KINETICS OF CO AND O_2 COMPLEXES OF RABBIT LIVER MICROSOMAL CYTOCHROME P_{450}.

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Summary: The equilibrium binding constant of the liver microsomal cytochrome P_{450}-CO-complex was found to be $K = 1.3 \times 10^6$ M^{-1}. The binding was not cooperative. A second order rate constant of the association reaction of $1_{12} = 2.4 \times 10^5$ M^{-1} sec^{-1} at 25°C was evaluated by flash-photolysis experiments. In the presence of oxygen, carbon monoxide and of hexobarbital an additional process with an absorption maximum at about 445 nm was observed whose rate was about 20 times faster than that of the CO-association reaction and proportionally related to the oxygen concentration.

Interaction of oxygen with cytochrome P_{450} is one of the several steps in the biotransformation reaction sequence catalysed by the liver microsomal electron transfer chain. The kinetics of the O_2-complex of cytochrome $P_{450\ CAM}$ of pseudomonas putida interchangeable with its CO-complex has been investigated in detail (1,2). In rabbit liver microsomes an intermediate with an absorption band around 440 nm has been reported (3) which has been attributed to the cytochrome P_{450}-O_2-complex. The aim of the present study was to investigate the binding kinetics of CO and O_2 to cytochrome P_{450} of phenobarbital stimulated rabbit liver microsomes mainly by application of the tech-

niques of flash-photolysis and rapid mixing. The results provide further evidence that the intermediate observed at around 440 nm might be due to the cytochrome P_{450}-O_2-complex. This complex is formed about 20 times faster than the CO-complex.

Experimental: Hexobarbital sodium was purchased from Merck, Darmstadt, NADPH from Boehringer, Mannheim. Gases used were high-grade purity. The gases N_2 and CO were further deoxygenated by chemisorption (oxisorb, Messer-Griesheim) and contained less than 0.02 ppm oxygen.

Rabbits received phenobarbital in drinking water (0.1%). Phenobarbital was withdrawn 48 hours before sacrifice of the animals. The livers were perfused in situ with 0.15 m KCl. Microsomes were prepared according to FOUTS (4). For the experiments washed microsomes (0.15 m KCl) were suspended in 0.1 m phosphate buffer pH 7.8. For the measurement of the CO-dissociation curve the method applied to myoglobin by ROSSI-FANELLI et al. (5) was adapted to cytochrome P_{450}. Absorbance spectra were recorded with a Cary 17 spectrophotometer. In the flash-photolysis experiments pulses of flashlight with a half-duration of 7 µsec and an energy of about 250 J were used. The monitoring light pathway of the sample cell was 5 cm (details of the apparatus are given by K.H. GRELLMANN et al. (6)). For the rapid mixing experiments a modified Durrum stopped flow apparatus was used. Protein was determined by the method of LOWRY et al. (7), cytochrome P_{450} as described by OMURA et al. (8).

Results: The CO-dissociation curve obtained was hyperbolic (fig. 1). From the double logarithmic plot of (Y/1-Y) vs. CO-

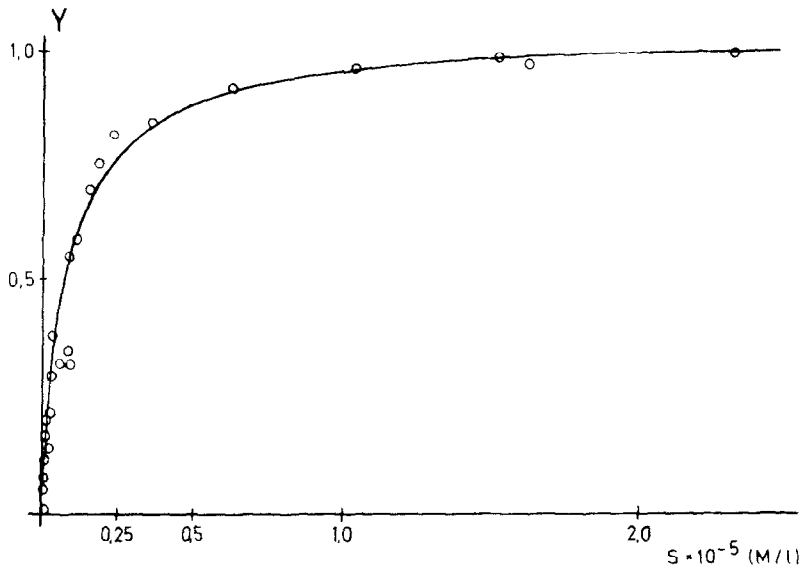

Fig. 1
Dissociation curve of the CO-complex of dithionite reduced cytochrome P_{450} of phenobarbital stimulated rabbit liver microsomes in 0.1 m phosphate buffer pH 7.8; 7.25×10^{-6}m cytochrome P_{450}, 1.9 mg protein/ml, 25°C; other experimental details see text. Y = fractional saturation of cytochrome P_{450} with CO. The circles are experimental values; the solid curve is the result of calculation for $K = 1.3 \times 10^{6} M^{-1}$.

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concentration, which was linear, a binding constant of K_{CO} = 1.3×10^{6} M^{-1} at 25°C was calculated.

In the presence of the high CO-concentrations used the recombination curve of the P_{450}-CO-complex after flash-photolysis (see fig. 2a) obeys the time course of a pseudo-first order kinetics, as the linearity of the plot of the logarithm of absorbance as function of time shows (see fig. 2b). The pseudo-first order constants have been found to be proportional to the CO-concentrations. The calculated second order rate constant (l_{12}) was 2.4×10^{5} M^{-1} sec^{-1} at 25°C.

In the presence of hexobarbital as a type 1 substrate, carbon

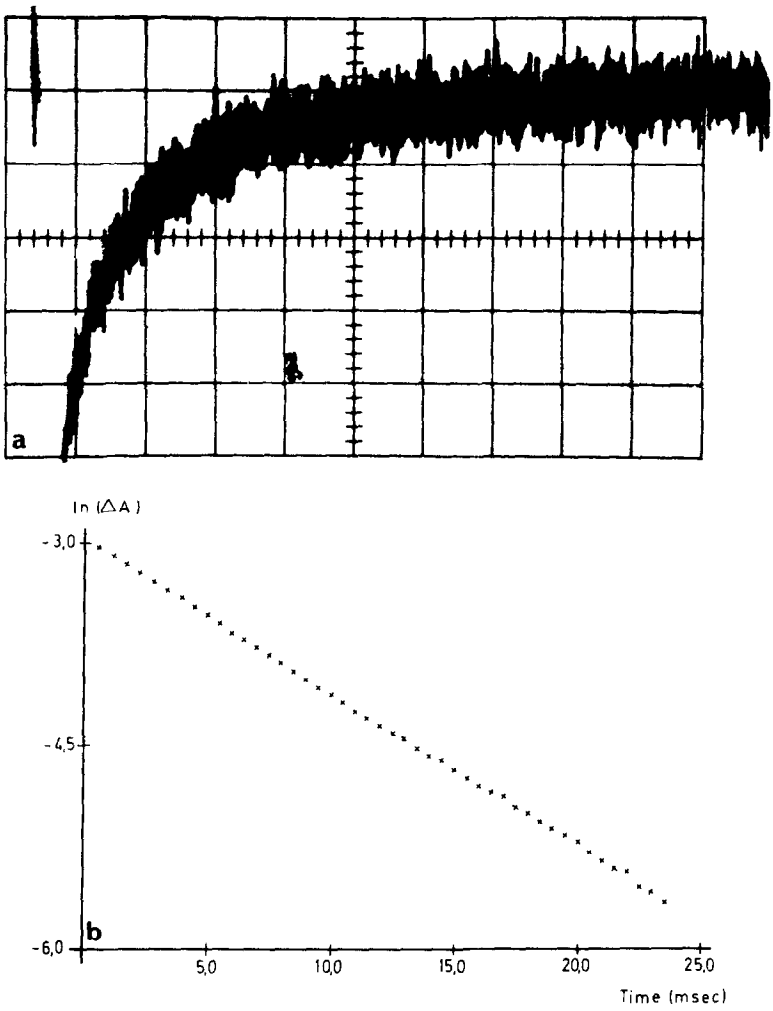

Fig. 2a

Time dependent transmission change at 450 nm after flash-photolysis of the CO-complex of NADPH reduced cytochrome P_{450} of stimulated rabbit liver microsomes in 0.1 m phosphate buffer pH 7.8; 1.36×10^{-6}m cytochrome P_{450}, 0.7 mg protein/ml, 5×10^{-4}m NADPH, 0.6×10^{-3}m CO, 25°C. UV-light was cut off by a filter (Schott GG 400). An upward deflection indicates a decrease in transmittance. Energy of the flash was about 250 J. Abscissa: 5 msec/div, ordinate: 0.2V/div..

Fig. 2b

Plot of logarithms of absorbancies of transmission changes shown in fig. 2a.

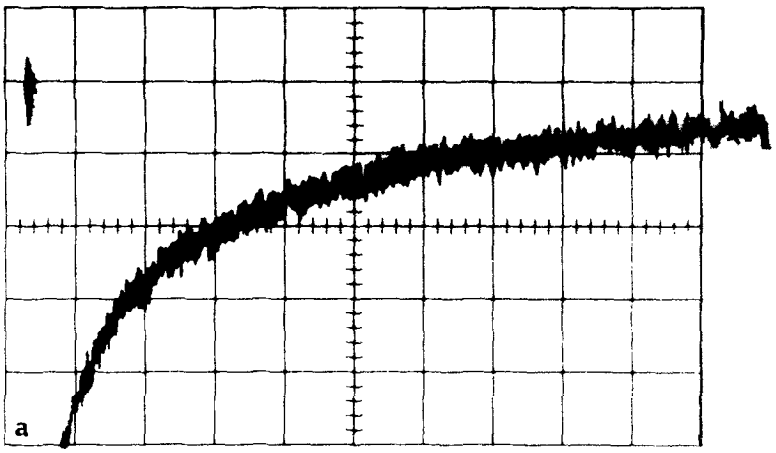

Fig. 3a

Transmission change at 445 nm after flash-photolysis of NADPH reduced cytochrome P_{450} of stimulated rabbit liver microsomes, which have been equilibrated with a CO-O_2-mixture, in the presence of hexobarbital in 0.1 m phosphate buffer pH 7.8; 7×10^{-7}m cytochrome P_{450}, 0.45 mg/protein, 0.83×10^{-4}m O_2, 2.1×10^{-4}m CO, 2×10^{-3}m hexobarbital, 5×10^{-4}m NADPH, 25°C, about 250 J flash-energy. Abscissa: 5 msec/div., ordinate: 0.2V/div..

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monoxide (2.1×10^{-4} m) and oxygen (0.83×10^{-4} m) a much faster absorbance change at 445 nm was observed in addition to the slower phase due to CO-recombination (figs. 3a and 3b). For this fast phase a first order constant of 367 sec^{-1} at 25°C could be evaluated which was found to be proportional to the O_2-concentration. If it is assumed that this fast phase is due to the association of O_2 with free cytochrome P_{450} a second order rate constant of $k_{12} = 4.4\times10^6$ M^{-1} at 25°C can be calculated.

The flash-photolysis difference spectrum of the CO-complex (fig. 4a hatched line) agrees well with the known optical absorbance difference spectrum, while the flash-photolysis difference spectrum obtained in the presence of carbon monoxide, of oxygen and of hexobarbital shows a broadening (fig. 4a solid

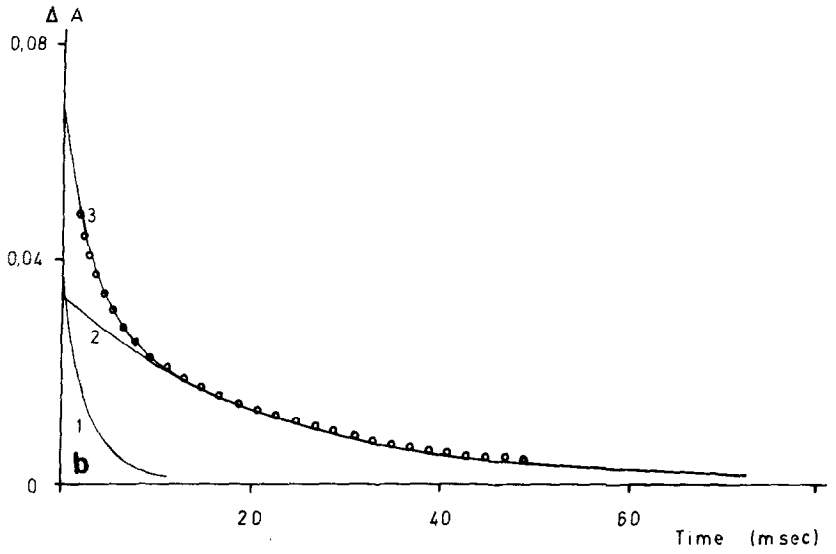

Fig. 3b

Curve fitting of the experimental curve of fig. 3a (open circles represent experimental values). Curve 3 is fitted by application of equation $\Delta A=A_{02}xe^{-1'xt}+ A_{01}xe^{-k'xt}$. The first term, drawn separately in curve 2, represents the recombination of CO with cytochrome P_{450}. The rate constant 1' was calculated from the measured association rate constant and from the CO-concentrations of $2.1x10^{-4}$m used. Curve 1 is the difference between curve 3 and curve 2 and represents an exponential function, from which a rate constant k' = 367 sec^{-1} was calculated.

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line) due to the appearance of an additional peak at about 445 nm, which is clearly demonstrated by plotting the difference of these spectra (fig. 4b). Hexobarbital had no effect on the reassociation rate of carbon monoxide in the absence of oxygen nor on the flash-photolysis difference spectrum of the CO-complex of cytochrome P_{450}.

Reduced microsomes equilibrated with carbon monoxide-oxygen-mixtures were oxidized by rapid admixture of $K_3[Fe(CN)_6]$. A two-phase curve of time dependent absorbance change at 445 nm was observed. The slow phase corresponds to the dissociation of

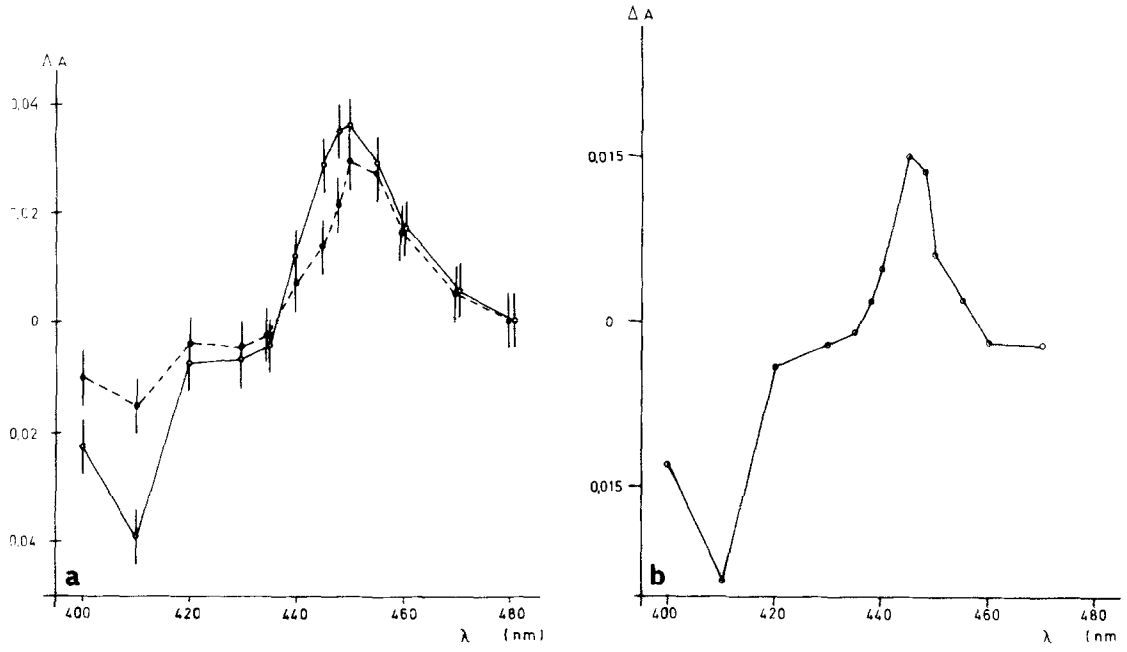

Fig. 4a

Flash-photolysis difference spectra of phenobarbital stimulated rabbit liver microsomes in the presence of hexobarbital equilibrated with CO (1.3×10^{-4}m, closed circles) and equilibrated with O_2 and CO (1.3×10^{-4}m CO, 0.25×10^{-4}m O_2, open circles); 4.8×10^{-7}m cytochrome P_{450}, 0.25 mg protein/ml, 5×10^{-4}m NADPH, 2.10^{-3}m hexobarbital, 25°C, about 250 J flash-energy. Absorbance changes were taken at 4.0 msec after the flash. Bars represent the half width of the oscilloscope trace at 4.0 msec. For each experimental value between 430 and 460 nm the trace of 3 flashes were nearly identical. Similar results were obtained with 3 different microsomal preparations.

Fig. 4b

Difference of the spectra shown in fig. 4a.

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the cytochrome P_{450}-CO-complex as shown by curve fitting (see fig. 5, curve 2 and legend). From this slow phase a dissociation rate constant of $l_{21} = 0.2$ sec^{-1} can be evaluated which is in agreement with the constant calculated from the measured equilibrium and recombination rate constants. The rapid phase, which

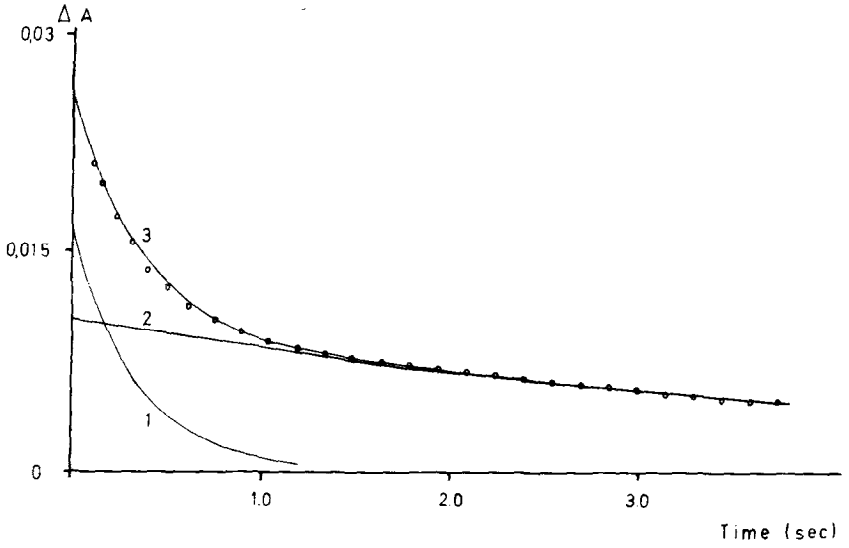

Fig. 5

Absorbance change at 445 nm (open circles) after mixing of equal vol. of a solution of $K_3[Fe(CN)_6]$ ($1x10^{-4}$m) and of reduced microsomes equilibrated with a $CO-O_2$-mixture ($2.58x10^{-6}$m cytochrome P_{450}, 1.5 mg protein/ml, $5x10^{-4}$m NADPH, $5.1x10^{-4}$m O_2, $3x10^{-4}$m CO) in a stopped flow apparatus (mixing time about 1 msec). Curve 3 is fitted by application of the equation $\Delta A = A_{03}xe^{-1}{}_{21}{}^{t} + A_{04}xe^{-k}{}_{21}{}^{t}$. The first term represents the dissociation of the cytochrome P_{450}-CO-complex calculated from the experimental values of the equilibrium and the recombination constants and is separately drawn in curve 2. Curve 1 is the difference between curve 3 and curve 2, from which a rate constant of $k_{21} = 3.0\ sec^{-1}$ was calculated.

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follows first order kinetics (fig. 5, curve 1), might be assumed to be due to the dissociation of the cytochrome P_{450}-O_2-complex. A dissociation rate constant $k_{21} = 3.0\ sec^{-1}$ could be calculated.

Discussion: There was no cooperativity of binding of CO to cytochrome P_{450} as the observed equilibrium kinetic indicates. The association with carbon monoxide was observed to be a first order reaction with respect to CO. In the presence of O_2 an

additional process appeared which was faster and of first order with respect to oxygen. Moreover as indicated by the flash-photolysis difference spectrum this fast process has its absorption maximum at 445 nm in accordance with the difference spectrum reported for the oxygenated form of microsomal cytochrome P_{450} (3).

A binding constant for oxygen of $K_{O_2} = 1.46 \times 10^6\ M^{-1}$ can be calculated on the assumption that the measured fast phases which appear on flash-photolysis and rapid mixing experiments are due to the processes of recombination and dissociation respectively of the cytochrome P_{450}-O_2-complex. Thus the affinity of cytochrome P_{450} of stimulated rabbit liver microsomes for oxygen is nearly as high as the affinity for carbon monoxide ($K_{CO} = 1.3 \times 10^6\ M^{-1}$). A roughly similar ratio was reported by PETERSON et al. (2) for cytochrome $P_{450\ CAM}$. The kinetic constants of the 2 types of cytochromes cannot be compared directly as they have been measured at different temperatures. Measurements of the activation energies of these reactions which are in progress will provide a basis for such a comparison which might be of value for an understanding of the mechanism of binding behaviour of these cytochromes.

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