrahedral to pseudotetragonal that does not involve the loss of the sulfur ligand(s) by the copper ion. The subsequent loss of the sulfur ligand(s), that may proceed through reduction/oxidation steps at the copper center, eventually leads to a tetragonal copper site in a 'normal', nitrogen/oxygen, environment. This is hardly distinguishable from that of the type 2 copper and actually lowers the apparent ratio of type 1/type 2 copper, as it is found in the EPR spectra of aged [3, 4] or impure [9] preparations of ascorbate oxidase.

Acknowledgement

The authors thank the Italian M.P.I. for financial support and the Italian C.N.R. for instrumentation facilities.

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