Spin equilibrium relaxation kinetics of cytochrome P450 LM2

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Received 7 September 1982; revision received 26 October 1982

The relaxation kinetics of the temperature-dependent spin equilibrium of cytochrome P450 LM2 has been resolved by means of a laser temperature-jump device. The Nd laser system in the Q-switched mode delivers 3 J/25 ns pulses which were Raman shifted to 1.89 μm by use of a high pressure H_2-gas cell. The LM2 equilibrium relaxation proceeds in the ns-range, the rate constants of the on- and off-reactions amount to about $10^6 s^{-1}$ in correspondence with respective processes of other hemoproteins and protein-free model compounds. Benzphetamine increases the on-rate constant (low-spin $\rightarrow$ high-spin) about 5 fold, the off-process is nearly unchanged.

Cytochrome P450 *Laser* *Temperature-jump* *Spin equilibrium* *Relaxation kinetics* *Reaction mechanism*

1. INTRODUCTION

The catalytic function of cytochrome P450 is determined by electron-transfer processes activating molecular oxygen [1]. The activities of single reaction steps, and of the overall reaction, as well, have been shown to be controlled by a ferric spin equilibrium. Therefore, the knowledge of the dynamic behaviour of the spin-state transition is of essential importance to understand the enzymatic mechanism of the P450-catalyzed oxidations.

The heme iron of P450 could be shown to establish a spin equilibrium, the two states of which exhibit high and low spin characteristics ($S = 5/2$, $S = 1/2$). Substrates control the composition of the respective fractions [2,3]. A distinct temperature dependence could be proved by different laboratories [4–8]. Correlations between the high spin content, the first electron-transfer reaction, and substrate turnover or NADPH oxidation, as well, have been observed [9–13]. The interpretations of the causal relationships therein differ mainly in assuming either the spin-state conversion as rate-limiting [11,12], based on temperature-jump investigations [14], or on the other hand, the conversion step not to be rate-limiting [10,13], as indicated by our earlier kinetic experiments [15]. Recently the spin-state conversion rates in the P450 CAM system have been proved not to be rate-limiting in the reduction process [16]. Here we present laser temperature-jump investigations of the P450 LM2 spin-state transition kinetics which confirm a reasonable rapid non-limiting process in the liver P450, too.

2. MATERIALS AND METHODS

Electrophoretically homogeneous P450 LM2 was prepared from liver microsomes of phenobarbital-treated rabbits [7,17]. Benzphetamine hydrochloride was obtained by extraction and crystallization from Didrex® (Upjohn Co, Kalamazoo MI). The measurements were performed in 0.1 M phosphate buffer (pH 7.4) with 20% (v/v) glycerol and 1 mM EDTA; in the substrate-bound experiments 2 mM benzphetamine was used, according to the K_s (28°C) = 0.2 mM [7] ~90% of the P450 was present as substrate complex.

The stimulated Raman laser temperature-jump techniques [18] has been employed by means of a self-constructed Nd-glass laser system. The oscillator running in a passive dye Q-switched mode was followed by an amplifier stage, which delivered 3 J/25 ns pulses. The primary 1.06 μm

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

laser radiation was Raman-shifted to 1.89 μm by use of a high pressure H_2-gas cell = (8–10 MPa). The thermostatted measuring cell contained 15 μl of the sample solution, the optical pathlength was 0.03 cm. The absorbance changes were monitored by use of the optical laser pumping pulse of a xenon flash lamp, spectral filters, and a multiplier system including a 30 MHz oscilloscope. The rise-time of the apparatus was determined by means of a dye system (phenol red) with ~30 ns. The delivered 10°C jump raised the initial sample temperature of 18°C to a final one of 28°C.

3. RESULTS AND DISCUSSION

The P450 LM2 spin equilibrium has been investigated by assuming a two-state model:

$$P450_{\text{low spin}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P450_{\text{high spin}}; \quad K = k_1/k_2$$

According to the respective band maxima 417 nm and 387 nm [15] the relaxation process was followed at 417 nm (graded interference filter), at 405 nm (isosbestic point, interference filter), and around 385 nm (filter combination). Fig. 1 shows the experimental traces for substrate-free P450 LM2. The temperature-induced shift towards the high spin state is exhibited in an absorption increase at 385 nm (a), and is synchronously reflected in a decrease at 417 nm (c). At the isobestic point no variation in the signal is observed (b). The signal amplitudes correspond to the statically determined spectral differences. A significantly increased amplitude was obtained with the substrate-bound cytochrome, corresponding to the altered spin equilibrium composition.

First order relaxation is evidenced by fig. 2 for the substrate-free ($n = 8$) and the substrate-bound ($n = 10$) P450, as well. The semilogarithmic plot of the relaxation amplitude *vs* time exhibits a homogeneous process. The determined kinetic parameters of the spin transition reactions are drawn in table 1. The rate constants k_1 and k_2 were calculated from the measured relaxation times τ and from the respective equilibrium constants K [7].

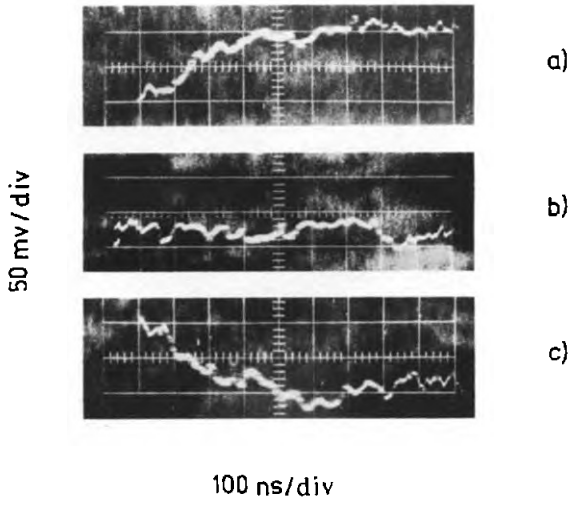

Fig. 1. Spin-state relaxation traces of rabbit P450 LM2 monitored at: (a) 385 nm (high-spin band); (b) 405 nm (isosbestic point); (c) 417 nm (low spin band); [P450] = 330 μM; temperature-jump, ΔT = 10°C; 50 mV corresponds to about ΔA = 0.010; abscissa, 100 ns/div.; ordinate, 50 mV/div.

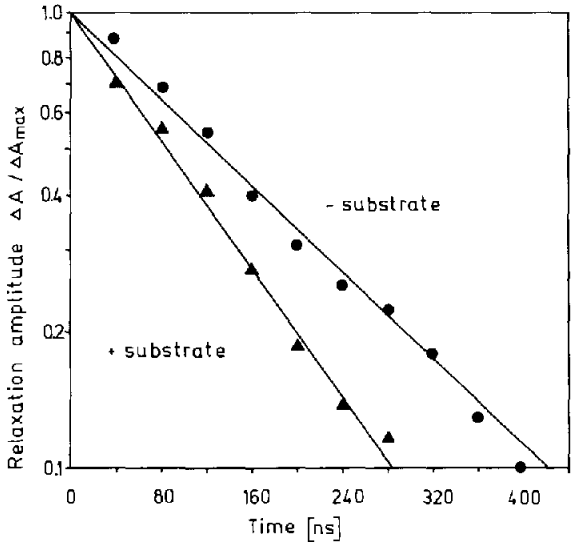

Fig. 2. Spin equilibrium relaxation of rabbit P450 LM2 for substrate-free and substrate-bound cytochrome: [P450] = 330 μM; [Benzphetamine] = 2 mM; temperature-jump, 18–28°C

Table 1

Kinetic parameters of the spin state relaxation of rabbit P450 LM2, 0.1 M phosphate buffer (pH 7.4), $T = 28°C$

Species	n^a	τ (ns)	$1/\tau = k_1 + k_2$ (s^{-1})	$K = k_1/k_2$	k_1 (s^{-1})	k_2 (s^{-1})
P450 LM2	8	182 ± 28	$5.5 \cdot 10^6$	0.12	$5.9 \cdot 10^5$	$4.9 \cdot 10^6$
P450 LM2 + benzphetamine	10	122 ± 24	$8.2 \cdot 10^6$	0.59	$3.0 \cdot 10^6$	$5.2 \cdot 10^6$

[a] Number of experiments; [b] selected from [7]

According to the preliminary experiments [15] the observed relaxations proceed in the ns time scale. That seems quite reasonable with respect to similar intramolecular changes in spin multiplicity in metmyoglobin (OH, N_3) [19,20] and in protein-free compounds [21]. The correspondence of the relaxation amplitudes as determined statically and in the kinetic experiments, respectively, proves that the observed effects represent the spin transition process, at least mainly. Any disturbance by means of the substrate equilibrium is excluded, because the substrate complex must relax in the ms range according to the respective kinetic and equilibrium constants k_{on} and K_S [7,22]. The relaxation data correspond to the P450 CAM investigations [16] in:

(i) An increase in the rate constant k_1 of the low-to-high spin transition in the presence of substrate;
(ii) In the exclusion of rate limitation towards the P450 reduction.

The deviation in the time scale could be due to some interference of the protein ligand in position 6 in the P450 CAM [23]. The slow relaxation in the presence of substrate as observed in liver P450 [14] must be questioned for clear-cut discrimination with respect to the substrate equilibrium, especially since no relaxation was observed with the substrate-free cytochrome in these investigations.

ACKNOWLEDGEMENTS

The authors are much indebted to Dr A. Lau and Dr K. Lenz, Central Institute of Optics and Spectroscopy, GDR Academy of Sciences, for advice and technical support in the construction of the laser device.

REFERENCES

[1] Werringloer, J. and Kawano, S. (1980) in: Biochemistry, Biophysics, and Regulation of Cytochrome P450 (Gustafsson, J.A. et al. eds) pp. 359–362, Elsevier Biomedical, Amsterdam, New York.
[2] Schenkman, J.B., Remmer, H. and Estabrook, R.W. (1967) Mol. Pharmacol. 3, 113–123.
[3] Jansson, I., Gibson, G.G., Sligar, S.G., Cinti, D.L. and Schenkman, J.B. (1980) in: Microsomes, Drug Oxidations and Chemical Carcinogenesis (Coon, M.J. et al. eds) pp. 139–142, Academic Press, New York.
[4] Lange, R., Bonfils, C. and Debey, P. (1977) Eur. J. Biochem. 79, 623–628.
[5] Pierson, W.C. and Cinti, D.L. (1977) Biochem. Biophys. Res. Commun. 78, 1139–1146.
[6] Rein, H., Ristau, O., Friedrich, J., Jänig, G.-R. and Ruckpaul, K. (1977) FEBS Lett. 75, 19–22.
[7] Ristau, O., Rein, H., Jänig, G.-R. and Ruckpaul, K. (1978) Biochim. Biophys. Acta 536, 226–234.
[8] Cinti, D.L., Sligar, S.G., Gibson, G.G. and Schenkman, J.B. (1979) Biochemistry 18, 36–42.
[9] Imai, Y., Sato, R. and Iyanagi, T. (1977) J. Biochem. 82, 1237–1246.
[10] Rein, R., Ristau, O., Misselwitz, R., Buder, E. and Ruckpaul, K. (1979) Acta Biol. Med. Germ. 38, 187–200.
[11] Backes, W.L., Sligar, S.G. and Schenkman, J.B. (1980) Biochem. Biophys. Res. Commun. 97, 860–867.
[12] Backes, W.L., Sligar, S.G. and Schenkman, J.B. (1982) Biochemistry 21, 1324–1330.
[13] Blanck, J., Rein, H., Sommer, M., Ristau, O., Smettan, G. and Ruckpaul, K. (1983) in preparation.
[14] Tsong, T.Y. and Yang, Ch.S. (1978) Proc. Natl. Acad. Sci. USA 75, 5955–5959.
[15] Ristau, O., Rein, H., Greschner, S., Jänig, G.-R. and Ruckpaul, K. (1979) Acta Biol. Med. Germ. 38, 177–185.

[16] Cole, P.E. and Sligar, S.G. (1981) FEBS Lett. 133, 252–254.
[17] Haugen, D.A., Van der Hoeven, T.A. and Coon, M.J. (1975) J. Biol. Chem. 250, 3567–3570.
[18] Ameen, S. (1975) Rev. Sci. Instrum. 46, 1209–1215.
[19] Beattie, J.K. and West, R.J. (1974) J. Am. Chem. Soc. 96, 1933–1935.
[20] Dose, E.V., Tweedle, M.F., Wilson, L.J. and Sutin, N. (1977) J. Am. Chem. Soc. 99, 3886–3888.
[21] Dose, E.V., Hoselton, M.A., Sutin, N., Tweedle, M.F. and Wilson, L.J. (1978) J. Am. Chem. Soc. 100, 1141–1147.
[22] Blanck, J., Smettan, G., Jänig, G.-R. and Ruckpaul, K. (1976) Acta Biol. Med. Germ. 35, 1455–1463.
[23] Sligar, S.G. (1976) Biochemistry 15, 5399–5406.