these latter enzymes are laccase and cytochrome oxidase. They contain four metal centers per functional unit. Although laccase contains only copper ions while cytochrome oxidase has two copper ions together with two heme irons, they show marked similarities with respect to the prosthetic metal groups. Thus, only two of the metal centers, type 1 and 2 Cu(II) in laccase and Cu_A(II) and cytochrome *a* in cytochrome oxidase are detected by EPR. These metal sites serve as primary electron-accepting sites which are reduced by one-electron donating substrates [1, 2].

In both enzymes the other two metal ions form a bimetallic unit, type 3 copper in laccase and cytochrome a_3-Cu_B in cytochrome oxidase. In the resting enzymes these metals are believed to be in the Cu(II) and Fe(III) states, respectively, which are ERP-silent due to strong exchange coupling between the contiguous metal ions. These sites form the dioxygen-reducing centers which are reduced by intramolecular electron-transfer from the two EPR-detectable metal sites.

Since the bimetallic units are EPR-nondetectable under most experimental conditions, their metal coordination and role in the enzyme mechanisms are little known but of great interest. However, new EPR signals, originating from one of the type 3 Cu(II) ions in laccase and from Cu_B(II) in cytochrome oxidase, have recently been generated [3, 4]. Further exploration of these metal sites has therefore been possible. Both proteins show very similar EPR signals which are characterized by a rhombic *g*-tensor and a hyperfine coupling which is intermediate between that of type 1 and 2 Cu(II). Rhombic Cu(II) EPR signals of a similar kind are also observed in other proteins with copper in bimetallic sites, such as superoxide dismutase and half-met hemocyanin. Together with a pronounced amino acid sequence homology between a blue oxidase (ceruloplasmin) and cytochrome oxidase with the copper-binding site in superoxide dismutase, the similarity in Cu(II) EPR signals suggests that the metals may be coordinated in a similar way in these different proteins.

The type 3 Cu(II) in laccase and the Cu_B(II) in cytochrome oxidase have been studied by the ENDOR technique [5]. The laccase data show the presence of at least three nitrogeneous ligands. At least one of these is an imidazole and this is further supported by pulsed EPR studies [6]. The hyperfine coupling to the three nitrogens differs significantly indicating a low coordination symmetry for this Cu(II) in agreement with the rhombic *g*-tensor. An exchangeable proton suggests a nearby H_2O or OH^-, perhaps as a fourth ligand. Comparative ^{14}N ENDOR studies of the Cu_B(II) site in cytochrome oxidase show that the resonances from three distinct nitrogeneous ligands with hyperfine couplings are essentially the same as those of the type 3 Cu(II) site in laccase. These results are therefore a further indication of a possible similar metal binding in both enzymes.

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I3

Cytochrome *c* Oxidase: A Short Review

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For some years now research on cytochrome *c* oxidase (EC1931) has been gaining momentum [see 1–3 for reviews]. Largely this is due to the important role this enzyme plays in cellular respiration but also it reflects the availability of new and sophisticated techniques which afford the opportunity of making rapid and significant progress in our understanding of both the structure and mechanism of the enzyme.

Cytochrome *c* oxidase is one of a small class of enzymes capable of catalysing the reduction of molecular oxygen to water. This reaction involves coupling the single electron donors of the mitochondrial respiratory chain to the 4 electron acceptor, dioxygen. The mechanism thus includes oxygen binding and activation, electron transfer steps and stabilisation of potentially harmful oxygen intermediates. Also, as protons are taken up from solution to produce water this terminal step in the respiratory chain is important for the maintenance of a proton gradient across the mitochondrial membrane and thus to ATP synthesis.

The complex and varied functions of which cytochrome *c* oxidase is capable are reflected in its structure which is itself complex and asymmetric. The enzyme isolated from eukaryote sources is made up of a number [7–12] of polypeptide subunits [4, 5] assembled to form a complex which spans the inner mitochondrial membrane [6, 7]. This complex contains four metal centres, two copper atoms and two haem *a* groups, all of which appear to be associated with the larger hydrophobic subunits.

A wide range of spectroscopic and kinetic techniques have now been applied to probe the nature of the metal sites and the route of electron transfer. The consensus view is that electrons enter the complex through one of the haem *a* groups (cytochrome *a*) and are rapidly transferred to a copper atom (Cu_A). Electrons subsequently pass to a binuclear centre consisting of a copper atom (Cu_B) and a haem *a* group (cytochrome a_3) in close association and which act as the oxygen binding site. The properties of these metal sites and the nature of their immediate environments have now been partially elucidated [*e.g.* 8–10].

The mechanism of reaction and the nature of bound intermediates have been investigated by coupling spectroscopic methods with low temperature trapping techniques thus allowing intermediates with short life times at *in vivo* temperatures to be captured and studied [11, 12].

A short review of the structure of cytochrome *c* oxidase and the nature of the metal sites will be presented together with an outline of the catalytic cycle as postulated from recent EPR measurements [13]. Experiments were performed in which samples of the enzyme during 'turnover', and whilst being monitored by optical methods, were rapidly frozen and prepared for EPR spectroscopy. In this way the optical and EPR signals associated with the metal sites could be related to each other and to the level of reduction maintained during steady-state. This technique has yielded information regarding which of the many known derivatives of the enzyme are populated during catalysis.

Attention will also be drawn to site–site interactions and to recent proposals suggesting that interconversion between forms of the enzyme (possibly conformational variants) play a role in the regulation of activity [14].

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I4

The Metal Centers of Cytochrome *c* Oxidase: Structure and Function

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Considerable progress has been made in recent years on the structure of the metal centers in cytochrome *c* oxidase. Most of these studies have naturally focused on the ligands of the metal center as they play a prominent role in electron transfer, oxygen reduction and possibly also energy conservation. The most unambiguous structural information on the ligands has emerged from EPR/ENDOR studies, particularly when these studies are undertaken in conjunction with isotopically substituted cytochrome *c* oxidases prepared by incorporating selectively isotopically substituted amino acids into the protein via biosynthetic procedures. These results will be reviewed.

Good progress has also been made towards elucidating the mechanism of dioxygen reduction. Preliminary evidence for a mechanism involving both a peroxo and a ferryl intermediate will be presented. The possible structural differences at the dioxygen reduction site between the resting oxidized enzyme and the pulsed enzyme will also be discussed.

While impressive progress has been made toward understanding the structure of the metal centers of cytochrome oxidase and their roles in dioxygen reduction, comparatively little is known about the mechanisms by which the free energy of this reaction is conserved in the form of a transmembrane electrochemical potential gradient. It appears likely that the protons consumed in the reduction of dioxygen to water are derived from the matrix side of the mitochondrial membrane. Since the electrons used in this reaction originate from the intermembrane space of the mitochondrion, a transmembrane electrochemical potential gradient will be generated. However, the spatial dispositions of the metal centers in the membrane profile (or along the transmembrane electrochemical potential profile) are not sufficiently known