Oxidation of 1,2,4,5-Tetramethoxybenzene to a Cation Radical by Cytochrome P450

Hideaki Sato and F. Peter Guengerich*

Department of Biochemistry and Center in Molecular Toxicology Vanderbilt University, Nashville, Tennessee 37232-0146

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Cytochrome P450 (P450) enzymes are found throughout nature and are of interest because of their ability to catalyze various oxidations, including those at chemically inert sites such as unactivated methyl groups.[1] A number of mechanistic possibilities have been presented, including mobile oxygen species and several high-valent Fe complexes, but the most generally accepted view is that an $Fe^{IV}=O$ porphyrin radical is usually the oxidant.[1a,d,2] This entity is the same as that generally accepted for peroxidases, for example, horseradish peroxidase (HRP).[3] Peroxidases have generally been considered to be inefficient at hydrogen atom abstraction, a process ascribed to P450s, but part of the reason may be the spatial inaccessibility of substrates to the FeO entity of peroxidases such as HRP and the tendency to use electron transfer via the porphyrin edge.[4] The view has been expressed that P450s are capable of hydrogen atom abstraction but not the $1e^-$ oxidation of low $E_{1/2}$ substrates, for example amines.[5]

Several lines of evidence indicate that P450s can catalyze $1e^-$ oxidations under some conditions. The evidence includes observed rearrangements,[6] radicals trapped from 4-alkyl-1,4-dihydropyridines,[7] linear free energy relationships,[8] several similarities with the electrochemical oxidation of amines (e.g., product distribution),[8a,9] and low intrinsic kinetic hydrogen isotope effects,[4b,10] However, all of this evidence is indirect. The production of stable cation radicals has been observed in P450 reactions supported by "oxygen surrogates" such as cumene hydroperoxide[11] and iodosylbenzene.[8c] However, the relevance to the normal reaction cycle, supported by NADPH and NADPH−P450 reductase, can be questioned.

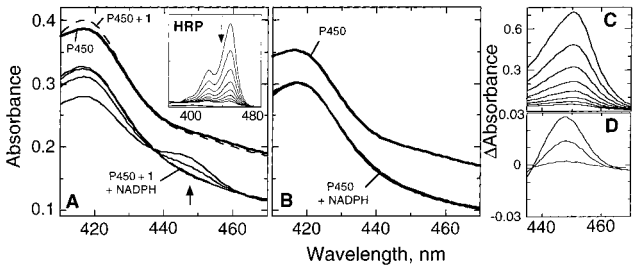

Figure 1. Optical absorption spectra of $\mathbf{1}^{\bullet+}$ formed during oxidation of **1** by rabbit P450 1A2. The incubation contained 2 μM P450 1A2, 4 μM rat NADPH−P450 reductase, 50 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine, 0.10 M potassium phosphate (pH 7.4), and an NADPH-generating system containing 0.05 mM $NADP^+$, 10 mM glucose 6-phosphate, and 2.6 U glucose 6-phosphate dehydrogenase mL^{-1} in the presence (A) or absence (B) of 5 mM **1**. Spectra were recorded every 40 s at room temperature. Difference spectra for the HRP (C) and P450 1A2 (D) reactions are derived from (A). The concentration was estimated to be 3.5 μM ($\epsilon_{450}=9800\ M^{-1}\ cm^{-1}$). The inset in A (labeled "HRP") shows spectra recorded with a mixture of 0.25 μM HRP, 0.44 mM H_2O_2, and 0.1 mM **1** in 100 mM potassium acetate buffer (pH 4.0), with spectra recorded every 1 min at room temperature (full scale absorbance 0.8).

Some polymethoxybenzenes have low $E_{1/2}$ values and yield stable cation radicals (at low pH) when oxidized by peroxidases such as HRP and lignin peroxidase.[12] When 1,2,4,5-tetramethoxybenzene (**1**)[13] (Scheme 1) was added to an aerobic system containing rabbit P450 1A2,[14] NADPH−P450 reductase, and NADPH, the formation of a stable spectral intermediate at 450 nm was observed, characteristic of the cation radical and similar to that seen in the reaction with HRP and H_2O_2 at pH 4 (Figure 1).[15] No band was seen when either P450, the reductase, or **1** was omitted.[16,20]

The characteristic ESR spectrum of $\mathbf{1}^{\bullet+}$ was observed in the HRP reaction,[12b] which is much faster that that of P450 1A2 (Figure 1). The level of the radical was too low to clearly observe the ESR spectrum in the P450 1A2 reaction, and a spin trap was used to accumulate radicals. 5,5-Me_2-1-pyrroline *N*-oxide (DMPO)

* Author for correspondence. Telephone: (615) 322-2261. Fax: (615) 322-3141, E-mail: guengerich@toxicology.mc.vanderbilt.edu.

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(14) Titration of P450 1A2 with **1** yielded a shift of the Soret band from 390 to 419 nm ("Reverse Type I" shift, transition from high- to low-spin iron), with a spectrally estimated dissociation constant (K_s) of 1.4 mM.

(15) No apparent cation radical was detected when *tert*-butyl hydroperoxide (5 or 15 mM) or H_2O_2 (0.44 mM) was used in place of NADPH. We also considered the use of iodosylbenzene as an oxygen surrogate, but it directly reacted with **1** to form $\mathbf{1}^{\bullet+}$ at a concentration of 0.2 mM.

(16) The development of a 450 nm spectral band in P450 reactions has been related to CO produced from trichlorethylene[17] or lipid peroxidation.[18] However, no unsaturated lipid was present here. Some destruction of the P450 heme occurred during the reaction, as described earlier.[19] The loss of the heme (which obscured observation of the cation radical at <430 nm) could be attenuated by the addition of catalase and superoxide dismutase; the changes in the spectra (Figure 1A,D) are not isosbestic.

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(20) No radicals (450 nm) were observed when 1,4-dimethoxybenzene ($E_{1/2}$ 1.34 V vs SCE) was used instead of **1** ($E_{1/2}$ 0.81 V vs SCE).[12b]

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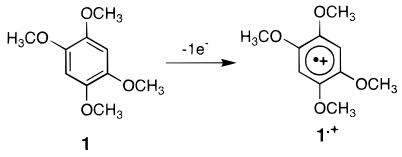
Scheme 1



1 $1^{\bullet+}$

did not produce a heme iron spin shift suggestive of binding in the substrate site of P450 1A2 when added at a concentration of 5 mM. This concentration of DMPO inhibited the production of $\mathbf{1}^{\bullet+}$ (450 nm band) by 30%. The values of the hyperfine coupling constants deduced from the six-line ESR spectrum observed under these conditions for $\mathbf{1}^{\bullet+}$ are typical for trapping of a carbon-centered radical[21] (Figure 2) and could arise from the adduction of $\mathbf{1}^{\bullet+}$ or a derived radical.

Some other P450s also formed $\mathbf{1}^{\bullet+}$, as judged by the extent of the change at 450 nm, but concentrations of $\mathbf{1}^{\bullet+}$ were less than with rabbit P450 1A2 (see Supporting Information).

Incubation of **1** with P450 1A2 also yielded 2,5-dimethoxy-1,4-benzoquinone ($v = 1.3$ min^{-1}) and 4,5-dimethoxy-1,2-benzoquinone ($v = 0.25$ min^{-1}) (expected for hydrolysis of $\mathbf{1}^{\bullet+}$ [12] and identified by HPLC retention, UV spectra, and mass spectrometry; see Supporting Information) and O-demethylation, as measured by the formation of formaldehyde[23] ($v = 2.8$ min^{-1}) (at a substrate concentration of 5 mM). Substitution of (d_{12}-O-*methyl*) **1** yielded a large kinetic isotope effect ($v \approx 0.1$ min^{-1}) for the O-demethylation reaction but only slightly decreased (<2-fold) the magnitude of the 450 nm band ascribed to $\mathbf{1}^{\bullet+}$ or quinone formation.[24,25]

We propose that the present work with $\mathbf{1}^{\bullet+}$ is the first report of direct observation of formation of a stable radical by a P450 in the normal catalytic system. These results have several mechanistic implications. P450s are definitely capable of 1e$^-$ transfer whenever the steric factors and the $E_{1/2}$ (actually a partial function of steric factors)[8b,28] are appropriate. This conclusion should apply not only to aromatic systems but also amines,[4b,6a,b,d,8a,c] strained cycloalkanes,[6c] and other low $E_{1/2}$ substrates, including several major polycyclic aromatic hydrocarbons (PAHs).[29] The detection of purine N^7- and C^8-PAH adducts in DNA has been used as evidence for roles of P450s in PAH 1e$^-$ oxidation because model 1e$^-$ oxidation systems generate the same adducts (other mechanisms are also plausible).[30]

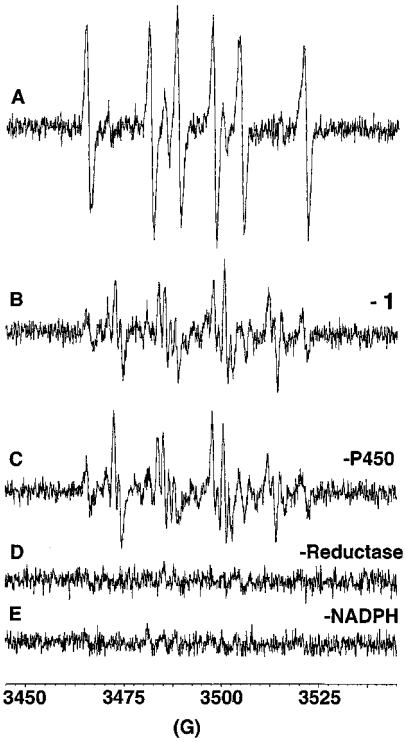


Figure 2. ESR spectra of DMPO-radical adducts produced by the reaction of P450 1A2 and **1**. A reaction (100 μL) similar to that described in Figure 1 was done in an ESR capillary (except that 30 mM DMPO was included). The ESR spectra shown here were all recorded 3.0 min after the reaction started. ESR analyses were carried out on aliquots of the mixture at room temperature and at X-band (9.81 GHz) microwave frequency with a Bruker EMX spectrometer operating with 100 kHz magnetic field modulation. Spectrometer conditions were: modulation amplitude, 1 G; microwave power, 10 mW; time constant, 0.041 s; scan time, 42 s; scan range, 100 G; receiver gain, 5×10^5. (A) Complete reaction system. Control experiments did not include either **1** (B), P450 1A2 (C), NADPH−P450 reductase (D), or the NADPH-generating system (E) Lower concentrations of what appear to be $O_2^{\bullet-}$, known to be produced by the reductase under these conditions,[22] and carbon-centered radicals were detected in the absence of **1** (B) or P450 (C). The latter radicals are attributed to flavin semiquinone radicals in NADPH−P450 reductase.

The significance of 1e$^-$ oxidation and rate-limiting events in P450 catalysis are under further investigation.

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Supporting Information Available: Analytical data for methoxybenzenes synthesized for use in this work, table of steady-state concentrations of $\mathbf{1}^{\bullet+}$ formed by various P450 enzymes, and HPLC/UV identification of quinone products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) When **1** was added to a reconstituted P450 1A2 system, the rate of NADPH oxidation increased from 26 min^{-1} to 57 min^{-1} (61 min^{-1} when d_{12}-**1** was used). The electron uptake is presumed to be involved in the cyclic formation of $\mathbf{1}^{\bullet+}$ and its subsequent reduction.

(25) The rate of 1e$^-$ oxidation (k_1) at pH 7.4 (k_2, a zero order constant) may be estimated from the first-order decomposition constant[26] and the concentrations of P450 1A2 and $\mathbf{1}^{\bullet+}$:[17b,27] $[\mathbf{1}^{\bullet+}] = k_1/k_2\,[\mathrm{P450}](1\text{-}e^{-k_2 t})$. Using data from Figure 1 at $t = 2$ min, $k_1 \approx 19$ min^{-1}.

(26) The **1** cation radical is relatively stable at pH 3.[12b] $\mathbf{1}^{\bullet+}$ was generated in the HRP/H_2O_2 reaction, and the kinetics of the stability of $\mathbf{1}^{\bullet+}$ were examined upon pH changes. The estimated first-order rate constants of its decay were 0.008 min^{-1} (pH 0.3), 0.57 min^{-1} (pH 4.0), 0.83 min^{-1} (pH 5.0), 4.3 min^{-1} (pH 6.4), and 14 min^{-1} (pH 7.4). $\mathbf{1}^{\bullet+}$ (generated with HRP/H_2O_2) was also found to be less stable in the presence of NADPH.

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