of free metal-ion 10^{-8}–10^{-3} *M* in the metal-ion buffers. The excess of activators inhibited the enzymatic reaction. The optimal pH values for I, II and III are 8.1, 7.9 and 7.3 respectively.

From the date presented, I, II and III may be identified respectively as aminopeptidase, tripeptidase and dipeptidase.

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Ascorbate Oxidase: Properties of the Copper Sites and Catalytic Activity of the Native and Type 2 Cu Depleted Enzyme

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Ascorbate oxidase (L-ascorbate: O_2 oxidoreductase, E.C. 1.10.3.3) is the subject of numerous biological investigations. Some of the recent developments in the purification of the enzyme, the number of Cu atoms per active molecule, the stoichiometry of the different Cu sites and the mechanism of reduction by L-ascorbate have been previously reviewed [1].

Ascorbate oxidase from squash Cucurbita p.m. contains 8 Cu atoms/140 000 M_r of which 50% are detectable by electron paramagnetic resonance (EPR), *i.e.* three type-1 Cu, one type-2 Cu and four type-3 Cu [2]. The number and stoichiometry of different Cu sites depend very much on environmental conditions maintained during the process of isolation and purification, such as pH, ionic strength or presence of metal chelators. Thus, dialysis of pure enzyme against acetate buffer, pH 4.5–5.5, may lead to drastic changes in the visible region of the electronic spectra (indicated by a decrease of the purity index A_{610}/A_{330} [1]) and of the EPR spectra (indicated by a decrease of the type-1 signal). In the presence of metal chelators, such as EDTA or dimethylglyoxime [3], the type-2 center is removed selectively (= t2d enzyme) as demonstrated by EPR spectrosocpy. Depending on dialysis conditions (pH, concentration of metal chelator, aerobic *vs.* anaerobic) up to four Cu atoms will be dialysed off. As epxected, the activity of these enzyme samples is lowered to less than 4% of the original activity present.

The so-called t2d enzyme (5.8 ± 0.3 Cu/140.000 M_r) is still reduced by L-ascorbate in the absence of dioxygen, approx. 5.5 electron equivalents are used for total bleaching of the absorbance at 610 nm. Upon exposure to air, enzyme reoxidation begins as indicated by the appearance of the typical blue color (λ_{max} 610 nm) and the type-1 Cu EPR signal. By comparison with native ascorbate oxidase this seems to be a relatively slow process, complete reoxidation is achieved after a few minutes at 20 °C, pH 7.0.

The time course of the reaction of reduced ascorbate oxidase, or reduced t2d enzyme, with dioxygen was followed by stopped-flow spectrophotometry and rapid-freeze EPR spectroscopy. Prior to reaction with molecular oxygen the protein is reduced by an excess of L-ascorbate (6–8 equivalents of reductant per enzyme), hereafter mixing with O_2/buffer solution is achieved under the strict exclusion of air at temperatures between 5 and 10 °C [1]. In the first rapid phase of the reoxidation of the native enzyme (2–4 msec) the characteristic absorption spectrum and EPR signal of the L-ascorbate radical (λ_{max} 365 nm, g = 2.005) can be detected [1], due to the turnover of the enzyme in the presence of reducing substrate and dioxygen. Total reoxidation of ascorbate oxidase occurs very rapidly, within less than 8 msec at 10 °C as shown optically by the appearance of absorbance at 610 nm and by EPR. By comparison with untreated ascorbate oxidase the reduced t2d enzyme is far less reactive towards dioxygen, the reoxidation process takes several minutes under identical conditions of pH, ionic strength, temperature and oxygen concentration.

From the kinetic experiments on the anaerobic reduction of ascorbate oxidase and oxidation of the reduced enzyme conclusions are drawn concerning the modes of internal and external electron transfer. Furthermore, mechanistic aspects of the catalytic function of the multicopper oxidase are discussed.

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