SUB-ZERO TEMPERATURE STUDIES OF MICROSOMAL CYTOCHROME *P*-450: INTERACTION OF Fe^{2+} WITH OXYGEN

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1. Introduction

Part of the mystery underlying the cycle of redox states undergone by the microsomal cytochrome *P*-450 during drug hydroxylation was solved by the earlier description by Estabrook et al. [1] of a new spectral intermediate appearing during the steady-state drug oxidation metabolism and attributed to the oxy–ferrous cytochrome *P*-450. This compound has a maximum at 440 nm in a difference spectrum and was obtained in the presence of hexobarbital as hydroxylisable substrate. Later on, an increasing interest was oriented towards the ability of cytochrome *P*-450 to bind and use organic hydroperoxides as combined source of electron and oxygen, either in the microsomes or in a reconstituted system [2–4].

The problem of the interaction of oxygen with reduced cytochrome *P*-450 was reactivated by the recent work of Guengerich et al. [5] describing the binding kinetics of O_2 to highly purified preparation of reduced cytochrome and the obtention of at least two distinguishable compounds.

The present work describes two spectrally detectable complexes, very similar to thoses of Guengerich et al. and obtained by the addition of O_2 to completely reduced intact microsomes at sub-zero temperatures. The advantages of such an experimental procedure is to allow a slowing down of reaction rates and the 'uncoupling' of certain processes from the preceding or following ones within a multi-reactions system. This procedure was already applied to study in the microsomes some reactions involving the cytochrome *P*-450, and previously described [6–8].

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2. Materials and methods

2.1. *Microsomal preparations*

Liver microsomes were prepared from male Wistar Albino rats, according to the method of Ernster et al. [9]. The animals were treated over 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 were stored frozen at −30°C before use.

These microsomes were used either directly, or as starting material for further solubilisation and purification of the cytochrome *P*-450, according to the method of Lu et al. [10]. In the final step of the preparation, the solubilized *P*-450 is stored at −30°C.

2.2. *Experimental procedure*

Microsomes are homogenized and then diluted in deoxygenated 0.1 M phosphate buffer pH_a 7 [11], to which an equal volume of deoxygenated ethylene glycol is added. The final suspension contains about 3.5 mg protein/ml and 10^{-4} M hexobarbital. The cytochrome *P*-450 is then reduced at + 10°C in the anaerobic cuvette of an Aminco-Chance DW 2 spectrophotometer by either dithionite or NADPH (5×10^{-5} M final). After full reduction followed by the appearance of the broad 445 nm band in the difference spectrum (reference reduced by NADH), the reference cuvette is also fully reduced and the baseline of zero absorbance recorded. The two cuvettes are then thermostated at the same sub-zero temperature which may be kept constant (± 0.5°C) through the use of an equipment already described [12].

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3. Results

When the two cuvettes are thermostated at a fixed sub-zero temperature, oxygen is bubbled through for 5–10 s in the sample cuvette and the solutions are allowed to stand 20 s to allow a perfect thermal and optical stability of the suspension. The first difference spectrum (Fe^{2+} + O_2 minus Fe^{2+}) thus obtained at −45°C is characterized by an increased absorption at 430 nm and 450 nm, as well as by a 415 nm peak and a 425 nm trough (fig.1a). The 430 nm peak decreases slowly with time, whereas the 455 nm absorbance remains stable within 30 min. In the mean time the amplitudes of the 415 nm peak and 425 nm trough increase continuously.

At −20°C (fig.1b) the 430 nm peak is very small on the first spectrum, if existing, and the 450 nm absorption decreases now quite rapidly and disappears within 30 min. At higher temperatures the 430 nm and 540 nm peaks cannot be detected by the present method which has a rather long dead-time (30 s) and the addition of oxygen results in a stable difference spectrum with a peak at 415 nm and a trough at 425 nm.

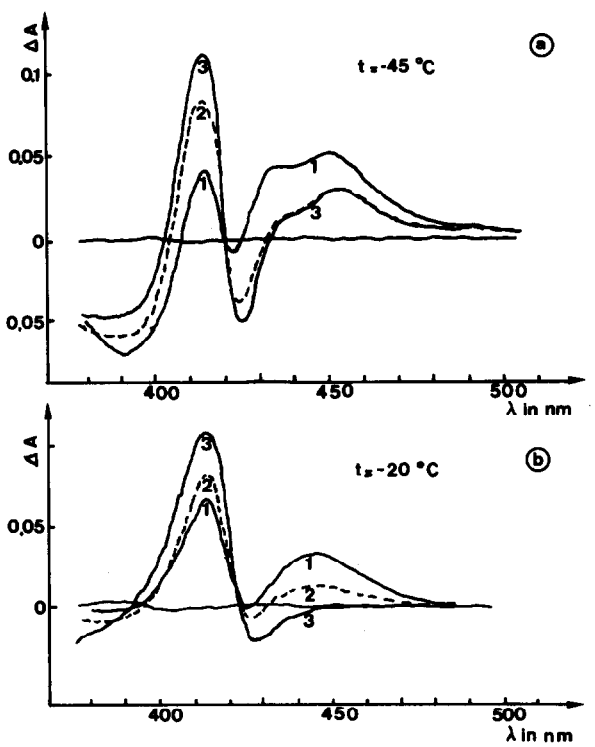

Fig.1. Difference spectra obtained after addition of oxygen to a reduced microsomal suspension (~ 3 mg protein/ml) ⓐ at −45°C, ⓑ at −20°C. Fig.1a. Spectra are obtained respectively at (1) 30 s, (2) 350 s, (3) 30 min after oxygen addition. Fig.1b. Spectra are obtained at (1) 30 s, (2) 170 s, and (3) 30 min after oxygen addition.

When hexobarbital is omitted, qualitatively identical, although less intense phenomena are observed. Identical spectra are also recorded with a partially purified preparation of soluble cytochrome *P*-450, according to the method of Lu et al. [10] and which contains 10% of cytochrome b_5 but no detectable NADH–DCPIP reductase nor NADH–Cyt. *c* reductase activities. Finally separate control experiments at the same temperatures show that this spectral evolution cannot be observed when N_2 is bubbled instead of O_2 and is not due to an evolution of the reference cuvette during the same time. In addition, if the same experiment is performed with a non anaerobically NADPH-reduced preparation where only cytochrome b_5 is reduced, no optical difference is recorded upon oxygen addition.

4. Discussion

The present optical spectra obtained at sub-zero temperature after addition of oxygen to fully reduced microsomes or solubilized cytochrome *P*-450 are noteworthy similar to those obtained recently by Guengerich et al. [5] at room temperature with highly purified ferrocytochrome *P*-450 from rabit-liver microsomes. These authors have describes two successive compounds; the first one (Complex I) is characterized by two absorption maxima at respectively 430 nm and 455 nm and is progressively transformed into a second one (Complex II) having only a broad maximum around 440 nm. Such a maximum in the difference spectrum is also characteristic of the new compound described by Estabrook [1] during the steady-state drug oxidation metabolism, and could be tentatively attributed to an oxy–ferro complex of cytochrome *P*-450.

The half time for Complex I formation is still less than 30 s at −40°C, since we do not detect its formation kinetics. This is not surprising since the *k*-value is estimated to be 6×10^4 M^{-1} s^{-1} at room

temperature. More surprising is the quite fast decomposition of Complex I into Complex II which occurs even at −40°C, as well as the instability of Complex II at −20°C, owing to the quite slow rates of these steps, 210 m^{-1} and 12 mn^{-1}, respectively, for the solubilized cytochrome at room temperature. Our results imply that, either the activation energies of both processes are relatively low, or other factors influence the decomposition rate, such as the source of microsomes, the state of the cytochrome (membrane-bound or solubilized), or yet the presence of other components of the hydroxylating chain in the intact microsomes (flavoproteins or cytochrome b_5). These could play a role similar to the effector role of the putidaredoxin in the bacterial methylene hydroxylase system [13]. Indeed the 415 nm peak and 425 nm trough in the difference spectra indicate that the cytochrome b_5 is reoxidized during the complex formation and decomposition process, a result not obtained with the soluble system [5].

If one continues the comparison with the bacterial system, the enzymatic decomposition of the oxygenated cytochrome *P*-450 by the reduced putidaredoxin is mono-phasic with a half-time of 80 s at −40°C, that is even faster than the decomposition of Complex I in the microsomes [15].

We must recall that at the temperature of the experiments, the electron-flow from NADPH towards either the oxidized cytochrome *P*-450 or cytochrome b_5 is stopped in the intact microsomes, so that only one turnover of the cytochrome is recorded [14]. This further strengthens the idea that the observed spectra are solely due to the interaction of cytochrome *P*-450 with oxygen, without interference of any other component. This property was already used to study the release of oxidizing species by cytochrome *P*-450 after its reaction with oxygen at sub-zero temperature under identical conditions [8]. Thus a direct comparison of these experiments with the present ones is easy and suggests that oxidizing species are released from the decomposition of Complex II and not from Complex I which would decompose at lower temperatures. The Complex I → Complex II transformation could thus correspond to a redistribution of electrons within the same complex, while Complex II decomposition would be related to changes in the redox state.

A closer analysis of the kinetics of these processes in connection with the cytochrome b_5 reoxidation are clearly needed and should help to the understanding of the exact structure and decomposition mechanisms of these compounds. For such studies in the intact microsomes as well as in the solubilized system, experiments at low temperatures appear quite useful.

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