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Superoxide Generation by an Iron–Tetraphenylporphyrin–Thiolate–Oxygen System and its Significance in Relation to the Coordination Site of Cytochrome P-450

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Iron–thiolate binding is especially important in cytochrome P-450 monooxygenases in that binding and activation of molecular oxygen occur at the active site of these enzymes [1]. Activation of oxygen by heme proteins such as cytochrome P-450 and some oxidases, involving electron transfer from iron, plays an essential role in the catalytic activities of the enzymes [2]. Thus a key to understanding these processes is the construction of a suitable chemical model in which one electron is transferred to oxygen using a metal ion. Recently we detected generation of superoxide in the reaction of Co(II)TPP and TGE in the thiolate form under air [3]: namely, a second example of superoxide generation with metalloporphyrin [3, 4] as the result of electron transfer from cobalt ion to dioxygen. A more faithful chemical model of natural heme iron containing proteins would, however, be an iron–porphyrin complex rather than osmium– [4] and cobalt–porphyrin [3]. Thus, in this work we tried to design a heme complex that could generate superoxide.

Here we report the generation by two methods of free superoxide in an artificial system containing heme–iron, Fe(III)TPP–TGE(thiolate)–O_2. Superoxide was detected by the usual method of ESR spectroscopy.

Materials and Methods

All experiments were made using a solution of Fe(III)TPP·Cl synthesized in our laboratory and other chemical reagents used were of reagent grade. Solvents were dried and distilled before use. The optical spectrum was measured with a Union SM-302 spectrometer at room temperature. The ESR spectrum was measured on frozen glass at 77 K with a JES-ME3X spectrometer operating at 100 KHz. ESR operating frequencies were measured with a Takeda-Riken microwave frequency counter, and the g-value was determined with Li–TCNQ (g = 2.0025) as a reference. The magnetic field was calibrated using as a base the splitting of Mn(II) in MgO (ΔH_{3-4} = 86.9 Gauss).

Results and Discussion

A mixture of TGE, Me_4NOH and Fe(II)TPP, which was prepared from Fe(III)TPP·Cl with zinc mirror *in vacuo,* showed no ESR signal at 77 K, indicating formation of the Fe(II)TPP–thiolate complex probably with a penta-coordinate geometry. When the ESR tube was opened and exposed to air for 3 min. at room temperature, ESR signals due to the concomitant formation of a ferric–heme complex in the low-spin state and superoxide were distinctly observed at 77 K (Fig. 1). The anisotropic ESR g-values (2.322, 2.228, 1.951 at 77 K) of the ferric low-spin species were similar to those of a hyperporphyrin-type complex, suggesting the formation of a dithiolate ferric heme complex [3, 5–8]. The observed anisotropic g-factors ($g_\perp = 2.007$ and $g_{/\!/} = 2.088$ at 77 K) provided clear evidence for the production of free superoxide and were akin to those observed in frozen solution reported previously [9, 10] and observed by us in the Co(II)TPP–TGE–O_2 system [3]. No ESR signal ascribable to the superoxide was detected after the mixture was allowed to stand, but when it was bubbled with oxygen or mixed with TGE, the ESR signal due to superoxide appeared again. This reaction cycle under air continued until all the added TGE had been fully oxidized to disulfide.

Generation of the superoxide was also demonstrated under air with the same components. Aerobic addition of Me_4NOH to a mixture of Fe(III)TPP·Cl and TGE in acetone at room temperature resulted in the formation of two new split Soret bands at 375 and 460 nm with broad bands at 562, 576 and 626 nm (Fig. 2)**. The half-life of this new complex was about 5 min. under air. A very similar

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Abbreviations: TTP, *meso*-tetraphenylporphyrinato; TGE, thioglycolic acid ethyl ester; Me_4NOH, tetramethylammonium hydroxide; ESR, electron spin resonance; P-450$_{cam}$, cytochrome P-450 from *Pseudomonas Putida*; MAA, mercaptoacetanilide; TN, thionalide; DTT, dithiothreitol; CTP, *para*-chlorothiophenol.

**Absorption maxima in the following systems: Fe(III)-TPP·Cl in benzene, 373, 414, 507 and 686 nm; Fe(III)-TPP·Cl in benzene–acetone, 374, 414, 500, 572 and 685 nm; Fe(III)TPP·Cl–Me_4NOH in benzene, 415, 573 and 613 nm.

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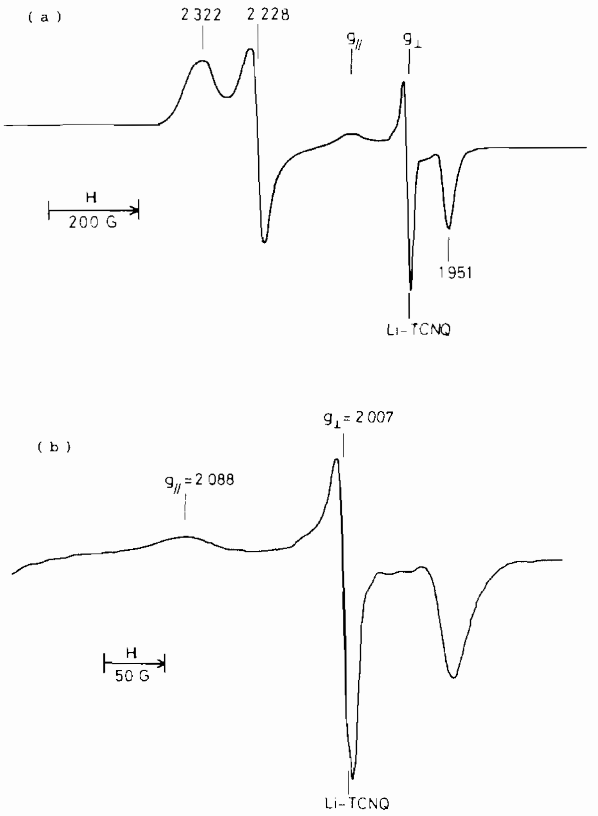

Fig. 1. ESR spectra of Fe(III)TPP–TGE–Me_4NOH–Oxygen system (a) and of superoxide (b) at 77 K. Zinc mirror, Fe(III)TPP·Cl (1 m*M* in CH_2Cl_2, 0.5 ml), TGE (0.5 *M* in acetone, 0.2 ml) and Me_4NOH (1 *M* in methanol, 80 μl) were prepared in four cuvettes connected to an anaerobic ESR tube. Fe(II)TPP, prepared from Fe(III)TPP with zinc mirror, was added to the TGE solution followed by Me_4NOH *in vacuo* at room temperature. The ESR spectrum was measured at 77 K. The ESR tube was opened and exposed to air at room temperature for about 3 min, and then the ESR spectrum was measured at 77 K (a and b). The solvent composition was thus CH_2Cl_2/acetone/methanol = 63.10/25.64/10.26.

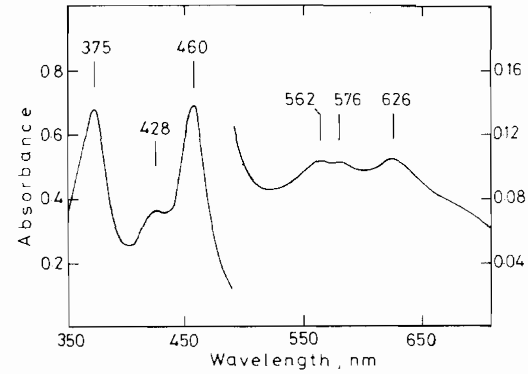

Fig. 2. Electronic spectrum of dithiolate–Fe(III)TPP complex at room temperature. A solution consisting of Fe(III)-TPP·Cl (0.25 m*M* in benzene, 0.1 ml) and TGE (0.1 *M* in acetone, 0.1 ml) in acetone solvent (2.8 ml) was mixed with Me_4NOH solution (1 *M* in methanol, 1 μl) under air at room temperature. The solvent composition was thus benzene/acetone/methanol = 3.33/96.63/0.03 (See footnote on p. L9)

spectrum has been reported for the dithiolate ferric– and cobalt–porphyrin complexes [3, 5, 6] and the dithiolate adduct complex of cytochrome P-450 [8, 11, 12] and cobalt-substituted P-450_{cam} [7]. This spectrum has been classified as a d-type hyperporphyrin spectrum containing two transitions: sulfur lone pair orbital $p^+ \rightarrow$ porphyrin π^* and porphyrin $\pi \rightarrow \pi^*$ [13, 14]. The hyperporphyrin spectrum was obtainable with various types of thiolesters and the spectral properties are summarized in Table I.

The ESR spectrum at 77 K of the hyperporphyrin-type complex prepared under air clearly indicated the concomitant formation of both the ferric heme complex in the low-spin state (g = 2.321, 2.227, 1.951) and superoxide ($g_{\perp}$ = 2.007 and $g_{\parallel}$ = 2.083), these ESR g-values being identical with those in Fig. 1.

TABLE I.

Hyperporphyrin Spectral Properties of TPP Complexes Containing Fe and Co and Cytochrome P-450.

heme complex	thiolate ligand	wavelength, nm					
FeTPP	TGE	375	(428)[a]	460	562	(576)[a]	(626)[b]
	MAA	373		459	562		(621)[b]
	TN	373		469	565		(622)[b]
CoTPP	TGE	381		472	610		660
	MAA	378		465	601		650
	TN	379		466	559		650
Fe–P-450_{cam}	CTP[d]	380		463	560		
	DTT[c]	374		461	557		
Co–P-450_{cam}	DTT[c]	375		467	573		

[a] The absorption bands in parentheses may be due to formation of an oxygen adduct complex. These absorption bands are very similar to those of $[Fe(O_2)TP_{piv}P(SC_6HF_4)](K\text{-}222)$ [19].

[b] The absorption bands in parentheses are due to formation of a ferric complex in the high spin state, whose structure is unidentified.

[c] Reference [7].

[d] Reference [8].

Thus the superoxide is generated by two methods in the FeTPP–thiolate–O_2 system. We are now measuring the superoxide quantitatively to evaluate the efficiencies of these two methods. Both methods are indispensable for determining the reaction mechanism of superoxide generation in our system. From careful ESR and optical studies on the model system, we deduced that the reaction mechanism for generating superoxide in the FeTPP–TGE(thiolate form)–O_2 system is as depicted in Fig. 3.

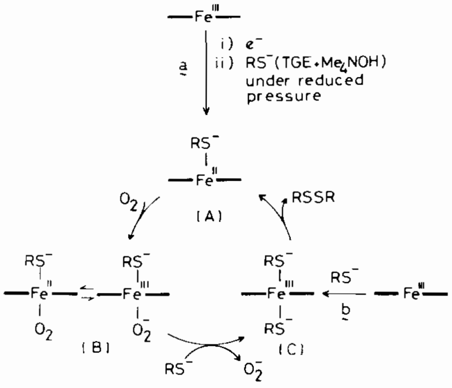

Fig. 3. Possible reaction mechanism in generation of superoxide by the Fe(II or III)TPP–thiolate–O_2 chemical system. Reactions *a* and *b* are processes in anaerobic and aerobic experiments, respectively. FeTPP is represented as –Fe–.

[A] is speculated to be a penta-coordinate thiolate–Fe(II)TPP complex. The structure of this complex that reacts with oxygen was recently confirmed by ESR spectroscopy in a CoTPP–TGE(thiolate form) system [15]. [B], which is a hexa-coordinate oxygen adduct complex of [A], is an equilibrium mixture involving electron transfer from iron to oxygen, and is suggested to be involved as an intermediate in the oxygen activating process of P-450 enzymes [1]. This form was also demonstrated in the CoTPP–TGE(thiolate form)–O_2 system by careful ESR spectrometry and analysis of its ESR parameters. Thus it was suggested that the metal–dioxygen d–π back bonding is weakened by the thiolate ligand, facilitating liberation of the bound O_2^- from the heme–iron site [15]. [C] is a hyperporphyrin complex with a dithiolate–Fe(III)TPP coordination mode, which was deduced by both ESR and optical spectroscopies.

This study provides the first direct evidence for the generation of superoxide in a model containing heme iron. This sytem provides not only a simple method for production of superoxide but also an insight into the mechanism of activation of oxygen by heme proteins. In fact, the production of superoxide was demonstrated with various types of P-450 [16–18]. This system may contribute further information on the two electron activation of dioxygen catalysed by P-450.

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