SUPEROXIDE ANION PRODUCTION BY THE AUTOXIDATION OF CYTOCHROME $P450_{cam}$

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SUMMARY - Chemiluminescence occurs on autoxidation of oxygenated ferrous cytochrome $P450_{cam}$ and is abolished by reagents that scavenge free radicals, by superoxide dismutase and singlet oxygen quenchers. We attribute the chemiluminescence to the decay of an excited singlet oxygen which arises from a superoxide anion radical precursor.

Camphor is hydroxylated in *Pseudomonas putida* by a soluble three component monoxygenase system consisting of 1) an FAD flavoprotein reductase, 2) a two-iron two-sulfur redox protein termed putidaredoxin, and 3) a b-type cytochrome $P450_{cam}$ (1, 2). Each of the proteins has been purified to homogeneity (3) and the cytochrome has been crystallized (4). This hydroxylase has been studied in great detail genetically, chemically and physically, and is the subject of a recent review (5).

In the reconstituted system, reducing equivalents are transferred from NADH through the reductase and redoxin and supplied to the cytochrome in two single electron events. The first electron transfer, responsible for the ferric-ferrous reduction of heme iron, is nonspecific in the sense that it can be supplied by chemical reducing agents such as dithionite or EDTA and light (6). The second redox reaction, however, is specific since putidaredoxin or a limited number of other effectors are required for product formation (5, 7). The specificity in the second electron transfer and coupled substrate hydroxylation allows the steady state formation of an oxygenated substrate-bound ferrous P450 intermediate (abbreviated $m_{O_2}^{rs}$) (8). The $m_{O_2}^{rs}$ has been identified as an obligatory intermediate in the overall hydroxylation reaction (9, 10, 11). In the absence of a specific effector molecule, $m_{O_2}^{rs}$ decays by an autoxi-

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Abbreviations: $m_{O_2}^{rs}$, oxygenated substrate-bound ferrous P450 intermediate; SOD, superoxide dismutase.

dation process that is formally identical to the conversion of oxygenated hemoglobin and myoglobin to the met form. The oxygen product of this autoxidation has not been previously determined. In this manuscript we present evidence that superoxide anions are produced in this reaction and that subsequent bimolecular dismutation of these radicals can yield oxygen in an excited singlet state. The autoxidation process can be observed by following the chemiluminescence from the radiative decay of the singlet oxygen. A preliminary report of this work has been presented (12).

MATERIALS AND METHODS

Cytochrome P450 was isolated as described previously (3). Superoxide dismutase purified according to reference 13 was a generous gift of S. Aust and T. Pederson; all other reagents are from Sigma.

The concentration of the oxygenated cytochrome $m_{O_2}^{rs}$ at any time can be determined spectrophotometrically from the Soret maximum at 418 nm and the extinction coefficient of 62 $mM^{-1}cm^{-1}$. This oxygenated intermediate is prepared by reducing the cytochrome anaerobically in the presence of substrate, then adding oxygen to initiate the reaction. For the chemiluminescence studies the cytochrome at 30 μM was dissolved in 3 ml of 50 mM KPO_4 buffer, pH 7.0, containing 10 mM EDTA and 420 μM camphor. The sample in an anaerobic cuvette is evacuated and exposed to white light from a 30 watt projection bulb with the water as a heat filter at a distance of 15 cm for 4 min (14). The reduced enzyme mixture is then equilibrated at the desired temperature in the luminescence apparatus described below and the reaction initiated by the admission of air to allow very rapid formation of $m_{O_2}^{rs}$. The measurements are made in a 0.5 x 2.0 cm quartz cell with one optical face silvered to increase the light collecting efficiency. The clear face of the cell is placed in close juxtaposition to a photomultiplier tube. The major radiative de-excitation pathway of singlet oxygen involves the dismutation of two singlet states to produce a photon with twice the excited state energy. As the corresponding wavelengths are in the region of 640 nm, a red sensitive (EMI 9558QB) phototube is employed (15).

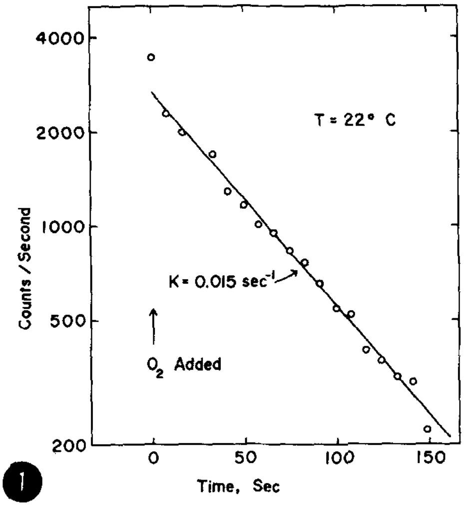
The excited singlet state of oxygen can decay to the normal ground triplet by emitting a photon. Radiative dipole transitions from pure states of different multiplicities are formally spin forbidden, hence the quantum yield of the chemiluminescence is very small and photon counting techniques were used. The current pulses from the anode of the phototube were fed to an Analog Devices 46J fast settling operational amplifier wired as a fast current-to-voltage converter. The output was connected to Texas Instrument 72710 voltage comparator used as a discriminator with the reference voltage set to give a background count of 50 to 100 sec^{-1}. Zero crossing of the comparator was sensed by a Texas Instruments 74121 one-shot multivibrator producing uniform output pulses of 100 nsec duration which were subsequently counted by a wide band scalar. The scalar output was latched at one-second intervals and the data converted to ASCII serial form which could drive a standard ASR33 teletype. The number of photon counts per one second timing interval was printed every second, yielding data on the time course for the decay of chemiluminescence.

The overall counting efficiency of the system for superoxide radicals was calibrated using xanthine and xanthine oxidase. The rate of O_2^- production was measured spectrophotometrically by the aerobic rate of cytochrome c reduction and concomitant xanthine oxidation. The same conditions were employed to generate a known concentration of superoxide anions in the photon counting apparatus.

All spectrophotometric measurements were made on a Heath EU 707 spectrophotometer system.

RESULTS

The results from a typical chemiluminescence experiment are shown in Fig. 1. With the log of the number of counts/sec plotted as a function of time, a singlet exponential decay is observed. Using the xanthine/xanthine oxidase calibration procedure, the calculated equivalent amount of superoxide generated during the autoxidation reaction represents at least 60% of the expected oxygen decay products. As mentioned earlier, it is also possible to follow the rate of the autoxidation reaction by monitoring the disappearance of 418 nm absorption



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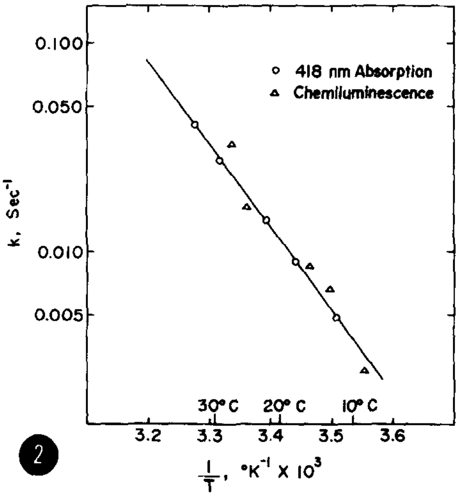

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Fig. 1. Chemiluminescence observed during the autoxidation of substrate-bound cytochrome m ($P450_{cam}$). The log of the counts/sec is plotted as a function of time. Reaction conditions are as described in Methods.

Fig. 2. Temperature dependence of the first order rate constants for $m^{rs}_{O_2}$ decay as determined by chemiluminescence and optical absorption measurements. Circles represent rates calculated by following the disappearance of the characteristic 418 nm absorption. Triangles are from the luminescence measurements.

(16). A correlation between the kinetics of $m^{rs}_{O_2}$ decay measured by chemiluminescence and quantitated by optical absorption can be obtained by a study of the temperature dependence of the rates.

In Fig. 2, the first order rate constant for the autoxidation reaction is displayed vs. inverse temperature in a standard Ahrennius analysis. Although the chemiluminescence data are prone to larger statistical error due to the low light levels observed, an obvious correlation exists between the measurements. The straight line represents an activation energy of 17 kcal/mole (0.74 ev/mol).

The photon production is inhibited to varying degrees by superoxide dismutase; cysteine, a known free radical scavenger (17); and azide, a quencher of singlet oxygen (15). The data in Table I show the inhibition in photon production by several singlet oxygen quenchers and free radical agents. The quenchers were added to the concentration indicated at the time or oxygen addition.

DISCUSSION

Superoxide radicals are known to dismutate to form peroxide and oxygen in

TABLE I

INHIBITION OF CHEMILUMINESCENCE

Reaction Mixture		Counts/sec at $t = 0^+$
30 µM $m_{O_2}^{rs}$		2,580
"	+ 0.3 µM superoxide dismutase	308
"	+ 6.3 mM cysteine	260
"	+ 0.1 mM azide	500

an excited singlet state (18, 19). Chemiluminescence from singlet oxygen has been observed, the major pathway for de-excitation being a dimeric relaxation of two excited states producing photons with a wavelength in the neighborhood of 530 nm (20, 21). We have observed low level chemiluminescence from the autoxidation of cytochrome m, $P450_{cam}$. The kinetics, which are first order, follow very closely the decay of $m_{O_2}^{rs}$ as determined spectrophotometrically. Thus, the rate limiting step in the production of a luminescent species is the autoxidation of the $m_{O_2}^{rs}$ precursor. The first order rate constant for the photon emission follows an Ahrennius temperature dependence paralleling the decay of $m_{O_2}^{rs}$, further supporting the postulate that the luminescence is associated with $m_{O_2}^{rs}$ decay.

The light emission is inhibitable by singlet oxygen quenchers and by various free radical scavengers. The main evidence for the involvement of superoxide anions as precursors for the chemiluminescent reaction arises from the inhibition of the light reaction using superoxide dismutase (SOD). Mayeda and Bard have recently shown (22) that SOD inhibits chemiluminescence associated with superoxide radical dismutation by catalyzing the dismutation of O_2^- without the formation of excited oxygen. Of particular importance is their finding that SOD does not directly quench singlet oxygen but only interferes with its production.

It is tempting to conclude, therefore, that the chemiluminescence we observe in the decay of $m_{O_2}^{rs}$ is due to singlet oxygen decay and that this excited state oxygen has a direct superoxide radical precursor. We are currently attempting to measure the spectrum of the emitted light and are extending these studies to the substrate-free form of oxygenated cytochrome m, with the hope of ultimately understanding the state of the oxygen molecule immediately prior to the attack on the carbon chain of the substrate.

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