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SPIN TRAP EVIDENCE FOR PRODUCTION OF SUPEROXIDE RADICAL ANIONS BY PURIFIED NADPH-CYTOCHROME P-450 REDUCTASE

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SUMMARY Previous evidence for superoxide radicals as initial reduction products of oxygen by NADPH cytochrome P-450 reductase has been indirect. In this paper a technique is described to spin trap radicals produced in incubations of oxygen and reductase. Reference spin trap adducts were synthesized by adding phenyl-t-butyl nitrone (PBN) to superoxide radicals (PBN-OOH) or to hydroxyl radicals (PBN-OH). Both PBN adducts are stable in water or ethyl acetate for hours. Electron Paramagnetic Resonance (EPR) spectra measured in N_2-saturated ethyl acetate allow clear resolution of the hyperfine extrema of PBN-OH and PBN-OOH (2.1 and 4.5 G splitting, respectively). Comparison of EPR spectra from reductase and oxygen incubations with those of synthetic PBN-OOH suggest that superoxide radicals are the major primary reduction product of oxygen.

INTRODUCTION. NADPH cytochrome P-450 reductase acts as an electron donor for the various forms of cytochrome P-450. In a membrane the phospholipid matrix is of importance for protein-protein interactions and may affect how efficiently electrons are transferred to cytochrome P-450(1). If the coupling is not optimal, reductase may reduce other electron acceptors such as molecular oxygen. Reduction of oxygen to superoxide by reduced reductase and the subsequent production of H_2O_2 and hydroxyl radicals is important as a side reaction in microsomes (2) and reconstituted systems (3). The high reactivity of these radicals makes it worthwhile to study their site of production, the conditions which increase their production, and their relative effect on cell toxicity. It has been postulated (4) that the observed production of hydroxyl radicals in liver microsomes during NADPH oxidation is entirely due to the dissociation of the cytochrome P-450-oxycomplex with intermediary superoxide radicals. However, it is possible that the production of superoxide by reductase alone is significant. In fact, an increased production of H_2O_2 from microsomes in the presence of substrates which are difficult to metabolize was

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reported by Hildebrandt et al. (1,2). This may occur because when electron transfer to cytochrome P-450 is blocked by the difficult "substrate," reductase reduces oxygen to produce superoxide radicals which then react with water to form H_2O_2 and subsequently decomposes to hydroxyl radicals. Although it is generally agreed that superoxide is the first reduction product of molecular oxygen by reductase, so far only hydrogen peroxide and hydroxyl radicals have been conclusively demonstrated (4-12).

Superoxide radicals have not been detected previously by direct spin trapping in reductase-cytochrome P-450 systems(13). In previous experiments, measurements of spectra were often carried out in oxygen-containing aqueous buffer. This results in a large increase in the EPR linewidth that make interpretaton of hyperfine splitting difficult. In this paper this problem is overcome by measuring spectra of the spin trap adduct in N_2-saturated ethyl acetate.

MATERIALS AND METHODS. NADPH cytochrome P-450 reductase was purified from microsomes from phenobarbital-pretreated rabbits by DEAE-cellulose and affinity chromatography (14) to a specific activity towards cytochrome c of 40 μmole/mg per min measured at 30° in 0.3 M potassium phosphate buffer pH 7.5 with 20% glycerol.

The superoxide adduct of phenyl-t-butyl nitrone (PBN-OOH) was prepared by adding solid KO_2 to an aqueous solution of PBN. If distilled water was used, the PBN-OOH adduct could only be obtained in solutions saturated with PBN at 50-70° and less than 10 mg KO_2/ml. However, a high yield of essentially pure PBN-OOH was produced within seconds in 0.14 M PBN containing 0.3 M potassium phosphate buffer pH 7.5 with less than 20 mg KO_2/ml at 20°. The products were immediately extracted into ethyl acetate to prevent hydrolysis of PBN-OOH to the PBN-hydroxyl radical adduct (PBN-OH) in the alkaline solutions resulting from reaction of KO_2 with water. With this technique it was not necessary to use tetramethylammonium superoxide as an intermediate (15). The presence of potassium phosphate, glycerol, and sodium cholate in the solutions had no effect on the splitting of the trapped radical. Identical reference spectra for PBN-OH could be obtained by several different reactions: decomposition of H_2O_2 in aqueous solutions containing 20 μM Fe EDTA; reduction of O_2-saturated aqueous solutions by sodium dithionite or NADPH; hydrolysis of superoxide before spin trapping by using spin trap concentrations below 10 mM ; or by alkaline hydrolysis of PBN-OOH.

In order to trap superoxide radicals produced by NADPH cytochrome P-450 reductase, 30 μM reductase was incubated in air-saturated 0.3 M potassium phosphate buffer pH 7.5 containing 20% glycerol, 0.15 M PBN, and 2 mM NADPH at 20°. An EPR spectrum identical to that of synthetic PBN-OOH could be measured in this aqueous solution after a reaction time of less than one minute.

EPR measurements were carried out on a Varian E-104A spectrometer at 22°. All spectra of ethyl acetate solutions were measured under N_2 to prevent O_2-broadening using a modulation amplitude of 1 G and 10 mW of microwave power. The EPR scan was calibrated to 0.2 G with tetracene.

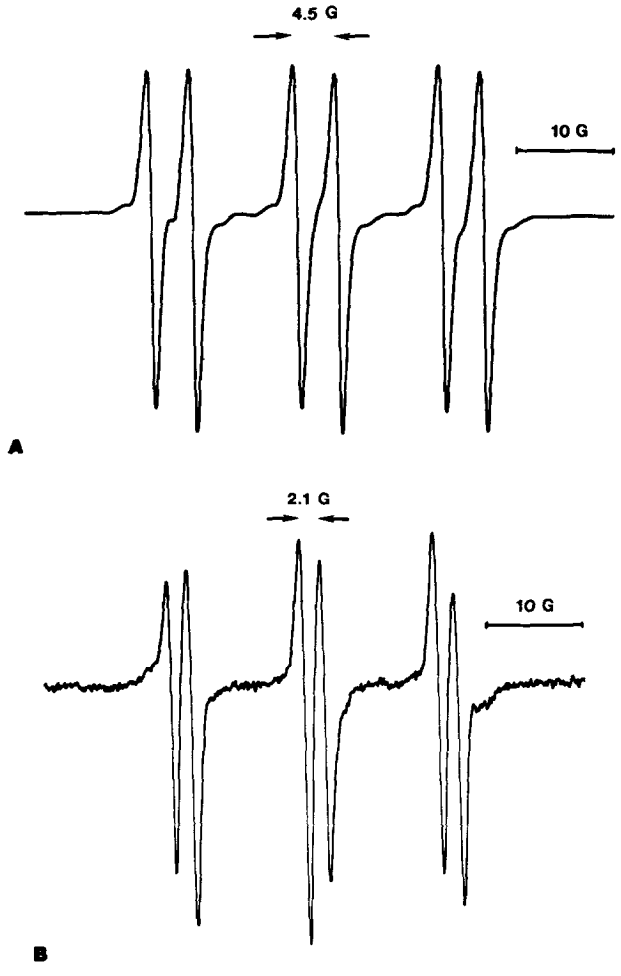

Figure 1: EPR-spectra of chemically-prepared PBN adducts after extraction and measurement in ethyl acetate under N_2. (A) PBN-OOH produced from KO_2. (B) PBN-OH prepared from 5% H_2O_2 and 20 µM Fe EDTA.

RESULTS AND DISCUSSION. Use of ethyl acetate had several advantages: The synthetic as well as the enzymatic reactions could be stopped by extraction of the spin trapped radicals. Although the nitroxide group of the PBN-adducts was reduced by reductase during the spin trapping process, it could be re-oxidized in air-saturated ethyl acetate. The sample was then concentrated and deoxygenated at low temperature by blowing N_2 over the solution. Spectra measured in ethyl acetate provided good resolution between PBN-OH and PBN-OOH, the doublets have 2.1 and 4.5 G splittings,

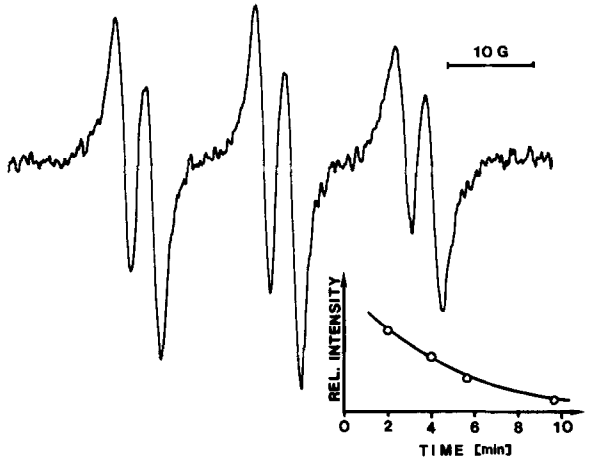

Figure 2: EPR-spectrum of PBN-radical adducts produced by 30 μM reductase in 0.3 M potassium phosphate buffer pH 7.5 with 20% glycerol, 0.1% sodium cholate, 2 mM NADPH, and 0.15 M PBN at saturation with air. NADPH was added and the spectrum recorded in 2 min. The insert shows the rate of reduction of the nitroxide group of PBN-OOH by the reductase which results in the disappearance of the spectrum.

respectively. Trapped radicals were stable for hours and their spectra had a high signal to noise ratio.

In Figure 1A a symmetric six-line spectrum with well-resolved hyperfine extrema is shown of synthetic PBN-OOH after extraction in N_2-saturated ethyl acetate. However, when the stringent conditions of temperature and concentration of components for spin trapping of PBN-OOH described in Methods were not adhered to, a nine-line spectrum which was a superposition of the PBN-OOH spectrum (Figure 1A) and the PBN-OH spectrum (Figure 1B) was obtained.

To determine if superoxide radical is a primary reduction product of oxygen by NADPH cytochrome P-450 reductase, we added NADPH to the air-saturated PBN-and-reductase-containing buffer system described in Methods. The aqueous suspension was immediately placed in the EPR cavity and a spectrum measured within one minute. This initial EPR spectrum produced in a phosphate buffer solution by NADPH cytochrome P-450 reductase was identical to that of synthetic PBN-OOH in buffer but the signal intensity decreased in minutes. The insert in Figure 2 shows the rate of loss of signal intensity. This loss

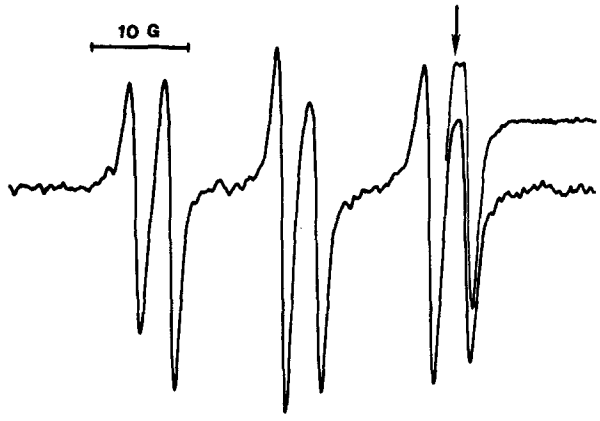

Figure 3: EPR-spectrum of PBN-OOH adducts produced in 15 min by 30 μM reductase (see legend Fig. 2) under O_2 saturation. Trapped radicals were extracted, reoxidized, and measured in ethyl acetate under N_2. The high field extrema indicated by the arrow shows the result of a longer, 30 minute incubation.

of signal is best explained by reduction of nitroxide group to the corresponding hydroxylamine by the reductase. After the aqueous incubation mixture described above had stood for 10 minutes and no longer exhibited an EPR spectrum, the spin trapped adducts were extracted into air-saturated ethyl acetate in which O_2 reoxidized the reduced nitroxide group. The extract was concentrated and O_2 was removed by a stream of N_2. The resulting EPR spectrum (Fig. 3) is nearly identical to the spectrum of synthetic PBN-OOH (Fig. 1A). The amount of superoxide radicals trapped per minute was shown to be oxygen dependent and quantitatively related to the concentration of reductase in the incubation.

A series of control experiments were then performed to validate the comparison of a spectrum of synthetic PBN-OOH (figure 3) with those obtained from the reductase and oxygen incubations. In a test of the stability of PBN-OH and PBN-OOH, they were individually incubated in pH 7.5 phosphate buffer at 20°. No change was observed within 3 hours. Spectra then were measured of a series of mixtures of synthetic PBN-OOH and PBN-OH. These spectra show that, under the incubation conditions described in figure 3, hydroxyl radical production was less than 5% of that of superoxide radicals. An anaerobic

incubation of synthetic PBN-OOH with reductase and NADPH in pH 7.5 phosphate buffer demonstrated that under the incubation conditions used in obtaining figure 3, a small amount of the synthetic PBN-OOH is converted into other compounds. We suggest that the presence of these secondary reduction compounds may explain why the spectrum in figure 3 is not exactly like that of the synthetic PBN-OOH reference in figure 1A.

CONCLUSION. From the close similarity of the spectrum in Figure 3, obtained from an incubation of O_2 with reductase, with the reference spectrum of the synthetic superoxide-spin trap adduct (PBN-OOH) in Figure 1A, superoxide radical anion appears to be a primary reduction product of oxygen by NADPH cytochrome P-450 reductase in the absence of cytochrome P-450. The small amount of underlying component in the spectrum of Figure 3 are apparently not due to trapped hydroxyl radicals but instead due to further reduction of PBN-OOH after it is formed. These results suggest that hydroxyl radicals are not a primary product of the reduction of O_2 by reductase. The production of superoxide radicals may be important in understanding other studies of lipoperoxidation, oxidation of nitrogen-containing compounds, and substrates that alter electron transfer from reductase to cytochrome P-450.

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