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Spectral Study of Ascorbate Oxidase

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Ascorbate oxidase (L-ascorbate:O_2 oxidoreductase, EC 1.10.3.3) is the most complex 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. We have undertaken an investigation of the spectral properties of ascorbate oxidase isolated from the green zucchini squash and wish to present here our preliminary results. The protein was purified according to the most recently published procedure [3]. The parameters M_r = 140000 and ϵ_{610} = 9700 M^{-1} cm^{-1} were assumed, while the ratio A_{330}/A_{610} for our preparation was approximately 0.90. The X-band EPR spectrum of a frozen solution of ascorbate oxidase in 0.1 *M* phosphate buffer, pH 6.8, recorded at −140 °C, was fitted according to the following computer simulation procedure. We minimized the sum of errors

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

where G(H) is the line-shape function sampled at 220 discrete points of the field. 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. 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 best fit of the EPR spectrum was obtained with the following parameters:

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

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

The molar fractions of type-1 and type-2 copper were estimated as 0.75 ± 0.02 and 0.25 ± 0.02, respectively. Double integration of the EPR signal revealed that 49.5% of the total copper was EPR-detectable. Our data are therefore in close agreement with those reported earlier for similar preparations of ascorbate oxidase [3, 4].

The visible CD spectrum of ascorbate oxidase displays extrema at 735 ($\Delta\epsilon$ = −15.85 M^{-1} cm^{-1}), 610 (+6.97), 475 (−4.85) and 330 nm (−2.08). Additional very weak positive activity may occur near 420, 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 [5], the location of the extrema occurs at slightly different wavelengths. In the near-UV region the CD spectrum of ascorbate oxidase is mainly contributed by the aromatic amino acid residues (tryptophan, tyrosine and phenylalanine) and by the disulfide bonds of cystine residues, while minor contributions may also be expected to arise from copper(II)–ligand charge transfer transitions. The near-UV maxima are located near 296 (+24.6), 291 (+28.4), 283 (+39.7) and 265 nm (+60.5, broad), while additional negative CD activity near 240 nm appears as a shoulder on the protein CD bands at higher energy. We note that the near-UV absorption spectrum of ascorbate oxidase is dominated by an intense band centered at 280 nm with shoulders near 290 and 260 nm. The far-UV CD spectrum between 200 and 240 nm contains a strong negative protein band at 218 nm (θ = −16700 deg cm^2 dmol^{-1} where θ is the mean residue ellipticity calculated on the basis of M_r = 140000 and 1085 amino acid residues per enzyme molecule) [3]. The presence of a single negative CD extremum at this wavelength indicates that, like ceruloplasmin [6], ascorbate oxidase exists predominantly in the β conformation, similar to that observed in the β form of poly(L-lysine).

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