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AN ANOMALOUS ESR SIGNAL FROM RAPIDLY FROZEN LIVER MICROSOMES

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SUMMARY

Rapidly frozen rabbit liver microsomes showed an ESR signal of several components near $g=2$, which disappeared upon warming to —60 °C. The signal could not be related to superoxide anion, but parts of it may have arisen from autoxidatively produced lipid peroxyl or protein-bound thio radical or from flavin semiquinone participating in a temperature-dependent equilibrium.

Attempts to show reduction of cytochrome P-450 by superoxide anion in presence or absence of substrate have failed.

INTRODUCTION

Cytochrome P-450, the oxygen-activating component of the liver microsomal mixed function oxidase system which catalyzes epoxidation and hydroxylation of drugs and steroids, is highly autoxidizable. It may therefore give rise to biologically significant free radicals derived from O_2, either functionally or adventitiously. In this study we have attempted to observe such radicals by allowing the native microsomal P-450 system to react with reductants and O_2, under various conditions, and interrupting the reactions by very rapid freezing. The captured states were examined by low temperature ESR spectroscopy. The free radical, superoxide anion, $O_2\cdot^-$, has in fact been shown to form from the reaction of O_2 with reduced flavoproteins[1,2], and a non-heme iron protein[3]. The requirement for a strongly reducing prosthetic group has been noted[4], and microsomal cytochrome P-450 has an apparent midpoint potential lying between −0.32 and −0.41 V[5,6] which is more negative than the O_2–$O_2\cdot^-$ couple[7]. In addition, an oxygen adduct of cytochrome P-450 has been observed[8,9] and since the electronic state of O_2 in an oxyhemoprotein may approach that of the superoxide anion[10], oxycytochrome P-450 may exist in equilibrium with superoxide and ferricytochrome P-450. These considerations prompted the present work.

MATERIALS AND METHODS

Whole microsomes, phenobarbital induced or as such, were prepared from rabbit liver perfused with 0.15 m KCl[11]. The cytochrome P-450 content varied between

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1.9 and 3.0 nmoles per mg protein. Superoxide was electrolytically produced in anhydrous *N,N*-dimethylformamide[12]. Solutions of $O_2^{\cdot -}$ were stored at liquid nitrogen temperature in sealed ampoules until use. Handling was done in an atmosphere of dry nitrogen. The presence of $O_2^{\cdot -}$ was indicated by its chemical properties[13] and by its ESR signal[14]. Electron spin resonance (ESR) spectra were measured with a Varian V-4500 spectrometer with variable temperature accessory. To quench reactions with oxygen the rapid mixing–freezing instrument of Bray[15], thermostated at 25 °C unless mentioned otherwise, was used equipped with two 0.14-mm exits[16]. Anaerobic samples for rapid mixing were prepared in tonometer flasks described by Beinert and Palmer[17]. Glucose oxidase was purchased from Nutritional Biochemicals. Crystalline catalase and NADPH:cytochrome *c* reductase[18] were preparations from beef and pig liver, respectively.

RESULTS AND DISCUSSION

Cytochrome P-450 exhibits a low level of catalytic activity during microsomal mixed function oxidation; the rate limiting step lies with the reduction of cytochrome P-450–substrate complex[19,20]. In a first series of experiments we tried to detect superoxide during the early phases of "single turnover" experiments starting with reduced microsomes and about 1 equiv of oxygen. Thus, microsomes, reduced under

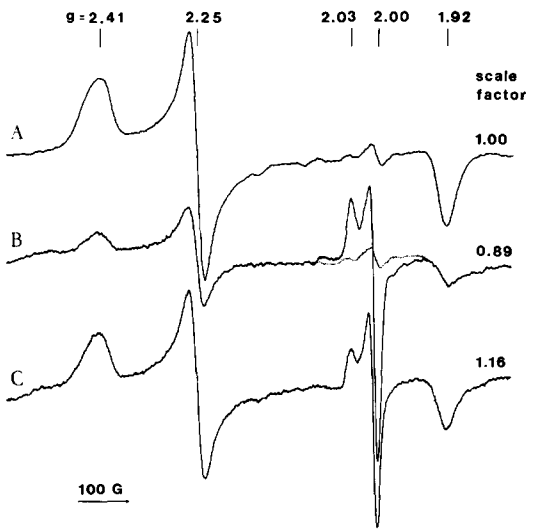

Fig. 1. ESR spectra of rabbit liver microsomes at 97 °K recorded with 10 mW power incident on the cavity and modulation amplitude of 12.5 G. (A) Normally frozen microsomes, 153 μM cytochrome P-450. (B) Microsomes anaerobically reduced for 30 min (153 μM cytochrome P-450, 10 mM NADPH) were rapidly mixed with 0.2 vol. of 1.2 mM oxygen at pH 10.6 and 25 °C, and frozen after 18 ms. Using CuEDTA as a standard for ESR quantitation we calculated that the radical signal accounted for 4.5 μM of unpaired spins. The dotted line shows the spectrum obtained after warming the sample to −60 °C for 3 min. (C) As (A) but rapidly frozen with the method of Bray[15]. The signal heights have to be multiplied by the appropriate scale factor indicated in the figure. This factor converts each signal to a standard gain of 100 and a standard performance of the ESR apparatus; to a common sample tube calibration; to a state of no dilution of the microsomes by reagents; and finally for the rapid freezing experiments to complete occupancy of the sensitive area in the ESR tube by sample, *i.e.* corrected for the volume fraction of isopentane.

anaerobic conditions with excess NADPH, were mixed at 25°C with 0.2 vol. of oxygen-saturated glycine buffer, pH 10.6, then quickly frozen to —130 °C. Besides a substantially reduced low-spin Fe signal of cytochrome P-450, a novel ESR signal was seen, with asymmetric characteristics not unlike those of $O_2^{\cdot -}$, having components at $g = 2.00$ and 2.03 (Fig. 1B). The signal faded almost entirely within seconds of warming to —60 °C, indicating that we were dealing with a transient species. The absorption which remained after warming resembled that observed in the ESR spectrum of microsomes as prepared (Fig. 1A). A $g_{||}$ value of 2.03 would be unusually low for superoxide in aqueous medium where one finds $g_{||} = 2.08$ to 2.12, and $g_{\perp} = 2.00$ (Fig. 2A)[1]. However, a large influence of the environment on the g-value anisotropy of superoxide is to be expected[1]. As demonstrated in Fig. 2B no increased absorption in the $g=2.09$ region was detected with microsomes. When microsomes were left out of the reaction mixture no appreciable ESR signals were found. We observed no change in radical concentration with a variation in reaction time between 18 and 234 ms and the presence of 10 mM phenobarbital or 10 mM aniline in the microsomal system did not affect the signal. When the pH of the oxygen-saturated buffer was lowered stepwise to 7.4 essentially no change in radical intensity took place. An increased stability of superoxide radical with alkaline pH has been reported in studies on xanthine oxidase[1], and a clear correlation of superoxide radical signal with oxygen concentration was established. However, with the present system, mixing reduced microsomes with anaerobic buffer did not obliterate the signal, but

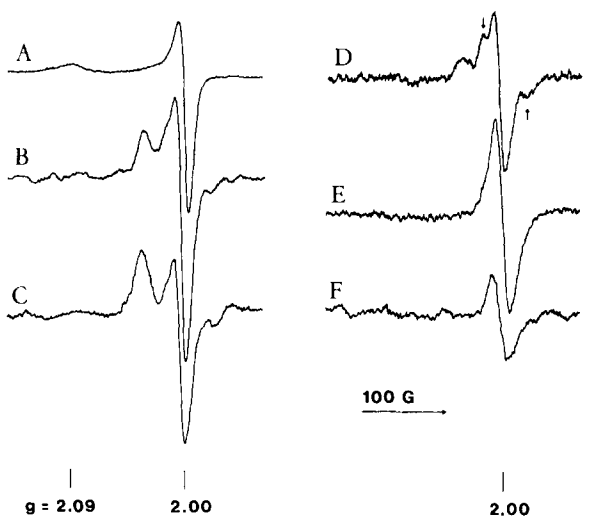

Fig. 2. ESR signals of $O_2^{\cdot -}$ and of microsomes in the $g = 2$ region recorded at 97 °K with modulation amplitude of 6.5 G. (A) Electrolytically produced $O_2^{\cdot -}$ (3.2 mM) in anhydrous *N,N*-dimethylformamide mixed with 5 vol. of 0.1 M glycine buffer, pH 9.4, and frozen after 245 ms. Microwave power, 1 mW. (B) Radical signal in rapidly frozen microsomes. Cytochrome P-450 concentration 219 μM at pH 7.4. Power 10 mW. (C) Radical signal obtained with microsomes (156 μM cytochrome P-450) anaerobically reduced with 1 mM NADPH, mixed with 0.2 vol. of *anaerobic* 1 M glycine buffer, pH 10.6, then rapidly frozen after 18 ms. Power 10 mW. (D) As (B), but at 0.4 mW microwave power and twice the gain. Shoulders in main signal appear at $g = 2.02$ and 1.98. (E) Signal observed with anaerobically reduced NADPH:cytochrome *c* reductase (46.3 μM flavin, 0.82 mM NADPH) after mixing with oxygen-saturated 1 M glycine buffer, pH 10.6, for 84 ms and freezing. Power 0.4 mW. (F) Remaining signal after warming (E) to —40 °C for 8 min. Same power and gain as for (E).

only slightly altered its appearance (Fig. 2C). Absence of a pH effect and lack of dependence on pO_2 make it probable that the radical signal was not due to superoxide. This was supported by the observation that microsomes did not need to be reduced to give the signal. As illustrated in Fig. 1C sudden freezing of a sample of oxidized microsomes with the Bray technique sufficed to develop the signal around $g = 2$. Power saturation curves (Fig. 3B) were identical for both components of the signal, but their relative intensities varied. In a large number of similar experiments we found an average signal amplitude ratio of 4.7 (range 3.7–5.9) while in the absence of oxygen this ratio was 2.9 (range 2.7–3.1), which indicates that in all likelihood there were two radical species with overlapping ESR signals. The spectrum is even more complex since, as shown in Fig. 2D, definite lines to the left and to the right of the main peak appear at very low microwave power.

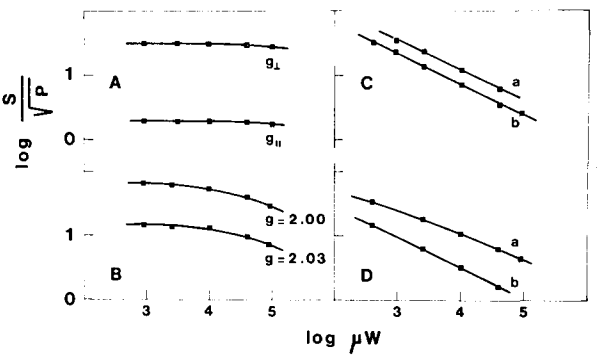

Fig. 3. Power saturation behavior at 97 °K of $O_2^{\cdot-}$ and of microsomal constituents in $g = 2$ region. (A) $g_{\parallel}$ and $g_{\perp}$ components of $O_2^{\cdot-}$ in aqueous medium (Fig. 2A). (B) Components at $g = 2.00$ and 2.03 observed in rapidly frozen microsomes. (C) a, Signal at $g = 2.00$ remaining after warming rapidly frozen microsomes to −60 °C. b, Signal at $g = 2.00$ in microsomes frozen in the usual manner. (D) a, Flavin radical in rapidly frozen NADPH:cytochrome *c* reductase (Fig. 2E). b, Stable signal after warming sample of (D) a to −40 °C. The saturation behavior here depicted is identical with that of the biradical form of NADPH:cytochrome *c* reductase (T. Iyanagi and H. S. Mason, personal communication).

We have considered three types of sources for the radicals giving rise to the anomalous ESR signals from liver microsomes: (1) an autoxidative process, involving unsaturated hydrocarbon chains or thiol groups, which leads to the formation of ESR-detectable alkyl peroxy or thio radicals; (2) an oxidation–reduction equilibration among microsomal redox components due to a sudden temperature drop; and (3) a metastable microsomal structure, trapped by sudden freezing, resulting in an increased relaxation rate of flavin radical. Alkyl peroxy radicals with anisotropic g values in the range 2.007–2.035, and with signal shapes similar to those of the observed signals have been described[21]. However, incubating the microsomes aerobically at 37 °C with 1 mM NADPH, which is known to stimulate autoxidative breakdown of lipids[22], was not effective in increasing the radical concentration, but this source of the anomalous signal cannot be ruled out. The $g = 2.03$ component may arise from sulfur radical, which in general shows g values above the free electron value[23]. The alternative hypotheses, that the radicals originate from a sudden temperature-dependent shift in either redox or conformational equilibrium sufficiently slow

to be trapped by low temperature quenching are consistent with the observation that considerably less intense signals were observed when microsomes were pre-incubated at 2 °C before being subjected to rapid freezing. The identity of the observed radicals is difficult to establish, and their relation to microsomal oxidative metabolism remains uncertain as some parts or the whole of the signal may arise from other cellular particles contaminating the microsomes. The $g = 2.00$ component has a peak to peak width of 15 G, and its saturation behavior (Fig. 3B) is characteristic of a flavin radical interacting with transition metal. The rest signal observed after warming the sample to −60 °C, identical with the signal observed in microsomes frozen in the usual manner, is like that of a non-interacting flavinsemiquinone having much slower relaxation (Fig. 3C). Easy saturation made it impossible to observe the rest signal entirely, even at the lowest power feasible with our ESR instrument. Therefore it cannot be decided whether unpaired spins were created in the sudden cooling process due to redox equilibration, or simply became more quickly relaxing in a transient environment.

We have investigated NADPH:cytochrome *c* reductase isolated from liver microsomes to check the assignment of at least part of the $g = 2.00$ component to flavin. Fig. 2E shows the ESR spectrum of a rapidly frozen mixture of NADPH-reduced reductase with oxygenated buffer. Here, too, a transient species formed which disappeared upon warming to −40 °C. This loss of signal again does not necessarily mean that electron transfer has taken place because the saturation patterns preclude quantitative observation of the signals (Fig. 3D). The remaining signal was indistinguishable from that of NADPH:cytochrome *c* reductase biradical. The above results with a purified microsomal component parallel the behavior of whole microsomes and support our tentative identification of the $g = 2.00$ component with a flavin radical of NADPH:cytochrome *c* reductase or NADH:cytochrome b_5 reductase associated with microsomes. Its efficient relaxation behavior in the rapidly frozen microsomes implies the nearness of an iron atom.

Our failure to detect $O_2^{\cdot -}$ during oxidation of microsomal electron carriers by oxygen does not disprove its involvement as a reaction intermediate because its concentration might not rise above our detection level. We supposed that $O_2^{\cdot -}$ would form by cleavage of oxygenated cytochrome P-450. Indirect evidence for this would be found if the reverse reaction could be shown. The ready availability of superoxide from electrolysis in aprotic solvents[12,13] made it possible to attempt the reduction of cytochrome P-450 by this reagent. Microsomes in 0.05 M phosphate buffer, pH 7.8, (0.3 or 2.8 mg protein per ml) were deaerated and saturated with 1 atm of CO. Glucose (40 mM), glucose oxidase (0.16 mg/ml) and catalase (6 μg/ml) were added to maintain anaerobiosis. The mixture (50 ml) was circulated through an optical cell and 4 mM solution of superoxide was infused at a rate of 2.5 μl per s. However, over a period of 6 min, no cytochrome P-450-CO spectrum appeared, in spite of the fact that in the same system cytochrome *c* was readily reduced. Adding 2 mM phenobarbital did not change the result. The spectrum of cytochrome P-450 nevertheless was modified under the influence of *N*,*N*-dimethylformamide to that of a Type II substrate–P-450 complex. The negative outcome of these experiments cannot be due to the low redox potential of cytochrome P-450 because the presence of CO greatly increases this potential. It must mean that there is a considerable kinetic barrier for reaction with $O_2^{\cdot -}$ and that this barrier exists also in the presence of sub-

strate. We conclude that we were unable to find evidence for superoxide involvement in microsomal hydroxylation. Instead, transient ESR signals were revealed associated with rapidly frozen microsomes. The participation of flavin in the reductive apparatus of microsomes is under further investigation in this laboratory (T. Iyanagi and H. S. Mason, personal communication).

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Recently, Strobel and Coon[24] have communicated that superoxide dismutase inhibits benzphetamine hydroxylation in the presence of NADPH, O_2 and a reconstituted liver microsomal enzyme system consisting of a cytochrome P-450 fraction, a NADPH:cytochrome P-450 reductase fraction and phosphatidylcholine. A superoxide-generating system was shown to substitute for the reductase and NADPH in supporting the hydroxylation. Significant effects of dismutase and of superoxide were, however, *not* observed with whole microsomes. The authors noted the possibility that $O_2\cdot^-$ acts as a reducing agent in the absence of NADPH and the reductase, and that ferrocytochrome P-450 then generates the true (enzymic hydroxylating) intermediate in the presence of O_2. In the light of our own failure to observe reduction of cytochrome P-450 in whole microsomes by $O_2\cdot^-$, and our failure to observe any ESR signal for $O_2\cdot^-$ from microsomes under hydroxylating conditions, we suggest that the system of Strobel and Coon is an "open" one in the sense that it does not represent a reconstituted microsomal hydroxylating membrane system but another one operating through free collision of its parts, whereas the results reported in this paper characterize the behavior of a "closed" or fully membranal system.

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