become coupled after addition of NO or O_2 to the exposed iron, but prior to formal oxidation of the second iron.

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PS1.3 — TH

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CHARACTERIZATION OF A NOVEL COPPER ENZYME: BACTERIAL NITROUS OXIDE REDUCTASE

The respiratory capability of bacteria is highly diverse considering the terminal oxidants being used other than dioxygen. About 25 genera of taxonomically different groups of bacteria respire nitrate to N_2, NO or N_2O. Three or four terminal oxido-reductases accomplish this respiratory redox process. When dinitrogen is the final product of nitrate respiration, it is formed from N_2O, involving catalysis by a novel Cu protein. The enzyme from denitrifying *Pseudomonas perfectomarina* (ATCC 14405) has been isolated and purified to homogeneity [1]. Nitrous oxide reductase contains about eight copper atoms per molecular weight 120 000. The protein is a dimer of two presumably identical subunits. Several spectroscopically distinct forms of the enzyme have been identified. A "pink" form of the enzyme is obtained when the purification is done aerobically. The specific activity of this species is 15-35 nkat per mg protein as measured by the nitrous oxide-dependent oxidation of photochemically reduced benzyl viologen. The spectrum of the pink form has absorption maxima at 480, 530, 620 and 780 nm (Fig. 1a).

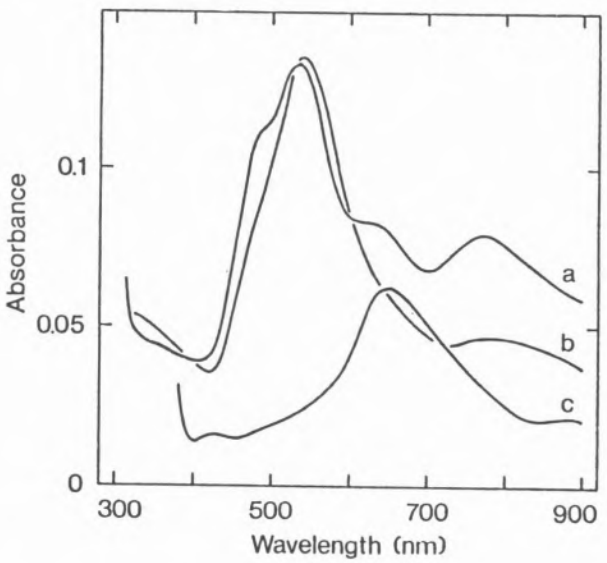

Fig. 1
Electronic spectra of (a) pink form of enzyme as isolated; (b) purple form as isolated; (c) dithionite-reduced form

Cells that were broken anaerobically and fractionated excluding oxygen from all chromatographic steps, yielded a "purple" form of the copper protein whose catalytic activity was consistently three to fivefold higher than that of the pink form. The spectrum of the purple form is shown in Fig. 1b. This form, as isolated, had absorption maxima at 540 and 780 nm. Maxima at 480 and 620 nm observed in the pink form were found only as slight shoulders.

EPR spectra of the pink and purple forms of the enzyme were similar, with the purple form providing better resolution. A representative spectrum of the oxidized purple protein is shown in Fig. 2a. The data suggest the presence of an unusual type 1 Cu. The type 1 Cu is unusual in the sense that although there is a relatively narrow hyperfine splitting ($A_{\parallel} = 35.33$ Gauss; $g_{\parallel} = 2.215$, $g_{\perp} = 2.033$) it does not appear to be associated

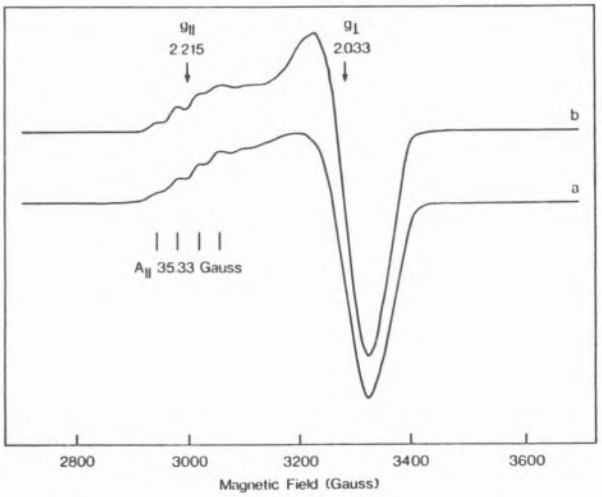

Fig. 2

EPR spectra of (a) purple form of enzyme as isolated in 50 mM phosphate, pH 7.1; (b) pink form after 28 hrs dialysis against 10 mM EDTA, Tris buffer, pH 7.5 (EDTA was removed by further dialysis)

with the absorption band at 620 nm. The 620 nm absorption is ruled out as the LMCT band from S → Cu because it is not bleached upon the addition of reducing agents.

Little change was observed in the absorption spectrum or copper content of the pink enzyme form that had been extensively dialyzed against EDTA. The EPR spectrum of this material, which is enzymatically inactive, is shown in Fig. 2b. Dialysis of the protein against KCN removed 80-90% of the copper and inactivated the enzyme. The addition of $Cu(en)_2SO_4$ to the cyanide-treated enzyme resulted in the partial regeneration of the electronic spectrum of the pink form. The regenerated form of the enzyme, however, remained enzymatically inactive.

A "blue" form of the enzyme was obtained by reduction of the purple or pink form by dithionite or ascorbate. The spectrum of the reduced form had a single absorption maximum at 640 nm and is shown in Fig. 1c. The pink form as isolated accepted eight electrons in an anaerobic titration with dithionite. Similar results were obtained when the absorbance decreases at 530 nm, 470 nm and 780 nm were monitored. Upon reduction with dithionite, the type 1 Cu EPR signal disappeared. A rather broad signal with no hyperfine structure ($g_{av} \approx 2.068$) remained even in the presence of excess reductant.

Attempts to remove part of the copper from the purple form of the enzyme by procedures which deplete type 2 Cu [2,3], produced a reduced species that showed no loss in copper content. The reoxidation of this species yielded the pink form of the enzyme.

The pink and purple forms of the enzyme can be activated by base. The activity increased about ten-fold from pH 6 to 10, to give a maximal specific activity around 330 nkat per mg protein. Enzyme inhibitors were cyanide, azide, EDTA, acetylene and fluoride. In addition to measuring activity by the nitrous oxide dependent oxidation of benzyl viologen, a direct measurement of nitrous oxide reduction and dinitrogen evolution was done by gas chromatography. Under these conditions the enzyme became inactivated or inhibited after a few turnovers. Further characterization of the copper chromophore should provide a better understanding of the structure and reactivity of copper sites in enzymes.

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PS1.4 — MO

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ANION BINDING SITES OF REDUCED BOVINE COPPER-ZINC SUPEROXIDE DISMUTASE: A Cl-35 AND HIGH-RESOLUTION H-1 NMR STUDY

Bovine erythrocyte Cu, Zn superoxide dismutase (Cu, Zn-SOD) in its oxidized form has been shown by X-ray crystallography [1] to be a dimer of two equivalent subunits, with one copper(II) and one zinc(II) ion per subunit. This protein is an extremely efficient catalyst of superoxide dismutation ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$) and it has been proposed that this activity is its primary physiological function *in vivo* [2]. Although the site of reactivity for superoxide and several anions with oxidized Cu, Zn-SOD has been established as being the copper(II) ion, the anion binding sites of reduced protein are still a matter of controversy [3]. This is an important question since the substrate, O_2^-, is also an anion and is known to react with the enzyme in both oxidized and reduced forms [2].

FEE and WARD [4] observed that the nuclear magnetic relaxivities of solutions of Cl^- was larger in the presence of reduced Cu, Zn-SOD than in the presence of apoprotein and that addition of CN^- lowered the relaxivity of reduced protein to that of the apoprotein. The binding of halides to reduced yeast Cu, Zn-SOD was also studied by high-field H-1 NMR [5]. Five resonances were assigned to C-2 protons of histidyl imidazoles at the active site of the reduced protein and three of these were observed to shift upon addition of halide ions. Although both of these observations are in agreement with coordination of halide ions to the copper(I) ion in reduced Cu, Zn-SOD [4,5], they are also consistent with binding of halide ions to a protein side chain close to the metal binding region.

We reanalyzed these data in light of the present understanding of electrostatic interactions in the reaction mechanism of bovine Cu, Zn-SOD. It has been shown from the SOD activity and anion affinity of proteins chemically modified at Arg-141 with phenylglyoxal [6,7] or at Lys-120 and Lys-134 with succinic anhydride, acetic anhydride or cyanate [8-11] that these residues are important anion binding sites in the oxidized protein [12].

We observed that reduced arginine-modified and reduced lysine-modified SOD (2.1×10^{-4} M) caused less broadening of the Cl^- resonance than did reduced native protein when measured under the same conditions. We also found that the C-2 proton resonances of histidyl imidazoles of reduced native SOD and reduced lysine-modified SOD were shifted upon addition of Cl^- whereas this anion had no effect in the H-1 NMR spectrum of reduced arginine-modified SOD (even at Cl^- concentrations as high as 1M). We therefore conclude that the major anion binding sites in reduced bovine Cu, Zn-SOD are Arg-141, Lys-120, and Lys-134 and not the copper(I) ion. Implications of these findings for the mechanism of superoxide dismutation of this protein will also be discussed.

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