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NADH- and Oxygen-Dependent Multiple Turnovers of Cytochrome P-450-CAM without Putidaredoxin and Putidaredoxin Reductase†

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ABSTRACT: Phenazine methosulfate (PMS) has been successfully used to mediate electron transfer from NADH to cytochrome P-450-CAM in the absence of putidaredoxin and putidaredoxin reductase under aerobic conditions. Identification and quantitation of *exo*-5-hydroxycamphor, the only product, has been accomplished by gas chromatography. In the absence of cytochrome P-450-CAM, or when other heme proteins (hemoglobin, myoglobin, horseradish peroxidase) are substituted for P-450-CAM, no *exo*-5-hydroxycamphor is detected. Product formation is not inhibited by the addition of catalase, superoxide dismutase, or hydroxyl radical scavengers; however, significant inhibition is observed with carbon monoxide and metyrapone, known inhibitors of the fully reconstituted P-450 system. Addition of 2,3-dimercaptopropanol to the NADH/PMS/P-450 system leads to a 4-fold increase in product formation; when putidaredoxin is added (without dimercaptopropanol), a 20-fold increase in product formation is observed. Constant bubbling with oxygen results in a further increase in the amount of product (150-fold increase overall). Our results show that PMS can substitute for the electron-transfer proteins putidaredoxin and putidaredoxin reductase in the transfer of electrons from NADH to P-450-CAM, resulting in multiple turnovers. Molecular oxygen dependent multiple turnovers of cytochrome P-450 have not been previously observed without the fully reconstituted, three-protein system.

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 mono-oxygenase activates molecular oxygen for insertion of one oxygen atom into organic substrates with concomitant reduction of the other oxygen atom to water. Pyridine nucleotides serve as the ultimate source of reducing equivalents. Since the hemoprotein itself cannot react directly with NAD(P)H, an electron-transfer system is required (Figure 1) (Gunsalus & Sligar, 1978; Ullrich, 1979; White & Coon, 1980).

The bacterial cytochrome P-450 isolated from camphor-grown *Pseudomonas putida* (P-450-CAM)[1] utilizes molecular oxygen and NADH to hydroxylate camphor at the exo-5 position and initiate camphor degradation (Katagiri et al., 1968). Figure 1 summarizes the current understanding of the reaction cycle of P-450 (Gunsalus & Sligar, 1978). Electrons

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[1] Abbreviations: P-450-CAM, the camphor-hydroxylating cytochrome P-450 isolated from *Pseudomonas putida* grown on camphor; CAM, 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 (phenazine methosulfate); NMR, nuclear magnetic resonance.

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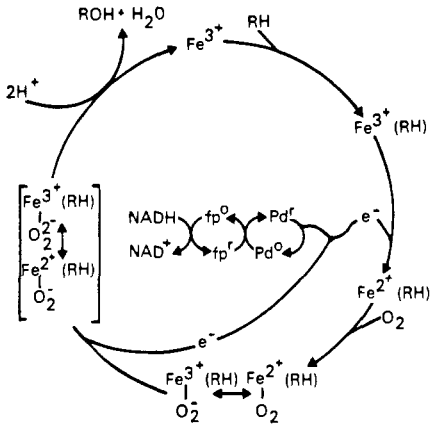

FIGURE 1: Reaction cycle of cytochrome P-450.

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). Reducing equivalents are transferred to P-450-CAM from putidaredoxin in two one-electron steps; the first occurs after the binding of camphor to ferric P-450, and the other follows the binding of oxygen and formation of the oxyferrous intermediate (Figure 1) (Gunsalus et al., 1971; Peterson et al., 1973).

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. Previous studies of the single and partial turnover of P-450 employing substitute, nonprotein electron sources have provided kinetic and mechanistic information regarding the normal electron-transfer sequence (Pederson et al., 1977; Sligar et al., 1980). Related experiments have suggested an effector role for putidaredoxin in the substrate hydroxylation reaction (Lipscomb et al., 1976). However, no multiple turnovers have been observed in previous studies of this type.

5-Methylphenazinium methyl sulfate, commonly known as phenazine methosulfate (PMS), is widely used as an electron mediator in biological systems. First used as an oxidant in electron-transfer reactions of mitochondrial proteins (Kearny & Singer, 1956), PMS has since been used to mediate the NADH-dependent reduction of a variety of protein electron acceptors including cytochrome *c* oxidase (Löw & Vallin, 1963), hemoglobin (Kajita et al., 1970), and liver microsomal cytochrome P-450 (Kulkarni & Hodgson, 1980).

In order to replace putidaredoxin and putidaredoxin reductase as mediators of electrons from NADH to P-450-CAM, we examined phenazine methosulfate as an artificial (nonprotein) electron mediator. Our study indicates that PMS can successfully mediate electron transfer from NADH to cytochrome P-450 in either the absence or the presence of putidaredoxin. Under optimized conditions, multiple turnovers are accomplished by the NADH/PMS/P-450 system.

Materials and Methods

All chemicals were reagent grade and were purchased from Aldrich or Sigma. Cytochrome P-450-CAM was purified from *Pseudomonas putida* grown on *d*-camphor by a modification (Dawson et al., 1982) of the reported literature procedure (Gunsalus & Wagner, 1978; O'Keeffe et al., 1978) and had an $A_{391}/A_{280} \geq 1.46$. Putidaredoxin was kindly provided by Prof. Stephen G. Sligar, Yale University. The concentrations of P-450-CAM and putidaredoxin were determined from published extinction coefficients (Gunsalus & Wagner, 1978). Catalase (bovine liver), superoxide dismutase (type I, bovine blood), and myoglobin (type II, sperm whale) were purchased from Sigma. Myoglobin was handled and stored as described previously (Sono & Dawson, 1982). Horseradish peroxidase (type VI, Sigma) was further purified by a literature procedure (Shannon et al., 1966) to an RZ (A_{403}/A_{275}) value greater than 3.5. Hemoglobin was prepared from fresh blood (hemoglobin A) in the oxygenated form by Dr. Masanori Sono, in our laboratory, according to the method described by Antonini & Brunori (1971).

Quantitation of *exo*-5-hydroxycamphor was accomplished by using a Varian 1400 series gas chromatograph equipped with a 3% OV-17 column ($^1/_8$ in. × 6 ft), a flame ionization detector, and a Hewlett-Packard 3390A reporting integrator. Samples were examined under conditions that resulted in base-line separation of the *exo*- and *endo*-5-hydroxycamphor standards (145 °C initial temperature, 2 °C/min linear temperature program). In Tables I and II, moles of *exo*-5-hydroxycamphor formed are expressed as average ± standard deviation (number of trials). The "optimized conditions" for incubations (2 mL) in the absence of putidaredoxin contained final concentrations as follows: 1 μM P-450-CAM, 600 μM *d*-camphor, 3 mM NADH, and 50 μM PMS, in 20 mM phosphate buffer (pH 7.40 and 100 mM KCl; with gentle oxygen bubbling). For samples containing putidaredoxin (3–5 μM), it was found that a NADH concentration of 5 mM was needed.

Experiments were performed at 27 °C and were protected from light. Incubation mixtures were preincubated for 5 min prior to the addition of NADH, and the reaction was then initiated by the addition of PMS. Oxygen bubbling was accomplished by passing a gentle stream (approximately 12 mL/min) of oxygen through a glass frit inserted in the reaction mixture. The reaction was terminated by a mixing with an equal volume of methylene chloride, at which time the internal standard (methyl *p*-hydroxybenzoate) was added. Samples were then centrifuged, and the organic layer was concentrated prior to gas chromatographic analysis. Identification of the product as *exo*-5-hydroxycamphor was accomplished by mass spectral analysis and comparison with authentic *exo*- and *endo*-5-hydroxycamphor purified from *P. putida* bacterial broth by a modification of the procedure of Bradshaw et al. (1959). The structures of the isolated hydroxycamphors were assigned from ^1H and ^{13}C NMR and mass spectral analysis. Both standards were greater than 99% pure by gas chromatography. The melting point of the exo alcohol (228.5–230 °C) is slightly higher than that previously reported (Bradshaw et al., 1959).

Control Experiments. Incubations were conducted for 10 min under optimized conditions, both with and without Pd, in the absence of either P-450, NADH, or PMS. Under these conditions, no significant product formation was observed. In control experiments using P-450 that was boiled for 10 min prior to use, within experimental error, no product was formed. When 1 μM myoglobin, hemoglobin, or horseradish peroxidase was substituted for P-450 in incubations under optimized conditions, again no product was formed. Anaerobic incubation samples were prepared by bubbling the reaction mixture with N_2 (12 mL/min) prior to starting the incubation and during the incubation. These anaerobic incubations resulted in less than 5% of the amount of product observed in aerobic incubations.

Inhibition Studies. Details and results of carbon monoxide and metyrapone inhibition studies are given in Table III. Incubations with catalase (1000 or 3000 Sigma units/mL), superoxide dismutase (20 Sigma units/mL), or hydroxyl

Table I: Product Formation[a]

(A) As a Function of P-450-CAM Concentration

P-450-CAM concn (μM)	nmol of *exo*-5-hydroxycamphor formed[b,c] with putidaredoxin (3 μM)	without putidaredoxin
0	0.01 ± 0.01 (2)	0.02 ± 0.01 (2)
0.5	23.6 ± 1.0 (2)	1.3 ± 0.1 (2)
1.0	49.8 ± 1.5 (6)	2.7 ± 0.2 (3)
2.0	97.0 ± 2.5 (2)	5.1 ± 0.2 (3)

(B) As a Function of Time

incubation (min)	mol of *exo*-5-hydroxycamphor formed per mol of P-450-CAM[c] with putidaredoxin (3 μM)	without putidaredoxin
1	28.8 (1)	1.5 ± 0.1 (2)
2	49.8 ± 1.5 (6)	2.7 ± 0.2 (3)
5	101.7 (1)	5.8 ± 0.1 (2)
10	188.7 ± 5.0 (2)	9.8 ± 0.3 (2)

[a] Incubations were done under optimized conditions (see text) unless otherwise indicated. See Materials and Methods for the control experiments. [b] Two-min incubations. [c] The number in parentheses is the number of trials of a particular experiment.

Table II: Effects of Continuous Bubbling with Molecular Oxygen

	mol of *exo*-5-hydroxycamphor formed per mol of cytochrome P-450-CAM in 2 min[a] without oxygen bubbling[b]	with oxygen bubbling[c]
standard incubation	0.4 ± 0.1 (5)	2.7 ± 0.2 (3)
plus 2,3-dimercaptopropanol (20 μM)	1.4 ± 0.1 (3)	2.7 ± 0.3 (2)
plus putidaredoxin (5 μM)	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 text) except as follows: 1.6 mM NADH, 250 μM PMS. [c] Optimized conditions (see text).

radical scavengers (hydroquinone, 200 μM; sodium benzoate, 500 mM) did not result in detectable inhibition of product formation.

Results

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. There is a linear relationship between the P-50-CAM concentration and the amount of hydroxylated product formed (Table I). The effect of time on product formation has also been examined (Table I). The addition of putidaredoxin to the NADH/PMS/P-450-CAM system results in approximately a 20-fold increase in the amount of product formed in a given time period. In the absence of P-450-CAM, PMS, NADH, or O_2, little or no product is formed either with or without putidaredoxin. When other heme-containing proteins (hemoglobin, myoglobin, horseradish peroxidase) or denatured P-450 (P-420) is substituted for cytochrome P-450-CAM, *exo*-5-hydroxycamphor is not formed. Product formation is not inhibited by the addition of catalase, superoxide dismutase, or hydroxyl radical traps (hydroquinone, sodium benzoate) in either the presence or the absence of putidaredoxin. In addition, oxygen bubbling (Table II) and 2,3-dimercaptopropanol (Figure 2) have been

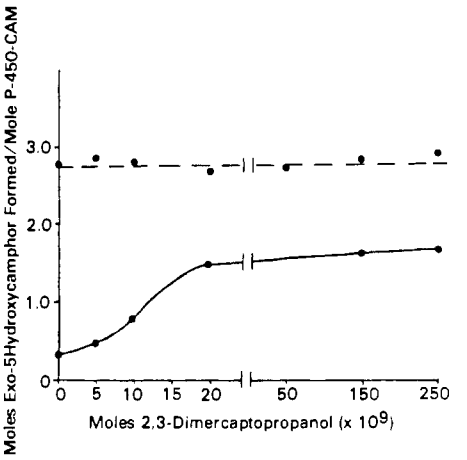

FIGURE 2: Product formation as a function of 2,3-dimercaptopropanol concentration: (--) with oxygen bubbling, 2-min incubations, optimized conditions (see text) except 250 μM PMS; (—) without oxygen bubbling, 10-min incubations, optimized conditions (see text) except 1.6 mM NADH and 250 μM PMS.

Table III: Inhibition Studies[a]

substance	amount	percent inhibition of product formation[b] without Pd	with Pd
metyrapone[c]	5 μM	23	30
	25 μM	41	42
	50 μM	48	53
	125 μM	60	60
carbon monoxide[d]	1:3	52 (2)	68 (1)
	1:1	83 (6)	87 (3)
	3:1	93 (10)	95 (10)
	9:1	99 (13)	97 (14)

[a] See Materials and Methods for information concerning inhibition studies with catalase, superoxide dismutase, and hydroxyl radical scavengers. [b] Percent inhibition = 100 − 100(moles of *exo*-5-hydroxycamphor formed with added inhibitor divided by moles of *exo*-5-hydroxycamphor formed in absence of inhibitor) (see data in Table IA). [c] Incubations without Pd were for 10 min under optimized conditions (see text) except as follows: 1.6 mM NADH, 200 μM PMS. Incubations with Pd (3 μM) were for 2 min under optimized conditions (see text). [d] Two-minute incubations under optimized conditions (see text). The solutions were bubbled with the indicated carbon monoxide to oxygen ratio. Two trials were done and the results averaged; the variability between trials was less than 5% of the number reported. Numbers in parentheses represent the results of control experiments in which solutions were bubbled with the indicated nitrogen to oxygen ratio.

separately found to increase product formation, while metyrapone (Table III) and carbon monoxide (Table III) inhibit product formation.

Constant bubbling of the PMS-mediated hydroxylation system with oxygen leads to increased product formation (Table II). This increase is approximately 6-fold for both the NADH/PMS/P-450 and the NADH/PMS/Pd/P-450 systems under optimized conditions. Bubbling with oxygen did not result in the conversion of P-450 to P-420; examination of protein samples after bubbling showed no P-420 formation as judged by the lack of a peak at 420 nm in the UV–visible absorption spectrum of the reduced + CO form. The effect of adding 2,3-dimercaptopropanol to the NADH/P-450-CAM system is seen in Figure 2. 2,3-Dimercaptopropanol has previously been shown to increase the amount of product formed from oxy-P-450 during partial turnover experiments done in the absence of putidaredoxin and was suggested to play an effector role in facilitating electron transfer (Lipscomb et al., 1976). Product formation increases by greater than 3-fold

upon increasing the concentration of dimercaptopropanol to 20 μM in the absence of oxygen bubbling. In the presence of oxygen bubbling, a corresponding increase in product formation upon the addition of dimercaptopropanol is not seen, although the actual amount of product formed is about twice as much due to oxygen bubbling as discussed above.

The effect of micromolar concentrations of metyrapone on the formation of *exo*-5-hydroxycamphor is shown in Table III. Metyrapone, a pyridine derivative that has been shown to inhibit cytochrome P-450 catalyzed reactions (Dominguez & Samuels, 1963; Leibman, 1969; Wood, 1979) including camphor hydroxylation (Peterson et al., 1971), binds competitively with camphor at the active site (Griffin et al., 1974; Mock et al., 1982). The concentration of metyrapone necessary to inhibit 50% of the original hydroxylation activity (I_{50}) in the presence and absence of the combination of putidaredoxin (3 μM) and oxygen bubbling is approxmiately 50 and 75 μM, respectively. When reaction mixtures were bubbled with carbon monoxide, inhibition of product formation was observed (Table III; $I_{50} \sim$ 20–25% CO). Carbon monoxide is known to inhibit P-450-catalyzed reactions by binding to the ferrous form of the enzyme (Peterson & Griffin, 1972).

In an attempt to maximize the amount of hydroxycamphor formed in a given time in the presence of oxygen bubbling, a series of experiments were conducted in which the concentration of either PMS, NADH, or putidaredoxin was varied at fixed P-450 (1 μM) and camphor (600 μM) concentrations in the absence of dimercaptopropanol. The lowest concentration of each variable component that gave a maximal yield of hydroxycamphor was then used in future experiments. Relative concentrations thus obtained were termed "optimized conditions" (see Material and Methods). The required amount of NADH was different for the systems with and without putidaredoxin (5 and 3 mM, respectively). Under optimum conditions, a turnover number of about 1.5 mol of product formed per mol of P-450-CAM per min was observed; with putidaredoxin and with oxygen bubbling, a turnover number of nearly 30 was found (Table I).

Discussion

The potential usefulness of phenazine methosulfate as an artificial (nonprotein) electron mediator between NADH and cytochrome P-450-CAM has been examined. Our results demonstrate that PMS can substitute for the electron-transfer proteins putidaredoxin reductase and putidaredoxin (Table I), although the yield of hydroxylated camphor is low. With addition of 2,3-dimercaptopropanol or putidaredoxin, with simultaneous oxygen bubbling, dramatic increases in product formation are observed (Table II). In the presence of putidaredoxin and oxygen bubbling, a turnover number of approximately 30 is achieved. This is approximately 50% of the value for the fully reconstituted three-protein system (White et al., 1984) and represents a substantial improvement over previous attempts to turnover the P-450 system without full reconstitution (Lipscomb et al., 1976). In fact, this study represents the first example of oxygen-dependent multiple turnovers of cytochrome P-450 without the fully reconstituted three-protein system.

Previously, Gunsalus and co-workers have presented evidence of an effector role for putidareodoxin in the formation of hydroxylated product form oxyferrous substrate-bound P-450-CAM (Lipscomb et al., 1976). In accord with these results, we find that addition of putidaredoxin to the NADH/PMS/P-450-CAM system results in an increased rate and amount of product formation (Tables I and II). Further, 2,3-dimercaptopropanol, a dithiol compound that can replace putidaredoxin in its effector role (Lipscomb et al., 1976), also stimulates product formation in our system (Table II). Neither putidaredoxin nor dimercaptopropanol was able to support hydroxylation by this system in the absence of PMS, confirming the need for an electron mediator.

The reaction of NADH with PMS to give the fully reduced form of PMS (5,10-dihydro-5-methylphenazine) and the interaction of reduced PMS with oxygen has been examined in several laboratories (Nishikimi et al., 1972; Halaka et al., 1982; Richter & Waddell, 1982; Richter et al., 1982); these studies have suggested that superoxide radical ($O_2^{-}\cdot$) or hydroxyl radical (HO$\cdot$) is formed from oxygen and reduced PMS. In fact, the NADH/PMS/O_2 system has been shown to be capable of hydroxylating aromatic substrates (Prema Kumar, 1972; Halliwell, 1977). The possibility that these types of nonenzymatically formed activated oxygen species are involved in the PMS-mediated hydroxylation of camphor reported here is eliminated by the control experiments. For example, when P-450-CAM is omitted from the reaction mixture or boiled prior to use, no product is detected. In addition, the formation of hydroxycamphor is not inhibited by catalase, superoxide dismutase, or hydroxyl radical scavengers, suggesting that free hydrogen peroxide, superoxide, or hydroxyl radical is not involved in the hydroxylation process. The lack of catalase or superoxide dismutase inhibition is characteristic of normal P-450-catalyzed reactions (Gorrod, 1978). These results do not preclude the formation of HO$\cdot$, H_2O_2, or $O_2^{-}\cdot$ in the reaction mixture. However, if such species are formed, they either are tightly bound to the enzyme or are not involved in the camphor hydroxylation reaction at all. Furthermore, since catalase is effective at decomposing alkyl hydroperoxides (Schonbaum & Chance, 1976), the lack of catalase inhibition of our system precludes the formation of an alkyl hydroperoxide from NADH/PMS/O_2 followed by cycling of P-450 by the peroxide shunt.[2] The NADH/PMS/O_2 system is significantly inhibited by metyrapone, a known inhibitor of cytochrome P-450-CAM catalyzed reactions, providing more evidence for an enzyme-dependent reaction. Although a direct comparison may not be appropriate since different sources of P-450 are involved, the values of I_{50} obtained in this study are, in fact, very similar to those seen in the metyrapone inhibition of hepatic microsomal P-450 coumarin hydroxylase activity (Wood, 1979). Inhibition of the NADH/PMS/P-450 system by carbon monoxide requires that the reaction pathway of the system involves the ferrous state (Figure 1), as carbon monoxide does not bind to the ferric heme iron. Finally, it is important to note that the only product formed, *exo*-5-hydroxycamphor, is the same product as is normally produced by P-450. Presumably, if a nonenzymatic mechanism were operating, this type of regio- and stereospecificity would not be seen.

The results of the oxygen bubbling experiments suggest that oxygen is being used up in a side reaction in competition with hydroxylation. Without oxygen bubbling, the formation of hydroxycamphor only occurs during the first few minutes of the incubation, after which little or no additional product is formed (data not shown). By contrast, in the presence of oxygen bubbling, product formation as a function of time is nearly linear for at least 10 min (Table I). This suggests that the oxygen dissolved in the buffer is rapidly used up, a result that cannot be accounted for solely by depletion of oxygen

[2] Numerous laboratories have shown that P-450 can be turned over with reduced oxygen equivalents (H_2O_2, ROOH, etc.) and the substate-bound ferric enzyme (Gunsalus & Sligar, 1978; White & Coon, 1980).

during product formation. Furthermore, when NADH disappearance is monitored by observing the loss in absorbance at 340 nm, the rate of NADH oxidation is found to be several times faster than product formation. This accounts for the relatively large amount of NADH necessary to achieve optimal results. Apparently, a significant fraction of the reduced PMS, formed from reaction with NADH, is involved in reducing oxygen rather than P-450. The ultimate fates of the products of these side reactions have not been determined.

The role of PMS in this system appears to be as a mediator of electrons from NADH to P-450-CAM (Figure 1). The reaction is catalytic in PMS, since 2.5 nmol of *exo*-5-hydroxycamphor were formed in incubations containing 1 nmol of PMS in the absence of putidaredoxin. In the presence of putidaredoxin, 31.6 nmol of product was formed with 1 nmol of PMS. Whether the reduction of PMS by NADH and the electron transfer from reduced PMS to P-450-CAM occur in one- or two-electron steps is unknown. While the one-electron-reduced semiquinone form of PMS is relatively unstable near neutral pH, stabilizing effects such as binding by an enzyme or combination with reacting molecules or ions in solution suggest that reduction or oxidation of PMS through the semireduced species in electron-transfer reactions of biological systems is possible (Zaugg, 1964; Chew & Bolton, 1980). An interesting feature of this system is the relative oxidation–reduction potentials of PMS and P-450-CAM. The two-electron reduction of PMS occurs at a potential of 80 mV (Dickens & McIlwain, 1938), approximately 250 mV more positive than that of substrate-bound P-450 ($E_0' = -0.170$V), indicating that the transfer of electrons from reduced PMS to P-450-CAM is a thermodynamically unfavorable process. The fact that the electron transfer does occur suggests that the redox potential of PMS is somehow altered, a phenomenon that has been observed in other PMS-containing systems (Ottaway, 1966; Zaugg et al., 1964) and may be attributed to interaction of the reduced or semireduced form of PMS with P-450 or another component of the incubation system.

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 with PMS serving as a mediator of electrons from NADH to P-450 (Figure 1). This system provides the first protocol for achieving oxygen-dependent multiple turnovers of P-450 in the absence of the fully reconstituted system. Just as the studies of the peroxide shunt mechanism of turning over the P-450 system[2] have led to important discoveries about the mechanism of oxygen activation by P-450 (Blake & Coon, 1980, 1981a,b; White et al., 1980), it is hoped that similar progress will result from further studies of the NADH/PMS/O_2 turnover of P-450.

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Registry No. PMS, 299-11-6; P-450, 9035-51-2; NADH, 58-68-4; oxygen, 58-68-4; carbon monoxide, 630-08-0; metyrapone, 54-36-4; 2,3-dimercaptopropanol, 59-52-9.

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Site-Specific Modification of *Escherichia coli* DNA Polymerase I Large Fragment with Pyridoxal 5′-Phosphate†

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ABSTRACT: Pyridoxal 5′-phosphate (PLP) is an inhibitor of DNA polymerase activity of *Escherichia coli* DNA polymerase I large fragment. Kinetic studies indicated that overall PLP inhibition was noncompetitive with respect to dNTP, and Hill plot analysis revealed that two molecules of PLP were involved in the inhibition. Reduction of the PLP-treated enzyme with sodium [^{3}H]borohydride resulted in covalent incorporation of 3 mol of PLP/mol of enzyme. This incorporation was at lysine residues exclusively, and the PLP-modified enzyme was not capable of DNA polymerase activity. The presence of dNTP during the modification reaction blocked the incorporation of 1 mol of PLP/mol of enzyme. Similar results were obtained in the presence or absence of template-primer. These data indicate that a PLP target lysine is in or around a dNTP binding site that is essential for polymerase activity and that this binding site is functional in the absence of template-primer. The enzyme modified in the presence of dNTP, containing 2 mol of PLP/mol of enzyme, was capable of DNA polymerase activity but was unable to conduct elongation of product molecules beyond a short oligonucleotide length.

Pyridoxal 5′-phosphate (PLP) modification of proteins is an effective approach toward active-site identification and dissection of distinct phases of the enzymatic mechanism and the ligand–receptor interaction. For example, Ohsawa & Gualerzi (1981) used this approach to identify the 30S ribosomal binding site of *Escherichia coli* intiatin factor IF3 and to distinguish this site from an AUG trinucleotide binding site. Benesch et al. (1982) found that PLP specifically modified the polyphosphate binding site of deoxyhemoglobin. Papas et al. (1977) used PLP modification to identify a dNTP binding site in the α-subunit of avian myeloblastosis virus DNA polymerase and to demonstrate that the β-subunit of the enzyme is devoid of a dNTP binding site.

We are using the PLP modification approach to examine the mechanism of *E. coli* DNA polymerase I large fragment (Pol I lf). It had been shown earlier (Modak, 1976) that PLP is an inhibitor of the polymerase activity of Pol I. In this paper, we report pyridoxylation of a lysine residue in or around a dNTP binding site that appears to be essential for polymerase activity. a template-primer was not required for dNTP to protect the enzyme against pyridoxylation at this binding site. In addition, we report that pyridoxylation in the presence of dNTP results in an enzyme containing 2 mol of PLP/mol of enzyme. This modified enzyme has altered polymerase properties such that each cycle of template-primer binding plus synthesis results in addition of only a few dNMP residues to the primer. These results are discussed in the context of current kinetic models for the polymerase activity of Pol I (McClure & Jovin, 1975; McClure & Chow, 1980; Detera & Wilson, 1982).

Materials and Methods

Materials. E. coli DNA polymerase I large fragment was from New England Nuclear Corp. (catalog no. NEE-102, lot no. 1607-023). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoretic analysis and Coomassie blue staining revealed that the only visible protein in the preparation was the M_r 68 200 enzyme polypeptide. The enzyme was stored at −20 °C in 100 mM KP_i buffer, pH 7.0, 1 mM dithiothreitol, and 50% glycerol. Tritium-labeled thymidine 5′-triphosphate and sodium borohydride also were from NEM. The latter was stored in crystalline form at 4 °C and was dissolved in 5 mM NaOH immediately before use. Polynucleotides and unlabeled deoxyribonucleoside 5′-triphosphates were from P-L Biochemicals. PLP and analogues were from Sigma, V8 protease was from Miles, and equipment and chemicals for gel elec-

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