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CYTOCHROME P-450 MEDIATED INTERACTION BETWEEN HYDROPEROXIDE AND MOLECULAR OXYGEN

A Proposed Method for P-450 Kinetic Studies

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SUMMARY. Rat liver cytochrome P-450 mediates a novel reaction between equimolar quanitities of dissolved oxygen and organic hydroperoxides. The reaction shares some of the properties of both NADPH-O_2 dependent hydroxylation and NADPH-O_2 independent peroxidase reactions, but does not require either NADPH, phosphatidylcholine, or any substrates other than hydroperoxide and oxygen. It proceeds at a rate approximately 100 times faster than other well known P-450 hydroxylation reactions. Monitoring the rate of O_2 consumption in this novel reaction may be a simple and rapid means for studying the kinetics of cytochrome P-450.

The microsomal NADPH dependent cytochrome P-450 mixed function oxidase system converts virtually any lipophilic compound via molecular O_2 into a variety of oxygenated products. Independent of NADPH and O_2, it can also act as a peroxidase, using organic hydroperoxides, iodosobenzene, $NaIO_4$ and peroxyacids in the following type of reaction: ROOH + R'H → ROH + R'OH (eq. 1). [For an overall review of P-450, see ref. 1]. In this paper, we report the observation of a third kind of P-450 mediated reaction in which molecular O_2 and organic hydroperoxides are apparently the only reactants.

MATERIALS AND METHODS

NADPH was obtained from P-L Biochemicals (Milwaukee, Wis); cumene and t-butyl hydroperoxides from Pfaltz and Bauer (Stanford, Conn.); P-450 inhibitors and antioxidants from Aldrich and all lipids from Applied Science Labs (State College, PA).

Microsomes were prepared from the livers of rats given saline, PB, and 3-MC according to Ortiz de Montellano and Kunze (2), essentially by the

ABBREVIATIONS: PB - phenobarbitol; 3-MC - 3-methyl cholanthrene; LOOH - linoleate hydroperoxide; PTC - phosphatidylcholine; CuOOH - cumene hydroperoxide; AA - arachidonic acid

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method of Mazel (3) except that the microsomes were more concentrated [ca. 10 mg protein/ml as determined by the method of Lowry with a BSA standard] and pH 7.2 phosphate buffer replaced Tris buffer. These microsome suspensions were stable indefinitely when stored in 5 ml plastic vials at -80°C. The method of Omura and Sato was used to determine total P-450 (4), and an absorptivity constant of 91 mM^{-1} cm^{-1} was used to determine P-450 concentrations (5) which were: 6 mM for uninduced microsomes, 17 mM for PB induced, and 15 mM for 3-MC induced (P-448).

Partial purification of P-450 was accomplished as described by Fisher, et.al.(6). Linoleate hydroperoxide was prepared as described previously (7).

Oxygen consumption was determined using a biological oxygen monitor equipped with a Clark oxygen electrode. Typically, 0.1 ml of microsome suspension (ca. 1 mg of protein) was injected into a constantly stirred air equilibrated mixture of 0.05 M pH 7.2 phosphate buffer and substrate hydroperoxide at 37°C. The 3.0 ml final volume contained 0.65 μ moles of O_2 (calculated). The oxygen uptake reaction was first order with respect to microsome concentration in the range studied at a given concentration of substrate. Reproducibility was on the order of ±10% or better.

Results

The catalyst for the reaction between hydroperoxide and molecular oxygen is induced by 3-MC and PB, and correlates with an increase in absorbance at 448 and 450nm respectively over control (uninduced) values. Velocity vs. hydroperoxide concentration curves (fig. 1A and 1B) demonstrate inducibility as well as the fact that the degree of induction is a function of the nature

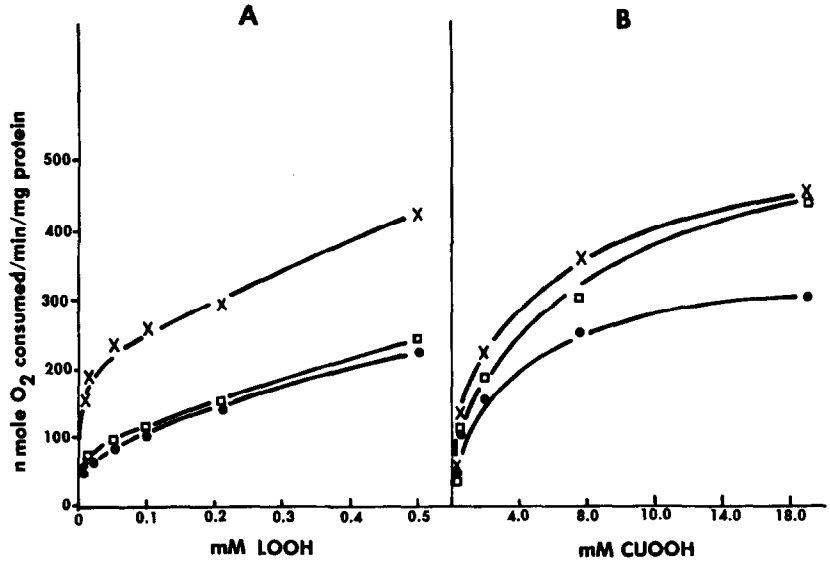

Figure 1 Cytochrome P-450 catalyzed rate of oxygen consumption as a function of: A - LOOH concentration; B - CuOOH concentration. x - PB induced microsomes. □ -3-MC induced microsomes. ● - uninduced microsomes

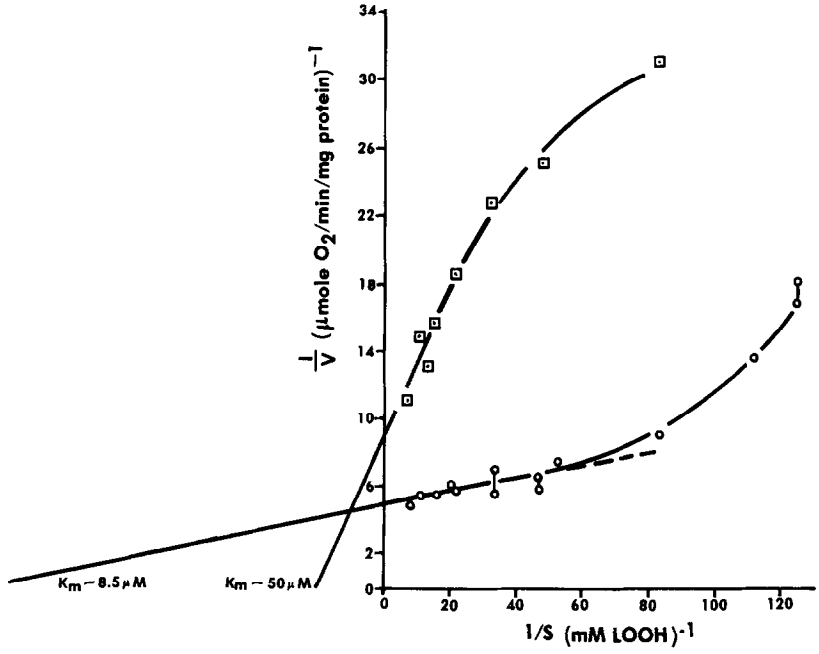

Figure 2 Inverse plots for rate of oxygen consumption vs. LOOH concentration catalyzed by: ⊡ - uninduced P-450. O -PB induced P-450.

of the hydroperoxide substrate. Microsomes from PB induced rats, for example, show markedly faster rates of O_2 uptake than microsomes from 3-MC induced or uninduced (control) rats when LOOH is the substrate (fig. 1A); but when CuOOH is the substrate, both 3-MC and PB induced microsomes show similar reaction velocities, although PB microsomes are more efficient at low CuOOH concentrations (1B). Comparing the concentrations of CuOOH and LOOH needed to give similar rates of O_2 uptake, it is apparent that P-450 utilizes LOOH much more efficiently than CuOOH.

An inverse plot using data obtained for concentrations of LOOH between 0.006 and 0.120mM (fig 2) yields apparent K_m values of 8-10mM for PB induced and 45-50mM for the uninduced microsomes. The apparent cooperative kinetics displayed may may only be due to the various P-450 isoenzymes having different affinities for LOOH.

In addition to CuOOH and LOOH, $NaIO_4$, t-butyl hydroperoxide and iodosobenzene can serve as oxygen donors in the P-450 peroxidase reaction (Ref. 1). In contrast, these same compounds, while also initiating measurable O_2 uptake

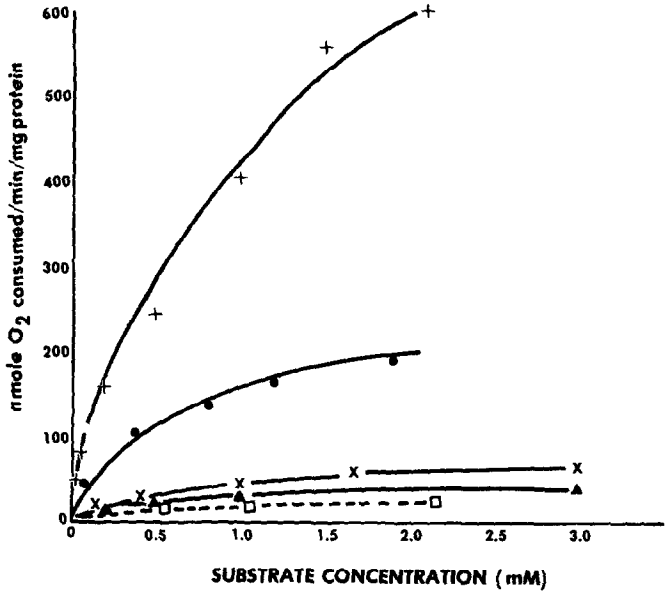

Figure 3 Uninduced P-450 catalyzed rate of oxygen consumption vs. concentration of various "substrates". + - LOOH. ● - CuOOH. Δ - $NaIO_4$. □ - t-butyl-OOH. x - iodosobenzene.

from solution in the presence of microsomes, do so to a practically insignificant degree compared to LOOH and CuOOH. (fig. 3)

The oxygen uptake reaction shares other well known properties of P-450 mediated oxidations as follows:

A. Both cimetidine and metyrapone, well known inhibitors of P-450 hydroxylation reactions, inhibit O_2 uptake from solution; but cimetidine is not nearly as potent as metyrapone in our studies. Typically, at LOOH concentrations approaching V_{max}, 10mM metyrapone inhibits O_2 uptake by 50-70%, while cimetidine at the same concentration inhibits only 25-35%.

B. The reaction is relatively insensitive to inhibition by CN^-. 2mM CN^- does not measurably inhibit O_2 uptake, and not until 20mM CN^- is the reaction inhibited 50%. Related to this, neither haemin nor catalayse show any O_2 uptake activity over the time span of a typical experiment (1-5 min.) when they are used in place of microsomes.

C. Treatments that convert P-450 to the inactive denatured P-420 form, such as preincubation with p-chloro mercuribenzene sulfonic acid or at pH 10, also destroy O_2 uptake activity.

Reaction inactivation of P-450 by "suicide" substrates has been described by various workers (2,8,9), and is thought to be achieved through highly specific substrate-enzyme interactions. This phenomenon is observed in our experiments when a relatively low concentration of P-450 in the microsomes is reacted with a high concentration of LOOH, e.g. 1.0 nmole P-450/5000 nmole

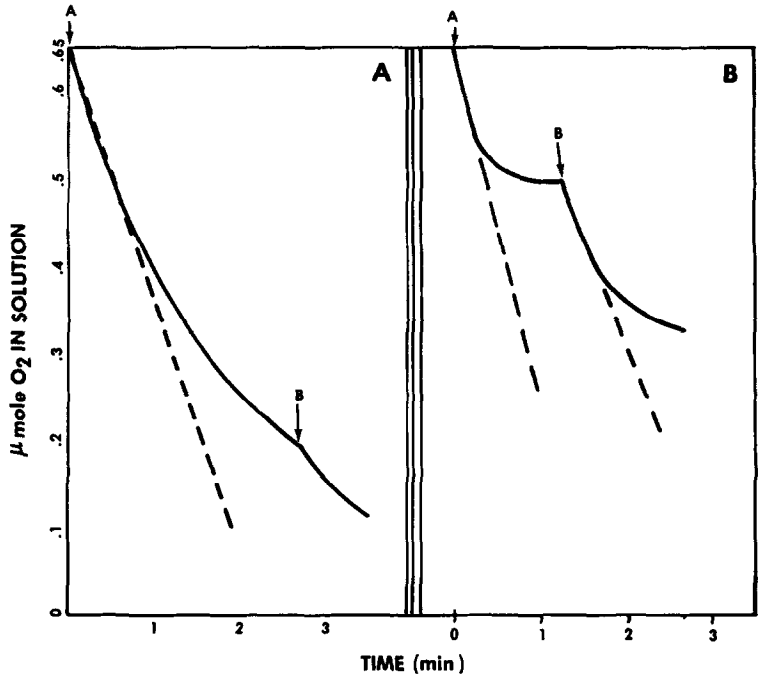

Figure 4 Time curves for oxygen consumption in mixtures of microsomes and LOOH. Part A - large excess of LOOH concentration over P-450 concentration. Part B - small excess.

LOOH, or CuOOH. After initiation at point A in figure 4A, the reaction is linear for about 1 min, then gradually falls off. Adding more LOOH at point B causes little or no additional O_2 uptake, indicating that P-450 has been largely inactivated. Figure 4B demonstrates the opposing case of a reaction with a relatively high P-450 and low LOOH concentration, e.g. 1 nmole P-450/100 nmole LOOH. Here, the linear O_2 uptake curve quickly leads to a zero slope (no further O_2 consumption) that corresponds to 1 mole O_2 consumed per mole of LOOH substrate added. In this case, adding more LOOH at point B results in renewed O_2 uptake, indicating that the reaction has reached completion, rather than that P-450 has been inactivated. The 1:1 ratio of O_2 consumed/LOOH added holds for reactions which are essentially complete within a few minutes. Longer (>5 min) reaction times, as when using low levels of microsomes, allow the myriad possible secondary lipid hydroperoxide oxidative reactions and oxidation of endogenous microsomal lipids to occur, thus 1:1 ratios are no longer observed. The reaction probably proceeds via a free radical meachanism

because the antioxidants BHA and t-butyl hydroquinone completely inhibit O_2 uptake at concentrations of just a few mg/ml.

P-450 treated with cholate and precipated by polyethylene glycol had the same specific activity and reaction characteristics as intact microsomal P-450. In contrast to both the NADPH dependent and the peroxide dependent substrate hydroxylations which require PTC for maximum activity (10), PTC had no effect on the LOOH induced rate of O_2 uptake using either intact or polyethylene glycol precipated microsomes as a catalyst. Work to identify products of the reaction is in progress.

DISCUSSION

When microsomes and NADPH are mixed, dissolved O_2 is consumed at about 20-40 nmole O_2/min/mg protein. Capdevila, et al. (11) noted an increase (extent not stated) in O_2 uptake over that level (20 nmole/mg P in their work) when AA was included in the mixture. We thought that this O_2 uptake perhaps could be used as a method for studying P-450 kinetics in lieu of the standard methods. No consistant or useful results were obtained, however, until it became apparent that contaminating hydroperoxides, not the AA per se, were initiating oxygen uptake. AA purified by TLC did not signifigantly increase oxygen uptake over the level due to microsomes plus NADPH alone in a reproducible manner. O'Brien and Rahimtula (12) also noted that the addition of hydroperoxides to microsomes initiated O_2 consumption, but their results were apparently due to peroxidation of endogenous microsomal lipids by cumene hydroperoxide and the heme moiety of P-450 acting in concert. Our results are in sharp contrast to theirs, in that LOOH induced O_2 uptake was observed using either soluabilized (lipid "free") or intact microsomes; and the amount of O_2 consumed was dependent on LOOH concentration and independent of microsome concentration. Thus endogenous lipids are not involved in the reaction.

Blake and Coon (13) presented evidence that the organic portion of the peroxy compound influences the hydrocarbon substrate related steps of the peroxidase reaction (eq.1). In the reaction discussed here, the organic por-

tion of the peroxy compound may in fact replace the hydrocarbon substrate. Witness that IO_4^- (no organic portion) and t-butyl hydroperoxide (contains only difficult to oxidize methyl groups) hardly react at all compared to CuOOH and, most effective of all, to the readily oxidized LOOH.

A typical reaction rate approaching the maximum for LOOH reactions in our experiments is ca. 500 nmole O_2 consumed/min/mg protein for the uniduced P-450. This is at least a 100 x greater reaction rate than has been reported for NADPH dependent drug and steroid metabolizing P-450 linked enzymes (14) and arachidonic acid hydroxylation (11). CuOOH dependent hydroxylations reported by Blake and Coon (13) showed, at best a K_m about 40 times less for substituted toluence substrates than the K_m found (fig. 2) for O_2 uptake by PB induced microsomes using LOOH as substrate. Such a high affinity for LOOH as compared to the various substrates for the NADPH dependent and the peroxidase reactions argues that the LOOH-O_2 P-450 mediated reaction has a physiological significance.

From the evidence presented here, we conclude that the microsomal P-450 system catalyzes a reaction between molecular O_2 in solution and organic hydroperoxides which forms some as yet unidentified product(s). Because of the ease with which O_2 consumption can be measured, and the rapid rate which eliminates interference of the myriad slower oxygen consuming reactions associated with microsomes and hydroperoxides, this reaction could possibly be used as a method for determining P-450 kinetics.

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