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ELECTRON TRANSFER FROM PYRIDINYL RADICALS, HYDRATED ELECTRONS, $CO_2^{\bullet -}$ AND $O_2^{\bullet -}$ TO BACTERIAL CYTOCHROME P450

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SUMMARY: Several rate constants for one-electron reduction of cytochrome P450 are more rapid in the absence than in the presence of the specific substrate. The respective values for methyl viologen, nicotinamide adenine dinucleotide and the 1-methyl-4-(and -3-)carbamidopyridinium radicals are 2.6, 3.4, 6 and 35 x 10^7 M^{-1} s^{-1} without camphor, and 0.15, 0.1, 1.8 and 110 x 10^7 M^{-1} s^{-1} for the camphor complex. Hydrated electrons react with cytochrome P450 with a rate constant of 3.0 x 10^{10} M^{-1} s^{-1} whether camphor is bound or not, but little of the reduction takes place at the haem iron. No reduction of the haem iron by $CO_2^{\bullet -}$ or $O_2^{\bullet -}$ could be detected, whether camphor is bound or not.

Haem-containing monoxygenases of the cytochrome P450 type are unique among haemoproteins in their capacity, having accepted one electron, to activate Fe^{2+}-bound oxygen through acceptance of a second electron. In the natural system used here the reduction is performed by the specific iron-sulfur protein putidaredoxin (1). The reduction potential of the cytochrome P450 of Pseudomonas putida is strongly modified (2) as are other physico-chemical parameters, by binding of the substrate camphor.

Pulse radiolysis has already been used to investigate electron transfer within proteins, especially haemoproteins (e.g. 3,4). This paper concerns the reduction of substrate-free and -bound cytochrome P450 by pyridinyl radicals, e^-_{aq}, $CO_2^{\bullet -}$ and $O_2^{\bullet -}$.

Abbreviations: Fe^{3+}: cytochrome P450; Fe^{2+}: haem-reduced cytochrome P450; P^+: pyridinium compound; $MV^{\bullet +}$; methyl viologen (paraquat) radical cation; $NAD^{\bullet}$: one-electron reduced nicotinamide adenine dinucleotide; RH: camphor; 4 $AM^{\bullet}$: one-electron reduced 1-methyl-4-carbamidopyridinium ion; 3 $AM^{\bullet}$: one-electron reduced 1-methyl-3-carbamidopyridinium ion.

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MATERIAL AND METHODS

Camphor-bound cytochrome P450 was prepared from Pseudomonas putida in the laboratory of Dr. Gunsalus. It was freed from camphor by chromatography on an LH20 column and elution with 50 mM Na-phosphate buffer (pH 7) at 4°C. Pulse radiolysis was carried out at 25°C, on 4 to 7 μM oxidised cytochrome P450 in 50 mM Na/K phosphate buffer (pH 7.3) containing 5×10^{-2} M Na formate. In the presence of 10^{-3} M pyridinium compounds all the primary radicals of water radiolysis are converted, within less than 1 μs, into pyridinyl radicals via the reactions (5) :

$$H_2O \longrightarrow e^-_{aq} + OH^\bullet + H^\bullet \quad (1)$$

$$H^\bullet + HCOO^- \longrightarrow H_2 + CO_2^{\bullet -} \quad (2)$$

$$OH^\bullet + HCOO^- \longrightarrow H_2O + CO_2^{\bullet -} \quad (3)$$

$$CO_2^{\bullet -} + P^+ \longrightarrow CO_2 + P^\bullet \quad (4)$$

$$e^-_{aq} + P^+ \longrightarrow P^\bullet \quad (5)$$

In the presence of oxygen (10^{-3}M) hydrated electrons and $CO^{\bullet -}$ give rise to $O_2^{\bullet -}$. When added, camphor concentrations were between 250 and 450 μM. The solutions were bubbled with pure Ar for at least 30 min at 4°C or, where indicated, with N_2O, O_2 or 10% CO in Ar. The pulse radiolysis set-up, located at the Christie Hospital, has been described (6). Capillary glass tubes and cylindrical cells with an optical path length of 1 cm and an inside diameter of 3 mm were used so as to minimise consumption of materials.

RESULTS

Fig. 1 gives the optical difference spectra Fe^{2+} minus Fe^{3+} and Fe^{2+} - CO minus Fe^{3+} for camphor-free and -bound cytochrome P450. The ferric forms are respectively low spin (λ_{max} 417 nm) and high spin (λ_{max} 392 nm) in absence and presence of camphor. The Soret spectra of the ferrous forms are independent of camphor binding, whether CO is bound or not (7).

1) Reaction with methyl viologen radical. Pulses generating between 25 and 110 μM of stable $MV^{\bullet +}$ were employed. These concentrations will consume any sub-μM residual concentration of oxygen in < 50 μs (5). Reaction with ~5 μM P450, monitored around 445 or 420 nm (cytochrome reduction and $MV^{\bullet +}$ consumption - see Fig. 1) or at 700 nm ($MV^{\bullet +}$ consumption alone), follows first order kinetics on a much slower time scale (Fig. 2).

(a) Without camphor. The pseudo first order rate constant for formation of Fe^{2+} is proportional to the initial $MV^{\bullet +}$ concentration

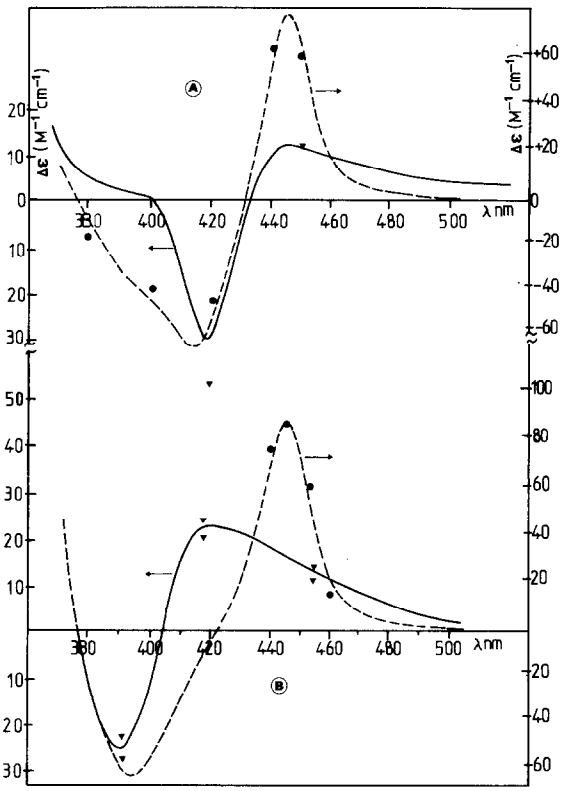

Figure 1. Difference spectra (—) Fe^{2+} minus Fe^{3+} and (- - -) Fe^{2+} - CO minus Fe^{3+} of (A) camphor-free cytochrome P450 and (B) camphor-bound cytochrome P450. Experimental points, obtained from Δ O.D. measured after $MV^{\bullet+}$ reduction (▼) in absence and (●) presence of carbon monoxide.

(Fig. 3) and leads to the rate constant $2.6 \pm 0.3 \times 10^7$ M^{-1} s^{-1}. Haem reduction was estimated from the absorbance changes at 445 or 420 nm, using $\Delta\epsilon$ values from Fig. 1, and $\epsilon_{MV^{\bullet+}}$ = 1.1 and 2.3 mM^{-1} cm^{-1} at 445 and 420 nm (5). The Fe^{3+} reduction, as determined from absorption changes in the Soret region, was stoichiometric with the concentration of $MV^{\bullet+}$ consumed, as measured at 700 nm ($\epsilon_{MV^{\bullet+}}$ = 3.4 mM^{-1} cm^{-1} (5)), but represented only 40 to 50% of the initial cytochrome concentration. The apparently non-reducible material increases with time and could represent protein denatured by the bubbling with argon and/or by standing at 25°C in the flow system. The stoichiometry found however excludes the possibility that denatured enzyme affects the

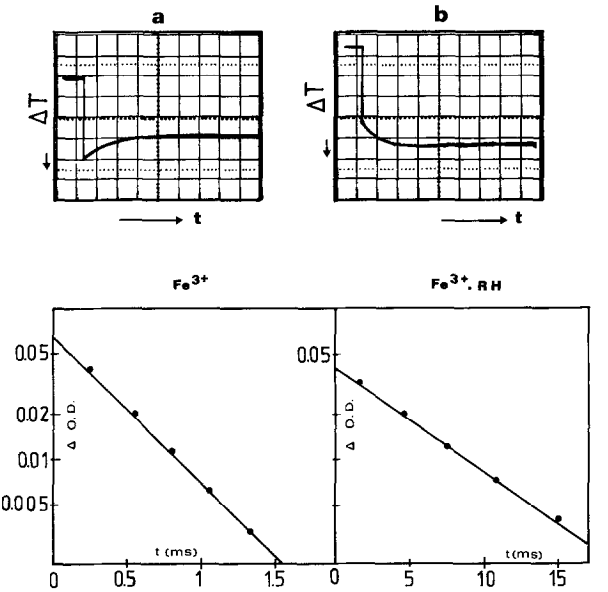

Figure 2. Reduction of camphor-free and -bound cytochrome P450 by methyl viologen radical (a) camphor-free cytochrome (Fe^{3+}); λ = 417.5 nm; [Fe^{3+}] = 5 μM; [$MV^{\bullet+}$] = 93 μM; 500 μsec/div.; Δ T (%) = 0.01/div.; temperature 25°C. Kinetic trace and semi logarithmic plot. (b) camphor-bound cytochrome (Fe^{3+} . RH); λ = 445 nm [camphor] = 450 μM; [Fe^{3+} . RH] = 5 μM; [$MV^{\bullet+}$] = 110 μM; 10 msec/div.; T (%) = 6.66 10^{-3}/div.; temperature 25°C; kinetic trace and semi logarithmic plot.

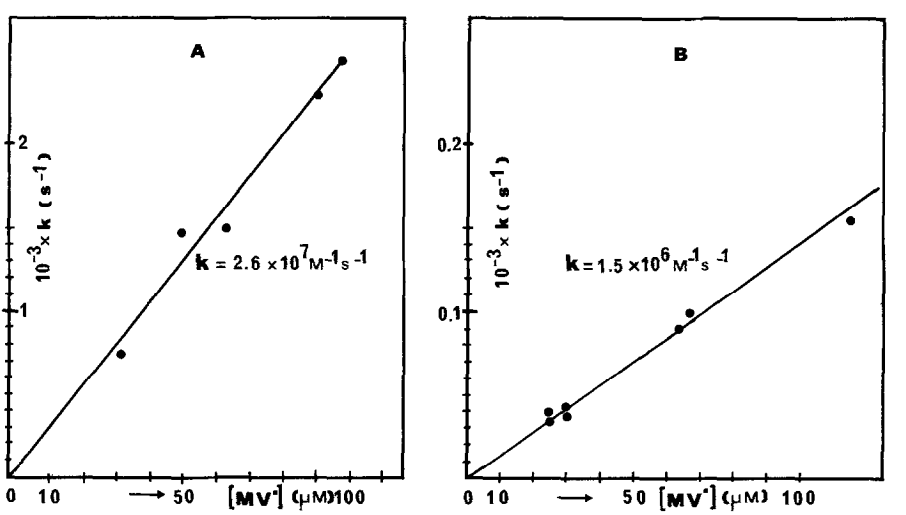

Figure 3. Concentration dependence of the pseudo first order rate constants for cytochrome P450 reduction by $MV^{\bullet+}$. Conditions as above except that dose varies. (a) Fe^{3+} (b) Fe^{3+} . RH.

rate constant obtained. The experiments with $MV^{\cdot+}$ were also performed in presence of 1.4×10^{-4}M CO, making it possible to follow the build-up of the Fe^{2+} - CO complex at 450 nm (Fig. 1). Such experiments confirmed the stoichiometry of the reaction of $MV^{\cdot+}$ with the enzyme.

(b) With camphor. Reduction of Fe^{3+}. RH by $MV^{\cdot+}$ in the presence of camphor show a much slower second order rate (Fig. 2b) with the rate constant $1.5 \pm 0.2 \times 10^{6}$ M^{-1} s^{-1}. Again Fe^{2+}. RH was stoichiometric with respect to the $MV^{\cdot+}$ consumed. The presence of camphor protects cytochrome P450 against the accumulation of non-reducible material, haem reduction measured in the Soret region indicating only 10 to 20% loss. $MV^{\cdot+}$ was found not to react with camphor itself. Again, addition of 1.4×10^{-4}M CO led to a build-up of 450 nm absorption (Fig. 1).

2) Reaction with NAD$^{\cdot}$ radical. NAD$^{\cdot}$ is unstable due to dimerisation (8). To slow down this process lower doses were used producing less radicals ($\sim$0.8 µM) than P450 (5 to 7 µM). Due to the traces of residual oxygen and the uncertainty in the concentration of reducible cytochrome, the rate constants carry a large error (up to 40 to 50%) and are only estimates. For the camphor-free enzyme the reduction was stoichiometric and followed pseudo first order kinetics, with second order rate constant 3.4×10^{7} M^{-1} s^{-1}. Fe^{3+} . RH reacted more slowly; $k = 10^{6}$ M^{-1} s^{-1} after allowing for competition between NAD$^{\cdot}$ - Fe^{3+}. RH reduction and NAD$^{\cdot}$ + NAD$^{\cdot}$ dimerisation.

3) Reaction with NAD$^{\cdot}$ analogues - 4 AM$^{\cdot}$ and 3 AM$^{\cdot}$ radical. 4 AM$^{\cdot}$ is stable under the conditions chosen (9) over the time scales of reaction with both Fe^{3+} and Fe^{3+} . RH. With the low doses described above, the reaction of 4 AM$^{\cdot}$ was pseudo first order with respect to the cytochrome concentration. The rate constant estimates were 6×10^{7} and 1.8×10^{7} M^{-1} s^{-1} with Fe^{3+} and Fe^{3+} . RH, respectively, haem reduction being stoichiometric.

3 AM$^{\cdot}$, like NAD$^{\cdot}$, is short-lived (9). Its reaction with cytochrome P450 was the fastest so far studied, rate constant estimates being 3.5×10^{8} M^{-1} s^{-1} with Fe^{3+} and 1.1×10^{9} M^{-1} s^{-1} with Fe^{3+} . RH. In both cases

haem reduction was found to be stoichiometric. Experiments with 3 AM$^{\bullet}$ + Fe^{3+} and Fe^{3+} . RH were also performed in presence of CO and led to CO complex at 450 nm, confirming the stoichiometry.

4) Reactions with e^-_{aq}, $CO_2^{\bullet -}$ and $O_2^{\bullet -}$ radicals. The reductions by e^-_{aq} were monitored in the absence or in the presence of camphor concentrations equimolar to Fe^{3+}, since previous experiments indicated moderate electron scavenging by camphor (10). The e^-_{aq} lifetime, measured at 700 nm, indicated a rapid reaction with protein, rate constant 3.0×10^{10} M^{-1} s^{-1}, independent of camphor presence. Haem reduction yield corresponded to ~10% of the e^-_{aq} reacting. The reaction of $CO_2^{\bullet -}$, formed in the presence of N_2O which converts e^-_{aq} into more OH$^{\bullet}$, and of $O_2^{\bullet -}$ in O_2 saturated solutions, failed to show haem reduction with or without camphor.

DISCUSSION

Rate constants for P450 reduction are collected in Table I. For $MV^{\bullet +}$, the only radical whose reaction with cytochrome P450 has been studied previously, the present value for Fe^{3+}. RH agrees well with the value obtained by dye-sensitised photoreduction, but for Fe^{3+} the present value is approximately 10 times faster than found before (11). The exact reason for this discrepancy is not clear; it could be due to different conditions of pa_H and salt concentration in the two types of experiment. The agreement for Fe^{3+} . RH excludes any fast Fe^{2+} . RH oxidation by H_2O_2 formed in the high energy radiation pulses.

It is worth noting that, except for 3 AM$^{\bullet}$, pyridinyl radicals reduce the free cytochrome faster than the substrate-complex, despite a more negative reduction potential (E'_o -340 mV and -170 mV for Fe^{3+} and Fe^{3+} . RH respectively (2)). This could be due to the lesser accessibility of the haem pocket to the radical when the substrate is bound, as already suggested by experiments with other probes (12-14). The high rate constant for reduction by 3 AM$^{\bullet}$ of both Fe^{3+} and Fe^{3+} . RH, the latter 10^3 times more rapid than the $MV^{\bullet +}$ reduction rate, implies a quite different route in this case.

TABLE I

Rate constants for reaction of various radicals with Fe^{3+} and Fe^{3+} . RH at 25°C.

Radical	Second Order Rate Constant $M^{-1} s^{-1}$ (x 10^{-7})	
	Fe^{3+}	Fe^{3+} . RH
$MV^{\bullet +}$	2.6 ± 0.3	0.15 ± 0.02
$NAD^{\bullet}$	3.4	0.1
4 $AM^{\bullet}$	6	1.8
3 $AM^{\bullet}$	35	110
e^{-}_{aq}	3000	3000

The high reactivity of e^{-}_{aq} with the low yield of haem reduction suggests that the electron, unlike other radicals, reduces other acceptors present in the protein, e.g. peptide bonds (15), aromatic amino acids (16) and protonated histidine or cysteine (16), which then act as "electron sinks" and do not transfer to the haem. Similarly, the lack of reduction by $CO_2^{\bullet -}$ and $O_2^{\bullet -}$, contrasting with their reactivity with other haemoproteins (3,4,17), reflects the unique character of cytochrome P450. Electrostatic effects could also be important since negative charges around the haem are known to modulate the spin state and perhaps redox potential of the ferric camphor complex (18).

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