LOW TEMPERATURE STUDIES OF MICROSOMAL CYTOCHROME P_{450}: I. STOPPED-FLOW EXPERIMENTS*

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Received 7 April 1973

1. Introduction

Interaction of carbon monoxide with cytochrome P_{450} has been studied by photodissociation of the microsomal cytochrome P_{450}–CO complex and the kinetics of recombination of reduced cytochrome P_{450} with CO [1], and by stopped-flow recording of carbon monoxide binding to bacterial reduced cytochrome P_{450} [2]. In these experiments, flash photolysis and rapid mixing techniques have respectively been used to determine equilibrium and kinetic constants of such fast recombination and combination reactions.

In the case of microsomal cytochrome P_{450}, complex kinetic results have been observed with at least two phases, one fast and the other slow. Some years ago a similar reaction between hemoglobin and carbon monoxide was studied in this laboratory [3] at low temperatures to make easier the resolution of the well-known concurrent fast and slow reactions and their kinetics were followed by a spectrophotometer of ordinary speed of response and sensitivity.

This first report describes results obtained at selected low temperatures for the reaction between liver microsomal cytochrome P_{450} and carbon monoxide studied by stopped-flow, and demonstrates that the binding occurs in a manner basically similar to binding in ordinary conditions, opening the way to the study of cytochrome P_{450} at low temperatures including by flash photolysis now under investigation in this laboratory.

* Number I of a numbered series.

2. Materials and methods

2.1. *Preparations*

Liver microsomes were prepared from phenobarbital treated rats according to the method of Ernster et al. [4] after perfusion of the liver with cold 0.9% NaCl "P_{450} subparticles" were obtained by the method of Nishibayashi et al. [5] and stored within 1 month frozen at −20° in concentrated suspension (about 40 mg/ml in 0.05 M phosphate buffer pH = 7.5 containing 25% glycerol).

Cytochrome P_{450} was further isolated and purified using the method of Mitani [6] except that the ammonium sulfate precipitation was replaced by a chromatography procedure which will be published later (C. Balny, personal communication). The preparation of isolated cytochrome P_{450} contained 10–15% cytochrome P_{420} and was stored in a 0.02 M Tris buffer pH = 7.5–ethylene glycol mixture (volume ratio 50:50) at −20°.

2.2. *Solvents*

The solvent used was a 0.02 M Tris buffer pH = 7.5 – ethylene glycol mixture (volume ratio 50:50, freezing point −45°), the pH of which at room and at low temperature was evaluated elsewhere [7].

Cytochrome P_{450} was reduced by few mg of sodium dithionite at room temp. and kept before use in a closed syringe at 0°.

Solutions of carbon monoxide were prepared by addition of definite volumes of buffer saturated with CO at 20° under 1 atm of pressure ($[CO] = 10^{-3}$ M) to a mixture of buffer and ethylene glycol, both having been previously deoxygenated and reduced with

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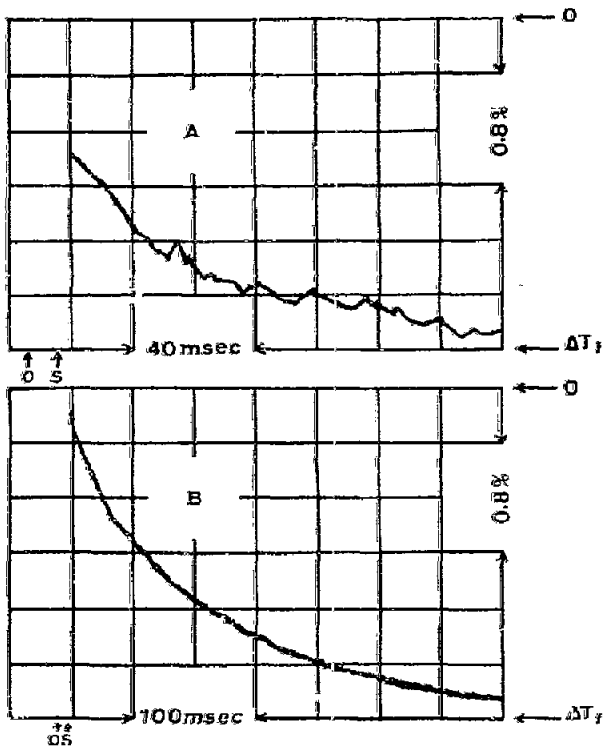

Fig. 1. Kinetic traces of the carbon monoxide binding to ferrous isolated cytochrome P_{450}. The vertical arrow indicates the stop of the flow. The final transmission is indicated by the horizontal arrow. For experimental procedure see Materials and methods. A) t = 20°. [CO] = 1.85×10^{-5} M. The initial concentration of ferrous cytochrome is 1.32×10^{-6} M. B) t = −19°. [CO] = 4.15×10^{-5} M. Same concentration of reduced cytochrome.

$Na_2S_2O_4$. The final volume ratio of the mixture is 50:50.

2.3. *Apparatus*

The stopped flow apparatus adapted for sub-zero temperatures was already described [8].

Static spectrophotometric measurements were performed on a Beckman Acta III type spectrophotometer. The reference and sample cuvettes may be thermostatically controlled to the same temperature (between 40° and −80°) according to a procedure already described [9].

3. Results

The reaction $Fe^{2+} + CO \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Fe^{2+}{-}CO$ was fol-

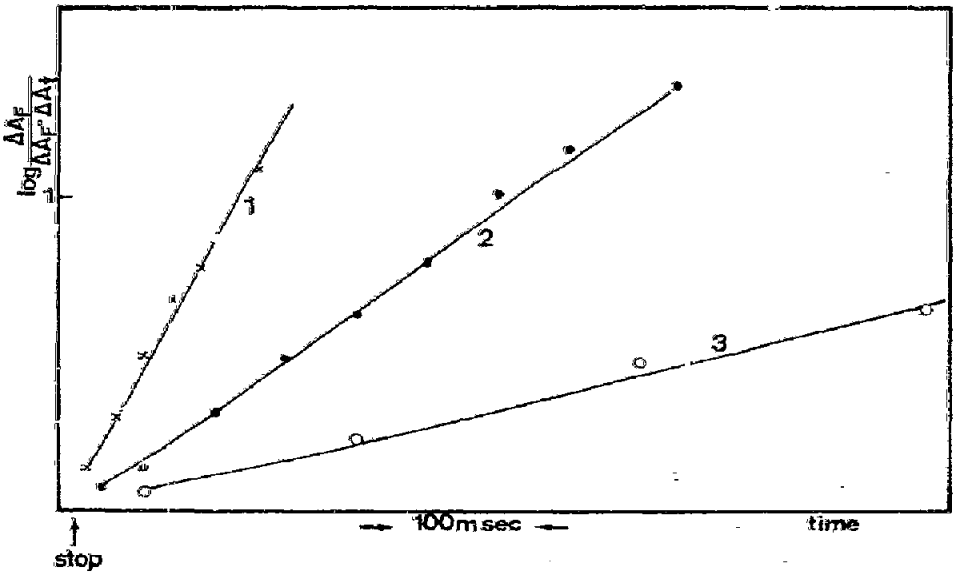

Fig. 2. Pseudo-first-order plots of the carbon monoxide binding to ferrous cytochrome P_{450}. ΔA_t and ΔA_∞ are respectively the changes in absorbance at time t and at infinite time. [CO] = 1.85×10^{-5} M and the initial concentration of cytochrome is 1.32×10^{-6} M. 1) t = 20°; 2) t = 0°, 3) t = −19°.

lowed by decrease in transmission at $\lambda = 450$ nm after mixing Fe^{2+} with a CO solution, the concentration of which is always higher than that of Fe^{2+} and may be considered as constant during the reaction. Fig. 1 shows kinetic traces obtained at 20° and −19°. As shown by a semi logarithmic plot of the change in absorbance versus time (fig. 2) the reaction is monophasic, the rate constant k depending on the CO concentration by the expression $k = k_1[CO] + k_{-1}$ (Eq. 1). Theoretically, the use of different CO concentrations allows to calculate both k_1 and k_{-1} at each temperature. Nevertheless k_{-1} is usually of the same order of magnitude as k_1 [CO] and any error on k or [CO] values leads to a great error on k_1 and k_{-1} values. In fact, results obtained by varying the CO concentration were somewhat dispersed and it seemed more accurate to obtain k_{-1} at different temperatures by the static measurement of $K_S = k_{-1}/k_1$. The saturation curve obtained with isolated cytochrome P_{450} at 20° yields to $K_S = (1.4 \pm 0.2)\ 10^{-6}$ M. After each addition of saturating or not saturating concentration of CO at 20°, the difference spectrum Fe^{2+}—CO minus Fe^{2+}, was recorded down to −40° and the saturation curve was then plotted at different temperatures. The saturation curve was also obtained by direct addition of CO solution to Fe^{2+} at low temperature, the fixation being within the response time of the apparatus (see table 1).

Both procedures yielded to the same curves and the dissociation constant K_S was found independent of the temperature ($K_S = 1.4 \times 10^{-6}$ M). Using this value of K_S in Eq. (1), k_1 and k_{-1} were calculated and plotted as a function of $1/T$ (fig. 3). The activation energy of the combination rate constant k_1 is

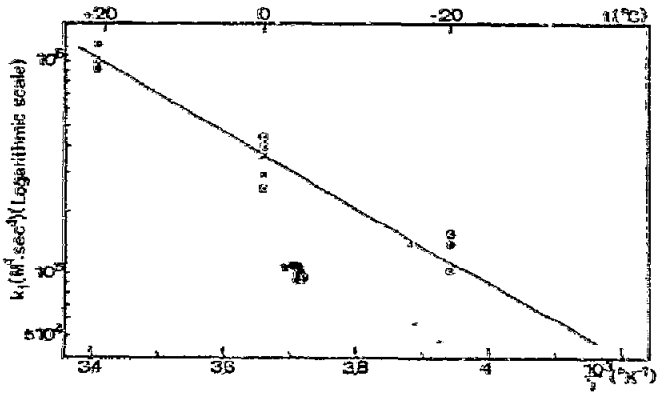

Fig. 3. Arrhenius plot of the rate constant k_1 for the fixation of carbon monoxide to ferrous cytochrome P_{450}. The experimental points refer to experiments performed the same day using the same preparation of soluble cytochrome.

E = 8.3 ± 1 kcal/mole. Since the dissociation constant K_S is independent of T, $\Delta H = 0$ for the overall fixation of CO and the activation energies of k_1 and k_{-1} are the same. Table 1 summarizes the values obtained for the different constants at several temperatures.

4. Discussion

Results obtained are in good agreement with those reported by Omura et al. by photodissociation on a "clarified" suspension of adrenal cortex microsomes (table 1). However, the very low value of K_S (2×10^{-7} M) explains the discrepancy in k_{-1} values at +4°. Omura and Sato [10] reported for liver microsomal cytochrome P_{450} a higher value (3×10^{-6} M), which is very close to the K_S obtained in this work on

Table 1

Temperature	k_1 (M^{-1} sec^{-1})	E (kcal/mole)	k_{-1} * (sec^{-1})	K_S (M)	ΔH (kcal/mole)
+20°	10^6	8.3	1.4	1.4×10^{-6}	0
+4°	4.5×10^5	8.3	0.63	1.4×10^{-6}	0
+4° **	3.4×10^5	–	0.068	2×10^{-7}	–
−20°	1.25×10^5	8.3	0.175	1.4×10^{-6}	0
−40°	2.6×10^4 ***	8.3	0.036	1.4×10^{-6}	0

* Calculated from $k_{-1} = K_S \cdot k_1$.
** Measured by Omura et al. [1] with adrenal cortex microsomes.
*** k_1 is extrapolated from the value of the activation energy E.

isolated cytochrome P_{450} (1.4×10^{-6} M). Since we found also $K_S = 3 \times 10^{-6}$ M with "P_{450} particles" both in aqueous buffer in the mixed solvent (Debey, unpublished observations), neither the isolation procedure nor the organic solvent seem to change the affinity of the cytochrome P_{450} toward carbon monoxide. It can be seen that combination of static spectrophotometric determinations with stopped flow technique, both used at sub-zero temperatures, yields to interesting results on thermodynamic parameters.

The fact that $\Delta H = 0$ is surprising when compared with the ΔH of ~ -10 kcal/mole observed with the same reaction on various monomeric hemoproteins such as myoglobins [11], but no good correlation seems to exist between free energy values and the structure of the protein or the electronic properties of heme fractions.

Over a broad range of temperatures the dissociation constant K_S and the activation energy do not vary. Therefore cytochrome P_{450} seems to undergo no temperature dependent structural change, and carbon monoxide binds to this protein at low temperature and in the ethylene glycol–water mixture in a manner basically similar to binding in ordinary conditions.

From a kinetic point of view, the monophasic combination of cytochrome P_{450} with CO observed in the present case (fig. 2) is in contradiction with the biphasic kinetics observed by M. Waterman and M Franklin, quoted by J.A Petterson and B.W. Griffin [2], but similar to the combination kinetics observed on soluble bacterial cytochrome P_{450} [2].

Further experiments at low temperatures with liver microsomal P_{450} are planned to try to clarify such contradictory results. Measurements made at widely different temperatures might in such a case make resolution easier, for the rates of different reactions would vary by amounts determined by their respective activation energies. Moreover, studies of the photodissociation and of the kinetics of recombination by flash-photolysis adapted to low temperatures and now in progress in this laboratory should help to confirm and complete the present results.

It is clear that the low temperature procedure is applicable to the study of reaction mechanisms involving soluble and membrane-bound cytochrome P_{450} and should represent a useful contribution to its knowledge.

Acknowledgements

We thank Claude Balny and Christian Le Peuch for their skillful assistance and their gift of isolated microsomal P_{450}. This work was supported by grants from the C.N.R.S. (Equipe Associée no. 262), the INSERM (contrat no. 72.4.030.2); the DGRST (convention no. 71 7.3091.01), and the Fondation pour la Recherche Médicale Française

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