The Roles of Putidaredoxin and P450 in Methylene Hydroxylation*

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SUMMARY

Putidaredoxin, the iron sulfur protein from the P450- mediated methylene hydroxylase system of Pseudomonas putida, is shown to be essential both as an electron transfer agent and as an effector of P450 necessary for product formation. P450 is recognized to progress through four well defined states in an ordered reaction cycle of oxidized, and, with substrate, oxidized, reduced, and oxygenated. Combination of reduced putidaredoxin with each of these states of P450, or of oxidized putidaredoxin with the reduced or oxygenated P450 in the presence of substrate and O2 yields product stoichiometrically. Putidaredoxin is not replaced efficiently by other iron sulfur proteins, nor by the phospholipid from the hepatic microsomal P450 system.

Rapid scan spectroscopy shows the oxygenated form of P450 to be dominant during NADH turnover in the reconstituted system. A scheme is developed for the reaction cycle in which the reaction intermediates are postulated to facilitate electron transfer and determine the nature of the product formed. Analytical treatment of steady state kinetic data suggests that a 1:1:1 equivalence of reductase to putidaredoxin to P450 in the catalytic complex.

Recent publications have been concerned with the role of iron-sulfur proteins in multicomponent hydroxylase systems (1–3). These proteins have been inferred to function as intermediate electron transfer agents between flavoprotein reductase and a specific terminal oxidase (4–10). The tendency to implicate iron-sulfur proteins as electron transfer agents for electrons from a flavoprotein reductase to a specific terminal oxidase (4–10). The tendency to implicate iron-sulfur proteins as intermediate electron transfer agents derives mainly from steady state kinetic, stereochemical, and substrate specificity studies that only 1 electron for oxygen reduction is transferred by way of the iron-sulfur component, the 2nd being transferred directly to an oxygenated form of the terminal oxidase by reduced pyridine nucleotides (4, 12). In drug and steroid hydroxylation in liver microsomes, cytochrome b5 has been postulated to transfer the 2nd electron to an oxygenated P450 complex (13). In fatty acid hydroxylation by P450 in liver microsomes, an iron-sulfur protein is not utilized for electron transfer but evidence suggests that a phospholipid is required (14). Clarification of the role of iron-sulfur proteins is pivotal to the development of a model for biochemical action of multicomponent hydroxylation systems.

This laboratory has been pursuing research directed toward understanding monoxygenase mechanisms by using the camphor hydroxylase system from Pseudomonas putida (10, 15). Reports on our discovery of a specific requirement for putidaredoxin in product formation and of the 418 nm oxygenated form of P450 have been given (16), and a reaction scheme for hydroxylation developed (17). The oxygenated form has also been reported independently elsewhere (18). One approach has been to dissect the reaction into the probable elements and to study the interactions of the separate elements. We have purified the three components of the monoxygenase system and have studied their interactions by chemical and kinetic techniques. The kinetics section of this work, including fast reaction and analytical kinetic methods applied to the reconstituted system, is reported in detail here.

EXPERIMENTAL PROCEDURE

Materials—All chemicals were reagent grade supplied by the Eastman Kodak Co., Mann Research Laboratories, and the Sigma Chemical Co. Water was deionized and glass distilled. The enzyme components (10) were purified from P. putida, strain P, C786 grown on n-camphor (17). The molecular weights of the reductase, putidaredoxin, and P450 have been given (16), and a reaction scheme for hydroxylation developed (17). The oxygenated form has also been reported independently elsewhere (18). One approach has been to dissect the reaction into the probable elements and to study the interactions of the separate elements. We have purified the three components of the monoxygenase system and have studied their interactions by chemical and kinetic techniques. The kinetics section of this work, including fast reaction and analytical kinetic methods, is reported in detail here.
pyridine hemochromogen technique (17, 19) and by stoichiometric transfer of electrons from the reduced cytochrome to ferricyanide and dichloroindophenol anaerobically. The concentrations of all components were determined routinely by visible spectroscopy using the extinction coefficients given in Table I.

Putidaredoxin was prepared free of the other two components by the purification steps outlined (10, 17). Controls with crystalline P450cam (20) showed that small amounts of the reductase did not alter the outcome of the results reported here. The amount of P450cam impurity in the reductase preparations was monitored spectrophotometrically and kinetically, and was ordinarily 2 to 3%. Two flavoprotein impurities, putidaredoxin reductase and an FMN-containing protein, presumably the E1 component of the ketolactonase system (21, 22), were found by fluorimetry to be present in the P450cam preparations in varying proportions. Both impurities were found to enhance the aerobic rate of autonomous turnover of NADH; the FMN-containing impurity was an order of magnitude more catalytic. Because of this, preparations with high FMN content were not used.

Repeated freeze-thaw cycles (20 or more) resulted in less than 5% loss in biological activity of P450cam as long as ≥100 μM camphor was present. Stock preparations of putidaredoxin, which suffer degradation on repeated freezing and thawing, were not used after three such cycles. Pure adrenodoxin, clostridial, and spinach ferredoxins and Azoto bacter vinelandii iron sulfur protein II were kindly donated by Dr. W. H. Orme-Johnson, University of Wisconsin, Pseudomonas oleovorans rubredoxin (1 iron atom per molecule) and rabbit liver phosphatidylcholine by Dr. M. J. Coon, University of Michigan, and rabbit cytochrome b₅ from Dr. P. Strittmatter, University of Connecticut Health Center, Farmington, Conn.

Kinetic Methods—Experimental conditions were 300 μM camphor, 200 μM NADH, 50 mM potassium phosphate buffer, pH 7.4, 25° under air, unless otherwise stated (23); the enzymes were added last. P450cam final concentrations in studies of product yield and fast reactions were 8 to 15 μM after mixing, based on hemec content. At these concentrations and in the presence of 300 μM camphor more than 95% of the P450 is in the camphor complex (K₆ = 5 μM at 6 μM P450cam) (17). Spectra were taken on a Cary 14 recording spectrophotometer in anaerobic cuvettes of 1 cm light path.

Analytical Methods—Stopped flow measurements employed a machine with 0.8 msec deadtime, provision for anaerobicity in one and/or both syringes, and rapid-scan spectrophotometry, permitting 25 scans per sec over the wave length range of 350 to 450 nm. The principal wave lengths were calibrated with a holmium oxide plate. The instrument was designed by Professor S. Smith of the Organic Division whose courtesy in making it available we wish to acknowledge. Temperature jump experiments employed a transient spectrometer, type SBA-T, made by Studiengesellschaft GmbH., Gottingen, Germany, using n Teflon cell and “jumping” from 22 to 25°.

Vapor phase chromatography was carried out with an Aerograph Hy-Yi model 600 Chromatograph and an SE-30 column at 80–120° to optimize speed and peak resolution. Products were extracted directly from 1.20 ml of reaction solution with 0.30 of dichloromethane. Polarographic measurements were made with a Gilson oxygraph (Gilson Medical Electronics, Inc., Middleton, Wis.).

Quantitative analyses for NAD⁺ and H₂O₂, respectively, were made with yeast alcohol dehydrogenase purchased from Sigma Chemical Co. and horseradish peroxidase from Worthington Biochemical Company, using o-dianisidine, with slight modifications in the procedures described in their catalogs. The disap-
The concentration of K+ phosphate buffer was 50 mM at pH 7.4 and 25°.

| Enzyme | Photoreductant | Concentration | λzem | λobserved | ksec⁻¹ |
|--------|----------------|---------------|-------|------------|--------|
| P450oam | None           | P450, 10 μM   | 408  | 391        | 0.006  |
|         | EDTA, 20 mM    | P450, 10 μM   | 408  | 391        | 0.07   |
|         | EDTA, 20 mM +  | P450, 10 μM   | 408  | 391        | 0.15   |
|         | proflavin, 5 μM| P450, 10 μM   | 408  | 391        | 0.006  |
|         |                 | P450, 10 μM   | 408  | 391        | <5     |

* Data from R. L. Tsai and C.-A. Yu, except for reduced + O₂.
* Dithionite, 5 μM excess.
* At 5°, O₂ or CO bubbled.

The hydroxylated product, here termed cam-OH, was shown to be exclusively the 5'-exo epimer. The three proteins, putidaredoxin reductase, putidaredoxin, and P450, were used as catalysts. No H₂O₂ has been detected. The d and l enantiomers of camphor, their 1,2-lactones, and several very similar bicyclic monoterpenes serve as substrates (24).

The function of each enzyme component was studied after purification and characterization. Kinetic measurements implied that the reductase is the NADH-reactive site (24). Visible and resonance spectroscopic studies showed the P450 to be the substrate and oxygen-reactive component (10, 15, 23). Physical and kinetic studies of the four stable states of P450 during the oxidation-reduction cycle, namely, oxidized without substrate and, with substrate, oxidized, reduced, and oxygenated (15-17), were chosen as the local starting point in an investigation of the hydroxylation mechanism.

### Reaction States of Cytochrome P450cam Oxidized Species and Substrates Complex

P450cam in the oxidized form exhibits a Soret peak at 417 nm, ε₉₂₅ = 105 (15, 17). It decays with a half-life of 180 min at 25° under air to an altered and catalytically inactive form of the enzyme called P420. If the substrate is present, this conversion does not occur rapidly. Camphor binds strongly and causes a shift in the Soret to 391 nm, ε₉₂₅ = 88 (15, 17). Camphor also causes the α and β bands of P450cam to coalesce and elicits a band at 645 nm attributed to formation of high spin ferric iron (25).

### Reduced Species

The P450cam camphor complex can be reduced quantitatively with NADH in an anaerobic titration to an end point of 1 electron per heme with the formation of the 408 nm peak, ε₉₂₅ = 72 (15, 17). Concurrent with this reduction, the ferric iron epr signal disappears (23). NAD⁺ concentration does not increase after the 1 electron end point. P450cam is also photoreducible to a species exhibiting the same spectral characteristics. The velocities and reactants are shown in Table II.

In the full system without camphor present, NADH will reduce the reductase flavoprotein and the redoxin but not the cytochrome P450cam. The P450cam complex, however, is readily reducible. The maximal rate constants for reduction by NADH of the components separately and in combination are given in Table III. With reductase and redoxin both present, the rate of the cytochrome-camphor complex reduction is 10⁶ to 10⁷ times faster than by any other pathway and is nearly double the turnover number for this component during steady state product formation under the same experimental conditions.


7 bimolecular rate constant of 1.7 \times 10^6 s^{-1} shortened as the NADH and oxygenated complex disappear. A jump from 22° to 25° after 90 sec. At this time, 60% of the rate in which NADH-reduced P450 Cam is exposed to air and then sag, at 25°, containing 10 mM K+ phosphate buffer, pH 7.4, at 25°, reduced flavine dinucleotide, oxygen pressure, putidaredoxin reductase, or ketolactonase reductase. Excess of dithionite, reduced flavine dinucleotide, oxygen pressure, putidaredoxin reductase, or ketolactonase reductase. The rate constant, k_{250}, is 0.01 s^{-1}, 2000 times smaller than that saturating and NADH as the electron source. The rate of decay of the oxygenated cytochrome is not significantly affected by an excess of dithionite, reduced flavine dinucleotide, oxygen pressure, putidaredoxin reductase, or ketolactonase reductase.

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FIG. 2. P450 Cam substrate complexes: Soret absorption, ferric, ferrous and oxygenated states in 50 mM K+ phosphate buffer, pH 7.4, at 25°, containing 10 mM P450 Cam and 300 mM d(+)-camphor. Reduction is with white light in the presence of 20 mM EDTA, 4 \mu M proflavine under argon. P450 Cam, oxidized P450 Cam; P450 Cam, reduced P450 Cam; S, d-camphor.

In contrast, the oxygenated species disappears within milliseconds after the addition of an excess of reduced putidaredoxin (t < 10 msec). The decay rate with oxidized putidaredoxin is only slightly slower as reported by Peterson et al. (28). With either 30 \mu M H_2O_2 or cytochrome c, the k_d is 0.02 sec^{-1}, and with 300 \mu M H_2O_2, 0.06 sec^{-1}. The low reactivity of H_2O_2 suggests a small rate constant for P450 Cam, deoxygenation, (k_d), for otherwise H_2O_2, which reacts very fast with the reduced P450, would accelerate the conversion to the oxidized form. A k_d of 0.01 sec^{-1} was calculated from the forward rate constant and a K_d for O_2 of about 5 mM, derived from the K_d for CO, 50% inhibition with a CO-O_2 (1:1) gas mixture (6) and the fact that CO is 40 times more soluble than O_2 at 25°.

Minimum Requirements for Hydroxylation

P450 Cam and Putidaredoxin—The participation of at least four states of P450 Cam as intermediates in the hydroxylation reaction can best be demonstrated by beginning with each state and defining the minimum conditions for hydroxylated product formation. Putidaredoxin appears to be essential and specific for efficient product yields from any P450 Cam state (Tables IV, V, and VI).

In the absence of putidaredoxin, NADH is catalytically oxidized (Table IV) but at a rate at least 100-fold slower. Unlike Equation 1, the major reaction corresponds to

\[
\text{NADH} + \text{O}_2 + \text{H}^+ \rightarrow \text{NAD}^+ + \text{H}_2\text{O} \quad (2)
\]

Regardless of the initial state of the P450 Cam, NADH or NADPH yields less than 0.5% of the theoretically expected yield of hydroxylated product based on reducing equivalents supplied. Standard electron donors, for example, dithionite and methyl viologen, will reduce P450 Cam but fail to produce hydroxylated product.

TABLE IV

Role of putidaredoxin in product formation

The conditions were as in Table II, plus 10 \mu M P450 Cam and air atmosphere.

| Reactants | Concentration | NADH | H_2O_2 | Cam-OH |
|-----------|---------------|------|--------|--------|
| NADH      | 200 \mu M     | 200  | 150    | 0.8 0.4|
| NADH + reductase + putidaredoxin | 200, 5, 10 | 200 | 0 | 100 95 |
| Putidaredoxin, reduced | 60 | | | 50 90 |

Yield of 3-exo-OH-camphor based on 2 e^- per molecule.

Photoreduced (Table II, 1 e^- per molecule. P450 Cam added from sidearm.

TABLE V

Product yields with oxygenated P450 Cam

The conditions are as in Table II. P450 Cam at 60 \mu M was photoreduced, then oxygenated at 3°.

| Addition from sidearm | 3-Exo-Cam-OH | % |
|-----------------------|--------------|---|
| NADPH                 | 200 \mu M    | 0 0 |
| NADPH                 | 200 \mu M    | 0 0 |
| NADH + putidaredoxin  | 200, 60      | 0 0 |
| Reduced putidaredoxin | 60           | 54 90 |
| Oxidized putidaredoxin| 60           | 24 80 |
TABLE VI

Product yields from electron carriers

| Additions       | Reduced carrier | Oxidized carrier | P450 cam + cam (800 μM) |
|-----------------|-----------------|------------------|-------------------------|
|                 | μM   | μM   | μM   | %   | μM   | %   |
| Putidaredoxin   | 60   | 20   | 27   | 90  | 20   | 80  |
| Ferredoxin      | 60   | 20   | 0    | 0   | 0    | 0   |
| Adrenodoxin     | 60   | 20   | 3    | 10  | 15   | 60  |
| Rubredoxin      | 60   | 20   | 7.5  | 25  | 6.2  | 20  |
| Clostridium acetici: Fd | 60 | 20   | 0    | 0   | 0    | 0   |
| A. vinelandii I811 | 60 | 20   | 0    | 0   | 0    | 0   |
| Clostridium pasteurianum, paramagnetic protein | 60 | 20   | 0    | 0   | 0    | 0   |
| Cytochrome b5 (rabbit) | 60 | 20   | 1.8  | 6   | 7    | 30  |
| Phosphatidyl choline | 200 | 0    | 0    | 0   | 0    | 0   |

a Reduced carrier is reacted with oxidized P450.

b Oxidized carrier is reacted with oxygenated P450.

* Yield of 5-exo-OH-camphor.

The oxygenated complex on decay, in the absence of the other hydroxylase enzymes or excess NADH, gives neither hydroxylated product nor more than 20% of the expected yield of H2O2. The high yields of H2O2 formed with NADH, P450cam, and camphor in the complete absence of putidaredoxin (Table IV) may then be due not to the formation and decay of the oxygenated complex but rather, may be attributable mainly, if not entirely, to side reactions resulting from the presence of trace impurities of proteins from the camphor ketalolase system (21, 22). The rate constant for NADH disappearance under air in a partially purified P450cam sample containing 8% of the FMN containing protein impurity was found to be 30 times faster than that for the anaerobic reduction of the sample. This reflects the 30-fold greater turnover number for the FMN containing reductase of the ketalolase system (21, 22). No loss of H2O2 titer is observed upon standing with equimolar oxidized P450cam for 10 min.

When reduced redoxin is the sole electron source (Table V), the reaction and stoichiometry corresponds to

\[ 2 \text{ Redoxin (reduced) + cam + O}_2 \rightarrow \text{P450}_{\text{cam}} \]

\[ \text{2 Redoxin (oxidized) + cam-OH} \]

Product is formed nearly stoichiometrically with the total reducing equivalents in the system regardless of the state of the P450cam (Tables IV and V). The autooxidation rate constant for putidaredoxin is 0.17 sec⁻¹ under air which is too small to compete with electron transfer to P450cam. When combined with the P450 cam oxygenated species, oxidized putidaredoxin also gives high yields of hydroxylated camphor based on the reducing equivalents supplied. The yield increases as the reaction temperature is lowered.

Replacement of Putidaredoxin—The dual protein requirement for hydroxylated product formation suggests for the putidaredoxin a role beyond electron transfer. Its specificity in this role is demonstrated by the general ineffectiveness of other iron sulfur proteins and other biological electron donors in product formation. This specificity applies to product formation both with ferrous oxygenated P450cam camphor and oxidized effector and with ferric P450cam-camphor and reduced effector as indicated in Table VI. Most of these proteins reduce the P450cam-camphor complex rapidly. Phosphatidyl choline from the liver microsomal P450 system is ineffective as an effector. The hepatic cytochrome b5 and rubredoxin from P. oleovorans both received electrons from reduced P450cam, thus giving low yields when tested as the electron suppliers. When the oxidized forms of these proteins are combined with ferrous oxygenated P450cam complex, both give product yields comparable to oxidized putidaredoxin.

Each of the donors was introduced in 10 μM concentration in a reaction mixture containing putidaredoxin reductase (0.5 μM), putidaredoxin (1.0 μM), P450cam (0.5 μM), plus the substrate and buffer as previously described. None of the donors tested had any effect on the reaction rate as measured by NADH turnover and none except putidaredoxin produced hydroxylated product.

P450cam Turnover during Catalysis—Kinetic and product yield studies are compatible with each of the four stable states of P450cam serving a role in the hydroxylation sequence. The dominant form of P450cam during NADH turnover with the reconstituted enzyme system was investigated by stopped flow and rapid-scan spectrophotometric techniques. At concentrations less than saturating for redoxin, in nearly equimolar concentration with reductase and P450cam, the 418 nm oxygenated form is dominant as shown in Fig. 3.2 When NADH is depleted, the 418 nm band still dominates under the given conditions, thereafter changing back to the 391 nm form at a rate far slower than the turnover number for P450cam.

Dependence of Rate on Ratio of Enzyme Components—The kinetic data (Figs. 4 and 5) and product yields given in Tables IV to VI demonstrate that both putidaredoxin and P450cam are required for substrate hydroxylase. The ratio of redoxin to the other components for hydroxylation is critical to optimize turnover. Previous reports of the ratio of the enzyme components for maximum turnover under assay conditions with other reductase-redoxin P450 hydroxylase systems were 1:20:1 and 1:50:1 (24, 23, 30). It is now found, however, that as the absolute concentrations of the components is increased, the relative amount of redoxin required to saturate the system decreases. Fig. 4 illustrates these results. The asymptotic value of redoxin to reductase-P450cam could not be obtained from these data, but the linearity of reciprocal plots and the 1:1 break in saturation curves with reductase and P450cam (not shown) suggest that the in vitro as well as in vivo ratio of components could be 1:1:1.
Simultaneous variation of the hydroxylase components in any ratio between 1:03:1 and at least 1:3:1 reductase to redoxin to P450cam results in a nonlinear rate dependence as shown in Fig. 5. If one of the components in Fig. 5, such as the reductase, is held constant, then the rate dependence is linear on simultaneous variation of the other two components well past the 1:1 saturation point for the reductase and P450cam. These results may be consistent either with an absolute increase in the amount of enzyme in the complexed form present at higher enzyme concentrations or possibly with a decrease in the mean free path between collisions needed to effect electron transfer.

Kinetic Evidence for Enzyme Complexes—The kinetic x-axis intercept of 1/v versus 1/[redoxin] plots at different, fixed reductase-P450cam concentrations shown in Fig. 4 clearly suggests effector kinetics. If it is assumed that some reductase-P450 (1:1) complex is vital to catalysis, KEC for this complex (the reciprocal of the intercept value) would be roughly 30 μM. One can take this value and use it to calculate constant turnover numbers from rate data at all concentrations of P450cam and nonsaturating reductin levels rather than requiring the reductin to be saturating, which supports the validity of the assumption of a 1:1 complex between the two components. If, however, the ratio of reductase to P450cam is increased, the x-axis intercept (although remaining constant for any fixed ratio) decreases ultimately to 4.2 μM, indicating that the intercept value contains other terms, the nature of which may be resolved in further study.

**DISCUSSION**

The kinetic and product yield studies presented here are designed to delineate the role of putidaredoxin and the four identified stable states of P450cam in camphor hydroxylation. Several points have been demonstrated.

**Putidaredoxin Is Required as Electron Transfer Agent to P450cam for Product Yield**—NADH and NADH plus the reductase in the absence of putidaredoxin neither reduce the P450cam camphor complex fast enough to account for the turnover of the reconstituted system nor yield product when combined with the ferrous-oxygenated P450cam complex. Reduced putidaredoxin, however, is quite capable of transferring both electrons to P450cam while giving quantitative product yields when combined with any state of P450cam plus camphor. The inclusion of putidaredoxin directly into the electron transfer scheme is the simplest explanation for the extremely rapid turnover observed in the reconstituted system.

**Putidaredoxin Appears to Function as Effector for P450cam, which Determines Course of Product Formation**—This contention is supported by product yield studies which indicate the need for an effector in hydroxylation and the specificity of putidaredoxin in performing this function. Also, steady state kinetic studies clearly show effector type kinetics only for the case in which the reductase-P450cam concentrations are fixed and the putidaredoxin concentration is varied.

**Oxygenated P450cam in the Absence of Putidaredoxin Spontaneously loses both an electron and oxygen to form the oxidized species directly**. Neither oxygenated camphor nor H2O2 is formed in significant quantities. To explain the fate of the electrons, one may postulate that some oxygen molecules are reduced to water, analogous to the spontaneous decomposition of oxyperoxidase (31). The presence of putidaredoxin then appears to modify the course of this reaction to give quantitative yields of hydroxylated camphor instead of H2O2 presumably by interacting directly with the P450-O2-camphor complex.

The results in Table VI suggest that this modifier role is something in addition to electron transfer to oxygenated P450cam. Spinach ferredoxin, adrenodoxin, dithionite, and methyl viologen have lower oxidation-reduction potentials than putidaredoxin
and can reduce oxidized P450cam very rapidly, but they will not reduced oxygenated P450cam to give hydroxycamphor. Rubredoxin and cytochrome b5, on the other hand, have oxidation-reduction potentials too high to reduce the P450cam-camphor complex (which consequently must have a redox potential between -0.20 and -0.100 volt), but can effect hydroxylation in appreciable yields. The decay rate of the oxygenated species is increased about 3-fold by the presence of either cytochrome b5 or rubredoxin. This is far less than the 1000-fold increase caused by putidaredoxin under the same conditions. The capacity to bring about hydroxylation irrespective of the rate of decay of the oxygenated intermediate may best be explained by an interaction between the modifier molecule and oxygenated P450cam during oxygen activation which affects the orientation of reactants within the active site. Putidaredoxin may have the correct structure and oxidation-reduction properties to carry out this function as well as electron transfer very efficiently in the bacterial system.

Data from Huang and Kimura (3) and from Schleyer et al. (30) lead to a similar conclusion with respect to adrenodoxin in the adrenal mitochondrial P450 system. Product yields in these two studies appear to be 15 to 20%, in good agreement, if the yield reported in Table II of Reference (30), is given on the basis of initial reducing equivalents present rather than P450. More effective competition of O2 for reduced adrenodoxin may account for lower product yields compared with those of the faster bacterial system.

Independent biophysical studies have already indicated that complexes of putidaredoxin and P150cam form and are detectable. Circular dichroism measurements in reduced and oxidized putidaredoxin-P450cam systems provided the first evidence of a possible interaction by demonstrating differences in the spectra of the individual components and their combination (32, 33). Electrofocusing experiments have been reported in which 6:1, 3:1, and 1:1 aggregates of putidaredoxin and P450cam were detected at high concentrations (34). A titration of P450cam with oxidized putidaredoxin in the EPR showed a slight but distinct shift in the position of the low spin ferric signals (about g = 2.0) and a decrease in intensity of the high spin bands up to equimolar concentrations, indicating that at least a 1:1 complex of the two could be formed (17, 34).

**Ferrous Substrate Oxidized Form of P450cam Dominates during Catalysis**—It has been demonstrated that each of the four identified stable states of P450cam will yield product if supplied with putidaredoxin and the required electrons and substrate. During turnover, however, rapid scanning techniques show the oxygenated species to dominate, suggesting that some step subsequent to oxygen binding is rate determining. The rate of anaerobic reduction of P450cam-camphor complex (k2) has been observed to exceed the rate of aerobic turnover (k3) under otherwise identical conditions which would support this observation (17). The continued dominance of the 418 nm intermediate after depletion of the NADH may be due to the sudden decrease in the level of reduced putidaredoxin which may serve both to replenish the 418 species and effect its decay. Association of k4 with an electron transfer, oxygen activation, complexation, enzyme modification, or another step cannot presently be made, and the steps subsequent to oxygenation in Fig. 1 are still not clearly delineated.

**1:1:1 Ratio of Hydroxylase Components Is Active and Is Inferred to Reflect Kinetically Significant Complex(es)—**P450cam and putidaredoxin reductase clearly saturate when their concentrations are equal. The failure of putidaredoxin to saturate under similar conditions could be due to the formation of multiredox-hydroxylase complexes. The linearity of the double reciprocal plots of NADH turnover versus redoxin concentration (Fig. 4), and the unique intercept on the abscissa for any given ratio of P450 to reductase suggest, however, that the active complex contains one putidaredoxin with a relatively high dissociation constant. Rapid dissociation could facilitate the transfer of the 2 required electrons for hydroxylation by the strictly 1 electron-reduced putidaredoxin, although not necessarily by the same molecule. The observation that quantitative yields of hydroxylated product are derived from combination of reduced putidaredoxin and either oxidized or oxygenated P450cam-camphor complex is the best experimental evidence to date that the electrons can be transferred in two steps. It is not clear whether reductase participates directly in the catalytic complex or transfers electrons to reductin which in turn transfers them to P450cam via two sequential binary complexes. Studies are continuing to see whether a physical significance can be more clearly attached to the x-axis intercepts obtained in Fig. 4 over a much broader range of conditions and whether a general rate law for the over-all reaction can be developed. Biophysical studies by Mössbauer, EPR, and ultra centrifuge studies are in progress to resolve the question of the capability of the complex to complex and the detailed mode of electron transfer and oxygen activation.

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