We have now studied the binding of ^{64}Cu to this enzyme in reconstitution and exchange experiments. High performance size-exclusion gel chromatography with the protein analysis column I-125 from Waters was used to separate the enzyme-bound and free ^{64}Cu, and the amount of ^{64}Cu bound to the protein was determined from the radioactivity eluting together with the protein. Experiments with binding of $^{64}Cu(II)$ to the apoenzyme give further evidence for a specific binding of 4 copper atoms per tetramer, but some weaker copper-binding sites were observed in the presence of an excess of copper. When the apoenzyme was incubated with 4 atoms of $^{64}Cu(II)$ per tetramer, about 3.5 copper atoms were eluted with the protein indicating that the binding of Cu(II) is not extremely tight. Similar amounts of ^{64}Cu were bound to the apoenzyme in the presence of ascorbate indicating the binding of Cu(I) is similar to that of Cu(II).

The exchanges of both Cu(I) and Cu(II) in the holoenzyme are rapid and a half-life of about 1 min was estimated for the exchange of the enzyme-bound Cu(II) in the presence of a two-fold excess of $^{64}Cu(II)$ at pH 6.1. Experiments in the presence of ascorbate revealed that the exchange of Cu(I) was complete in 1 min at similar conditions. The exchange of the copper atoms in dopamine β-monooxygenase are thus much more rapid than reported for other copper proteins, and the present results point to a unique copper-binding site in this protein.

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O6

Zinc in Aminolevulinic Acid Dehydratase

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5-Aminolevulinic acid dehydratase (EC 4.2.1.24) catalyzes the formation of the tetrapyrrole precursor porphobilinogen from 5-aminolevulinic acid. The isolated enzyme contains variable amounts of zinc but binds a maximum of 8 Zn(II) per octameric protein. Removal of zinc by chelators increases K_M 60-fold and decreases V_{max} to about 10 to 20% of its original value. After removal of zinc the enzyme activity may be restored to its original value by Zn(II) or Cd(II). The ^{113}Cd-NMR spectrum of the enzyme reconstituted with $^{113}Cd(II)$ exhibits a single sharp resonance signal at 79 ppm which is not changed when substrate is added. Obviously there is no immediate interaction between the metal and the substrate. On the other hand zinc is located in the substrate binding domain since the enzyme is specifically inhibited by the affinity reagent 2-bromo-3-(5-imidazolyl) propionic acid.

It is concluded that zinc in 5-aminolevulinic acid dehydratase is a constituent of the active centre but does not have an immediate catalytic function.

O7

Preliminary Crystallographic Data for the Copper Enzyme Ascorbate Oxidase

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Ascorbate oxidase (EC 1.10.3.3) is a copper enzyme belonging to the group of so-called 'blue oxidases' together with laccases and ceruloplasmin. The enzyme, widely distributed in several plant species, catalyzes the oxidation of L-ascorbate, transferring the reducing equivalents to molecular dioxygen. The biological function of the enzyme is still in question. Ascorbate oxidase activity is highest in those parts of plants which grow faster; on the other hand some authors suggested a possible role of the enzyme in plant respiration [1].

The native enzyme is a non-covalent dimer, whose subunits (respectively 75,000 and 72,000 Mr) contain 8 Cu^{2+} ions; these can be classified, according to their coordination environments, as of type-1, type-2 and type-3 [2, 3]. Ascorbate oxidase is known to undergo fully reversible association-dissociation phenomena. Its ultracentrifuge pattern changes as a function of pH and buffer media, while the spectroscopic properties and the activity towards ascorbate remain unchanged.

Although the information available at present is not sufficient to fully elucidate the sequence of redox events which take place within the protein, there exists some evidence that the three classes of copper ions fulfil different functions. Type-1 copper is the primary site of electron acceptance; type-2 and type-3 coppers are implicated respectively in ascorbate and O_2 binding [4]. Ascorbate oxidase is thus an ideal model enzyme for the study of biochemistry and biophysics of vegetal copper proteins.

In consideration of its physico-chemical properties, the elucidation of ascorbate oxidase three-dimensional structure may also contribute to the comprehension of the association-dissociation phenomena and of their biological significance.

The protein employed for the crystallization experiments was purified from green zucchini squash according to the method of Avigliano *et al.* [5], showed absorption ratios $A_{280}/A_{610} = 25 \pm 1$, $A_{330}/A_{610} = 0.8 \pm 0.05$ and had a turnover number of 5×10^5 mol/min. Several micro-buttons filled with a 15 mg/ml solution of the enzyme were used in parallel dialysis experiments against different buffers and precipitating agents, in the pH range 5–9. Under the following conditions the same characteristic blue crystals of the enzyme could be grown:

(a) 1.8 *M* ammonium sulfate, at pH values 6.7 through 8.4

(b) 1.0 *M* sodium citrate, at pH 7

(c) 1.9 *M* potassium phosphate, at pH 7

The crystals obtained were subsequently used for a preliminary crystallographic investigation. From the analysis of the diffraction pattern symmetry and of the systematic absences it was possible to conclude that ascorbate oxidase crystallizes in the orthorhombic space group $P2_12_12_1$ with unit cell edges $a = 125.4$, $b = 189.8$, $c = 112.2$ Å. The asymmetric unit can thus accommodate a dimer of the enzyme (Mr 294,000) and the (volume) solvent content of the crystals is 45%. The crystals diffract to 3.0 Å resolution; this crystalline modification is isomorphous with that reported by Ladenstein *et al.* [6] for the same enzyme, but grown under different physico-chemical conditions.

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O8

EPR-Detected Interaction between Cytochrome a_3 and Cytochrome a in Cytochrome c Oxidase

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A prevailing view of cytochrome *c*-oxidase's mode of action is that the cytochrome *a* moiety of the oxidase is first reduced by electrons from ferrocytochrome *c*, and then electron transfer occurs from cytochrome *a* to cytochrome a_3 and finally to oxygen [1]. We address the question of how this functional interaction is related to structural perturbation of the a_3 center by the *a* center or to simple proximity of the two centers. We have investigated this problem with NO complexed to heme a_3 in fully reduced oxidase ($a^{2+}\cdot Cu_a^{1+}\cdot a_3^{2+}\cdot Cu_{a3}^{1+}$) and with NO and CO complexed to heme a_3 in a mixed valence (MV) state oxidase ($a^{3+}\cdot Cu_a^{2+}\cdot a_3^{2+}\cdot Cu_{a3}^{1+}$).

Bovine cardiac cytochrome *c* oxidase was prepared by methods of Refs. 2 and 3. Fully reduced and mixed valence complexes were prepared in a modified Thunberg cell under strictly anaerobic conditions. EPR (electron paramagnetic resonance) spectra were recorded under non-saturating microwave powers with a Bruker ER 420 (9.0–9.8 GHz) spectrometer over a 12–77 K temperature range.

The EPR of nitrosylferrocytochrome a_3 (*i.e.*, a_3-NO) in both fully reduced and MV forms showed detailed hyperfine structure from nitrogen nuclei of NO and proximal histidine. The EPR features encompassed a g-value range of g_x, g_z, g_y = 2.09, 2.006, 1.98. The MV-a_3-NO form, but not the fully reduced, showed additional temperature-dependent spectral changes setting in below 40 K. The most striking change occurred near g = 2.09 and smaller line-broadening changes occurred near g = 2.00. In MV-a_3-NO there thus appears to be an internal magnetic interaction that shows rapid, temperature-dependent fluctuations down to 40 K but below 40 K slows sufficiently to allow resolution by EPR. This internal interaction has a magnitude of 12, 1.5, and 5 Gauss at g_x, g_z and g_y, respectively.

At present we have two separate explanations for the phenomenon which both indicate a–a_3 interaction. Either, as a result of redox changes at cytochrome *a*, a changed ligand-binding environment, which has conformations that rapidly convert down

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