PHOTODISSOCIATION OF Fe^{2+}–CO BY CONTINUOUS IRRADIATION *

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Received 21 December 1973

1. Introduction

Recently we reported some results concerning the binding of carbon monoxide to the reduced microsomal cytochrome *P* 450, studied by stopped-flow [1] and flash photolysis methods [2] in fluid solvents, above and below zero degrees.

Under similar conditions of medium and temperature, Banerjee and Douzou showed that carboxyhemoglobin may be fully photodissociated at −55°C by a continuous irradiation (several seconds) and that the following recombination kinetics, though much slower, are basically similar, to the kinetics obtained under normal conditions [3].

This paper deals with such a procedure of long irradiation applied to carboxy-cytochrome *P* 450, and reveals unexpected kinetic behaviour.

2. Materials and methods

2.1. *Preparations*

Liver microsomes are prepared from phenobarbital treated rats according to the method of Ernster et al. [4] after perfusion of the livers with cold 0.9% NaCl to remove hemoglobin. '*P* 450 particles' are obtained by digestion of the microsomes with protease (Baterial type VII Sigma) or trypsin (from bovine pancreas type III Sigma) [5]. The different preparations are stored as concentrated pellets at −20°C and used within 1 month. The samples used contain no hemoglobin and 0–15% cytochrome *P* 420.

* Number 3 of a numbered series on Low Temperature Studies of Microsomal Cytochrome *P* 450.

2.2. *Solutions*

The solvents used are mixtures of 0.1 M or 0.05 M phosphate buffer (pH 6.2–8) and ethylene glycol (50% by volume) or glycerol (66.6% by volume). The pH of the ethylene glycol–buffer mixtures has been evaluated elsewhere [6].

The '*P* 450 particles' are diluted in the above mixtures reduced at room temperature by a few milligrams of sodium dithionite and a known concentration of carbon monoxide is produced by addition of a definite volume of water saturated with CO at + 20°C under 1 atm, of pressure.

2.3. *Apparatus*

Kinetics are recorded on a Cary 15 or an Aminco–Chance dual wavelengths spectrophotometer, both sample and reference cuvettes being thermostated at any temperature between + 50°C and −100°C as already described [7].

Irradiation of the sample is performed perpendicular to the monitoring light by a continuous Xenon lamp of 450 W (Osram X BO 450 type). The wavelengths are selected by interference filters centered around 450 nm or 535–555 nm. A mechanical shutter allows the choice of irradiation times between 2.10^{-3} sec and several minutes.

3. Results

Under our conditions of irradiation the yield of photodissociation of *P* 450–CO is very small above zero degrees but increases at lower temperatures so that recombination kinetics can be easily recorded

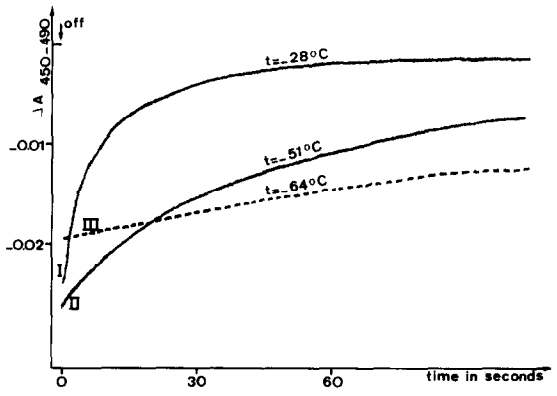


Fig. 1. Kinetic traces of the increase of absorbance between 450 nm and 490 nm recorded after a long irradiation of the Fe^{2+}– CO complex. '*P* 450 particles' are diluted in a mixture of 0.1 M phosphate buffer pH = 7.5 and glycerol (66.6% by volume). The irradiation times are respectively: I, 20 sec, II, 30 sec, III, 2 min. The arrow indicates the end of the irradiation.

and studied with a spectrophotometer with an ordinary speed of response. Fig. 1 shows such kinetic traces obtained at 450 nm at several temperatures in fluid ($t \geqslant -50$°C) or even glassy medium ($t = 60$°C).

On increasing the illumination time, (0.5 sec to 1 min), the yield of photodissociation increases and reaches a steady state value ($\Delta A/A$ = 10–20%, depending on the conditions), which depends on both CO concentration and temperature. It decreases with increasing CO concentration from 10^{-5} M to 5.10^{-4} M, which is the limiting value for any accurately recordable recombination, the original enzyme concentration being usually $\sim 10^{-6}$ M. When the temperature is lowered, the steady state yield increases but seems to reach a maximum at about – 45°C, which is then the optimal temperature for a precise recording.

The recombination rate is proportional to CO concentration within experimental error, but is surprisingly much slower than the corresponding rate measured either by stopped-flow [1] or after flash photolysis [2] at the same temperature.

Furthermore, as shown by a semi-logarithmic plot of ΔA versus time (fig. 2), the kinetics appears to be a mixture of at least three first order, CO dependent phases, which may not be separated in time, even at low temperature.

The faster and slower rate constants, as measured by the slope of the semi-logarithmic plot, have a similar activation energy of approximately 9 kcal/mole.

After different irradiation times, the kinetics remain identical, except that the initial yield may vary as described above.

As shown by fig. 3, the spectrum after irradiation is similar to the difference spectrum Fe^{2+}–CO minus Fe^{2+} at the same temperature, and kinetics recorded at any wavelength are identical to the kinetics at 450 nm.

Finally, the phenoma remain essentially the same when several parameters are varied, like the pH of the buffer (6.2–8), the concentration of cytochrome (6 10^{-6} M to 5 10^{-7} M), when ethylene glycol (volume ratio 50%) is used as organic solvent, and when cytochrome *P* 450 from crude microsome is enzymatically reduced by NADPH.

4. Discussion

In contrast to the results obtained with Hemoglobin at –55°C, the recombination kinetics observed after long irradiation of the Fe^{2+}–CO complex of cytochrome *P* 450 take place in a time scale approximately 20 times greater than that of the recombination reactions after flash photolysis, which should be almost completed within the deadtime of the method ($\sim$ 1 sec).

Such a slow reaction cannot be explained by any viscosity or solvent effect since it is also observed, though with very small amplitude, in aqueous buffer at 4°C, and is identical in glycerol–water (70–30) or ethylene glycol–water (50–50) mixtures, despite quite different viscosities. Furthermore, a similar behavior was found with partially purified cytochrome *P* 450 [2] free of membranes.

Thus, the results reported here deal with a reaction essentially different from the recombination after a flash, and support the idea of the existence of a new species, Fe^{2+} X, which is not produced in sufficient concentration after a flash, but accumulates during the long irradiation and combines with CO through a very slow, CO dependent reaction. The following scheme may thus be proposed, which is consistent with the fact that the 'slow reaction' is favored at low

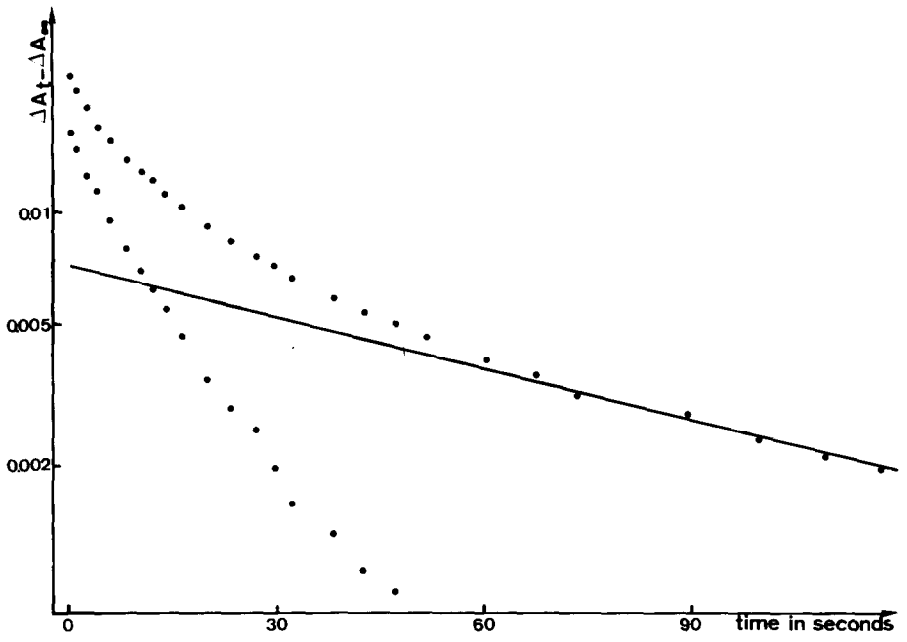
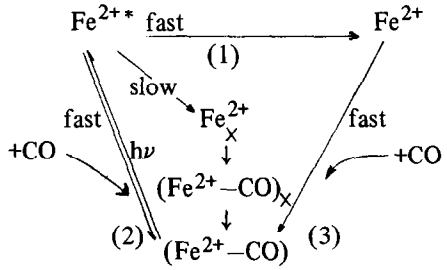


Fig. 2. Semi-logarithmic plot of curve I from fig. 1. The entire curve as well as the curve obtained after substraction of the final slow phase are shown on the same figure. ΔA refers to the absorbance between 450 nm and 490 nm at time t, ΔA_∞ refers to the same change in absorbance at ininite time.

concentrations of CO and low temperatures, whereas reactions (2) and (3) are slower.



However, the existence of $(Fe^{2+}–CO)$ X is as yet hypothetical and the complexity of the recombination reaction remains to be explained. None of these new species is detectable by any new spectral property, except perhaps a change in the ϵ value, though small spectral changes could have escaped the investigation owing to tne rather low yield of photodissociation. Small conformational changes of the protein could be responsible for this kinetic behavior.

It must be recalled that a long irradiation technique was used by Imai and Mason, who proposed that photodissociation of Fe^{2+}–CO produces an abnormal form

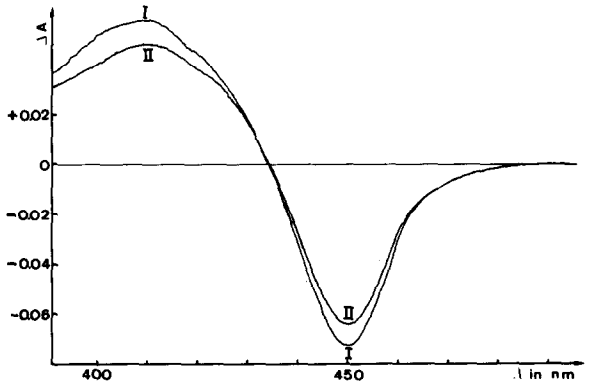


Fig. 3. Difference spectra Fe^{2+}–CO minus Fe^{2+} of '*P* 450 particles' in a 0.1 M phosphate buffer (pH 7.5)–glycerol (66,6% by volume) mixture I before and II after irradiation. The initial concentration of carbon monoxide is 1.33×10^{-5} M, the irradiation time 30 sec, and the temperature – 45°C. The spectra are taken with an Aminco–Chance spectrophotometer (scanning speed 20 nm per sec).

of the reduced microsomal cytochrome *P* 450 [8]. Experiments involving photodissociation in the pre-

sence of competitive ligands are now under investigation in order to correlate our results with those of Imai and Mason.

Acknowledgements

We thank Miss Evelyne Campion for her skilful technical assistance. We are grateful to Drs T. Yonetani and M.D. Kamen for advice and discussions.

This work was supported by grants from the CNRS (Equipe Associée no. 262), the INSERM (Contrat no. 72 40 302) and the DGRST (Convention no. 71 73091 01).

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