a) There are probably two distinct and physiologically operative electron transfer sites. b) One of these sites is centered around the respective Cr(III) labeled region. c) By elimination, the second is at the exposed, homologous imidazole of His-87 or 117 in Pc and Az, respectively.

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THE BINUCLEAR COPPER CENTER AND THE REACTION MECHANISM OF TYROSINASE

Tyrosinase is a copper-containing monooxygenase catalyzing the formation of dark colored melanin pigments [1]. Various forms of the enzyme can be obtained (met-, halfmet-, oxy- and desoxytyrosinase), depending on the oxidation state of the active site copper. Met- and oxytyrosinase contain two tetragonal Cu(II) ions antiferromagnetically coupled through an endogenous bridge with the exogenous oxygen molecule in oxytyrosinase bound as peroxide. In halfmettyrosinase the two coppers are present in a mixed-valence state [2]. The chemical and spectroscopic properties of the different forms are surprisingly similar to those reported for the oxygen-binding hemocyanins [3]. However, differences between the tyrosinase and hemocyanin active sites are apparent from peroxide displacement and binding studies of tyrosinase substrate analogues [4]. Binding of L-mimosine and various derivatives of benzoic acid to halfmettyrosinase results in very unusual Cu(II) spectral features. They relate to a significant distortion of the Cu(II) site as shown by the rhombic splittings and perpendicular hyperfine structure of the EPR spectra [5]. In addition, these competitive inhibitors are found to bind to the enzyme with an equilibrium constant higher by one order of magnitude relative to aqueous Cu(II) [6]. It is suggested that the protein environment of the binuclear copper complex contributes significantly to the stabilization of substrate analogues binding to the active site. This stabilization and the concomitant change from a tetragonal toward a trigonal bipyramidal geometry of the Cu(II) site seem to greatly assist the catalytic hydroxylation reaction of tyrosinase. It is proposed that the binding of a monophenol substrate to oxytyrosinase leads to a distortion of the Cu(II) complex, thus labilizing the peroxide. This then leaves a reactive, polarized peroxide which in turn can hydroxylate the monophenol most likely via an electrophilic attack on the aromatic ring.

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ASCORBATE OXIDASE: FURTHER INVESTIGATIONS OF ITS STRUCTURE AND CATALYTIC PROPERTIES

Ascorbate Oxidase (L-ascorbate:O_2 oxidoreductase, AO, E.C. 1.10.3.3) is a multicopper enzyme widely distributed in plants. For a review of the literature prior to 1982 see ref. [1]. This article describes some recent developments in the number of Cu/M_r, the selective depletion of the type-2 Cu (t2d-AO), the preparation of the apoprotein and its reconstitution to the holoenzyme. In addition redox reactions of AO and t2d-AO with L-ascorbate, thiomolybdate(VI) and dioxygen are reported as studied by stopped flow spectrophotometry and rapid freeze EPR spectroscopy. Finally, a model is presented describing the active site of the multicopper oxidase. On the basis of this model some mechanistic aspects of the reaction of reduced AO with molecular oxygen are discussed.

Cu content, stoichiometry of the Cu types, and stability towards H^+, OH^-

The number of Cu atoms/M_r and stoichiometry of the three Cu types strongly depend on several environmental factors maintained during the process of purification, such as pH, ionic strength, buffer, metal ions and chelating agents in the medium. Below pH 5.5, upon dialysis against acetate buffer, the absorbance at 610 nm slightly decreases, whereas an increase in the region 300-400 nm is observed. At pH 4.0, the blue color of AO has completely disappeared accompanied by a slow precipitation of the protein. These results are confirmed by EPR which clearly show that the type-1 signals have been replaced by signals characteristic of non-prosthetic Cu. The acid induced denaturation of AO is irreversible below pH 4.5, dialysis against buffer, pH 7.0, does not restore the original spectral properties. Above pH 8.0, the optical density at 610 nm also diminishes, but no concomitant turbidity due to protein denaturation is observed. At pH > 10.5 the typical blue color of AO has faded completely, again an increase at 330 nm (type-3 region) can be detected. The reaction at alkaline pH is biphasic with $t_{1/2}$ 5 and 45 min. at pH 11.5, 25°C. Despite the drastic changes in the optical spectrum of AO the Cu remains bound to the protein as shown by EPR and AAS. Up to pH 10.5, this process is fully reversible, dialysis against buffer, pH 7.0, restores both the spectral properties of AO and its activity towards L-ascorbate/dioxygen. According to CLARK *et al.* [2] AO dissociates into two subunits (65,000 M_r) at pH > 10.0. Our studies on the reversibility of the OH^- induced processes seem to indicate that one (or several) ligand(s) of the type-1 Cu are replaced by OH^- producing a type-2 ligand field. Alternatively, prosthetic groups close to the Cu sites might be deprotonated, i.e. -NHCO-, $-NH_3^+$ or imidazole, inducing changes of the protein conformation and the Cu coordination spheres.

Dialysis of AO against phosphate buffer, pH 7.0, containing less than 10^{-9} M Cu (determined by AAS), leaves the optical density at 610 nm unchanged but decreases the intensity of the 330 nm chromophore (A_{330}/A_{610} 0.56 vs. 0.75 [1]). After addition of extraneous Cu to the dialysis medium (0.31 mM $CuSO_4$ corresponding to 20 Cu/AO) this effect is reversed.

t2d-AO, apo-AO and reconstitution.

Depending on pH, time of dialysis and the presence of metal chelating agents (EDTA, DMG) the type-2 Cu can be removed giving the so-called t2d-AO. Starting with 7.8±0.3 Cu/M_r in native AO a t2d-AO is obtained with 6.5±0.2 Cu, and a specific activity of 13%. The EPR spectrum of t2d-AO documents the loss of the type-2 Cu, the optical purity index A_{330}/A_{610} decreases to 0.36 (*vs.* 0.75 in AO). The intensity of the fluorescence emission at 335 nm increases by 55±5% compared to native AO.

Dialysis against CN^-, at basic pH, in the absence of O_2, causes the complete loss of AO (0.8±0.3