LOW TEMPERATURE STUDIES OF MICROSOMAL CYTOCHROME P_{450}. RELEASE OF OXIDIZING SPECIES*

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1. Introduction

It is known that the functionning of the multienzyme hydroxylating system of rat liver microsomes produces oxidizing species [1–3] identified as O_2^- and H_2O_2 [3–5], but it remains unknown whether such species are normally or incidentally released by the NADPH cytochrome P_{450} reductase [6], or by cytochrome P_{450}, or both.

The demonstration that such a release of oxidizing species can occur from cytochrome P_{450} would give decisive evidence of its oxygenated intermediate, as has been postulated by Eastabrook [7].

The present paper shows that, after enzymic reduction of cytochrome P_{450} at room temperature, and subsequent cooling (which inhibits the enzymatic electron transport activity of the system), addition of oxygen in the presence of luminol determines an emission of luminescence due to the thermal decomposition of an oxygenated intermediate of cytochrome P_{450}.

2. Materials and methods

2.1. *Microsomal preparations*

Liver microsomes are prepared from male Wistar Albino rats, according to the method of Ernster et al. [8]. The animals are treated during 4 days by intraperitoneal injection of phenobarbital (70 mg/kg body), and the livers perfused with cold 0.9 % NaCl to remove hemoglobin. The pellets of microsomes are stored frozen at −30° C before use.

* Number 4 of a numbered series.

2.2. *Products*

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and luminol are from Sigma. Other reagents are Merck products. The aniline is purified by distillation and kept under nitrogen atmosphere at 0° C.

The deoxygenation of the solutions is performed by bubbling pure nitrogen (N_{48}, Air Liquide) for at least 30 min.

2.3. *Experimental procedure*

Enzymatic reduction of cytochrome P_{450} was carried out in anaerobic conditions. Microsomes (protein concentration of about 3.5 mg/ml) were diluted in 2 ml of a deaerated mixture of 0.1 M Tris buffer pH 8.5 and ethylene glycol (volume ratio 1:1). The accurate pH* value was estimated according to a procedure published elsewhere [9]. The medium contained luminol at a final concentration of 2.8×10^{-4} M.

150 μl of NADPH (10^{-2} M) were added to each aliquot (2 ml). The time required for the reduction of cytochrome P_{450} under these conditions has been previously recorded spectrophotometrically on the combination P_{450}–CO (Aminco-Chance DW 2 apparatus). This reduction requires about 30 min at 20° C. Preparations were free of any exogenous substrate.

When the enzymic reduction of cytochrome P_{450} was completed (30 min of incubation at 20° C), the samples were cooled to −30° C. They were then transfered in a luminescence apparatus [10] and progressively rewarmed in the presence of oxygen. The temperature variation during heating of samples was controled with a thermocouple.

3. Results

Upon injection of oxygen at −30° C, the microsomal preparations previously enzymatically reduced and containing luminol give a light emission during warming, with an optimum at −16 ± 2° C, and a rapid decay (fig. 1).

When the temperature reaches −5–0° C, a new emission occurs stabilized over minutes at 20° C (fig. 1) and can be identified as the 'normal' emission previously reported in similar conditions by several authors [1,2].

Both emission processes are reproducible. They are suppressed when O_2 is omitted or when the 30 min incubation at room temperature is carried out in absence of NADPH, even if O_2 is added at −30° C. It has been verified that when oxygen is added to microsomal preparations in anaerobiosis just before cooling, the 'low temperature' luminescence is suppressed but the second is unaffected.

Upon addition of deoxygenated aniline (10 mM final concentration) just after reduction and before cooling, both luminescences are totally quenched.

Moreover, if the last 5 min of the enzymic reduction are performed under CO atmosphere followed by a 30 sec CO bubbling, the emission intensity at −16° C diminishes by a factor ~ 3 and the second emission is only affected by a reduction intensity of a few percent.

Finally, the addition of catalase (0.5 μM) before cooling suppresses totally the 'low temperature' emission, whereas the second is only 50% quenched.

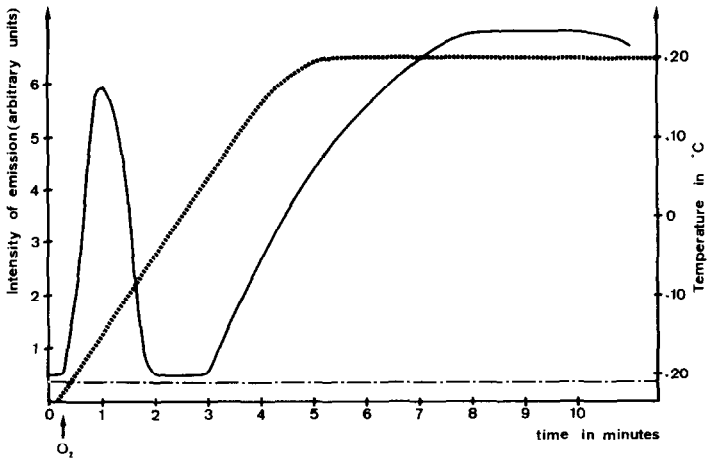

Fig. 1. Evolution of intensity of luminescence as a function of time and temperature (the concentrations are indicated in the text): (——) intensity of light (left hand scale); (—) temperature (right hand scale); (–·–) background in total anaeroby. The arrow indicates the introduction of oxygen.

4. Discussion

It should be pointed out that when luminescence occurs at sub-zero temperatures, the enzymic reduction of the NADPH cytochrome P_{450} reductase is still possible, although presumably at a very reduced rate, whereas the transfer of electrons to endogenous cytochrome P_{450} or to exogenous cytochrome *c* is practically inhibited [11]. Under these conditions, there are two requirements to the induction of the transient luminescence: firstly the microsomal preparations must have attained strict anaerobiosis and must be fully reduced; secondly the oxygen must be injected at low temperature (−30° C).

The luminescence then induced is not a 'burst' but an emission the intensity of which varies both as a function of time and temperature, suggesting the formation and decomposition of some unstable species, the products of which react with luminol.

The fact that no emission occurs when reduced microsomal preparations are warmed from −30 to 20° C in absence of oxygen rules out the occurence of thermoluminescence from the decomposition of peroxidative compounds accumulated during the functionning of the

enzymic systems [12]. On the other hand, it is highly improbable that the emission is due to a reaction between the reduced form of the NADPH cytochrome P_{450} reductase (FP_1) and oxygen since such a reaction is already very slow at room temperature [13] and complete reduction of FP_1 does not require the anaerobic conditions which must be fulfilled to induce the transient luminescence.

On the contrary, the reduced form of cytochrome P_{450} cannot be accumulated unless oxygen is totally consumed. This ferrous form should be at the origin of the 'low temperature' luminescence as evidenced by the quenching observed in presence of aniline as a substrate [14] and in presence of carbon monoxide as a ligand. In the latter case about 30% of the combination ferrous P_{450}-CO disappear after oxygen bubbling at $-30°$ C, and might be responsible for the remaining luminescence.

Thus, it can be assumed that after reduction of cytochrome P_{450} and upon addition of oxygen, an unstable oxygenated intermediate is obtained

$$\text{Cyt}\,P_{450}{}^{2+} + O_2 \rightarrow \text{Cyt}\,P_{450}{}^{2+} \cdot O_2$$

and thermally decomposed, yielding oxydizing species which react with luminol to produce the transient luminescence.

It is not yet possible to identify the oxidizing species, except for the H_2O_2 as demonstrated by the quenching effect of the catalase. Further experiments might indicated whether superoxide ions are among the oxidizing species.

It is obvious that the luminescence observed in the range of normal temperatures is identical to that already reported during the normal enzymic functionning of microsomes in the presence of NADPH and oxygen [1,2]; such an emission occurs when the reduction of cytochrome P_{450} by the NADPH cytochrome c reductase is resumed ($-5°$ C) [11]; it is quenched by aniline (100%) and catalase (50%), could be due to the same oxygenated intermediate, and deserves further experiments to understand its quantitative differences with the 'low temperature' emission.

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