THE ROLE OF OXYGENATED CYTOCHROME P-450 AND OF CYTOCHROME b5 IN HEPATIC MICROSONAL DRUG OXIDATIONS

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The occurrence of an oxygenated form of cytochrome P-450 was first proposed by Estabrook and his colleagues in 1968 (1, 2), and the presence of this species of cytochrome P-450 in hepatic microsomes was recently confirmed (3). Subsequently, Schleyer et al. (4), using a partially purified preparation derived from adrenocortical mitochondria, also observed the presence of oxygenated cytochrome P-450 and demonstrated its function in steroid hydroxylations. Oxygenated cytochrome P-450 has been prepared from cytochrome P-450 purified from Pseudomonas putida, and its properties have been characterized by Peterson et al. (5) and by Gunsalus et al. (6).

Oxygenated cytochrome P-450 is presumably a ternary complex of ferrous cytochrome P-450, substrate, and oxygen (5, 6). It is this complex which is proposed to accept the second electron required for the transfer of one atom of molecular oxygen to the organic substrate molecule. This report describes some of the properties of oxygenated cytochrome P-450 in hepatic microsomes and discusses the proposed role of a second hemoprotein, cytochrome b5, in hepatic microsomal drug oxidations.

Properties of Oxygenated Cytochrome P-450

When the spectral changes associated with alterations in pigment reduction in hepatic microsomes are examined during the aerobic steady state of drug oxidation, changes in the extent of reduction of cytochrome b5 are observed together with the appearance of a new spectral species (fig. 1) that has been identified as the oxygenated form of cytochrome P-450 (3). In the difference spectrum, oxygenated hepatic microsomal cytochrome P-450 exhibits absorption band maxima at about 440, 556, and 589 nm. This spectrum is quite similar to the difference spectrum reported for the oxygenated form of the comparable pigment isolated and purified from P. putida (5).

The appearance of the oxygenated form of cytochrome P-450 in hepatic microsomes is dependent, in part, on the presence of a type I substrate which can be oxidized by the microsomes. While oxygenated cytochrome P-450 is observed in the presence of such type I substrates as hexobarbital, ethylmorphine, and aminopyrine, the presence of type II substrates, such as aniline, does not result in the appearance of the spectral changes indicative of oxygenated cytochrome P-450 (3). The small spectral changes attributable to oxygenated cytochrome P-450 that do appear in hepatic microsomes in the absence of exogenous type I substrates presumably reflect the presence of endogenous substrates in these microsomal preparations.

The magnitude of the spectral changes ascribable to oxygenated cytochrome P-450 depends on the concentration of the substrate present, as seen in fig. 2. There is a striking correlation of the spectral dissociation constant (Kd) for the binding of hexobarbital to hepatic microsomal cytochrome P-450 and the concentration of hexobarbital required for half-maximal formation of oxy-
FIG. 1. Difference spectra of hepatic microsomes during the aerobic steady state in the presence and absence of hexobarbital.

Hepatic microsomes from phenobarbital-treated rats were diluted to 2 mg of protein per ml in a buffer mixture containing 50 mM Tris-chloride buffer, pH 7.4; 150 mM KCl; 10 mM MgCl₂; 7 mM sodium isocitrate; and excess isocitrate dehydrogenase. When included in this buffer mixture, the concentration of hexobarbital was 2 mM. The diluted microsomal suspension was divided equally between two cuvets and a baseline of equal light absorbance was recorded. TPNH (0.2 mM final concentration) was then added to the sample cuvet and the resulting difference spectrum was immediately recorded. The solid line represents the difference spectrum obtained in the presence of 2 mM hexobarbital. The dashed line represents the difference spectrum obtained in the absence of hexobarbital. The temperature was maintained at 8°C throughout the experiment.

Fig. 2. The relationship between hexobarbital binding to hepatic microsomal cytochrome P-450 and the magnitude of oxygenated cytochrome P-450.

Hepatic microsomes from phenobarbital-treated rats were diluted to a protein concentration of 2 mg/ml in a buffer mixture containing 50 mM Tris-chloride buffer, pH 7.4, with 150 mM KCl and 10 mM MgCl₂. After establishing a baseline of equal light absorbance, various concentrations of hexobarbital were added to the sample cuvet and the magnitude of the resulting type I spectral change was determined from the difference in absorbance between 385 and 422 nm (●—●). The magnitude of oxygenated cytochrome P-450 in the presence of the various concentrations of hexobarbital was determined from the difference in absorbance between 440 and 500 nm (○—○) immediately after the addition of TPNH, as described in fig. 1.

More significantly, these values approximate the Kₘ concentration of hexobarbital for its oxidation by hepatic microsomes (0.1 mM). The choice of substrate employed also determines, in part, the magnitude of the spectrum of oxygenated cytochrome P-450 (fig. 3). The maximal spectral change in the presence of a saturating concentration of ethylmor-
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![Graph showing the absorbance of different spectra](image)

**Fig. 3.** Comparison of the magnitude of oxygenated cytochrome P-450 in hepatic microsomes during the aerobic steady state in the presence of hexobarbital and ethylmorphine.

The conditions of the experiments were the same as described in fig. 1, except that the temperature was maintained at 25°C. Curve A (-----) represents the difference spectrum obtained in the presence of 8 mM ethylmorphine (Dionin). Curve B (-----) represents the spectrum obtained in the presence of 2 mM hexobarbital. Curve C (-----) represents the spectrum observed in the absence of exogenous substrate.

Ethylmorphine is about one-half of that produced in the presence of a saturating concentration of hexobarbital. This observation may be related to the fact that, since ethylmorphine is oxidized at a faster rate than hexobarbital by hepatic microsomes, the steady-state level of reduced and/or oxygenated cytochrome P-450 is presumably reduced in the presence of ethylmorphine.

In addition to the presence of a type I substrate which can be oxidized by hepatic microsomes, the appearance of oxygenated cytochrome P-450 in hepatic microsomes during the aerobic steady state also depends on the presence of oxygen and the use of TPNH as the source of reducing equivalents. Indeed, when DPNH is employed as the electron donor rather than TPNH, the spectral changes indicative of oxygenated cytochrome P-450 are not observed. This observation is consistent with the findings (7) that DPNH very slowly reduces cytochrome P-450 and only poorly supports mixed function oxidation reactions catalyzed by hepatic microsomes. Furthermore, the concentration of TPNH employed influences both the magnitude of the spectral change observed and the decay of the oxygenated species of cytochrome P-450 formed during the aerobic steady state of hepatic microsomal drug oxidations. As shown in fig. 4, at 25°C and in the presence of hexobarbital and a relatively high concentration of TPNH (0.2 mM), the spectral change due to oxygenated cytochrome P-450 begins to decrease immediately after formation. Under these conditions, this decrease parallels the oxidation of TPNH, and oxygenated cytochrome P-450 can once again be formed upon another addition of TPNH. When the same experiment is carried out in the presence of limiting concentrations of TPNH (i.e., 3-25 μM TPNH), the magnitude of the spectral change is less and the oxygenated cytochrome P-450 decays much faster.

Employing stopped flow spectrophotometric techniques, the kinetics of formation of the oxygenated form of hepatic microsomal cytochrome P-450 have been examined (8). Computer analysis of the data shown in fig. 5 reveals that the formation of this spectral species is complex and appears to proceed by means of two first-order phases (8). The first phase accounts for approximately 90% of the oxygenated cytochrome P-450 formed in hepatic microsomes in the presence of ethylmorphine and TPNH. This phase is extremely rapid with a half-time of formation of about 0.16 sec and a first-order rate constant of approximately 4.3 sec\textsuperscript{-1} at 25°C. The second phase of formation is considerably slower than the initial phase and accounts for only a small portion of the spectral change observed. Since the initial rate of formation of oxygenated cytochrome P-450 is considerably faster than the overall rate of drug (ethylmorphine) oxidation, these data support the conclusion that this spectral species is indeed an intermediate formed during the functioning of cytochrome P-450 as the terminal oxidase in drug oxidations.

The Role of Cytochrome b\textsubscript{5} in Hepatic Microsomal Drug Oxidation

While examining the spectral changes occurring in hepatic microsomes during the aerobic steady state of drug oxidation, alterations in the extent of the TPNH-dependent reduction of cytochrome b\textsubscript{5} are observed (3, 7). As shown in fig. 1, the spectral contribution of reduced cytochrome b\textsubscript{5} in the presence of substrate is quite different from that in the absence of substrate: the
The rate of formation of oxygenated cytochrome P-450 was determined using an Aminco-Morrow stopped flow apparatus in combination with an Aminco-Chance dual wavelength spectrophotometer. The output of the photomultiplier tube was converted from percent transmission to absorbance using a rapid response log amplifier circuit, and the changes in absorbance were recorded with a Tektronix storage oscilloscope. Hepatic microsomes from phenobarbital-treated rats were diluted to 4 mg of protein per ml in a buffer mixture containing 50 mM Tris-chloride buffer, pH 7.4, with 150 mM KCl, 10 mM MgCl₂, and 8 mM ethylmorphine. This suspension was placed in one syringe of the stopped flow apparatus, and a 0.4 mM solution of TPNH in the same buffer mixture was placed in the other syringe. At zero time, the contents of the two syringes were mixed and the change in absorbance between 440 and 482 nm was recorded. The temperature was maintained at 25°C throughout the experiment.

FIG. 5. Kinetics of formation of oxygenated cytochrome P-450 in hepatic microsomes.

The rate of formation of oxygenated cytochrome P-450 was determined using an Aminco-Morrow stopped flow apparatus in combination with an Aminco-Chance dual wavelength spectrophotometer. The output of the photomultiplier tube was converted from percent transmission to absorbance using a rapid response log amplifier circuit, and the changes in absorbance were recorded with a Tektronix storage oscilloscope. Hepatic microsomes from phenobarbital-treated rats were diluted to 4 mg of protein per ml in a buffer mixture containing 50 mM Tris-chloride buffer, pH 7.4, with 150 mM KCl, 10 mM MgCl₂, and 8 mM ethylmorphine. This suspension was placed in one syringe of the stopped flow apparatus, and a 0.4 mM solution of TPNH in the same buffer mixture was placed in the other syringe. At zero time, the contents of the two syringes were mixed and the change in absorbance between 440 and 482 nm was recorded. The temperature was maintained at 25°C throughout the experiment.

Absorbance bands of reduced cytochrome b₅ at both 426 and 527 nm are markedly depressed. Moreover, when the spectral changes occurring during the aerobic steady state in the presence of TPNH and substrate are scanned repetitively, fluctuations in the extent of reduction of cytochrome b₅ are found (3, 7). These observations have led to the suggestion that cytochrome b₅ is involved in hepatic microsomal drug oxidations (3, 7).

Since hepatic microsomal cytochrome b₅ appears to be only partially reduced during the aerobic steady state in the presence of both TPNH and substrate, the possibility exists that the drug substrates either inhibit the reduction of cytochrome b₅ by TPNH or stimulate the re-oxidation of the reduced hemoprotein. Inhibition of the TPNH-dependent reduction of cytochrome b₅ by the drug substrates, however, would not be consistent with the suggestion that cytochrome b₅ is involved in these mixed function oxidation reactions. Indeed, as shown in fig. 6, the initial rate of the TPNH-dependent reduction of hepatic microsomal cytochrome b₅ is not affected by a drug substrate, although the steady-state level of reduction is depressed. Furthermore, the rate of cytochrome b₅ reduction is sufficiently rapid to allow for the participation of reduced cytochrome b₅ in drug oxidations.

On the other hand, Hildebrandt and Estabrook (7) observed that the addition of a drug substrate, such as ethylmorphine, to hepatic micro-
Hepatic microsomes from phenobarbital-treated rats were diluted to 2.4 mg of protein per ml in a buffer mixture containing 50 mM Tris-chloride buffer, pH 7.4, with 150 mM KCl and 10 mM MgCl₂. When included in this buffer, the concentration of ethylmorphine was 1.33 mM. The diluted microsomal samples were cooled to 4°C, and the changes in absorbance between 556 and 540 nm upon the addition of TPNH were recorded.

Fig. 6. Effect of ethylmorphine on the initial kinetics of cytochrome b₅ reduction by TPNH.

These results indicate that the concentration of ethylmorphine required to produce half-maximal decrease in the steady state of TPNH-reduced cytochrome b₅ corresponds very well with the experimentally determined Kₘ for ethylmorphine in the N-demethylation reaction (0.4 mM) catalyzed by hepatic microsomes. The observation that DPNH-reduced cytochrome b₅ in hepatic microsomes is not modified in the presence of the drug substrate (Fig. 7) is consistent with the fact that these drug oxidation reactions are not supported very well by DPNH (7). These results, together with observation that the magnitude and, therefore, the concentration, of oxygenated cytochrome P-450 observed in hepatic microsomes during the aerobic steady state is related to the ratio of cytochrome P-450 to cytochrome b₅ concentrations, indicate that reduced cytochrome b₅ may serve as the donor of the electron to oxygenated cytochrome P-450 which is required for the oxidation of the drug substrate.

Summary

An examination of the spectral changes occurring in hepatic microsomes during the aerobic steady state of oxidative drug metabolism has revealed the presence of a spectral species which has been identified as the oxygenated form of ferrous cytochrome P-450. The appearance of this species in hepatic microsomes depends on 1) the presence of a type I substrate which can undergo oxidation, 2) the presence of oxygen, and 3) the use of TPNH as the source of reducing equivalents. The rate of formation of this oxygenated form of cytochrome P-450 is much faster that the overall rate of substrate oxidation and is consistent with the inclusion of this intermediate in the sequence of cyclic reduction and oxidation transitions which cytochrome P-450 undergoes during substrate oxidation.
The observation that the presence of drug substrates produces a partial reoxidation of TPNH-reduced cytochrome \( b_s \), thereby resulting in a lower aerobic steady-state level of reduction of this hemoprotein, indicates that cytochrome \( b_s \) may function in hepatic microsomal drug oxidations. It is proposed that reduced cytochrome \( b_s \) may serve in the capacity of an electron transfer intermediate which donates the electron to oxygenated cytochrome P-450 required for substrate oxidation.

References

Discussion

**Dr. Schenkan, Yale U.**: I am just curious about how long it takes before the 440 nm peak which you showed in one of your earlier slides reaches the size almost as large as the cytochrome \( b_s \) absorbance band. Your slides showed them to be approximately of the same magnitude, unless I was misreading it. In the presence of NADPH, do you see a disappearance of the \( b_s \) peak in the spectrum and a formation of the 440 nm peak?

**Dr. Baron**: The spectrum to which you are referring (fig. 1 in text) reflects a very interesting phenomenon. When these steady-state experiments are carried out at low temperatures, such as at \( 8^\circ C \) in this case, the magnitude of the spectral change attributable to the oxygenated form of cytochrome P-450 is markedly enhanced. Under conditions at which one normally determines enzymatic activity, that is, at \( 25^\circ C \), the rate of formation of oxygenated cytochrome P-450 is very rapid, although the spectral change due to this form of cytochrome P-450 at \( 25^\circ C \) is only about half as large as that produced at low temperatures. We have not measured the rate of formation of oxygenated cytochrome P-450 at these lower temperatures.

**Dr. Schenkan**: So, in other words, you are slowing down the reaction at low temperature. You are really trapping this intermediate.

**Dr. Baron**: That is correct.

**Dr. Schenkan**: Now, from what your group is showing, NADH facilitates the input of a second electron. does NADH discharge that 440 nm peak?

**Dr. Baron**: This is a difficult question to answer. We have investigated the effect of NADH on the decomposition of oxygenated cytochrome P-450, but, to be completely honest, many results of these experiments depend on the individual microsomal preparations. All we can say at this time is that, in general, NADH does not appear to be able to discharge the oxygenated form of cytochrome P-450.

**Dr. Gunsalus, U. Illinois**: I believe a number of these reactions are also potential-dependent and, in part, potential-regulated. Please recall also, as pointed out earlier in this symposium, that among the P-450 systems under study, there are differences in the type and number of components, the dynamics, regulation, substrate selectivity, the turnover numbers, and the cellular organization. Three figures will illustrate for a specific system the observations I wish to make. These data concern a single specific cytochrome-containing monooxygenase system, composed of three enzyme components—each prepared in pure homogeneous conditions. I shall focus first on the reaction cycle of the cytochrome which is the site of the substrate and oxygen-binding and of two single-electron reductions. The iron sulfide protein performs an effector and an electron

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**FIG. 1.** Cytochrome P-450 oxygenation and reduction cycle.
transport role. Both proteins are essential to product formation. Their separation permitted us to observe, in static condition, each of the intermediate states of the reaction cycle for the cytochrome P-450.

In fig. I we show each of the details, in particular at the lower right-hand corner of the reaction of the ferrous oxy-substrate form of P-450. Here we are interested in the transfer of the second electron required for the reduction of a single atom of oxygen to O₂, during which the other oxygen atom from a single molecule is transferred to the substrate, forming a hydroxyl or secondary alcohol where previously a methylene group existed. The process is stereoselective, always yielding the exo-alcohol. The most nearly representative mammalian system is that of the adrenal mitochondria, which is also known to carry out a specific hydroxylating process—forming the alcohol of sterols.

The addition of the second electron to the ferrous oxy system requires, in our studies so far, specifically the iron-sulfur protein putidaredoxin, which undergoes a one-electron redox reaction, as is also characteristic of the cytochrome. The overall rate of this process, denoted on the slide as k₄, is clearly a complex of several individual steps.

If the rates of the individual processes are very different, k₄ must be the rate-limiting reaction of the sequence. On the top of the slide, the transfer of the first electron to the cytochrome prior to the oxygenation, following substrate addition, is referred to as k₂. This reaction is more simple, though it also requires putidaredoxin as the electron donor. Its rate is usually non-rate-limiting and occurs at a rather negative potential, relative to the second electron transfer reaction.

Table I indicates the potential of the reactants which enter into the cytochrome oxygen cycle. If you will run your eye down the column about halfway, you will notice a compound referred to here as P-450, meaning the ferrous substrate form of the protein. It has a redox potential at pH 7 indicated as ~ 170 mV. Further down the list you will see P-450,S₃O₄—that is, sulfur dioxide, but the ferrous oxy form of the cytochrome. You will note that the potential is listed as approximately 0 mV at pH 7. This is the addition of the second electron (i.e., the reaction referred to of which one step occurs in the constant k₄).

I should like in a moment to talk about the effectors which bring about the formation of product. For the moment I will refer to the putidaredoxin as the natural effector. You will notice at the top of the slide that it has a redox potential at pH 7 of approximately ~ 240 mV. Another protein which has an effector capacity—cytochrome b₅—you will notice, is even more positive than the potential for the addition of the second electron to the cytochrome (i.e., somewhere in the neighborhood of 30 mV). This again is one-electron transfer. Other cytochromes listed which are of even higher potential are ineffective. They are not of interest to us at this moment, for they do not act as effector proteins.

Starting with the ferrous oxy-substrate P-450, addition of putidaredoxin in the oxidized form will catalyze the formation of 0.5 mol of product per mol of P-450. This is equivalent to a stoichiometry of two electrons per mol of product. A similar effect is found with cytochrome b₅, although a higher concentration is required. In this case, if the oxidized cytochrome b₅ is added, it will be reduced at the expense of the intermediate (i.e., the potential is 30 mV higher and the yield will be somewhat penalized). If the reduced b₅ is added, only 0.5 mol of the product is formed so that it acts as an effector, but not as an electron-transfer protein. These data are indicated in table I, together with the electron transport reaction (i.e., cytochrome b₅ and the rubredoxin will each catalyze the formation of 0.5 mol of product as the limiting value).

The concentration of cytochrome b₅ required is approximately 200 times that of the natural effector, although the reciprocal plots indicated similar reaction order. The second reaction—that resulting in the formation of a full mole of product per molecule of cytochrome P-450—occurs in our experience so far only with the reduced putidaredoxin.

Speaking on a related subject in answer to the questions of Dr. Schenck, I should like to show you one slide (fig. 2) depicting the binding of CO by the ferrous form of cytochrome P-450 and the effect of the substrate camphor on this binding. As discussed earlier in this symposium, not all information required can be obtained from spectral measurements, especially with the ferrous form of the cytochromes. In particular, the ferrous form of the cytochromes and the ferrous substrate form of the cytochromes have very similar

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*Cooper et al.: 305 mV, 1972 data; Kimura et al.: 260 mV, personal communication.
spectra. When one adds CO to these compounds, the final spectrum is similar. The question arises: has the substrate been displaced, or is it still present? This information was obtained by carrying out an equilibrium dialysis with $^{14}$C-camphor in the presence of CO—as you will see from the Scatchard plot in fig. 2—shows that the substrate is still bound to the enzyme in a stoichiometry of 1 mol/mol of heme.

Based on spectral evidence, I had earlier made the statement—which now proves to be untrue—that the product of the hydroxylation reaction did not appear to bind. It does, in fact, have a similar binding constant as the substrate, though it does not shift the position of the Soret band. This observation has further implications in terms of the nature of the binding and its exact position.

Dr. Peterson, U. Texas Southwestern Med. Sch.: I would like to show results we have obtained with the purified bacterial cytochrome P-450 since the rate constants which we have determined better support Dr. Baron's results than do those reported by Dr. Gunsalus. There are two particular points which I want to focus on (fig. 3), and these are the rate constants for the introduction of the first and second electrons in the overall hydroxylation reaction of camphor. The rate and equilibrium constants which are shown in fig. 3 were determined at 4°C because of the experimental limitations which are imposed by the 3-msec dead time of our stopped flow apparatus. The upper right-hand portion of this figure illustrates the introduction of the first electron by putidaredoxin, the iron-sulfur protein (ISP), and the first-order reaction rate constant is 10.6 sec$^{-1}$. In this reaction, the rate of reduction of cytochrome P-450 is zero order with respect to iron-sulfur protein concentration. We have varied the concentration of reduced iron-sulfur protein over a 10-fold range with respect to cytochrome P-450 from a 1:1 ratio of iron-sulfur protein to cytochrome P-450 to a 10:1 ratio. In other words, varying the concentration of iron-sulfur protein over this range has no effect on the rate of reduction of cytochrome P-450. Looking at the lower left-hand corner of this figure, the introduction of the second electron and the hydroxylation reaction are illustrated. The rate of this reaction is dependent upon the concentration of iron-sulfur protein. However, the reaction is not first order with respect to iron-sulfur protein concentration, which is what we had initially expected to observe. A plot of the reciprocal of the pseudo-first-order rate constant vs. the reciprocal of the iron-sulfur protein concentration gave a straight line relationship. The $K_w$ determined from this plot is the value which is shown in this slide, $7.4 \times 10^{-4}$ M. The $V_{max}$ determined from this plot is 240 sec$^{-1}$.

Thus, as can be seen, the bacterial cytochrome P-450 system is ideal for studying the interaction of the various electron carriers and their effect on the steady-state level of the various intermediates of cytochrome P-450 during hydroxylation reactions. If the concentration of putidaredoxin reductase is high and not rate-limiting, increasing the concentration of iron-sulfur protein will cause the rate of introduction of the second electron to approach $V_{max}$. The introduction of the first electron will then be the rate-limiting step. Therefore, under these conditions, there will be no oxycytøme-P-450 Camphor. The figure shows that the rate of the reaction is zero order with respect to the iron-sulfur protein concentration.
chromo P-450 accumulated in the steady state. However, with a low iron-sulfur protein concentration relative to cytochrome P-450, oxycytochrome P-450 will accumulate as an intermediate and there will be little if any of the ferric camphor complex of cytochrome P-450 in the steady state. Thus, the system can be poised to accumulate either oxycytochrome P-450 or the camphor complex of ferric cytochrome P-450 in the steady state. This can be illustrated by some experiments we have done with carbon monoxide inhibition of oxygen consumption by bacterial cytochrome P-450. A central requirement for this analysis is that the ferrous cytochrome P-450 camporph complex is the form which combines with either carbon monoxide or oxygen and that these two reactions are competitive.

A couple of years ago when we first investigated the carbon monoxide inhibition of oxygen consumption of camphor hydroxylation by bacterial cytochrome P-450, the experiments were done in the presence of a large excess of putidaredoxin and putidaredoxin reductase. Referring to fig. 3 you will see that with these conditions there should be little if any of the oxocomplex of cytochrome P-450 in the steady state, and we found very little carbon monoxide inhibition. However, utilizing these rate constants we have re-investigated the problem of carbon monoxide inhibition. In the new experiments, we have lowered the iron-sulfur protein concentration so that now the ratio of iron-sulfur protein to cytochrome P-450 is 2:1. With the conditions illustrated in fig. 4, we were able to obtain good carbon monoxide inhibition. These experiments were done with a tightly coupled system in which oxygen consumption was dependent on the presence of each of the three protein components as well as on added camphor. The dashed line illustrates the control reaction mixture in the absence of carbon monoxide where the ratio of oxygen to nitrogen was 1:9 and the rate of oxygen consumption was 975 nmol of oxygen per min per reaction mixture. The solid line illustrates a similar reaction mixture in which the nitrogen was replaced by carbon monoxide, giving initially a 1:9 oxygen/carbon monoxide ratio. We initially observed greater than 50% inhibition with 450 nmol of oxygen consumed per min per reaction mixture in the dark. Light was turned on, indicated by the arrow, and the carbon monoxide inhibition of oxygen consumption was reversed almost completely. A high-pressure xenon arc lamp with narrow band-pass interference filters was used to produce monochromatic light of 450 nm. From the dashed trace in the absence of carbon monoxide, it can be seen that the amount of light on and off has no effect on the rate of oxygen consumption. I might add that in the experiment done in the absence of carbon monoxide but in the presence of nitrogen and oxygen mixture, approximately 80% of the cytochrome P-450 is present as the oxygen complex in the steady state.

Therefore, knowing the rate constants for the enzyme has permitted us to poise the reaction so that the enzyme will exist in the steady state essentially all as the oxy complex as indicated here or all as the camphor-bound ferric substrate complex.

Dr. Gaukhas: I do not understand what you mean in this context by the term "poising the system." If I read your figures correctly, you are stating that you observed that oxygen and the CO-binding constants are not very different. Thus, if you add a higher concentration of CO to the ferrous oxy-P-450erm, it binds CO and the
oxygen is released. Or, if you start with the ferrous substrate P-450 and add CO, you do not then bind oxygen. The reaction, in other words, seems to be competitive for these two ligands. You are also stating that the reaction velocity for the first and the second electron into the system is dependent on the concentration of the reducing agent putidaredoxin. You will recall that we have previously published data and we have discussed the stoichiometry of binding of P-450 and putidaredoxin in the reaction system. Thus, we are not in disagreement as to the properties of this hydroxylation system.