LOW TEMPERATURE STUDIES OF MICROSOMAL CYTOCHROME P_{450} FLASH PHOTOLYSIS EXPERIMENTS*

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1. Introduction

The interaction of carbon monoxide with ferrous cytochrome P_{450}, which was purified from rat liver microsomes, was investigated previously by a stopped–flow technique at sub zero temperature [1]. Both association and dissociation rate constants of the reaction and their respective activation energies were determined in a temperature range from +20°C– – 20°C.

The present paper deals with a flash-photolysis study of the recombination of the photolyzed carbon monoxide (CO) with cytochrome P_{450} in microsomal preparations under conditions similar to those used in the previous study [1].

2. Materials and methods

2.1. *Preparations*

Liver microsomes were prepared according to the method of Ernster et al. [2] starting from male Wistar Albino rats (weighing 150–200 g) which received a daily injection of phenobarbital (80 mg/g of net wt.) for 4 days and fasted for 1 day before sacrifice. The livers were perfused with a cold solution 0.14 M NaCl before homogenisation, in order to remove most of the hemoglobin.

'P_{450} subparticles' were obtained from the microsomes by the method of Nishibayashi et al. [3] and stored frozen at −20°C in concentrated solution (about 40 mg protein/ml) in 0.05 M phosphate buffer, pH 7.5, containing 25% glycerol. The suspension was thawed just before use and the contamination of cytochrome P_{420} in preparations after thawing was less than 5%.

2.2. *Solvents and solutions*

The solvents used are 0.05 M phosphate buffer, pH 8 (above 0°C), or mixed with ethylene glycol in the volume ratio 1:1 or with glycerol in the volume ratio 3:7. The freezing points of these solvents are −45°C and −50°C, respectively. The pH of the ethylene glycol–buffer mixture was evaluated elsewhere and was shown to vary only slightly with the temperature [4].

The 'P_{450} particle' suspension was diluted by addition at 0°C of first the buffer, then the organic solvent, both of them having been deoxygenated by bubbling of nitrogen. A definite volume of buffer saturated with CO at room temperature under 1 atmos. pressure ($[CO] = 10^{-3}$ M), and a few mg of sodium dithionite were rapidly added to the solution which was then transferred in the closed cell of the flash apparatus. The volumes of buffer, organic solvent, and CO solution were adjusted to insure the desired composition of the medium.

2.3. *Apparatus*

Photodissociation was carried out by a flash-photolysis apparatus which will be published elsewhere (C. Balny, to be published). Xenon filled flashes develop 1 kJ in an effective time of 120 μsec. The observation cell may be thermostated at any temperature

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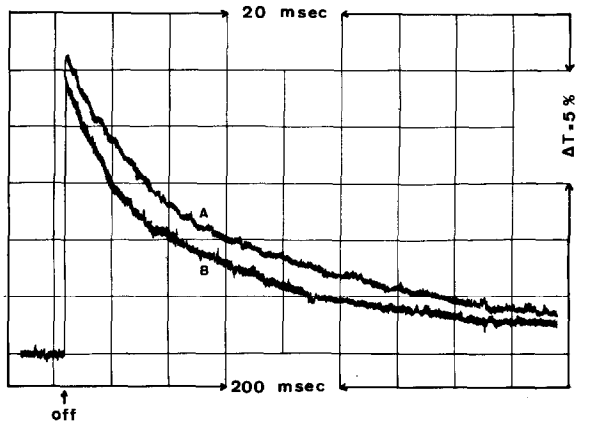

Fig. 1. Kinetic traces of the recombination of carbon monoxide with the ferrous cytochrome P_{450} of 'P_{450} particles' after flash photolysis in ethylene glycol–buffer mixture (volume ration 1:1) at different temperatures. The photolysis was performed at 1.6×10^{-6} M cytochrome P_{450} and 2.8×10^{-5} M CO. Traces A and B were obtained at 11°C and −21°C respectively.

between +20°C and −60°C (± 0.05°C). The recording of recombination kinetics is started 3 msec after the flash.

3. Results and discussion

3.1. The combination kinetics following the flash dissociation of cytochrome P_{450}–CO (Fe^{2+}–CO) in presence of a given concentration of CO is recorded by the decrease in transmission at λ_{max} = 450 nm.

Fig. 1 illustrates kinetics of the recombination of ferrous cytochrome P_{450} after a flash photolysis at different temperatures in the fluid mixture of ethylene glycol and aqueous buffer (volume ratio 1:1).

Between +11°C and −15°C, the value of Δ T extrapolated to the time t = 0 represents 100% of photodissociation. Below −15°C, the initial yield of the photodissociation decreases markedly with decreasing the temperature. A similar behavior was observed at the same temperature in the mixture of glycerol and aqueous buffer (volume ratio 7:3) and thus does not seem to depend of the viscosity.

The recombination kinetics are biphasic at any selected temperature, as shown by the semi-logarithmic plot of the change in absorbance (Δ A) as a function of time (fig. 2).

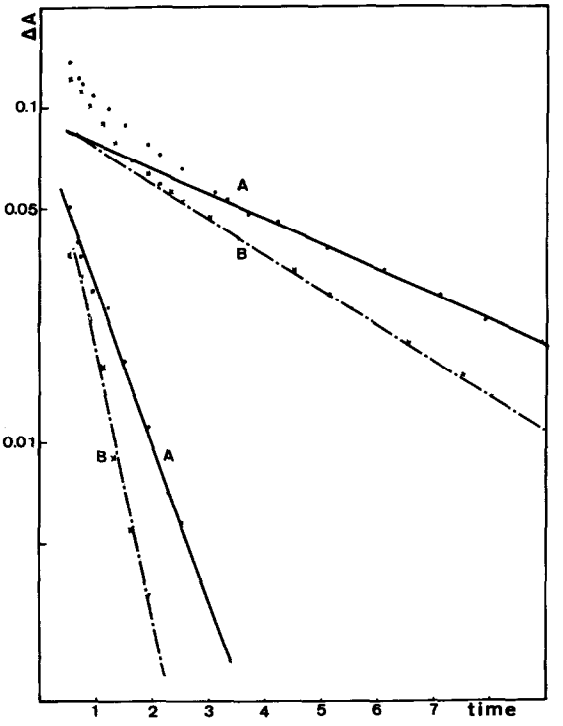

Fig. 2. Semi logarithmic plots of the change in absorbance at 450 nm for the kinetic traces of fig. 1. The entire curve as well as the first rapid phase are plotted. ΔA is the difference between the absorbance at infinite time and the absorbance at time *t*. A. and B are plotted with 10 msec/division and 100 msec/division respectively.

A rapid phase representing 1/3–1/2 of the total absorbance change is followed by a 3-times slower first order reaction. Both rates are dependent on the CO concentration and the temperature, but their ratio does not vary significantly with the above parameters or the nature of the solvents used (ethylene glycol–water in the volume ratio 1:1, glycerol–water in the volume ratio 7:3 or water above 0°C).

Similar kinetics are obtained with isolated cytochrome P_{450}, which was solubilized as already described [1] by treatment of 'P_{450} particles' with Lubrol and further purified by chromatography. This result rules out a membrane effect.

The biphasic recombination observed by the present photolysis technique is not consistent with the monophasic kinetics obtained by the stopped–flow method with the isolated P_{450} [1]. Since the dead time for the stopped–flow experiments was about

Table 1
Characteristic constants of the combination of CO with ferrous cytochrome P_{450} in a 1:1 (v:v) mixture of ethylene glycol and buffer at +4°C.

	k_1 ($M^{-1}sec^{-1}$)	k_{-1} (sec^{-1})	Activation energy E_a (Kcal/mole)	K_S (M)	k_1^* ($M^{-1}sec^{-1}$)	k (sec^{-1})
Stopped–flow on isolated cytochrome P_{450} [1]	$4.5 \cdot 10^5$	0.63	8.3 ± 1[a]	$1.4 \cdot 10^{-6}$[d]	–	–
Flash-photolysis on 'P_{450} particles'[e] (this work)	$4 \pm 1.5\ 10^5$	3 ± 1.5	8 ± 1[b]	$3 \cdot 10^{-6}$[d] $10 \pm 7.6 \cdot 10^{-6}$[c]	$2.3 \pm 0.7\ 10^6$	35 ± 15

a) Measured between +20°C and −20°C.
b) Measured between +11°C and −30°C.
c) Calculated from $K_S = k_{-1}/k_{-1}$.
d) Measured by static titration (see [1]).
e) Mean values from 3 sets of experiments using 3 different preparations.

10 msec [5], the rapid phase could have been detected if such a phase existed. One of the most plausible explanations of this contradiction may be the formation of a 'new' form of the ferrous cytochrome upon photolysis, which may be analogous to the quickly-reacting form of deoxyhemoglobin formed upon photolysis of its carbon monoxy form [6]. Such a new species was already postulated by Imai and Mason [7] in the case of the cytochrome P_{450}.

In our conditions of temperature and medium other monomeric hemoproteins such as horse radish peroxidase and myoglobin give a monophasic recombination (unpublished results). Recently the cytochrome P_{450} extracted from bovine adrenocortical mitochondria was reported to be a polymer [8]. This property, as well as the existence of several forms of the microsomal P_{450} [9, 10] might explain the biphasic kinetics observed both in mixed solvents and in aqueous solution.

3.2. Assuming the following equation to be valid for the slow first order phase:

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

the values of the association and dissociation constants were calculated at different temperatures by using different CO concentration high enough to be considered as constant during the recombination. These values at 4°C (see table 1) are very similar to those obtained by the stopped–flow technique in the same solvent and temperature conditions, using purified cytochrome P_{450} [1] (table 1). Furthermore the activation energy E_a = 8 ± 1 kcal/mole calculated between +11°C and −34°C and the constant over this range of temperature is identical within the experimental errors to the value E_a = 8.3 ± 1 kcal/mole obtained by the stopped–flow method [1].

It seems reasonable then to compare the two sets of experiments and to assume that the slow first order kinetics do represent the combination of CO with the 'normal' species of ferrocytochrome P_{450}.

3.3. The rapid phase obtained by subtracting the slow reaction from the experimental curve follows a first order equation, except eventually in its very initial segment where the absorbance seems to increase more slowly. The significance of such an initial curvature – if representing a real phenomenon or an artefact – is now under investigation. It is nevertheless possible to evaluate, although with less certainties, the characteristics of the linear portion of the rapid phase.

If a new ferrous species Fe^{2+*} is produced by the photodissociation process, as discussed above, the situation after a flash may be described, as in the case of hemoglobin [6], by the subsequent set of competitive reactions:

$$Fe^{2+*} + CO \underset{k_{-1}^*}{\overset{k_1^*}{\rightleftharpoons}} Fe^{2+} - CO \quad (1)$$

$$Fe^{2+*} \xrightarrow{k} Fe^{2+} \quad (2)$$

$$Fe^{2+} + CO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe^{2+} - CO \quad (3)$$

In presence of high concentrations of carbon monoxide, the dissociation reactions may be neglected and the time constant of the rapid phase is [6]:

$$t = \frac{1}{k + k_1^* [CO]}$$

Measurements at different CO concentrations at 4°C yield to the values:

$k_1^* = 2.3 \pm 0.7 \ 10^6 \ M^{-1} sec^{-1}$

$k \quad = 35 \pm 15 \ sec^{-1}$.

Although k is obtained with great uncertainty, it may be seen that the conversion of Fe^{2+*} into the normal cytochrome is slower than the conversion of the 'quickly reacting' hemoglobin into normal hemoglobin [6].

Further experiments, especially with the use of other ligands, are now needed to investigate the exact significance of this biphasic recombination.

During this study we saw that the kinetics in mixed solvents below 0°C are in perfect continuity with those observed above 0°C, which are identical in both aqueous solutions and the above-mentioned mixtures. This demonstrates the validity of experiments performed under these conditions.

The presence of ethylene glycol or glycerol [11], and the use of low temperature, are found to have a protective effect on the transformation of the cytochrome P_{450} to cytochrome P_{420}, even after several cooling–warming cycles.

From a technical point of view, the lower light scattering of the microsomes in ethylene glycol–water or glycerol–water mixtures allows the increase of the concentration and thus the precision of the recordings, while the low temperature, increasing the viscosity of the media, decreases very much the fluctuations due to the particulate nature of the suspensions.

On the other hand, it was possible to calculate the activation energy of the combination with a good precision over a wide range of temperature and obtain thus a good basis for the comparison with the stopped–flow experiments.

However, because of the analogous activation energies of the two phases, a 'resolution' of the kinetics is not possible as in the case of hemoglobin [12] and the method is somehow limited by the decrease of the initial photodissociation with temperature, for which we do not have, up to now, any satisfactory explanation.

Further experiments with the same technique are now in progress in order to clarify some of the present preliminary results.

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