AN EPR SIGNAL FROM THE "INVISIBLE" COPPER OF CYTOCHROME OXIDASE

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SUMMARY: Sulfide is both an inhibitor and a slow reductant of oxidized cytochrome c oxidase. When the enzyme is exposed to sulfide for short times (one minute or less) and frozen, the resultant electron paramagnetic resonance (EPR) signals show clearly: low spin heme a, low spin heme a_3, the usual "EPR detectable" Cu^{2+} signal ($g_{\parallel} = 2.17$, $g_{\perp} = 2.03$), and a new Cu^{2+} signal superimposed on the same region, with ($g_{\parallel} \sim 2.19$, $g_{\perp} = 2.05$). This new signal presumably arises because the antiferromagnetic coupling postulated to exist between the iron atom of heme a_3 and this copper is disrupted when heme a_3 is driven to a low spin state by sulfide. The implications of this result with respect to models of the O_2-binding site and redox geometry of oxidase are briefly discussed.

Several studies (1,2,3) have confirmed the unusual behavior of sulfide as a ligand for cytochrome oxidase. It binds strongly to both oxidized and reduced forms of the enzyme (1), and specifically binds to oxidase several hundred times more strongly than to most other heme proteins (4). Additionally, it acts as a slow reducing agent for the enzyme; at sufficiently long incubation times all redox centers except the heme a_3-SH compound can be seen to be reduced (1,2). The "on" constant for binding is sufficiently fast compared to the rate of reduction that the enzyme can be prepared in a state in which all redox centers of the enzyme are oxidized and detectable by EPR. That is, the presence of a low-spin heme a_3 signal in the spectrum, indicating that the a_3-Cu antiferromagnetic coupling has been abolished, and the simultaneous presence in the spectrum of those components which are reduced rapidly in reductive titrations (5,6), imply that the usually undetectable copper atom of the antiferromagnetically coupled pair should, under these conditions, contribute to the spectrum. This effect is observed here experimentally. (The effect explains a broadening of the Cu region in the EPR spectra of Wever et al., (2), but these investigators concerned

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themselves with the heme binding properties of a series of ligands and did not explore the implications for antiferromagnetic coupling).

MATERIALS AND METHODS

The method of Sun et al. (7) for purification of cytochrome oxidase dispersed in Triton X-100 was used, with the addition of several hours dialysis against EDTA at 4°C to remove "adventitious" copper. The absence of such copper was checked by a series of studies at different temperatures and microwave power settings (8). A cytochrome oxidase preparation using sodium deoxy cholate according to the method of Yonetani (9) gave virtually identical results spectroscopically, and the Triton results were chosen for presentation only because of the greater purity of the preparation when examined by gradient-gel electrophoresis in the presence of SDS and 6 M urea (10). Detergents and other chemicals were purchased from Sigma Chemical Company and used as received. Heavy layer beef heart mitochondria used in the preparation were the kind gift of Dr. Y. Hatefi of Scripps Clinic and Research Foundation of La Jolla, California.

The enzyme was stored under liquid nitrogen and sulfide titration with fresh sulfide solution was carried out rapidly upon thawing of the stored enzyme. The variable rates of reduction of the enzyme reported occasionally (1,11) correlate with the loss of specific activity noted if the enzyme is kept on ice for several hours.

EPR spectra were recorded on a Varian E-12 spectrometer equipped with an Air Products liquid helium cryogenic system, under the conditions noted in the figure ligands.

RESULTS AND DISCUSSION

Figure 1 shows a comparison of the EPR spectrum of resting cytochrome oxidase and the EPR spectrum of cytochrome oxidase treated with sulfide at 4°C for 40 seconds. The spectrum is scaled to present the copper region of the spectrum more prominently, rather than the heme region which is usually emphasized. A large new copper signal has appeared which is displaced from the normal resting Cu^{2+} signal (Cu_A in the notation of Palmer et al. (12)). Figure 2 displays the resting oxidase spectrum against that of enzyme treated with sulfide at 4°C for 10 min. Extensive reduction of the enzyme has taken place. The kinetics of reduction are temperature and pH dependent. (The copper signal can be removed more rapidly by incubation at room temperature, as in ref. 2.).

The new signal has a clearly discernible $g_\perp = 2.05$ and a less clear $g_{\parallel} \sim 2.19$, partially obscured by the low spin heme a_3 signal at $g_y = 2.22$.

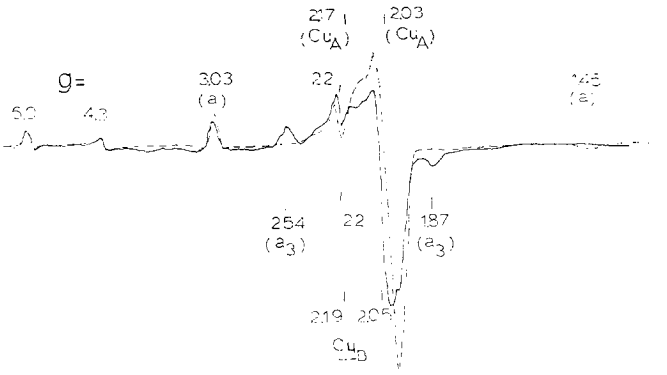

Fig. 1. EPR spectra of cytochrome oxidase at 5°K. Microwave power = 10 mW, modulation amplitude = 8.2 G, Microwave frequency = 9.307 GHz. Dashed line: Native enzyme, Triton X-100 suspension, 0.1 M phosphate buffer, pH 7.0. Solid line: enzyme in same detergent and buffer in presence of 0.01 M Na_2S in solution for 40 seconds. Cu_A and Cu_B as defined in text. Signals at g = 6.0 and 4.3 are signals typically present in small amounts in oxidase preparations (2, 5, 8, 16).

The signal is notable, as is the Cu_A signal, for lack of resolved copper hyperfine splitting $A_{//}$, and for its breadth at the low temperature of 5°K. The lack of hyperfine splitting may be attributed, as for Cu_A, to extensive electron delocalization (13,14), and the linewidth to proximity to the paramagnetic center heme a_3. Assuming that sulfide induces no gross conformational changes, the Cu_B (i.e. previously undetected (12)) is within $\sim$5 Å of the Fe^{3+} of heme a_3, demanding extensive electron-electron dipolar broadening (this broadening should be orientation-dependent, a point under investigation in this laboratory). Consistent evidence for the proximity of the two metals is the breadth of the low spin heme a_3-SH signal compared to that of other heme-thiol derivatives (15). The g values for Cu_B obtained in this study are somewhat abnormal compared to those for copper proteins in

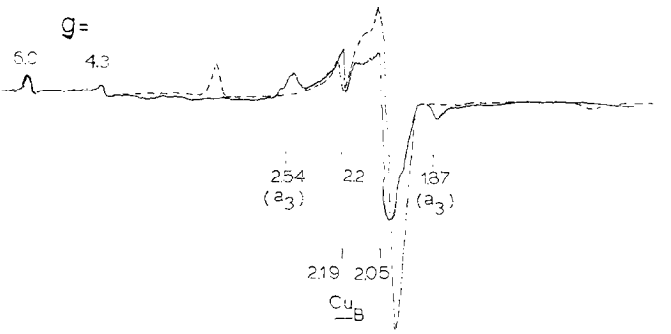

Fig. 2. Conditions as in Fig. 1 except that sulfide exposure allowed for 10 minutes.

general (13), having a $g_{//}$ similar only to oxidase Cu_A, but a $(g_{\perp} - 2)/(g_{//} - 2)$ ratio which is at least similar to that of copper in other oxidases. A study of the linewidth of the Cu_B signal at very low temperatures ($\sim 1.5^{\circ}K$) for improved resolution is under way.

A point of great interest with respect to the geometry of O_2 reduction by oxidase is the contrast of sulfide binding studies with those employing cyanide. Cyanide bound to the isolated oxidized enzyme produces no new low spin heme a_3 signals (16), suggesting that the a_3-Cu_B antiferromagnetic coupling is intact. Sulfide, for which the enzyme has as high an affinity as for cyanide, obviously eliminates this coupling. Since both cyanide and sulfide can be shown to drive heme a_3 exclusively to a low-spin state, it is difficult to reconcile the recurring suggestion (12,17) of a histidine ligand-bridge between the heme a_3 iron and Cu_B with the EPR data. That is, in this model of the inhibitor and O_2 binding site it is most unlikely that the a_3-CN compound bridged through histidine to Cu_B^{2+} would be coupled while the a_3-SH compound in the same configuration would not. A more plausible explanation, derived from CO binding studies (18,19), is that the a_3 iron and Cu_B are close enough to form an effective two-metal-center binding site for O_2 and inhibitors. In this case the π orbitals of a cyanide at the O_2 site would

mediate the antiferromagnetic coupling, whereas sulfide, while also driving the heme a_3 iron to a low-spin state, would provide no such pathway for electron-spin coupling. Thus the EPR data presented here may be taken as supporting the idea of an initial two-electron reduction of oxidase-bound O_2 (20,21).

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