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DIRECT OBSERVATION BY EPR OF A REDUCTIVELY DECOUPLED TYPE 3 SITE IN TYPE 2 COPPER DEPLETED LACCASE

Patrick Frank and Israel Pecht

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

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SUMMARY: New epr features consistent with a novel type of Cu(II) are observed in partially reduced Type 2 copper depleted laccase molecules. Cu(II) hyperfine lines appear near 2590 G and 2770 G, and a rhombic $g_{\perp}$ feature is also observed. These reflect a Cu(II) emergent on reductive disruption of the binuclear Type 3 site in T2D laccase. Additionally, much of the new, magnetically isolated Cu(II) is retained on full reoxidation of partly reduced Type 2 copper depleted laccase. The proportion of disrupted Type 3 Cu(II) sites remaining after reoxidation appears to depend on the prior distribution of electrons within T2D laccase.

Rhus laccase is one of the most thoroughly studied members of the class of copper oxidases containing four prosthetic copper ions in three characteristic sites (1,2). These include a single "blue" Type 1 site copper, a single Type 2 site copper and the Type 3 site antiferromagnetically coupled (3,4) Cu(II) pair. It is at this latter site where the initial binding and reduction of dioxygen occur (1,2,5-8).

The preparation of a derivative of Rhus laccase depleted of Type 2 copper was reported some time ago (9,10) and the complexity of this material is attested to by the fact that even the resting state spectroscopy and redox status of the remaining copper ions has been a matter of some controversy (10-15).

We have been investigating the redox behaviour of T2D laccase, by means of anaerobic reductive (14,16) and re-oxidative (16) titrations. From the results, it was proposed (16) that the Type 3 site pairwise dicopper interaction is disrupted during reduction, and is restored on reoxidation by H_2O_2. Accompanying Type 3 site structural rearrangements were also inferred.

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A consequence of the proposed model is the prediction that a population of half-reduced disrupted Type 3 sites be formed during reduction (Figure 1, inset). Since the Type 3 site Cu(II) ions were found to be 35 mV more reducing than the Type 1 copper ion (16) any emergent paramagnetism should dominate the epr spectrum of highly, but incompletely, reduced T2D laccase. The results of experiments testing this prediction are reported below.

MATERIALS AND METHODS

Type 2 copper depleted Rhus laccase was prepared as described (16) and was found to contain 3.04 $\pm$ .02 copper ions/molecule by the biquinolyl method. T2D laccase concentrations were determined from $\varepsilon(615) = 5700\ M^{-1}\ cm^{-1}$ (14).

Anaerobic methods and reagents used for reductive and oxidative titrations were described previously (14,16).

Electronic spectra were taken, and reaction progress monitored at 615 nm, on a Cary 118 equipped with thermostatted cell holders. EPR spectra were obtained near liquid nitrogen temperature using a Varian E-3 X-band spectrometer.

Reduction and reoxidation reactions were carried out at 20 $\pm$ 1°C in 0.05 M sodium acetate pH 5.2 buffer, in a special anaerobic quartz vessel equipped with both an optical and an epr cell.

RESULTS AND DISCUSSION

The visible absorption spectrum of oxidized T2D laccase is shown in Figure 1-1, and the frozen solution epr spectrum in Figure 2a. On anaerobic addition of 2.3 equivalents of hydroquinone to a solution of T2D laccase the slow Type 1 site reduction described previously (16) was observed. The calculated distribution of reductant electrons within the Type 3 site (Fig. 1, inset) indicates that 29% of the Type 3 sites be half reduced, and constitute about 75% (16) of the paramagnetic Cu(II) present at equilibrium. In Figures 1-2 and 2b-b" are presented the visible electronic and frozen solution epr spectra respectively, of the partly reduced protein. The latter shows a new rhombic feature convolved with the Type 1 Cu(II) signal near 3240 G. Appearance of this signal on reduction reflects emergence of a previously undetectable Cu(II) ion; the Cu(II) of a reductively disrupted (vide infra) half reduced Type 3 copper pair.

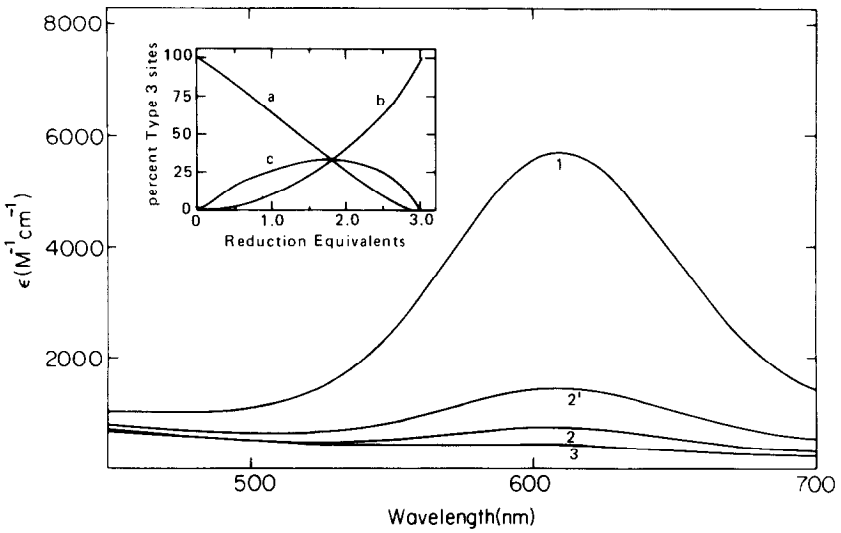

FIGURE 1. Visible spectrum in 0.05 M sodium acetate, pH 5.2 of : 1.T2D Rhus laccase; 2. As in 1, after 2.16 equivalents of hydroquinone; 2' As in 2 after freezing at about 100°K, then thawing; 3. As in 1, fully reduced with 4.3 equivalents of hydroquinone. Inset: Calculated equilibrium mole-fraction % of: a. fully oxidized; b. fully reduced; and c. half-reduced Type 3 sites within T2D laccase during reductive titration, based on the model given in ref. 16.

The partially reduced T2D laccase sample was found to have been significantly reoxidized upon freezing as indicated by the enhanced absorption intensity at 615 nm (Fig. 1-2'), observed upon thawing after acquisition of the epr spectrum. On standing, slow re-reduction of Type 1 Cu(II) commenced, tending to the prior position of equilibrium. T2D laccase displayed a rhombic epr spectrum (Fig. 2c) even after fully reduced (Fig. 1-3) indicating the formationon freezing of an isolated Cu(II) within the reduced Type 3 site. On thawing, absorption intensity at 615 nm was again found to have reached a level near that of Fig. 1-2'. In partly reduced T2D laccase, the observed reoxidation of Type 1 copper may possibly be ascribed to intramolecular electron redistribution induced by freezing. However, in the frozen solution of fully reduced protein, both Type 1 and Type 3 copper must clearly have been reoxidized by benzoquinone. The reoxidation of reduced native laccase on freezing in the presence of other redox agents has been reported previously (17,18). Interestingly, it was shown in the case of NO (18) that the reoxidation phenomenon was not a

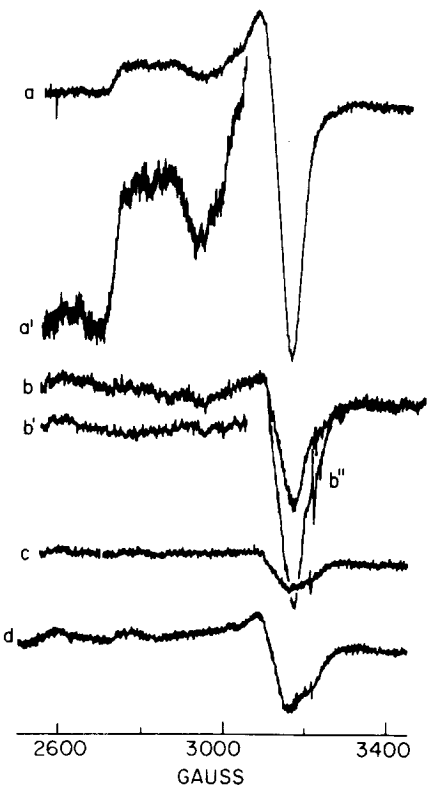

FIGURE 2. EPR spectrum of: a. T2D laccase, 0.15 mM in .05 M sodium acetate pH 5.2, power 25 mW; lower trace, gain x 5; b. As in 1, after 2.16 equivalents of hydroquinone added; b'. cavity scan. b''. Power 50 mW, gain x 4; c. As in 1 after addition of 4.3 equivalents of hydroquinone. d. T2D laccase partially reoxidized with 1 mole-equivalent of H_2O_2 after full reduction with 4.8 equivalents of hydroquinone. The sharp line at g=2 originates within the quartz EPR tube

function of freezing itself, but of lowered temperature. The suggested mechanism, involving electron transfer through Type 2 copper to a bound acceptor, is clearly untenable in the case of the T2D protein. However, the observed reoxidation of both Type 1 and Type 3 copper implies the possible utility of the latter in an alternative pathway. No obvious epr signal attributable to the presence of a trapped semiquinone radical (19) was observed.

After full reduction with 4.8 electron equivalents of hydroquinone T2D laccase was partially reoxidized on addition of 1 mole-equivalent of H_2O_2 (20). In the epr spectrum of the equilibrated product mixture (Fig. 2d), the Cu(II) hyperfine lines and assymetric lineshape near $g_\perp$ again indicate

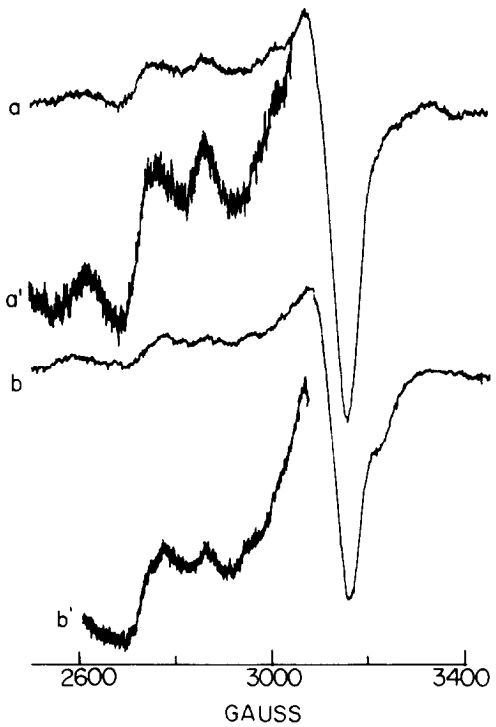

FIGURE 3. T2D laccase in .05M sodium acetate pH 5.2 after: a. Anaerobic reduction with 1.1 equivalents of hydroquinone followed by aerobic reoxidation. Lower trace, gain x 3. b. Anaerobic reduction with 2.2 equivalents of hydroquinone followed by either aerobic, or anaerobic $K_3Fe(CN)_6$, reoxidation. Lower trace gain x 3.

a disrupted Type 3 site in the partly reoxidized protein (20). Although the resolution of the experiment does not permit an unambiguous assignment, the epr spectrum in Fig. 2d does not appear to have the seven lines (21,22) expected for a delocalized electron within a mixed valence half-oxidized dicopper site.

Two separate T2D laccase samples were anaerobically reduced with 1.1 and 2.2 equivalents, respectively, of hydroquinone, then fully reoxidized by exposure to air (Fig. 3a,3b) or to excess anaerobic $K_3Fe(CN)_6$ (Fig. 3b). The prominent non-Type 1 Cu(II) features indicate that much of the Type 3 sites have remained in a disrupted state in the reoxidized T2D protein samples. The spectrum in Fig. 3a is essentially identical to that of the reported "half-met" Type 3 site derivative of T2D laccase(24,25). The electron distribution within the two T2D samples prior to reoxidation are

TABLE[a]

Total Electrons Added	Reduced Type 1[b], and			Oxidized Type 1, and			Total electrons in T2D laccase			
	T3(00)[c]	T3(01)[c]	T3(11)[c]	T3(00)[c]	T3(01)[c]	T3(11)[c]	$3e^-$	$2e^-$	$1e^-$	None
$1.1e^-$	35%	16%	7%	26%	11.5%	5.5%	7%	21.5%	43%	26%
$2.2e^-$	17%	27%	44%	2%	3.5%	6%	44%	33%	20.5%	2%

[a]The relative distribution of electrons within the manifold of T2D laccase reduction intermediates at the specified levels of reduction.

[b]Total % reduced Type 1 site after 1.1. electrons: 59%; after 2.2 electrons: 88%.

[c](00): oxidized; (01) half-reduced; and (11) fully reduced Type 3 sites. The value for percent Type 3 sites in each case was calculated from the reductive titration curve in (16) and the Type 3 site electron distribution plot in Figure 1, inset.

summarized in the Table. We have shown (16) that peroxide reoxidation of fully reduced T2D laccase restores the antiferromagnetism of the Type 3 Cu(II) site. Therefore the observed novel Cu(II) magnetism must derive from the respective manifolds of T2D laccase molecules in intermediate stages of reduction. The intensity of rhombic Cu(II) epr feature appears to follow the relative population of doubly reduced T2D laccase within which the Type 3 site is half-reduced.

However, a straightforward analysis is complicated since the distribution of electrons within partly reduced T2D laccase is in a dynamic equilibrium, and the exact mechanism of oxidation obscure. Nevertheless, it may be suggested that the status of the Type 3 site copper in reoxidized T2D laccase is dependent on the prior degree of reduction and the consequent intersite electron distribution within the protein molecule. That is, a half-reduced Type 3 site in redox equilibrium with an oxidized Type 1 site is probably structurally distinct from such a site in similar equilibrium with a reduced Type 1 site.

The similarity of the non-Type 1 Cu(II) epr features in the spectra of the partially reduced proteins in Fig. 2 with those of the reoxidized protein sample in Fig. 3b suggests a corresponding similarity in the ligand environment of the Cu(II) ions within the disrupted Type 3 sites regardless

of oxidation state. Preliminary experiments (23) have indicated that the rhombicity in the Fig. 3b spectrum can be titrated by N_3^-, and that an essentially axial spectrum is obtained with a 1000-fold excess of this anion. Therefore, at least one of the Cu(II) ligands in the disrupted Type 3 site is probably solvent derived water, occupying a coordination position uncovered through loss of a ligand.

The above results have confirmed the hypothesis advanced previously (16) that the pairwise interaction between the Type 3 site copper ions is disrupted during reduction in T2D laccase.Finally, half-reduction of the Type 3 site must be accompanied by a significant structural change, as required by the reductive disruption model. That is, if electron uptake were not accompanied by the physical separation of the previously antiferromagnetically exchange coupled Cu(II) pair, reoxidation would have undoubtedly restored this interaction. It may be that Type 3 site disruption involves loss of the dicopper bridging ligand.

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REFERENCES

1. Malmstrom, B.G. (1982) Ann. Rev. Biochem. 51, 21-60.
2. See monographs in "Copper Proteins" T.G. Spiro, ed. (1981) Wiley-Interscience and references therein.
3. Moss, T.H., and Vanngard, T. (1974) Biochim. Biophys. Acta 371, 39-43.
4. Dooley, D.M., Scott, R.A., Ellinghaus, J., Solomon, E.I., and Gray, H.B. (1978) Proc. Natl. Acad. Sci. USA 75, 3019-3022.
5. Goldberg, M., Farver, O., and Pecht, I. (1980) J. Biol. Chem. 255, 7353-7361.
6. Farver, O., Goldberg, M., and Pecht, I. (1980) Eur. J. Biochem. 104, 71-77.
7. Andreasson, L.-E., Branden, R., and Reinhammar, B. (1976) Biochim. Biophys. Acta 433, 370-379.
8. Aasa, R., Branden, R., Deinum, R., Malmstrom, B.G., Reinhammar, B., and Vanngard, T. (1976) Biochem. Biophys. Res. Commun. 70, 1204-1209.

9. Graziani, M.T., Morpurgo, L., Rotilio, G., and Mondovi, B. (19765) FEBS Lett. 70, 87-90.
10. Morpurgo, L., Desideri, A., Rotilio, G., and Mondovi, B. (1980) Biochem. J. 187, 361-366.
11. Morpurgo, L., Desideri, A., Rotilio, G., and Mondovi, B. (1980) FEBS Lett. 113, 153-156.
12. Reinhammar, B., and Oda, Y. (1979) J. Inorg. Biochem. 11, 115-127.
13. LuBien, C.D., Winkler, M.E., Thamann, T.J., Scott, R.A., Co, M.S., Hodgson, K.O., and Solomon, E.I. (1981) J. Am. Chem. Soc. 103, 7014-7016.
14. Frank, P., Farver, O., and Pecht, I. (1983) submitted for publication.
15. Farver, O., Frank, P., and Pecht, I. (1982) Biochem. Biophys. Res. Commun. 108, 273-278.
16. Frank, P., and Pecht, I. (1983) Manuscript in preparation.
17. Morpurgo, L., Graziani, M.T., Desideri, A., and Rotilio, G. (1980) Biochem. J. 187, 367-370. From these authors' data a solution potential of 396 mV can be estimated for the laccase sample showing reoxidation on freezing. In comparison, from the known hydroquinone-benzoquinone couple, a solution potential of 388 mV can be calculated for the fully reduced T2D laccase sample, prior to freezing.
18. Martin, C.T., Morse, R.H., Kanne, R.M., Gray, H.B., Malmstrom, B.G., and Chan, S.I. (1981) Biochemistry 20, 5147-
19. Pethig, R., Gascoyne, P.R., McGlaughlin, J.A., and Szent-Gyorgyi, A. (1983) Proc. Natl. Acad. Sci. USA 80, 129-132.
20. The hydroquinone was present in 61% equivalent excess, therefore the partly reoxidized T2D laccase molecules still contained 2.8 electrons on the average. From the final A(615), the Type 1 site was 9% reoxidized by this procedure. The corresponding presence of up to 11% half-oxidized Type 3 copper sites, would contribute up to 55% of the epr intensity. The presence of a significant population of fully oxidized Type 3 sites, although not strictly ruled out, is not likely at so high a level of reduction.
21. Sigwart, C., Hemmerich, P., and Spence, J.T. (1968) Inorg. Chem. 7, 2545-2548.
22. Gagne, R.R., Koval, C.A., Smith, T.J. and Cimolino, M.C. (1979) J. Amer. Chem. Soc. 101, 4571-4580.
23. Frank, P. and Pecht, I. Unpublished results of spectroscopic titrations with N_3^- indicate a complex binding pattern with up to three distinct modes or sites. The fully azide complexed protein shows nitrogen superhyperfine lines imposed on the copper hyperfine portion of the now axial epr spectrum.
24. Spira, D.J., Winkler, M.E., and Solomon, E.I. (1982) Biochem. Biophys. Res. Commun. 107, 721-726.
25. The reported treatment with aerobic iron hexacyanide would have reoxidized this T2D protein sample. We have observed very efficiently mediated reoxidation with .05 electron equivalents of $K_3Fe(CN)_5$.