REDUCTION KINETICS AND CONTENT OF CYTOCHROME P-450 BY APPLICATION OF DUAL WAVELENGTH TECHNIQUES TO HEMOGLOBIN-FREE PERFUSED RAT LIVER

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Received 2 December 1968

1. Introduction

For a better understanding of the function of the CO-binding pigment, cytochrome P-450, more basic knowledge of its properties is required. The pigment has so far resisted purification attempts by conversion to a substance called P-420 [1]. We shall try here to contribute to the characterization of the CO-binding pigment within the whole rat liver, employing the hemoglobin-free perfused liver as a model of the intact organ. Recent advances in instrumentation allow transmittance spectrophotometry of the peripheral area of a lobe of perfused liver [2]. The method permits to obtain the overall spectrum of the liver as well as the monitoring of individual pigments by a dual wavelength technique.

2. Experimental

Hemoglobin- and protein-free liver perfusion from female Wistar rats, 100–200 g weight, fed on stock diet, was performed in closed circuit at 30–33°C with dextran 40 *-Krebs-Henseleit solution as described [3]. No substrate was added. Oxygenated and argonated perfusion fluid was provided by two separate "oxygenators" which received a gas mixture of 91.5% oxygen or argon, respectively, 5% carbon dioxide, and 3.5% carbon monoxide. This allows to switch over from normoxic to anoxic perfusion, and vice versa, instantaneously. The perfusion medium (total of 100 ml per oxygenator) passed through the liver at a speed of 25 ml/min by way of the portal vein and the vena cava superior.

The left lower liver lobe, in an area about 2 mm thick, was used for transmittance spectrophotometry with a method [2] based on the "Rapidspektroskop" (Howaldt-Werke, Kiel). The method is in its final result comparable to the dual wavelength method developed by Chance [4].

* Rheo-Macrodex, kindly supplied by Knoll Co., Ludwigshafen.

3. Results and discussion

3.1. *Difference spectrum*

The registration of the overall difference spectrum of perfused liver is yet somewhat laborious. However, the feature important in this context, i.e., the demonstration of the appearance of the 450 nm Soret peak and the 397 nm trough in the reduced-oxidized (anoxia minus normoxia) spectrum, is clearly possible (fig. 1) when the gas mixture equilibrated with the perfusion medium contains 3.5 vol% CO. The position of these absorption bands is similar to that found with cytochrome P-450 from adrenal cortex mitochondria [5]. Fig. 1 was recorded at the end of the second phase of P-450 reduction kinetics (see below).

3.2. *Dual wavelength readings*

An insight into the reduction kinetics of individual respiratory pigments is directly provided in fig. 2 for cytochrome P-450 (trace IV) and cytochrome ($a+a_3$) (trace III). 1 min 20 sec after switching to argonated medium (Ar-anoxia), cytochrome ($a+a_3$) has reached

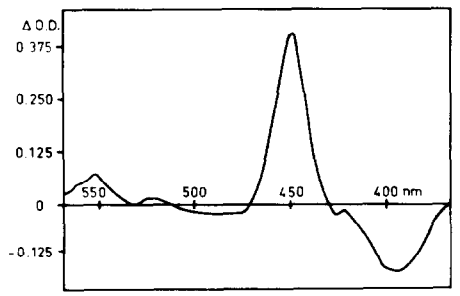

Fig. 1. Difference spectrum of whole liver (anoxia minus normoxia). Female rat, 150 g, after two successive daily phenobarbital injections i.p. (50 mg/kg). Appearance of P-450-CO compound is demonstrated.

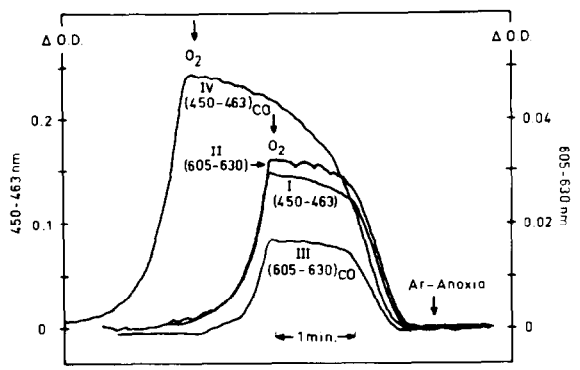

Fig. 2. Normoxia-anoxia transitions of 2 min (and 3 min) duration, without and with 3.5 vol% CO. Original records from the same lobe area, registered sequentially, about 10 min apart, as numerated. Phenobarbital application as in fig. 1. Note difference in kinetics after CO addition. Calculation of ratio (cytochrome P-450/cytochrome $(a+a_3)$) from this experiment according to the equation given in the text:

$$\frac{\text{P-450}}{(a+a_3)} = \frac{(0.240 - 3.6 \cdot 0.017)}{0.017} \cdot \frac{11.5}{91} = 1.33.$$

95% of its final O.D. increment, while cytochrome P-450 has reached only 65%. After 3 min (when oxygen was restored in trace IV) the O.D. increment arrived at about 90% as judged from other experiments; the P-450 reduction is completed after about 8 min. This behavior distinguishes cytochrome P-450 from mitochondrial cytochromes which together reach a plateau essentially after 2 min. The second, slow phase in P-450 reduction kinetics may be due to slow supply of electrons and to lack of substrate saturation, i.e., the flux in the reaction chain. Further experimentation to this point is required.

3.3. *Ratio of tissue content of cytochrome P-450 to cytochrome $(a+a_3)$*

This ratio is calculated with the equation

$$\frac{\text{Cytochrome P-450}}{\text{Cytochrome } (a+a_3)} = \frac{11.5 \cdot (\Delta E_{(450-463)_{CO}} - 3.6 \cdot \Delta E_{(605-630)_{CO}})}{91 \cdot \Delta E_{(605-630)_{CO}}},$$

where ΔE represents the O.D. increments from normoxia to anoxia followed between the indexed wavelengths. In the mean of 6 measurements on non-induced livers, the in situ ratio (P-450)/$(a+a_3)$ was found to be 0.7 ± 0.3. An example for induced liver is given in the legend of fig. 2. ΔE's are read after 3 min anoxia for P-450, after 2 min for cytochrome $(a+a_3)$, thus leading to a value about 10% too low for P-450, as mentioned above. Longer anoxia, however, may damage the organ's functional state.

The extinction coefficients used are 91 mM^{-1} cm^{-1} for P-450 [1], and 11.5 mM^{-1} cm^{-1} for $(a+a_3)$ as calculated from Yonetani's spectra of purified cytochrome $(a+a_3)$ in the presence of CO [6]. The contribution of cytochrome $(a+a_3)$ to the $\Delta E_{(450-463)_{CO}}$ was taken into account in the numerator with the help of the mentioned spectra [6]. (See also next section.)

Cytochrome $(a+a_3)$ is used as a suitable cellular concentration indicator because it is fully oxidized in normoxia [3,7] and fully reduced in anoxia; hence, $\Delta E_{(605-630)_{CO}}$ is proportional to the content of that pigment in the lobe area studied. The liver content of cytochrome $(a+a_3)$ is calculated from data on isolated mitochondria [8] to be about 15 nMoles/g wet weight, when the mitochondrial protein is 50 mg/g wet weight.

A comparative figure for P-450 content in female rat liver, as measured with isolated microsomes, is 8.8 nMoles/g wet weight [9]. The ratio (P-450)/$(a+a_3)$ from these data is thus about 0.6.

3.4. *Carbon monoxide effects*

The addition of CO to the gas mixture equilibrated with the perfusion medium serves two purposes; firstly, to eliminate spectral interferences due to traces of

residual hemoglobin [10], and secondly, to produce the P-450-CO compound. The partition coefficients between CO and O_2 differ suitably: 5×10^{-3} for Hb, 1 for P-450, and 15 for ($a+a_3$) [11]. The addition of 3.5 vol% CO blocks the small amount of hemoglobin still present in perfused liver, and allows to monitor the P-450-CO compound, and hence the redox state of the microsomal hydroxylating system, without inhibition. The non-inhibition of ($a+a_3$) is demonstrated by the unchanged overall respiration after CO–addition.

With respect to the spectral changes, the addition of CO causes a normoxia-anoxia O.D. increment at (605–630) 0.85 times the increment in the absence of CO in purified ($a+a_3$), again as calculated from [6]. The experimental records in fig. 2, traces III and II, yield the smaller factor of 0.53. In 12 perfusion experiments, this factor was found to be 0.68 ± 0.11. The difference to 0.85 can be attributed to the traces of residual hemoglobin.

Additional experimental evidence supporting the above calculation is provided by azide reduction of cytochrome ($a+a_3$). At a concentration of about 2 mM sodium azide, the reduction is essentially complete. This is demonstrated when read at $(605–630)_{CO}$. At $(450–463)_{CO}$, an O.D. increment was observed approximately as predicted by the above equation.

In a perfused liver respiring endogenous substrate, the CO/O_2 ratio in the used experimental setup is about 0.1 when the oxygen concentration in the vena cava sup. is considered. From titrations with carbon monoxide, we concluded that P-450 is maximally 6% reduced in normoxia [10], contrasting the assumption of Sih and Whitlock [12] that it should be mainly reduced. The above calculation therefore seems to be justified for P-450, too.

3.5. K_m(oxygen) of P-450

A partial reduction of the oxygen-consuming pigments was achieved by a normoxia-hypoxia transition; the hypoxic circuit then contained high argon and low oxygen concentration, with CO and CO_2 held constant as above. The ratio of the reduction in hypoxia to that in anoxia allows a comparison of the oxygen control regions in different pigments. As an example, P-450 was 30% reduced and ($a+a_3$) was 15% reduced when Ar/O_2 was 3; in another experiment, when Ar/O_2 was 7, these figures were 80% and 50%, respectively. The K_m(oxygen) for the two pigments may thus range in the same order of magnitude. K_m(oxygen) for ($a+a_3$) was reported to be 0.042 μM in isolated rat liver mitochondria [13]. Estabrook's result of 0.1 μM for K_m(oxygen) of P-450 from experiments with isolated rat liver microsomes [14] is therefore grossly reconfirmed *in situ*.

Acknowledgement

The skillful technical assistance of Mrs. S.Schnitger and Miss E.Rehn is gratefully acknowledged.

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