Minisymposium: Cytochrome Oxidase and Blue Oxidase

Convener: BO G. MALMSTRÖM; Gothenberg, Sweden

I1

Redox Investigations of the Type 3 Site in Type 2 Copper Depleted *Rhus* Laccase

PATRICK FRANK and ISRAEL PECHT*

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

We report the results of experiments designed to characterize the Type 1 and Type 3 copper sites in *Rhus* laccase depleted of Type 2 copper (T2D). Use of the Lowry method for determining protein concentration, with native laccase as a standard, established the value 5680 ± 710 M^{-1} cm^{-1} for the extinction at 615 nm for T2D laccase. Anaerobic reductive titrations of T2D laccase have been performed using Cr(II)aq ions, $Ru(NH_3)_6^{2+}$ and hydroquinone as reductants. For five independent titrations on three independent T2D laccase preparations, 3.1 ± 0.25 reduction equivalents were required to reduce the protein completely, as judged by the absorption at 615 nm. Thus the fact that the Type 3 copper ions are in the +2 oxidation state in fresh T2D laccase is unequivocal.

The data from equilibrium reductive titrations with hydroquinone fit a model in which the Type 1 site copper in T2D laccase is 35 mv more oxidizing than those of the Type 3 site, and the Type 3 site coppers are reduced in equipotential single electron steps. Both these findings contrast with the case for the native enzyme. In addition theoretical considerations governing the electron distribution in multinuclear equivalent site species during reduction imply that reduction of the Type 3 site coppers is accompanied by a protonation or conformational transition within the site itself in T2D laccase.

Treatment of T2D laccase with a 70-fold excess of H_2O_2 induced a new shoulder at 330 nm ($\Delta\epsilon$ = 660 M^{-1} cm^{-1}), minor intensity enhancement of the ultraviolet absorption band and a decreased absorption at 615 nm ($\Delta\epsilon$ = −900 M^{-1} cm^{-1}). The following difference spectra were constructed from experimental data: (native laccase minus T2D laccase) and (peroxy native laccase minus peroxy T2D laccase). Both were found to exhibit maxima at 325 nm, ($\Delta\epsilon$ ~1200 M^{-1} cm^{-1}) and shoulders near 370 nm ($\Delta\epsilon$ ~500 M^{-1} cm^{-1}). The similarity of these spectra throughout the near ultraviolet region confirms previous CD results that peroxide is bound to the Type 3 copper site of T2D laccase in a fashion similar to that of the native protein, despite the diminished binding constant (10^4 M^{-1} *vs* > 10^8 M^{-1}, respectively). The lack of any other new near-UV feature on treatment with peroxide corroborates the reductive titrations in demonstrating the oxidized state of the Type 3 copper ions in resting T2D laccase.

Dioxygen reoxidation of ascorbate reduced T2D laccase produced new difference bands at 330 nm ($\Delta\epsilon$ = 770 M^{-1} cm^{-1}) and 270 nm ($\Delta\epsilon$ = 13000 M^{-1} cm^{-1}), the former assigned to a bound peroxide dioxygen reduction intermediate. In the corresponding epr spectrum of this material new low intensity Cu(II)$g_{\|}$ features ($A_{\|}$ ~130 G) indicative of a magnetically isolated copper ion, as well as a new triplet signal near 3400 Gauss were observed. Reoxidation by dioxygen as mediated by iron hexacyanide, or by ferricyanide alone, produced a reoxidized protein having the original spectral determinants. Thus not only does reduced T2D laccase react directly with dioxygen, but the magnetism of the reoxidized Type 3 site may occasionally be perturbed during turnover. Conformational transitions occurring in T2D laccase during turnover may therefore diminish and perhaps obviate the binuclear coupling of the Type 3 copper ions.

In summary, removal of the Type 2 copper from *Rhus* laccase has produced an altered Type 3 site. It appears that T2D laccase is conformationally labile around the Type 3 site, and therefore that direct analogies relating the chemistry or spectroscopy of native and T2D laccase should be drawn with caution.

Acknowledgements. We thank Dr. Ole Farver for his critical interest in this work. P. F. gratefully acknowledges the support of a Bergmann Postdoctoral Fellowship Grant administered by the United States–Israel BNSF, Jerusalem, Israel.

I2

Cu(II)-Coordination in the Bimetallic Sites of Laccase and Cytochrome Oxidase

BENGT REINHAMMAR

Department of Biochemistry and Biophysics, University of Göteborg and Chalmers Institute of Technology, S-412 96 Göteborg, Sweden

Molecular oxygen plays a key role in many biological oxidation reactions. Dioxygen is reduced to peroxide by most oxidases but a few enzymes can reduce it all the way to two water molecules. Among

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.

Acknowledgements. This study has been supported by grants from the Swedish Natural Science Research Council and benefited from a grant from EMBO.

1 B. Reinhammar, 'Copper Proteins', Vol. II, R. Lontie, Ed., CRC Press, Boca Raton, Florida, in press.
2 B. G. Malmström, *Ann. Rev. Biochem., 51,* 21 (1982).
3 B. Reinhammar, *J. Inorg. Biochem.*, in press.
4 B. Reinhammar, R. Malkin, P. Jensen, B. Karlsson, L.-E. Andréasson, R. Aasa, T. Vänngård and B. G. Malmström, *J. Biol. Chem., 225,* 5000 (1980).
5 J. Cline, B. Reinhammar, P. Jensen, R. Venters and B. M. Hoffman, submitted for publication.
6 W. B. Mims, J. Peisach and B. Reinhammar, unpublished.

I3

Cytochrome *c* Oxidase: A Short Review

M. T. WILSON

Department of Chemistry, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, U.K.

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.