LOW TEMPERATURE STUDIES OF MICROSOMAL CYTOCHROME P_{450}. DETERMINATION OF DISSOCIATION CONSTANT OF THE [Fe^{2+}–CO] FORM*

Claude BALNY and Pascale DEBEY

U-128, INSERM, BP 5051, 34033 Montpellier Cedex, France, and Ecole Pratique des Hautes Etudes, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

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1. Introduction

The combination of carbon monoxide (CO) with reduced rat liver microsomal cytochrome P_{450} was previously studied with various techniques in fluid hydro-organic solvent at subzero temperatures [1–3]. Stopped flow and flash photolysis allowed the determination of the association rate constant k_1 for the reaction:

$$Fe^{2+} + CO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Fe^{2+}–CO]$$

in the + 20°C, –40°C temperature range. However the usual static measurement of $K_S = k_{-1}/k_1$, which could provide an evaluation of k_{-1} is difficult owing to the very low value of K_S [1] and the simultaneous existence, in a given preparation, of various forms of cytochrome P_{450} [4,5].

Furthermore cytochrome P_{450} is unstable in very dilute solutions and is gradually transformed into cytochrome P_{420} during the time necessary for a saturation curve recording.

A procedure for measuring k_{-1} was thus developed, based on the competitive binding of CO with both cytochrome P_{450} and hemoglobin and on the possibility of monitoring the transfer of CO on cytochrome P_{450} following carboxyhemoglobin photodissociation.

When two reduced hemoproteins are mixed in presence of a definite low CO concentration, an equilibrium between the reduced and carboxy forms of both hemoproteins is established. If one of them has a higher quantum yield of photodissociation and a lower combination rate constant, CO may be transferred from this protein to the other by photodissociation. The dark reaction represents the dissociation of CO from the later protein.

The present communication deals with such a method and gives more complete and precise values for dissociation constants of [$P_{450}{}^{2+}$–CO] [1].

2. Materials and methods

2.1. *Preparations and solutions*

Rat liver microsomes were prepared according to the method of Ernster [6], and treated according to our previous descriptions [1–3]. Oxyhemoglobin (Hb) was extracted from fresh sheep blood collected over heparin (20 U/ml) [7]. This compound was stored under nitrogen at + 4°C and used the day after preparation.

The mixed solvent was ethylene glycol (EGOH)/aqueous 0.1 M phosphate buffer pH 8.0 (volume ratio 50/50). The protonic activity of this mixture was determined at different temperatures [8]. Comparative measurements were performed in pure aqueous phosphate buffer.

CO solutions were prepared by appropriate dilution of a saturated stock solution in pure water at + 20°C (1 mM).

2.2. *Apparatus*

Kinetics were recorded on a Amino-Chance DW2 spectrophotometer specially equipped for experiments at sub zero temperatures [9]. The sample was irradiated

*Number 5 of a series.

in the spectrophotometer by a perpendicular beam of light from a Xenon source (Osram XBO, 450W); MTO or interferential filters were used where appropriate.

Different irradiation times were provided by a mechanical shutter. An automatic electronic device switched off the high voltage of the photomultiplier during irradiation, and allowed the signal to be recorded some msec after the end of the irradiation.

3. Results and discussion

3.1. *Experimental results*

When cytochrome $P_{450}{}^{2+}$ (usual concentration 0.8 μM) and Hb^{2+} (20 μM) are mixed in the presence of both excess of dithionite and 0.05 μM to 5 μM of CO, the following equilibrium is rapidly obtained:

$$Hb^{2+} + [P_{450}{}^{2+}\text{–CO}] \rightleftharpoons [Hb^{2+}\text{–CO}] + P_{450}{}^{2+} \quad (1)$$

The concentration of the constituents may be measured by spectrophotometry. Equal amounts of this mixture were placed into the two cuvettes of the spectrophotometer and the sample cuvette irradiated at 421 nm for 10 sec at 4°C. The difference spectrum (fig.1) recorded just after irradiation shows an increase of absorbance both at 450 nm ($P_{450}{}^{2+}$–CO) and 430 nm (Hb^{2+}) and a concomitant decrease in the 418–420 nm region (Hb^{2+}–CO). The same figure shows the progress of the reaction in the dark until the initial spectrum is restored. This change has an isosbestic point at 465 nm. Since 452 nm is an isosbestic point for the transformation $Hb^{2+} \rightleftharpoons [Hb^{2+}\text{–CO}]$, the kinetics are measured with the dual wavelength mode of the spectrophotometer between 452 and 465 nm and only reflect changes affecting cytochrome P_{450}.

As shown in fig.2, the kinetics are biphasic under different conditions of temperature, solvent and CO concentration. The slow phase is not completely reproducible and depends on the microsomal preparation and ageing. But the rapid phase obtained after subtraction of the slow phase is reproducible within 10% and absolutely independent of CO concentration from 0.05 to 5 μM. (The Hb and cyt. P_{450} concentrations being respectively 20 and 0.8 μM).

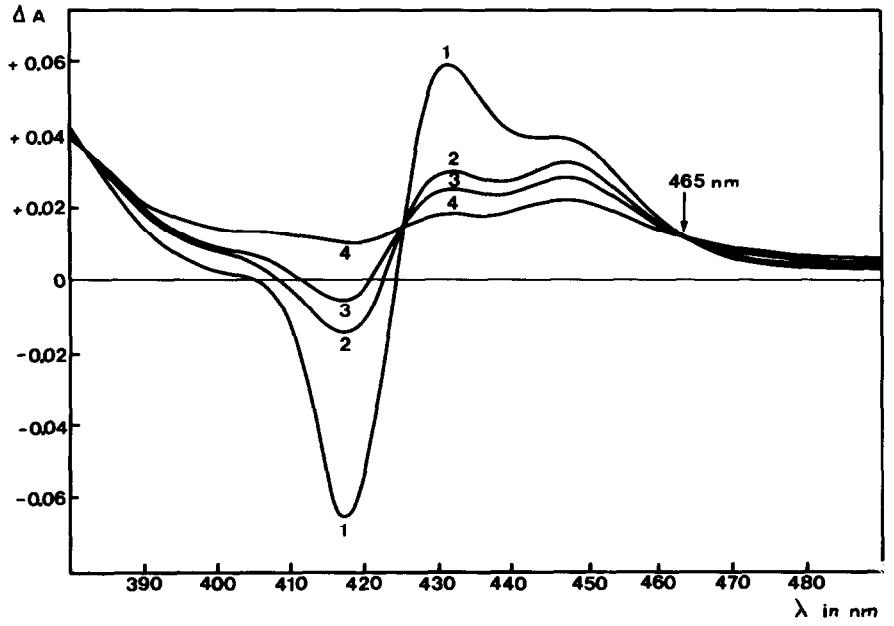

Fig.1. Typical evolution of difference spectrum of a mixture of Hb^{2+} (10 μM), P_{450}^{2+} (5 μM) and CO (3 μM) in a EGOH/phosphate buffer pH 8, 0.1 M, in presence of an excess of dithionite. The hemoprotein concentration are different from those usually used in kinetic measurements, in order to increase the spectral differences. The scan speed is 20 nm/sec. Temperature + 4°C. 1, just after 10 sec irradiation; 2,3,4, after respectively 1 min, 2 min, 8 min in the dark.

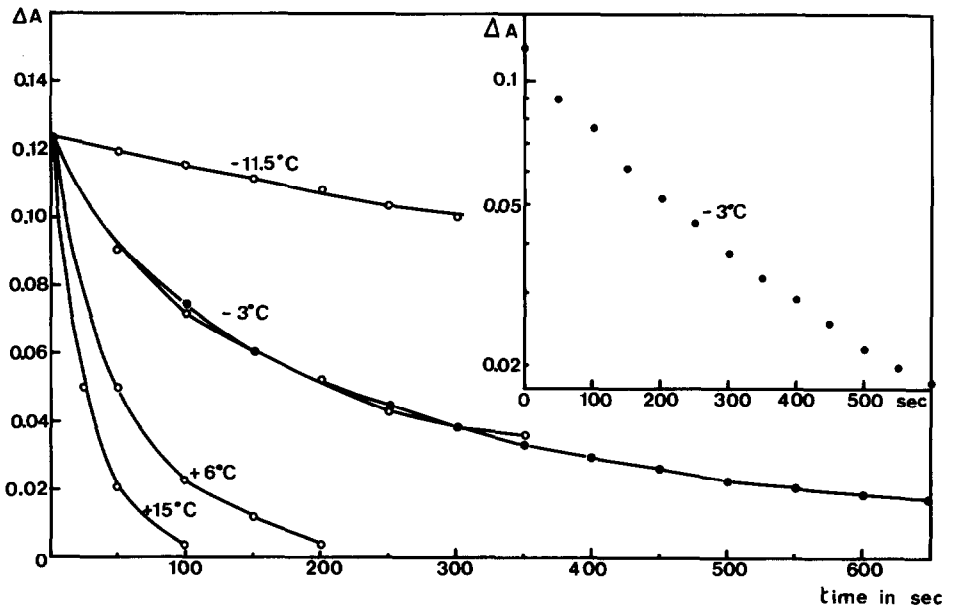

Fig.2. Kinetic traces of the decrease of absorbance (452 nm minus 465 nm) recorded after 10 sec of irradiation in EGOH/phosphate buffer. The concentrations are: Hb 20 μM; P_{450}, 0.8 μM; CO, 0.5 μM, the dithionite being in excess. The temperatures are as indicated. The inset shows the kinetic trace at −3°C redrawn as a semi-logarithmic plot.

At + 20°C, in pure aqueous buffer and in mixed solvent, the half life-times are respectively 15 ± 1 sec and 100 sec for the fast and the slow phases, the last value being very imprecise. Under identical conditions of CO and Hb concentration, a 10 sec irradiation at 421 nm produces no recordable effect. The change is either too small or too fast.

The above results clearly show that during the irradiation some photodissociated CO combined with cyt. P_{450}^{2+}. Thus the reaction (1) is displaced to the left by irradiation and then returns to equilibrium in darkness. The CO-independent dark kinetic should thus represent the dissociation of CO from $[P_{450}^{2+}\text{–CO}]$. The k_{-1} thus obtained at + 20°C is $k_{-1} = 0.07\ \text{sec}^{-1}$ which gives, using the previously determined value of $k_1 = 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ [1], $K_S = 0.07\ \mu$M.

We will now discuss the validity of the method used, in the chosen conditions of concentrations.

3.2. *Validity of the method*

The above results show that the dissociation constant K_{S_1} for $[P_{450}^{2+}\text{–CO}]$ is of the same order of magnitude as the corresponding value for $[\text{Hb}^{2+}\text{–CO}]$ ($K_{S_2} = 0.2\ \mu$M) [10].

Thus in a mixture of P_{450}^{2+} (concentration C_1) and Hb^{2+} (concentration C_2) in the presence of CO with $C_1 \ll C_2$ and $[\text{CO}] \ll C_1 + C_2$, neither hemoprotein is saturated with CO but the concentration of free CO in the medium is extremely low. For example, rough calculations using the above values of K_{S_1} and K_{S_2}, with $C_1 = 0.8\ \mu$M and $C_2 = 20\ \mu$M, show that the concentration of free CO in the mixture is approx. $5\cdot10^{-16}$ M and $5\cdot10^{-14}$ M respectively when 0.05 μM and 5 μM CO are initially included in the solution.

During the photodissociation of $[\text{Hb}^{2+}\text{–CO}]$, the CO produced combines rapidly with the free P_{450}^{2+} owing to its higher association constant with P_{450}^{2+} ($k_1 = 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ at 20°C) than with Hb^{2+} ($k_2 = 2.3\cdot10^5\ \text{M}^{-1}\ \text{sec}^{-1}$ at + 20°C) [11], so that the overall equilibrium (1) is displaced to the left. Since the concentration of free CO is almost negligible with regard to that of $[P_{450}^{2+}\text{–CO}]$, the rate of return to equilibrium is the CO independent rate of the limiting step, that is the dissociation of CO from $[P_{450}^{2+}\text{–CO}]$.

3.3. *Existence of two phases*

The existence of two phases could be explained by different forms of microsomal cytochrome P_{450},

as already postulated [4,5]. Indeed different values of k_1 have already been found, both by flash photolysis [2] and by photodissociation by long irradiation [3]. The rapid phase seems to concern the 'normal' cytochrome P_{450} since the amplitude of the slow phase increases with ageing, and could represent more or less degraded cytochrome. The Arrhenius plot of the rate constant between + 20 and + 4°C for the aqueous medium or between + 20 and −10°C for the mixed solvent allows the calculation of E = 20 ± 2 Kcal mole^{-1} for both media. The value of k_1 being 10^6 M^{-1} sec^{-1} at + 20°C, with an activation energy of 8.2 Kcal mole^{-1} [1], $K_S = 0.07 \cdot 10^{-6}$ M and the enthalpy ΔH of the reaction is then $\triangleq -12$ Kcal mole^{-1}.

4. Conclusion

The above method is based on the fact that both hemoglobin and cytochrome P_{450} are able to combine with carbon monoxide with very similar dissociation constants, so that conditions may be obtained where both hemoproteins are in equilibrium with their carboxy form and CO may be transferred to cyt. $P_{450}{}^{2+}$ by photodissociation of [Hb^{2+}–CO]. It thus allows the direct measurement of dissociation rate constant for cyt. $P_{450}{}^{2+}$, a protein for which the static determination of K_S is difficult. This method could be applied to other couples of CO binding hemoproteins fullfilling the properties defined above.

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