Single Turnover Kinetics of the Reaction between Oxycytochrome P-450_{cam} and Reduced Putidaredoxin*

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A study of the single turnover kinetics of the reaction between oxycytochrome P-450_{cam} and reduced putidaredoxin was performed using the inhibitor metyrapone to trap the cytochrome immediately after release of the product, 5-exo-hydroxycamphor. EPR determinations of the concentrations of reduced putidaredoxin and ferric metyrapone-bound cytochrome at the same time points showed that there is no time lag between the oxidation of reduced putidaredoxin and the appearance of metyrapone-bound cytochrome. This implies that the rate constant for electron transfer is smaller than the rate constant for the later processes involved in product formation and release, lumped into a single step. Taking this restriction into account and doing computer simulation of absorbance versus time curves, previously obtained at various putidaredoxin concentrations using stopped-flow spectrophotometry, allowed bounds to be determined for rate constants of the processes within the reaction. At 4°C in buffer at pH 7.4 with 0.50 M KCl, the rate constant for the bimolecular association of the two enzymes is between 5 and 20/µM·s; the rate constant for dissociation is between 12 and 600/µM·s; the rate constant for electron transfer is between 60 and 100/µM·s; and the rate constant for the later processes is at least 200/µM·s.

Although the basic groups of reactions catalyzed by the family of enzymes called cytochrome P-450 were discovered more than 2 decades ago, there is still very little known about the precise chemistry of these reactions. In an effort to define more clearly the chemistry of this important enzyme, our laboratory and others have attempted to investigate the individual steps which constitute the catalytic cycle.

In the normal catalytic cycle of cytochrome P-450_{cam}, the enzyme first binds camphor, its substrate, then accepts one electron from the iron-sulfur protein putidaredoxin, and then reversibly binds molecular oxygen to form oxycytochrome P-450_{cam}. This species, in the presence of reduced putidaredoxin, accepts another electron and catalyzes the 5-exo-hydroxylation of the bound camphor molecule. The product is then released, and the enzyme is ready to bind another camphor molecule (Estabrook et al., 1972; Gunsalus et al., 1972).

In our study of the kinetics of the oxygcnating step of this reaction, we have made separate solutions of oxycytochrome P-450_{cam} and reduced putidaredoxin and then rapidly mixed them. The putidaredoxin solution contained a high concentration of metyrapone to trap rapidly and irreversibly the camphor-free enzyme (Peterson et al., 1971; Griffin and Peterson, 1972) after product release, preventing the continuation of the reaction cycle. In previous experiments using this experimental scheme, we have demonstrated the hyperbolic dependence of initial reaction rate upon putidaredoxin concentration (Brewer and Peterson, 1986). This relationship shows that the reaction mechanism includes the formation of a complex of reduced putidaredoxin and oxycytochrome P-450_{cam} and that its formation is not rate-limiting, at least at higher putidaredoxin concentrations. This finding is in accord with the "effector" kinetics of reactions of oxycytochrome P-450_{cam} with the nonphysiological reactant dihydrolic acid or oxidized putidaredoxin (Lipscomb et al., 1976). It is also in agreement with the results of experiments done at subzero temperatures in a mixture of phosphate buffer and ethylene glycol (Hui Bon Hoo et al., 1978). These authors concluded that the catalytic step of the reaction cycle is rate-limited by the first-order decomposition of an intermediate formed by the rapid binding of reduced putidaredoxin and oxycytochrome P-450_{cam}.

We now address the question of which of the first-order processes in this "final" step of the cycle is rate-limiting: electron transfer, product formation, or product release. Cleland (1975) has argued convincingly that enzyme-catalyzed reactions are generally rate-limited by product release; the argument is supported both by known examples and by some theoretical considerations. On the other hand, preliminary experiments from two laboratories have suggested that electron transfer is rate-limiting in the catalytic reaction step of cytochrome P-450_{cam} (Peterson and Mock, 1975b; Pederson et al., 1977). In this paper, we report experiments which show that electron transfer is indeed rate-limiting. We also present a model reaction sequence with approximate rate constants obtained by curve-fitting procedures.

MATERIALS AND METHODS

Commercial Reagents—Crystallized Tris base and lyophilized glucose oxidase (Grade I) were obtained from Boehringer-Mannheim. Metyrapone, (1S)-(+)-camphor, and MOPS were products of Aldrich. Catalase in 0.1% thymol, myoglobin, and the disodium salt of NADH and ferric metyrapone-bound cytochrome at the same temperatures in a mixture of phosphate buffer and ethylene glycol (Hui Bon Hoo et al., 1978). These authors concluded that the catalytic step of the reaction cycle is rate-limited by the first-order decomposition of an intermediate formed by the rapid binding of reduced putidaredoxin and oxycytochrome P-450_{cam}.

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study had a 392 to 280 nm absorbance ratio of greater than 1.3 (Yu et al., 1974). Putidaredoxin was purified from the same cell cultures essentially as described by Mock (1977) with minor modifications. The diluted putidaredoxin fraction was not concentrated by pressure ultrafiltration as before, but was loaded directly onto the second DEAE-cellulose column. Since it contained a significant amount of glucose, glucose oxidase, and catalase (Peterson and Mock, 1975a), aliquots from at least two preparations of cytochrome P-450, and at least two preparations of putidaredoxin. For each of the experiments using 28 WM putidaredoxin and complete reduction required 15-20 min. After complete reduction, the reduction was initiated by the addition of NADH inside the glove bag after mixing in the stopped-flow spectrophotometer. Putidaredoxin and were deaerated. Each putidaredoxin solution contained the desired amount of putidaredoxin, about 0.1 M putidaredoxin reductase, and a concentration of NADH which was at least 20 times the concentration of putidaredoxin. The preparation for putidaredoxin solutions also included a catalytic oxygen-scavenging system consisting of glucose, glucose oxidase, and catalase (Peterson and Mock, 1975a), and it contained metyrapone to give a final concentration of 2.5 mM inhibition of the enzyme. Putidaredoxin and cytochrome P-450, were made using the following instrument parameters: frequency = 9.152 GHz, power = 100 milliwatts, modulation amplitude = 12.5 G, and time constant = 0.3 s. Gain was either 400, 800, or 2000. Magnetic field was repeatedly scanned from 3175 to 3575 G at 2 min/scan, and from 2 to 8 scans were computer-averaged for each determination. The amount of metyrapone-bound cytochrome P-450 was determined by measurement of the peak-to-peak height of the signal at g = 1.94 (3372 G), dividing by the instrument gain, and comparing the result with a standard curve.

**EPR measurements of ferric metyrapone-bound cytochrome P-450** were made using the following instrument parameters: frequency = 9.152 GHz, power = 100 milliwatts, modulation amplitude = 12.5 G, time constant = 0.3 s, and gain = 6200. Field strength was repeatedly scanned from 2100 to 3100 G at 1 min/scan, and 50 scans were computer-averaged for each measurement. The method of quantitation of ferric metyrapone-bound cytochrome P-450 was similar to that used for reduced putidaredoxin. Because the signal from the cytochrome is inherently much weaker than the putidaredoxin signal, a higher receiver gain was required for the cytochrome. Therefore, in experimental samples containing a mixture of the two enzymes, part of the EPR spectrum of the cytochrome which overlaps the putidaredoxin signal was not scanned. Since the amount of ferric metyrapone-bound cytochrome P-450 was determined by measurement of the peak-to-peak height at g = 2.25 (2900 G), not having the entire spectrum did not present a problem.

**RESULTS AND DISCUSSION**

As previously reported (Brewer and Peterson, 1986), the time course of the reaction between oxyctochrome P-450 and reduced putidaredoxin in the presence of metyrapone consists of two spectrophotometrically observable phases. The first phase, which is quite rapid, includes in sequence the formation and release of metyrapone-bound cytochrome P-450, transfer of an electron from putidaredoxin to the cytochrome, formation and release of 5-endo-hydroxycamphor, and the more rapid irreversible binding of metyrapone to the ferric cytochrome. The second phase is much slower and includes the reversible reduction of ferric metyrapone-bound cytochrome by excess reduced putidaredoxin in the solution. A reaction scheme representing the physiologically relevant first phase of the reaction is shown in Fig. 1. In this scheme, the product formation and release and the metyrapone binding were
Oxidized putidaredoxin, Fe(III), and reduced putidaredoxin, Fe(II), proposed complex of oxycytochrome P-450, and reduced putidaredoxin, Fe(II), same complex after one electron has been transferred from putidaredoxin to the cytochrome active site; Pd, oxidized putidaredoxin; HO-cam, 5-exo-hydroxycamphor; Fe(III), ferric metmyrapone-bound cytochrome P-450,.

Jumped into one reaction step because they are kinetically indistinguishable. Initially, the electron transfer step was treated as irreversible for the sake of simplicity. The correctness of that treatment will be discussed later. A set of rate equations for the given reaction sequence is shown: 

\[
\begin{align*}
\text{d}[A1]/dt &= k_{-a}[B] - k_a[A1] \cdot [A2] \cdot [B] - k_{-r}[B] - k_r[B] - k_c[C]; \text{and} \text{d}[D1]/dt = k_c[C].
\end{align*}
\]

A trial-and-error curve-fitting procedure was used to find sets of rate constants \(k_a, k_r, k_c, k_{-a}, k_{-r}, \text{and} k_{-c} \) that were consistent with the experimental data previously reported (Brewer and Peterson, 1986) for a range of putidaredoxin concentrations from 4 to 60 \(\mu M\). Using the computer program MLAB (obtained from the National Institutes of Health and run on a DEC System 10 computer) to perform numerical integrations, we found several sets of suitable rate constants are given in the legend to Fig. 3; the figure shows both experimental and calculated results. From a double-reciprocal replot of the experimental data were calculated a \(k_{cat}\) of 53 \(\mu M/\mu M\)-s and a \(K_m\) of 33 \(\mu M\). The term "\(K_m\)" as used here refers to the maximum turnover rate of the cytochrome in the partial reaction starting with oxycytochrome and ending with the dissociation of hydroxycamphor from the cytochrome. Its value is a composite of the values of \(k_a\) and \(k_c\) in Fig. 1. The \(K_m\) for reduced putidaredoxin represents the concentration at which the turnover rate of the cytochrome is half the maximal value. Its value is a function of all of the

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**Fig. 1. Reaction model.** Fe(II)-cam-O\(_2\) oxycytochrome P-450\(_{cam}\), Pd\(_{red}\), reduced putidaredoxin; Fe(II)-cam-O\(_2\)-Pd\(_{red}\), proposed complex of oxycytochrome P-450\(_{cam}\) and reduced putidaredoxin; Fe(II)-cam-O\(_2\)-Pd\(_{ox}\), same complex after one electron has been transferred from putidaredoxin to the cytochrome active site; Pd\(_{ox}\), oxidized putidaredoxin; HO-cam, 5-exo-hydroxycamphor; Fe(III), ferric metmyrapone-bound cytochrome P-450\(_{cam}\).

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**Fig. 2. Examples of an acceptable and an unacceptable fit to the first-phase data.** The two lines are the results of two separate experiments at 420 nm using 3.2 \(\mu M\) cytochrome and 32 \(\mu M\) putidaredoxin. The open circles represent a successful fit to the data using the following parameters: \(k_a = 3.4/\mu M\cdot s, k_{-a} = 12/s, k_b = 70/s, \text{and} k_c = 500/s\). The filled circles represent the fit obtained using these parameters: \(k_a = 2.4/\mu M\cdot s, k_{-a} = 12/s, k_b = 110/s, \text{and} k_c = 240/s.\) This set of parameters was rejected because the calculated initial slope is more than 10% higher than it should be.
rate parameters shown in Fig. 1.

Comparison of the Time Course of Electron Transfer with the Time Course of the Overall Reaction—We then narrowed down the possibilities for rate constants by determining whether electron transfer was slower than a subsequent step of the reaction. To do this, we compared the time course of electron transfer with the time course of appearance of metyrapone-bound cytochrome, i.e., the time course of the overall product-forming reaction.

The general design of this experiment was to freeze-quench reaction samples at several times less than 0.1 s and to measure by EPR the amount of reduced putidaredoxin remaining and the amount of ferric metyrapone-bound cytochrome formed in each sample tube. If a relatively slow process were occurring after the electron transfer, there would be, at early reaction times, a lag between the electron transfer and the completion of the overall reaction. The sets of possible rate constants obtained from the modeling results were used to aid in choosing enzyme concentrations at which to perform these experiments. Enzyme concentrations were required which were as close as the possible sets of rate constants, would ensure that the hypothetical lag could be measured if it existed. Thus, concentrations were to be high enough for accurate measurements of their changes but not so high that any lag would occur within the 15 ms dead time of the freeze-quench method. Concentrations of 28 μM putidaredoxin and 30 μM cytochrome were chosen based on computer calculations of the time courses of accumulation of species D2 in Fig. 1 (oxidized putidaredoxin) and of species D1 (metyrapone-bound cytochrome) using several different sets of rate constants, all compatible with the stopped-flow data. With these enzyme concentrations, for any values of $k_a$ and $k_c$, that were consistent with the stopped-flow data, there were calculated to be measurably different ratios of D2 to D1 at times of 15, 25, 40, and 80 ms, depending only on the relative magnitudes of $k_a$ and $k_c$.

Fig. 4 shows the computer-calculated time courses for these two species and the resulting ratios for four sets of rate constants which are representative of cases in which 1) $k_a < k_c$, 2) $k_a < k_c$, 3) $k_a = k_c$, and 4) $k_a > k_c$. The experiment was then performed to allow some of these four cases to be ruled out.

Reactions between 28 μM reduced putidaredoxin and 30 μM oxyacytochrome P-450$_{cam}$ were freeze-quenched at 15, 26, 40, or 82 ms and packed into EPR tubes. The concentration of camporph was 40 μM, and the metyrapone concentration was 7.4 mM. In each EPR tube, the amount of metyrapone-bound ferric cytochrome and the amount of reduced putidaredoxin were measured as described under “Materials and Methods.” The initial amount of reduced putidaredoxin in each experiment was determined by freeze-quenching each putidaredoxin solution mixed with buffer instead of the oxyacytochrome just before the freeze-quenching of the actual reaction mixture. The amount of reduced putidaredoxin oxidized was calculated as the difference between the amount measured in the buffer-mixed sample and the amount measured in the reaction sample. A control experiment was performed to determine how much, if any, metyrapone-bound cytochrome could be detected in a 15 ms mixture of buffer containing metyrapone with the oxyacytochrome under the usual reaction conditions, with no putidaredoxin present. The amount of metyrapone-bound cytochrome present was above the limit of detection but was too small for very accurate quantitation. It was estimated to be approximately 1 μM by comparison with the 4 μM standard, and this concentration was subtracted from the final concentration found in each reaction mixture to obtain the change due to the reaction. The presence of metyrapone-bound cytochrome in the absence of catalytic turnover ideally would not occur and probably did not occur when the experiments were performed at low concentrations of cytochrome. But, in the experiments at 30 μM cytochrome, the camporph concentration (40 μM) presumably was not sufficient to saturate the high concentration of cytochrome. Thus, it is reasonable to suppose that 1 μM cytochrome may have remained substrate-free and would have bound metyrapone immediately upon being exposed to it. The results of the freeze-quench experiments, performed in triplicate, are shown in Table 1. Comparison of these experimental results with the expected results for different relative values of $k_a$ and $k_c$ (Fig. 4) reveals that the conditions $k_a > k_c$ and $k_a = k_c$ are inconsistent with the results. Thus, $k_a$, the rate constant of electron transfer, is at least 2-fold smaller than $k_c$, the rate constant of product formation and release, and is most likely several-fold smaller.

A stopped-flow, spectrophotometric measurement was made at the same concentrations of reduced putidaredoxin and oxyacytochrome as were used in the freeze-quench experiments. A wavelength of 555 nm was used for this measurement, which gave a total absorbance change of almost 0.1 with a background absorbance of about 0.5. Wavelengths in the 400-500-nm range were not suitable because of extremely high background absorbances at such high enzyme concentrations. Although the absorbance at 555 nm decreases during the reaction, a conversion factor of $-0.005/\mu$M was used to convert the absorbance change into a positive change in concentration of metyrapone-bound ferric cytochrome P-450$_{cam}$. The time course of this change, along with the average values of putidaredoxin change and cytochrome change from the freeze-quench experiments, is presented in Fig. 5. There is good agreement between the spectrophotometric and the EPR results; however, it may be noted that the overall reaction rate shown in Fig. 5 is less than the rate predicted in Fig.
back reaction.

The independence of these parameters is as follows: \( k_b \) is between 12 and 20 \( \mu M \cdot s \); \( k_a \) is between 12 and 600/s; \( k_b \) is between 60 and 100/s; and \( k_c \) is at least 200/s. The fact that step b in Fig. 1 (the electron transfer step) is followed by a relatively fast and practically irreversible step means that the possibility of the reversibility of step b is no longer important; that is, even if step b is theoretically reversible, the next step will minimize the accumulation of species C (in Fig. 1) and thus prevent any significant rate of back reaction.

**Effects of Varying KCl Concentration**—The effect of KCl concentration upon the reaction was examined by determining the relationship between initial rate and putidaredoxin concentration at 20 and 100 mM KCl, for comparison to the earlier data at 500 mM KCl. The results of these initial rate measurements are given in Figs. 6 and 7, and again they show a hyperbolic dependence of initial rate on putidaredoxin concentration. Calculated \( k_{cat} \) and \( k_{cat}/K_m \) values at the three concentrations of KCl are plotted together in Fig. 8. Fig. 4A shows in\((k_{cat})\) plotted against the square root of KCl concentration. The relationship is not linear but deviates from linearity in a manner which is consistent with the expected deviation of electrostatic effects from Debye-Hückel theory at ionic strengths from 0.1 to 0.5 M (Barrow, 1979). This suggests that the mechanism of the intramolecular portion of this reaction step, which determines \( k_{cat} \), may involve an electrostatic interaction. Since parallel experiments using salts other than KCl have not yet been done, the possibility of a specific ion effect upon \( k_{cat} \), as opposed to a general ionic strength effect, has not been ruled out.

Fig. 4B shows \(\ln(k_{cat}/K_m)\) plotted against the square root of KCl concentration. These data are indicative of the effects of KCl upon \( k_{cat} \), the rate constant for the association of oxycytochrome P-450 \(_{ox} \) with reduced putidaredoxin. There is no significant change in \( k_{cat}/K_m \) between 20 and 100 mM KCl, but there is a large decrease between 100 and 500 mM KCl. In contrast with these results, an increase might be expected based on the fact that both of these proteins have rather acidic pI values, 4.7 and 3.4 (Dus et al., 1970; Gunsalus and
TABLE I
Results of the freeze-quench experiments using 28 μM putidaredoxin and 30 μM cytochrome

In each case, camphor concentration was 40 μM and metyrapone concentration was 7.4 mM. The reactions were in standard buffer at 4°C. For each time point, the results of measurements of two species in each of three separate experiments using different reactant solutions are shown, along with the averages of the three. Also shown are the ratios of the changes in the two species and the average ratio for each time point. Pd\text{red}, reduced putidaredoxin; met, metyrapone.

| Time (ms) | Loss of Pd\text{red} (μM) | Increase in P450-met (μM) | ΔPd\text{red} ΔP450-met |
|-----------|---------------------------|---------------------------|-------------------------|
| 15        | 7.6                       | 12.2                      | 0.62                    |
| 15        | 6.7                       | 12.2                      | 0.78                    |
| 15        | 6.4                       | 10.1                      | 0.68                    |
| 26        | 12.4                      | 11.5                      | 1.08                    |
| 26        | 9.6                       | 10.3                      | 0.93                    |
| 26        | 10.8                      | 10.9                      | 0.99                    |
| 40        | 11.3                      | 13.4                      | 0.84                    |
| 40        | 11.1                      | 12.5                      | 0.89                    |
| 40        | 12.4                      | 12.5                      | 0.99                    |
| 82        | 20.8                      | 18.7                      | 1.11                    |
| 82        | 16.7                      | 17.3                      | 1.02                    |
| 82        | 16.4                      | 16.8                      | 0.98                    |

Fig. 5. Time course of reaction between 28 μM reduced putidaredoxin and 30 μM oxyctochrome P-450\text{cam}, measured in different ways. In each case, camphor concentration was 40 μM and metyrapone concentration was 7.4 mM. The reactions were in standard buffer at 4°C. The line represents the time course of appearance of ferric metyrapone-bound cytochrome calculated from a stopped-flow experiment at 555 nm. The diamonds represent the average at each time point of EPR measurements of putidaredoxin oxidized in freeze-quench experiments, and the squares represent the averages of EPR measurements of metyrapone-bound ferric cytochrome P-450\text{cam} in the same freeze-quench experiments.

Fig. 6. Dependence of initial reaction rate upon concentration of reduced putidaredoxin at 0.02 M KCl. Concentrations after mixing were 3.2 μM oxyctochrome P-450\text{cam}, 20 μM camphor, and 2.5 mM metyrapone. The reactions were performed at 4°C in standard buffer. From the linearized plot in B were calculated $K_{cat} = 9 \text{μM}$ and $k_{cat} = 109 \text{μM/μM·s}$. Pd, putidaredoxin.

Lipscomb, 1973); and therefore, both are negatively charged at pH 7.4. Such an increase in rate constant with increased ionic strength has been seen, for example, in the oxidation of Chromatium vinosum high-potential iron-sulfur protein by ferricyanide (Feinberg and Johnson, 1980). The negative charges on both species cause electrostatic repulsion at low ionic strength, but these charges are shielded at high ionic strength, permitting a faster reaction rate. In the case of reduced putidaredoxin and oxyctochrome P-450\text{cam}, there is apparently no net electrostatic repulsion limiting the rate of association. Indeed, the decrease in $k_{cat}/K_m$ at high KCl concentration might be interpreted as arising from small regions of opposite charges on the surfaces of the two enzymes, which would provide local electrostatic attraction at low, but not high, ionic strength. Alternatively, the decrease might be attributed to either a specific ion effect or an electrostatic effect on the conformation of one or both of the proteins, resulting in a loss of binding efficiency. In any case, the nonlinear relationship seen in Fig. 8B argues against electrostatic shielding as the sole effect of KCl upon $k_{cat}$.

Modeling of the experiments at 20 and 100 mM KCl was performed as described for the experiments at 500 mM. The
modeling results imply a minimum $k_a = 17 \mu M/s$ for the low and medium salt cases, compared with a minimum of $3/\mu M/s$ in high salt. This difference is consistent with the higher values of $k_{cat}/K_m$ in low and medium KCl. The minimum calculated values of $k_b$ are $200/s$ at $20 \text{mM KCl}$ and $140/s$ at $100 \text{mM KCl}$, compared with $60/s$ at $500 \text{mM KCl}$. These values are consistent with the pattern of $k_{cat}$ variation with KCl. A minimum value of $k_c = 200/s$ is estimated for all three salt concentrations. Maximum values of the rate constants could not be found for the low and medium salt cases.

Comparison with Rate Data for the First Electron Transfer Step in the Cytochrome P-450 Cycle—The rate data for the first cytochrome P-450 reduction step (Hintz, 1981) have several points of dissimilarity to the rate data obtained for the second electron transfer. First, the time courses of the first reduction reactions were exponential, apparently indicative of first-order kinetics. Second, the effect of KCl upon $k_{cat}$ for the second reduction (Fig. 8A) is quite different from its effect upon $k_{cat}$ for the first reduction. In that case, experiments done at a higher range of KCl concentrations showed no effect upon $k_{cat}$ until the KCl concentration reached about $0.5 \text{M}$, above which the ln($k_{cat}$) declined linearly with the square root of KCl concentration. Third, the relationship between concentration of reduced putidaredoxin and rate constant was not hyperbolic in the case of the first reduction, although saturation was reached at about $20-30 \mu M$ putidaredoxin. This saturation behavior permits the identification of a putidaredoxin concentration at which the rate is half-maximal, even though no formal $K_m$ can be calculated.

For reaction conditions of $10^\circ C$ and ionic strength of $0.16 \text{M}$, a half-maximal reaction rate was achieved at slightly greater than $1 \mu M$ putidaredoxin. This compares with a $K_m$ of about $7 \mu M$ for the second reduction at $4^\circ C$ and $0.1 \text{M KCl}$. Comparing the maximal turnover rates under the same two sets of conditions, the rate of the first reduction at saturating putidaredoxin was about $10-15/s$, and the rate of the second reduction at saturating putidaredoxin was about $75-80/s$. At $4^\circ C$ under optimal conditions, the turnover rate for the entire catalytic cycle is $10-20/s$ (Ishimura et al., 1971). The differences between the kinetic characteristics of the first and second reductions support the argument (Peterson and Mock, 1975b) that at high reduced putidaredoxin concentrations, the first reduction may limit the overall turnover rate of the...
catalytic cycle; whereas at lower reduced putidaredoxin concentrations, with the same concentration of the cytochrome, the second reduction may be no faster than the first. As mentioned by Peterson and Mock (1975b), carbon monoxide inhibition of the overall reaction would not be expected when the first reduction is rate-limiting but should be observed when the second reduction is partially rate-limiting. The maximal rates of the two electron transfer steps \((k_{cat})\) are significantly different, whereas the ratios of maximal rate to the putidaredoxin concentration at half-maximal rate \((k_{cat}/K_m)\) are not. This suggests that kinetically the most significant difference between the mechanisms of these two steps is probably in the electron transfer process and not in the cytochrome-putidaredoxin binding process.

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