TEMPERATURE-JUMP MEASUREMENT OF THE SPIN STATE RELAXATION RATE OF CYTOCHROME $P450_{cam}$

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Received 10 August 1981

1. Introduction

Cytochrome P450 is known to catalyze a wide variety of biological oxidation processes including alkane and aromatic hydroxylation, epoxidation, dealkylation, and halogen oxygenation [1–4]. The hydroxylation cycle involves sequential steps of substrate binding, ferric–ferrous reduction of the heme group, dioxygen binding, second electron input and coupled oxygen–oxygen bond scission, substrate oxygenation, water release, and regeneration of the ferric, substrate-free cytochrome. In many instances the rate-limiting step in the overall hydroxylation cycle is the first electron input into the hemoprotein and, as such, serves as a metabolic control step in the hepatic microsomal electron-transport scheme [5,6]. Cytochrome P450 isolated from the soil bacterium *Pseudomonas putida* has for many years served as an excellent system for the study of P450-catalyzed oxidations [1–4]. The regulation in the oxidation–reduction potential of the hemoprotein via substrate binding was documented in [7], wherein the normally quite low redox potential of the substrate-free hemoprotein of −300 mV was raised to −173 mV upon the addition of camphor to saturating levels. Inasmuch as the normal reductant protein for this cytochrome P450, putidaredoxin, has an oxidation–reduction potential of −196 mV when bound to P450 [7], this regulation in oxidation–reduction potential via substrate association conceptually serves as a 'switch' to prevent unwanted electron flow to the terminal oxidant in the absence of oxidizable substrate. Understanding of this regulation process was further advanced by the description of substrate binding, oxidation–reduction reactions and the $S = 1/2$ to $S = 5/2$ ferric spin-state transition in terms of fundamental microscopic constants [8]. Through [8], the importance of the thermal ferric spin equilibrium in dictating the observed oxidation–reduction potential of the heme group was documented. Extension of this model to the hepatic P450 illustrated similar mechanisms of substrate-induced alteration in the P450 redox potential [6], and resulted in a general, simplified model for the linkage between spin state and reduction of P450 [5,9]. Here, we present the experimental determination of the thermal spin-state relaxation rate for substrate-free and camphor-bound cytochrome $P450_{cam}$, as well as an estimation of the overall reduction rate of the cytochrome by putidaredoxin in a putidaredoxin–P450 complex.

2. Materials and methods

Cytochrome $P450_{cam}$ was purified from *Pseudomonas putida* essentially as in [10,11]. Camphor was removed by passage over a Biogel P2 column equilibrated in Tris–HCl buffer (50 mM at pH 7.4) followed by extensive dialysis against 50 mM potassium phosphate buffer (pH 7.0). The instrument used for temperature-jump measurements was built according to [12] with minor modifications. The temperature-jump cell had a volume of 1.4 ml and an optical pathlength of 0.7 cm [13]. The putidaredoxin–P450 electron-transfer rate in the P450–putidaredoxin complex was measured by flash photoreduction using a 100 J Xenon flash lamp with a half-width of 50 μs and a photo-sensitizing solution of proflavin and methyl viologen with EDTA as electron donor follow-

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Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

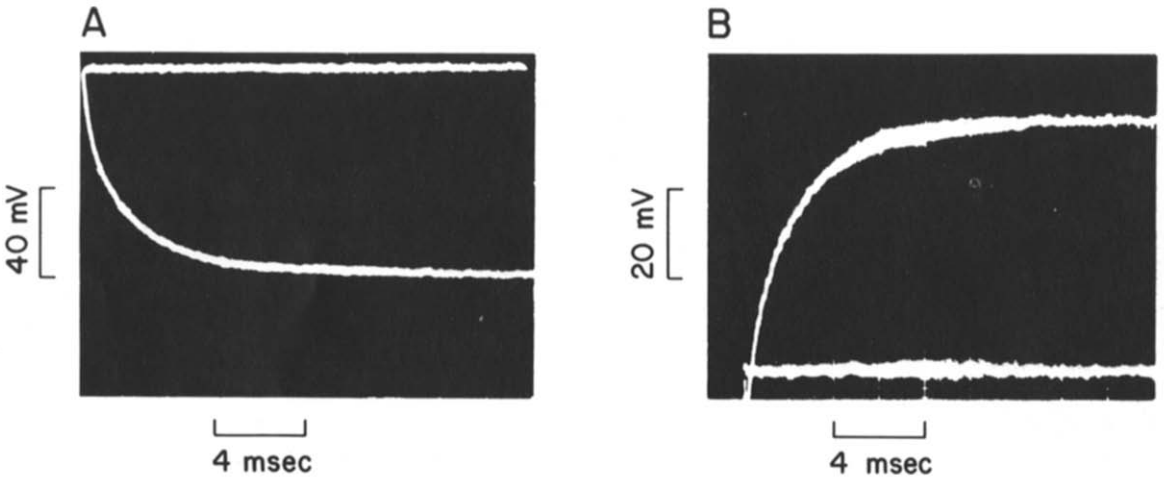

Fig.1. Temperature-jump relaxation of substrate-bound cytochrome $P450_{cam}$. Initial temperature in both cases was 17.2°C with a 2.4°C temperature-jump. Cytochrome P450 at 10 μM in 100 mM potassium phosphate (pH 7.0) containing 400 μM camphor was monitored at 417 nm (A) and 390 nm (B).

ing [14]. Because the putidaredoxin–cytochrome bimolecular complex was preformed prior to the addition of reducing equivalents, this measurement of electron transfer kinetics does not quantitate the multiprotein binding reaction in [15]. Camphor saturation was verified by optical spectroscopy as in [10]. Spin-state equilibrium constants were redetermined using the buffer conditions of kinetic measurements and agreed with those in [8].

3. Results and discussion

Fig.1A illustrates a typical temperature-jump trace obtained when substrate-bound cytochrome is subjected to a 2.4°C jump with a final temperature of 19.6°C. In this case the optical absorbance was followed at the wavelength of maximal absorption of high spin cytochrome, 390 nm. Fig.1B illustrates an identical jump with optical monitoring at the wavelength of maximal absorption of low-spin cytochrome, 417 nm. The rigorous first-order dependence of the relaxation rate, the complementary behavior at 390 nm and 417 nm, and the clean isosbestic behavior at 406 nm (not shown), indicate a simple two state equilibrium diagramed below:

$$P450(Fe^{3+}, S = 1/2) \underset{k_2}{\overset{k_1}{\rightleftarrows}} P450(Fe^{3+}, S = 5/2)$$

Similar results were obtained with substrate-free cytochrome. Semilogrithmic plots of representative traces are presented in fig.2. Table 1 summarizes the observed relaxation times, τ, for substrate-bound and -free cytochrome together with the rate constants k_1 and k_2 derived from the equilibrium constants shown. Clearly the presence of bound substrate serves to drastically shift the observed ferric spin equilibrium constant and increase the forward rate of the reaction.

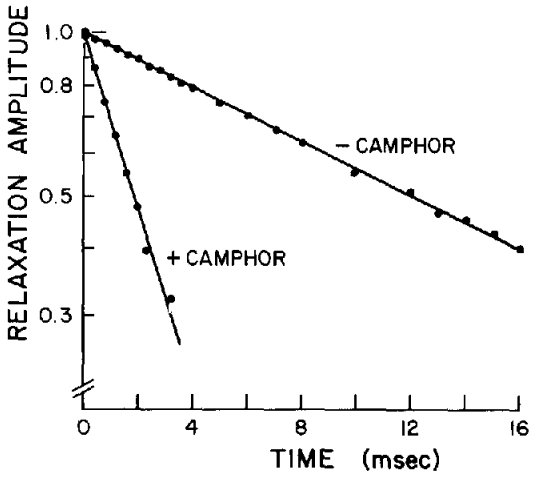

Fig.2. The relaxation signal amplitudes from experiments exemplified by fig.1 are plotted for both camphor-bound and camphor-free forms of the enzyme. Conditions are as described in the text and legend to fig.1.

Table 1
Spin state and reduction rates of cytochrome $P450_{cam}$[a]

	$P450(Fe^{3+}, S = 1/2) \underset{k_2}{\overset{k_1}{\rightleftarrows}} P450(Fe^{3+}, S = 5/2)$			$Pd^r \cdot P450(Fe^{3+}) \underset{k_B}{\overset{k_A}{\rightleftarrows}} Pd^o \cdot P450(Fe^{2+})$			
	τ (ms)	$1/\tau = k_1 + k_2$	$K_{eq} = k_1/k_2$[b]	k_1	k_2	k_A	k_B
−Camphor	17	59	0.086	4.6	53	0.22	22
+Camphor	2.7	365	15	342	23	41	17

[a] All rates are s^{-1}; [b] Measured as in section 2

Table 1 also indicates the observed rate constants for the overall reduction of cytochrome P450 by putidaredoxin as determined by flash photolysis:

$$Pd^r \cdot P450(Fe^{3+}) \underset{k_B}{\overset{k_A}{\rightleftarrows}} Pd^o \cdot P450(Fe^{2+})$$

(where Pd^o and Pd^r are oxidized and reduced putidaredoxin, respectively)

Clearly evident is the drastic reduction in the ferric–ferrous reduction rate of the P450 heme in the absence of camphor, confirming the kinetic control of electron transfer between putidaredoxin and P450 via substrate association.

The overall model of spin state regulation of cytochrome P450 reduction is the scheme:

$$P450(Fe^{3+}, S = 1/2) \underset{k_2}{\overset{k_1}{\rightleftarrows}} P450(Fe^{3+}, S = 5/2) \underset{k_4}{\overset{k_3}{\rightleftarrows}} P450(Fe^{2+}, S = 2)$$

The regulation of electron transport in this system is through k_3, the inherent rate constant for the reduction of high spin heme, because in no case is the forward spin-state interconversion rate, k_1, limiting in the overall reduction velocity (table 1). The dominant contribution to the modulation of the ferric spin-state equilibrium constant via substrate association is through alteration of the low-spin to high-spin transition rate, k_1, with the reverse reaction rate, k_2, changing significantly less between the camphor-bound and -free forms of the hemoprotein. This situation in the bacterial P450 system contrasts with that of the purified rat hepatic protein wherein the spin state transition rates may be to some extent rate-limiting in the overall observed reduction velocity [9].

Acknowledgements

This research was supported by research grants from the National Institutes of Health, GM 24976 (SGS) and GM 26109 (PEC), and NIH Research Career Development Awards AM 00778 (SGS) and GM 00471 (PEC).

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