on the leucocyte membrane and about its modifications concerned with the metabolic concomitants.

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The redox potential of fungal laccase

Fungal laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) has been found to contain four copper atoms per molecule[1]. Two of these are in a diamagnetic state, presumably Cu^{+}, while the other two exist as Cu^{2+} in the resting enzyme[2,3] and are reduced by substrate during catalysis[4]. However, only one cupric ion has the unique bonding properties which account for the high absorbance at 610 mμ and the narrow hyperfine structure splitting in the EPR spectrum[5]. We wish to report here that this cupric ion is further distinguished by having an exceptionally high redox potential.

In a previous communication it was shown that the enzyme undergoes a reversible loss of blue color at pH values greater than 6 (see ref. 6). EPR experiments confirmed that the reduction of one Cu^{2+} occurred concomitantly with loss of absorbance at 610 mμ. Such a pH-dependent reaction could be due to a reducing group on the enzyme or to reduction by solvent. Failure to detect sulfhydryl groups on the enzyme led us to a more serious consideration of the latter possibility. Assuming that one copper of laccase is coupled to the half-reaction

$$O_2 + 4H^+ + 4e^- = 2H_2O \qquad E_0 = 1.23\ \text{V (see ref. 7)}$$

and using the pK of 7.4 determined previously[6] for the pH-dependent reduction, it can be computed that the redox potential must be about 0.78 V.

The half-reaction

$$Mo(CN)_8^{3-} + e^- = Mo(CN)_8^{4-}$$

is known to have a redox potential[8] in the range of that calculated on the basis of the above assumption. A further indication that the molybdenum octacyanide complexes could be used as a redox buffer was the similarity of their solution chemistry and that of the hexacyanoferrate substances[9–11].

Potassium octacyanomolybdate(IV) dihydrate was prepared by a standard procedure[12]. Potassium octacyanomolybdate(V) was prepared according to the method of KOLTHOFF AND TOMSICEK[8]. The purity of these substances was determined by potentiometric titration with standard solutions of potassium permanganate and potassium hexacyanoferrate(II). Laccase A was prepared by the method of FÅHRAEUS AND REINHAMMAR[13], and ceruloplasmin was obtained from AB Kabi, Stockholm. Deionized water and reagent grade chemicals were used throughout.

The redox potential of the octacyanomolybdate(IV)–(V) system was determined by a potentiometric method to be 0.778 V at I 0.2 which is in good agreement with previous results[8]. Since the cyanide complexes of most metals are known to be sensitive to light[10] all experiments were carried out in a darkened room or in vessels wrapped with aluminum foil.

Experiments designed to determine the redox potential of laccase were carried out as follows: Laccase solution (2 ml), which had been dialyzed against phosphate buffer (pH 6.2, I 0.2) and filtered through a 0.2-μ Millipore filter, was placed in the cuvette portion of a modified Thunberg tube. A solution (1 ml) containing known proportions of octacyanomolybdate(IV) and octacyanomolybdate(V) ions was added to the upper compartment of the vessel. The tube was weighed, evacuated to remove oxygen, and weighed again to determine water loss. After equilibration at 25° the contents of the two compartments were mixed and the absorbance recorded at short intervals for several minutes. The pH was determined at the end of each experiment. The final concentration of laccase was 10^{-4} M and the initial concentration of the molybdenum cyanide solutions (known to $\pm$ 2–3 %) was $2 \cdot 10^{-2}$ M.

Upon mixing the enzyme solution with that of the molybdenum cyanide complexes there is a very rapid drop in the intensity of the blue color, the extent of which is determined by the proportion of low valent form present, followed by a slow, nearly linear decrease. An example of the time dependency is given in Fig. 1. This secondary process is undoubtedly caused by a small amount of cyanide released from the complexes[9,10] which is known to decrease the blue color of the enzyme[5]. The precise reversibility is therefore lost due to this protracted side reaction. Operationally, the absorbance value obtained by extrapolation to zero time, corrected for water loss, was taken to be that of equilibrium. The maximum attainable absorbance at 610 mμ was determined by mixing 1 ml of $2 \cdot 10^{-2}$ M octacyanomolybdate (V) solution with 2 ml of laccase solution as described above and recording the absorbance. The minimum value was taken as that found at pH 9 in the presence of oxygen. Neither oxidation state of the molybdenum complexes absorbs light of 610 mμ wavelength.

The results are presented in Fig. 2 in the form of a Nernst plot. The solid line was obtained by the method of least squares and has a slope of 0.8. The dashed line, drawn through the experimental intercept, is theoretical with a slope of unity. The deviation from theory is not great and most certainly reflects the complicated

chemistry of the system. Using the value of 0.778 V determined for the octacyanomolybdate system and the log of the ratio of Mo(IV) to Mo(V) at 50% reduction taken from Fig. 2 the redox potential is

$$E_0 = 0.778 - 0.059\,(0.185) = 0.767\ \text{V}$$

It is difficult to assess the error associated with this figure as there may be some systematic contributions. However, the agreement between the value calculated assuming oxidation of water and that determined experimentally shows that a reduction of the specifically bound copper by water at higher pH values is thermodynamically feasible. Kinetic considerations aside, a cupric complex possessing a redox potential of 0.767 V would be half reduced to the cuprous complex at pH 7.6. This is in good agreement with the observed pH of half-reduction for laccase of 7.4 (see ref. 6).

In similar experiments with human ceruloplasmin it was found that the molybdenum cyanide complexes do not serve as a redox buffer since the low valent form is not oxidized to a sufficient extent. However, ceruloplasmin reduced by hexacyanoferrate(II) ion was readily reoxidized by octacyanomolybdate(V) ion indicating a lower redox potential than that found for laccase. The experimental finding of a 12% reduction in the blue color of a solution initially $4.6 \cdot 10^{-5}$ M in ceruloplasmin and $6.67 \cdot 10^{-3}$ M in octacyanomolybdate(IV) allows one to calculate (assuming the transfer of one electron) that the potential of ceruloplasmin is in the range of 0.5–0.6 V. A more precise value cannot be obtained from this type of experiment because the low but unknown concentration of oxygen enters the calculation. It is clear, however, that the redox potential is some 0.15–0.25 V lower than that of laccase.

Two fundamental questions which are raised by these findings are: What factors contribute to these unusually high redox potentials for the $Cu^{2+} + e^- = Cu^+$

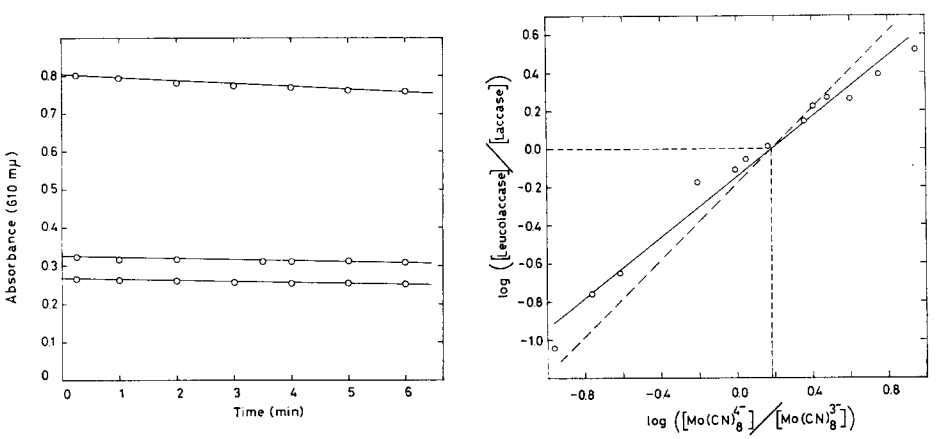

Fig. 1. Time dependence of absorbance at 610 mμ after mixing laccase with solutions containing various ratios of octacyanomolybdate (IV) to (V) ions under anaerobic conditions, showing the slow secondary reaction. The top curve was obtained with only octacyanomolybdate(V) present and the value estimated by extrapolation represents the initial absorbance in all cases. The ratio, (IV)/(V), was 3.00 and 5.67 for the center and bottom tracings, respectively.

Fig. 2. Plot of log [reduced laccase]/[oxidized laccase] against log [octacyanomolybdate(IV)]/[octacyanomolybdate(V)]. The experimental procedures are described in the text. The ratio of total octacyanomolybdate concentration to that of laccase varied from 33 to 41. The ionic strength was 0.2 and the pH was 6.25 ± 0.05.

half-reaction; and how is it possible for two cupric complexes having almost identical optical and magnetic properties[5,14] to have markedly different oxidation potentials? BRILL, MARTIN AND WILLIAMS[15] have discussed a number of factors which can lead to high potentials for the cupric–cuprous couple. Those which seem to be of importance here are binding of the copper to sulfur or other unsaturated ligands and a steric environment in the high valent form which is other than a tetragonally distorted octahedron.

The finding of unusually high redox potentials for these copper proteins is entirely consistent with recent results from this laboratory on laccase[5] and previous findings of BLUMBERG[16] on ceruloplasmin indicating that the cupric ions are in a state of lower than square planar symmetry. No direct experimental evidence for binding to sulfur has so far been obtained.

It is possible that secondary processes, such as conformational changes of the protein, may account for the differences in the redox potentials[16] found among the copper proteins characterized by an intense blue color and narrow hyperfine structure splitting in their EPR spectra.

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