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EPR-spectroscopy of reduced oxyferrous-P450$_{cam}$

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X-irradiation of the ternary complex of P450:substrate:O_2 at 77 K produces a reduced intermediate by electron addition to the Fe:O_2 complex which can be studied by EPR-spectroscopy. The EPR spectrum of the new species exhibits rhombic symmetry with g-factors of 2.27, 2.17 and 1.95, respectively. Increasing the temperature of the sample to 190 K results in loss of intensity of the intermediate signals. X-irradiation of oxymyo- and oxyhemoglobin produces similar EPR signals indicating that the added electron is resident on the Fe:O_2 complex (Kappl, R., et al. (1985) Biochim. Biophys. Acta 870, 20–30).

Cytochrome P450; Oxygen activation; EPR-spectroscopy; One-electron reduction; Heme-oxygen complex

1. INTRODUCTION

The reduction of the ternary complex of ferrous P450:O_2 is an important step in the catalytic cycle of P450. Little structural information has been gained so far from the intermediate species due to the instability of both the ternary complex and, even more, its one-electron reduced successor [1]. Kobayashi and co-workers have applied pulse-radiolysis to study one-electron reduction of the oxy-form of various hemoproteins by means of optical spectroscopy [2,3]. In particular, the reduced ferrous oxygen complex in 2,4-diacetyldeutero-cytochrome P450$_{cam}$ was found to exhibit absorption maxima at 370 nm and at 470 nm and a half-life of 2 μs [4]. This lifetime impedes the use of Electron Paramagnetic Resonance (EPR)-spectroscopy, which is expected to provide additional structural information. A way to overcome this obstacle is the application of X-irradiation-generated electrons in situ, at 77 K. We have shown previously that kinetically stabilized adduct complexes with heme-oxygen complexes as appropriate electron acceptors can be formed by irradiation of frozen aqueous solutions of oxymyo- and oxyhemoglobin [5,6]. Applying this technique to study the structural features in the initial product of the reduction of the ternary complex of cytochrome P450$_{cam}$ we wish to present the first EPR-results of the observation of a new intermediate.

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2. MATERIALS AND METHODS

P450$_{cam}$ was prepared according to the methods described previously [7]. Oxygenated ferro-P450$_{cam}$ (≈ 0.6 mM) was formed in degassed water/glycerol-buffer (35% glycerol v/v), 5 mM camphor, 50 mM potassium phosphate buffer, pH 7.4, 5 mM methylviologen [8] by adding 0.70 mM sodium dithionite and by subsequent careful bubbling with oxygen. The procedure was carried out at −20°C. The formation of the oxygen complex was monitored spectrophotometrically. Frozen solutions of the complex were exposed to X-irradiation (Seifert, 100 kV, 25 mA) at 77 K with doses ranging up to 4 Mrad. EPR-measurements were performed on a Bruker ESP 380 spectrometer at 77 K.

3. RESULTS AND DISCUSSION

Fig. 1 shows the optical spectra of the reduced (solid line) and the oxygenated complex of P450$_{cam}$ and substrate (dashed line). The wavelengths for the maximum absorptions indicated as well the difference in extinction coefficients, for both species align well with literature data [9]. The spectra are identical at 77 K. After irradiation at that temperature there is loss of intensity in the 552 nm band which amounts, e.g. for a dose of 3 Mrad, to about 15%.

The EPR spectrum of the ternary complex of substrate:P450$_{cam}$:O_2 formed after irradiation at 77 K is given in Fig. 2 (top). Besides the intense signals centered at around g = 2 which are due to organic radicals as well as to H$^{\cdot}$-atoms, the latter distinct by their 50.8 mT hyperfine interaction, 2 characteristic rhombic, low-field components at g_1 = 2.27 and g_2 = 2.17 can be observed. In ferrous P450$_{cam}$ without an O_2-ligand, as well as in the CO-liganded species, these EPR signals are absent. Upon increasing the temperature to 178 K, the decrease in the magnitude of the organic radical signal allows us to observe a third component at g =

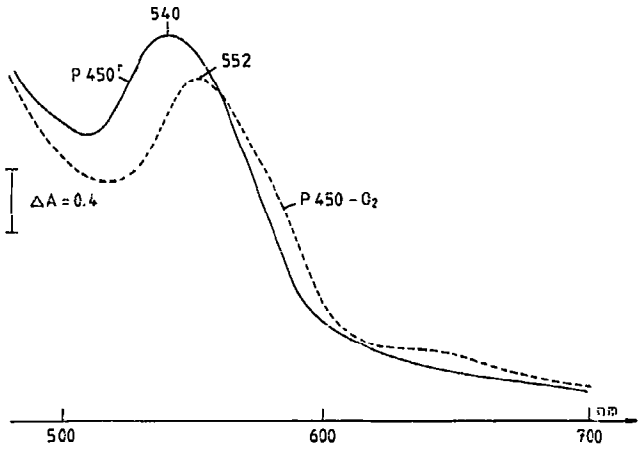

Fig. 1. Optical absorption spectra of reduced $P450^{r}_{cam}$ (solid line) and the ternary substrate:$P450_{cam}$:O_2 complex (dashed line). The wavelengths of maximal absorptions are indicated.

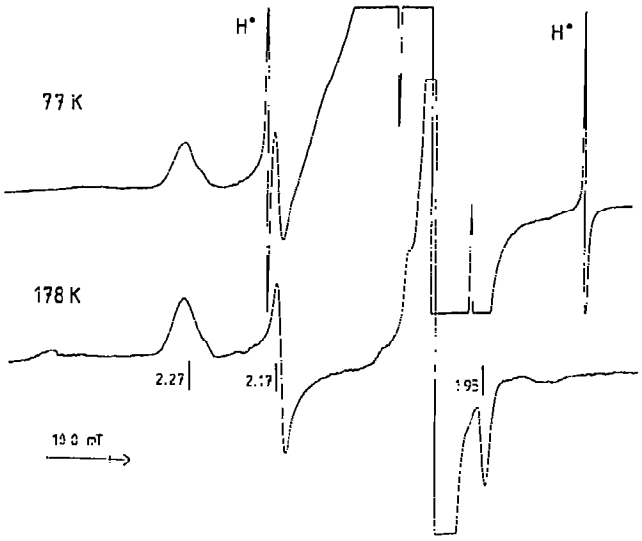

Fig. 2. EPR spectra of the substrate:$P450_{cam}$:O_2 complex X-irradiated at 77 K (top) and after annealing at 178 K (bottom). The three rhombic g-factors of the electron adduct center are given for the annealed sample. No loss in intensity is observed for the electron adduct center, but the obstructing signals of H - and organic radicals (g = 2), which were present at 77 K, had decayed to a large extent.

1.95. The intensity of the signal at both g = 2.27 and 2.17 is unchanged so that we assign the parameters with g-factors of 2.27, 2.17 and 1.95 to the same species denoted compound I. Increasing the temperature to 190 K results in a complete and concerted loss of the spectral features of I. At this stage a small amount of a low-spin ferric species II, with g-factors of 2.44, 2.24 and 1.90, is visible (Fig. 3). This is due to ferricytochrome P450 [9] which is present in the spectra before irradiation.

For the binary complex $P450_{cam}$:O_2 without camphor, the initial spectra after irradiation are identical in g-factors, and the annealing behaviour of species I is the same until about 190 K, at which temperature the sample from the ternary complex could not be annealed further for technical reasons. The substrate-free intermediate which was annealed to 273 K showed that there was, again, no relation detectable between the loss of the intermediate I and the ferric species II.

The EPR parameters for the initial reduced intermediate in $P450_{cam}$, observed in the present study, closely resemble those found for the primary electron adduct center in X-irradiated oxymyo-(MbO_2) and oxyhemoglobin (HbO_2) [5,6]. Furthermore, in X-irradiated samples of ferrous $P450_{cam}$ without oxygen or with carbon monoxide ligation, the characteristic rhombic EPR signal with g-factors of 2.27, 2.17 and 1.95 due to species I is absent. We therefore conclude that the latter is due to the addition of electrons produced by ionizing radiation to the Fe(II)–O_2 complex in $P450_{cam}$, yielding a very similar spin distribution as in MbO_2^- and HbO_2^-. This is somewhat surprising since the nature of the axial ligand in the trans-position to O_2 differs between the 2 groups of hemoproteins. On the other hand, isotopic substitution (^{57}Fe, $^{17}O_2$) carried out for the MbO_2^- complex indicated that most of the spin-density is located on these two nuclei giving little contribution from other positions. We mention that in MbO_2 there are 2 primary MbO_2^- species at 77 K which have different bonding geometry [5,6]. Also, for one of the species we have been able to observe a strong H-bond to the distal histidine (E7) by ENDOR-spectroscopy [5]. Further investigations will be necessary for the intermediate in $P450_{cam}$ in order to elucidate structural details of its stereochemistry and the mechanism of its conversion to hydroxylated product and ferric enzyme.

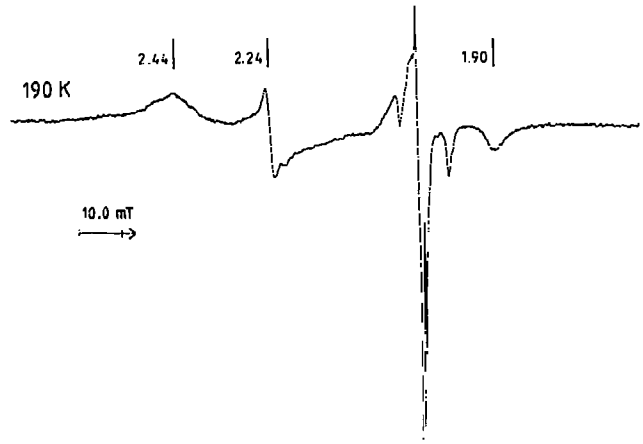

Fig. 3. EPR spectrum of the substrate:$P450_{cam}$:O_2 complex after annealing at 190 K with the rhombic g-factors typical of a low-spin ferric species. The electron adduct center, which was present at lower temperatures, has been lost completely.

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