pH 7 results either from a spin conversion or interaction between high and low spin hemes. EPR, NMR and optical spectra show that this is a different type of heme-heme interaction than observed with diheme cyt $c'$.

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## The Electron Transfer Reactivity of Cytochrome P-450-CAM. Molecular Oxygen Dependent Multiple Turnovers Using Chemical Reductants

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Cytochrome P-450, unlike most other cytochromes, does not function merely as an electron carrier but is also an enzyme capable of catalyzing oxygenation reactions. This heme-containing monooxygenase activates molecular oxygen for insertion of one oxygen atom into organic substrates with concomitant reduction of the other oxygen atom to water. Bacterial P-450, isolated from camphor-grown *Pseudomonas putida* (P-450-CAM)†, utilizes molecular oxygen and NADH to hydroxylate camphor at the exo-5 position and initiate camphor degradation [1]. Because the hemoprotein itself cannot react directly with NADH, electrons are transferred from NADH to P-450-CAM via first a FAD-containing flavoprotein (putidaredoxin reductase, fp) and then an iron-sulfur protein (putidaredoxin, Pd).

†Abbreviations: P-450-CAM, the camphor hydroxylating P-450 isolated from *Pseudomonas putida* grown on camphor; fp, the flavoprotein (putidaredoxin reductase) that accepts electrons from NADH; Pd, the iron-sulfur protein (putidaredoxin) that accepts electrons from fp and delivers them to P-450-CAM; PMS, 5-methylphenazinium methyl sulfate (phenazene methosulfate).

TABLE I. Product Formation.[a]

A. *As a Function of P-450-CAM Concentration*

| | P-450-CAM Conc. ($\mu M$) | *Exo*-5-hydroxy-camphor formed[c] (nmol) |
|---|---|---|
| With Putidaredoxin ($3\ \mu M$) | 0 | 0.01 ± 0.01(2) |
| | 0.5 | 23.6 ± 1.0(2) |
| | 1.0 | 49.8 ± 1.5(6) |
| | 2.0 | 97.0 ± 2.5(2) |
| Without Putidaredoxin | 0 | 0.02 ± 0.01(2) |
| | 0.5 | 1.3 ± 0.1(2) |
| | 1.0 | 2.7 ± 0.2(3) |
| | 2.0 | 5.1 ± 0.2(3) |

B. *As a Function of Time*

| | Incubation (min) | *Exo*-5-hydroxy-camphor formed[c] (Mol/Mol of P-450-CAM) |
|---|---|---|
| With Putidaredoxin ($3\ \mu M$) | 1 | 28.8(1) |
| | 2 | 49.8 ± 1.5(6) |
| | 5 | 101.7(1) |
| | 10 | 188.7 ± 5.0(2) |
| Without Putidaredoxin | 1 | 1.5 ± 0.1(2) |
| | 2 | 2.7 ± 0.2(3) |
| | 5 | 5.8 ± 0.1(2) |
| | 10 | 9.8 ± 0.3(2) |

C. *Control Experiments*

| | *Exo*-5-hydroxycamphor formed[c] (nmol) | |
|---|---|---|
| | Without Pd[d] | With 5 $\mu M$ Pd[e] |
| 1. No P-450 | 0.02 ± 0.01(2) | 0.01 ± 0.01(2) |
| 2. No NADH | 0.01 ± 0.01(2) | 0.01 ± 0.01(2) |
| 3. No PMS | 0.02 ± 0.01(3) | 0.01 ± 0.01(3) |
| 4. Boiled P-450[f] | 0.03 ± 0.02(2) | – |
| 5. Myoglobin (no P-450)[g] | None | – |

[a]Incubations were done using optimized conditions unless otherwise indicated. Optimized conditions: 1 $\mu M$ P-450-CAM, 600 $\mu M$ d-camphor, 3 m$M$ NADH, and 50 $\mu M$ PMS, in 20 m$M$ phosphate buffer (pH 7.40, and 100 m$M$ KCl), 2 ml total volume, with gentle oxygen bubbling. In the presence of Pd (3–5 $\mu M$), 5 m$M$ NADH was used. [b]Two minute incubations. [c]The number in parentheses is the number of trials of a particular experiment. [d]Ten minute incubations without oxygen bubbling using optimized conditions except as follows: 1.6 m$M$ NADH, 250 $\mu M$ PMS. [e]Same conditions as described in footnote d, plus oxygen bubbling. [f]P-450-CAM was boiled for 10 minutes prior to use. [g]1 $\mu M$ myoglobin.

TABLE II. Inhibition Studies.

| Substance | Amount | Inhibition of product formation[a] (%) | |
|---|---|---|---|
| | | Without oxygen bubbling[b] | With oxygen bubbling and Pd[c] |
| Metyrapone | 5 $\mu M$ | 23.3% | 29.5% |
| | 25 $\mu M$ | 40.9% | 42.0% |
| | 50 $\mu M$ | 47.5% | 53.3% |
| | 125 $\mu M$ | 59.8% | 59.6% |
| Catalase | 1000 $\sigma$ units/ml | none[d] | – |
| | 3000 $\sigma$ units/ml | – | none[d] |
| Superoxide dismutase | 20 $\sigma$ units/ml | none[d] | none[d] |
| Carbon monoxide | 1:3[e] | – | 67.8 ± 1.7(2) |
| | 1:1 | – | 87.1 ± 3.7(2) |
| | 3:1 | – | 94.8 ± 0.2(2) |

[a]% inhibition = 100–100 [Mol *exo*-5-hydroxycamphor formed with added inhibitor divided by Mol *exo*-5-hydroxycamphor formed in absence of inhibitor (see data in Table IA)]. [b]Ten minute incubations under optimized conditions (see Table I) except as follows: 1.6 m$M$ NADH, 200 $\mu M$ PMS. [c]Two minute incubations under optimized conditions (see Table I), plus 3 $\mu M$ Pd. [d]Within experimental error, no inhibition was seen. [e]Two minute incubations, optimized conditions, bubbled with the indicated carbon monoxide to oxygen ratio.

Since reconstitution of the P-450-CAM system requires the isolation and purification of three proteins, we have sought to develop new methods to replace one or both of the electron transfer proteins (fp, Pd) with artificial electron mediators. We have found that phenazine methosulfate (3-methylphenazinium methyl sulfate, PMS), a widely used electron mediator in biological systems, can successfully mediate electron transfer from NADH to cytochrome P-450-CAM in the absence of putidaredoxin reductase and in either the absence or presence of putidaredoxin (Table I). The addition of PMS to aerobic reaction mixtures containing NADH, camphor and cytochrome P-450-CAM results in the formation of *exo*-5-hydroxycamphor as the only product detected by gas chromatography. The relationship between the P-450-CAM concentration and the amount of hydroxylated product formed is linear. The effect of time on product formation has also been examined. The addition of putidaredoxin to the NADH/PMS/P-450-CAM system results in approximately a twenty-fold increase in the amount of product formed in a given time period. In the absence of P-450-CAM, PMS, or NADH or when myoglobin is substituted for P-450-CAM, no product is formed. Product formation is not inhibited by the addition of catalase, superoxide dismutase or hydroxyl radical traps

TABLE III. Effects of Continuous Bubbling with Molecular Oxygen.

| | *Exo*-5-hydroxycamphor formed (Mol/Mol of Cytochrome P-450-CAM)[a] | |
|---|---|---|
| | Without Oxygen Bubbling[b] 2 min | With Oxygen Bubbling[c] 2 min |
| Standard incubation | 0.4 ± 0.1(5) | 2.7 ± 0.2(3) |
| Plus 2,3-dimercaptopropanol (20 $\mu M$) | 1.4 ± 0.1(3) | 2.7 ± 0.3(2) |
| Plus Putidaredoxin (5 $\mu M$) | 8.2 ± 2.1(2) | 49.8 ± 1.5(6) |

[a]The number in parentheses is the number of trials of a particular experiment. [b]Optimized conditions (see Table I) except as follows: 1.6 m$M$ NADH, 250 $\mu M$ PMS. [c]Experiments were carried out under optimized conditions (see Table I).

(hydroquinone, sodium benzoate). However, significant inhibition is observed upon addition of carbon monoxide or metyrapone, both of which are known to inhibit P-450-catalyzed reactions (Table II).

Constant bubbling of the PMS-mediated hydroxylation system with oxygen leads to increased product formation (Table III). This increase is approximately six-fold for both the NADH/PMS/P-450-CAM and the NADH/PMS/Pd/P-450-CAM systems under optimized conditions. Addition of 2,3-dimercaptopropanol, a dithiol compound which has previously been shown to increase the amount of product formed from oxy-P-450 during *partial* turnover experiments done in the absence of Pd [2], results in a greater than three-fold increase in product formation in the absence of oxygen bubbling. Dimercaptopropanol has no effect in the presence of oxygen bubbling.

Reaction conditions were optimized by quantitating the amount of hydroxycamphor formed in a given time while varying the concentration of either PMS, NADH, or putidaredoxin. Relative concentrations thus obtained were termed 'optimized conditions' (Table III) and were used in subsequent experiments. Under optimum conditions with oxygen bubbling, a turnover number of about 1.5 mol of product formed per mol of P-450-CAM per minute was observed. Addition of putidaredoxin increases the turnover number to nearly thirty (Table I). While a turnover number of thirty is only approximately two percent of the value for the fully reconstituted three protein system [2, 3], it represents a substantial improvement over previous attempts to turnover the P-450 system without full reconstitution [4].

In summary, the formation of *exo*-5-hydroxycamphor as the only product along with the results

of the inhibition and oxygen bubbling studies support the hypothesis that hydroxylation by the NADH/PMS/P-450-CAM system occurs by an oxygen-dependent enzymatic pathway. The role of the PMS in the system appears to be as a mediator of electrons from NADH to P-450. This system provides the first protocol for achieving molecular oxygen dependent multiple turnovers of P-450 in the absence of the fully reconstituted three protein system.

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## The Active Sites of Chloroperoxidase and Cytochrome P-450-CAM: Comparative Spectroscopic and Ligand Binding Properties

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Spectral similarities between chloroperoxidase (CPO) and cytochrome P-450 (P-450) have previously been used to suggest endogenous thiolate axial ligation to the CPO heme iron [1–6] as has been established for P-450. Both enzymes exhibit unique hyperporphyrin ('split Soret') spectra in their ferrous CO forms [1]. However, no free sulfhydryl groups available for ligation to the heme iron have been found in chemical studies of CPO [7]. The MCD [4] and EPR [6] spectroscopic properties of analogous CPO and P-450 derivatives are generally similar, although some differences do exist. To obtain additional information about the active site structure of CPO, we have carried out extensive optical

Abbreviations: CPO, chloroperoxidase; P-450, cytochrome P-450; CAM, camphor; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance.

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TABLE I. Soret Absorption Maxima (nm) and Spin States of the Ligand Complexes of Ferric CPO and P-450-CAM.[a]

| Ligand | CPO[b] | | P-450-CAM[c] | |
|---|---|---|---|---|
| | $\lambda(\epsilon_{mM})$ | Spin[d] State | $\lambda(\epsilon_{mM})$ | Spin[d] State |
| $SH^-$ | 449(e) | ls | 467(43)[f] | ls |
| $CH_3COS^-$ | 446(62)[g] | ls | 459[e] | ls |
| $CN^-$ | 439(92) | ls | 439(78) | ls |
| Pyridine | 439(82) | ls | 421(111) | ls |
| NO | 437(114) | ls | 430.5(105) | ls |
| $CH_3(CH_2)_3NC$ | 435(105) | ls | 429.5(98) | ls |
| $N_3^-$ | 432(110) | ls | 427(81) | ls |
| $SeCN^-$ | 432(80) | ls | 425(90) | ls |
| $SCN^-$ | 429(112) | ls | 425(98) | ls |
| Imidazole | 429(100) | ls | 425(98) | ls |
| $NO_2^-$ | 427.5(101) | ls | [e] | |
| $OCN^-$ | ~427[e] | ls | 418.5(98) | ls |
| $HCOO^-$ | 425(121) | ls | 419(106) | ls |
| $HOCH_2CH_2SH$ | 418(69) | ms | 464(58)[f] | ls |
| $CH_3CH_2CH_2SH$ | 416(74) | ms | 417(~60), 464(~33)[f] | ls |
| $CH_3SCH_3$ | 416(93) | ms | 424(91) | ls |
| Thioxane | 416(88) | ms | 418(97) | ls |
| $CH_3SSCH_3$ | 416(93) | ms | 418(98) | ls |
| $CH_3COO^-$ | 413(97) | ms | 420(98) | ls |
| $F^-$ | 409(98)[g] | hs | no binding | |

[a]0.1 *M* $K^+$ Phosphate, 4 °C. [b]pH 6.0. [c]pH 7.0, (Refs. 9–11). [d]ls, low spin; ms, high-low mixed spin; hs, high spin. [e]Unstable. [f]Red Soret of hyperporphyrin spectrum (Ref. 11). [g]pH 3.0.

TABLE II. Soret Absorption Maxima (nm) of the Ligand Complexes of Ferrous CPO and P-450-CAM.

| Ligand | CPO[a] $\lambda(\epsilon_{mM})$ | P-450-CAM[b] $\lambda(\epsilon_{mM})$ |
|---|---|---|
| $Me_2PhP$[c] | 459(d) | 460(98) |
| $CN^-$ | 454(~140) | Not observed |
| $CH_3(CH_2)_3NC$ | 452(141) | 452(106) |
| CO | 444(167) | 446(120) |
| NO | 440(114) | 438(83) |

[a]pH 6.0. [b]pH 7.0. [c]Dimethylphenylphosphine. [d]Not determined.