THE PHOTOCHEMICAL ACTION SPECTRUM OF THE CARBON MONOXIDE INHIBITED HYDROXYLATION OF CYCLOHEXANE BY RAT LIVER MICROSOMES

H.DIEHL, S.CAPALNA and V.ULLRICH
Biochemisches Institut der Universität Gießen, 63 Gießen, G.F.R.

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1. Introduction

Recently we have shown that cyclohexane can be regarded as a typical substrate of the unspecific monooxygenation system in rat liver microsomes [1]. It has been suggested that cytochrome P 450 is the oxygen-activating component of this microsomal hydroxylating system. This, however, has been shown definitely only for three substrates by means of a photochemical action spectrum; the monooxygenations of of acetanilide, aminopyrine and codein which are inhibited by carbon monoxide and can be reversed by irradiation. Between 400 and 500 nm, the reactivation is maximal with light of 450 nm wavelength where the cytochrome P 450 – CO complex has its absorption peak [2]. As this technique gives the only definite proof for participation of cytochrome P 450 as a terminal oxidase, it was applied to the cyclohexane-monooxygenation system. A direct and sensitive test for cyclohexanol also allowed an investigation of the visible part of the action spectrum.

2. Methods

Male Wistar rats (strain Han F) were treated by intravenous injection with phenobarbital (80 mg/kg/day) for two days. The liver microsomal fraction was isolated

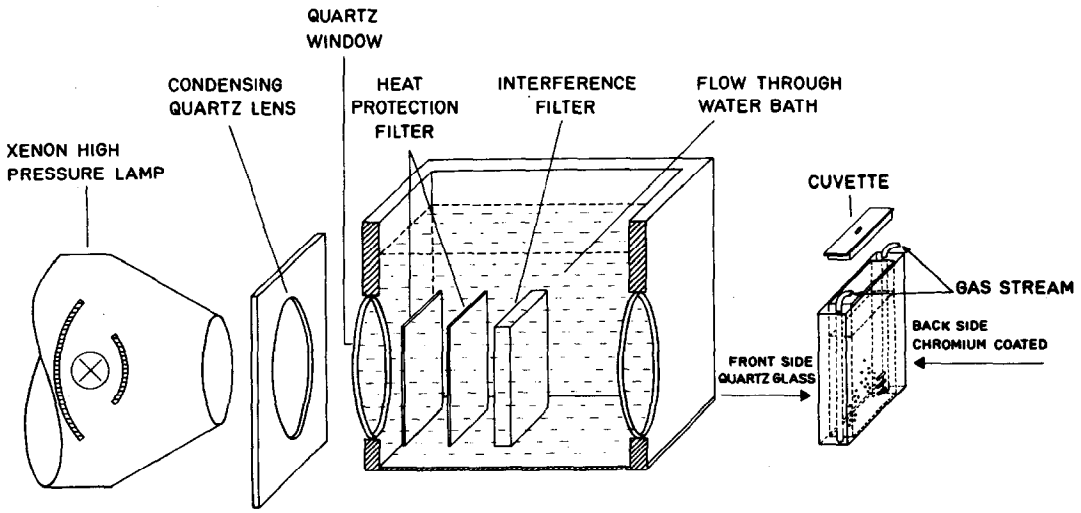

Fig. 1. Arrangement for determining the photochemical action spectrum.

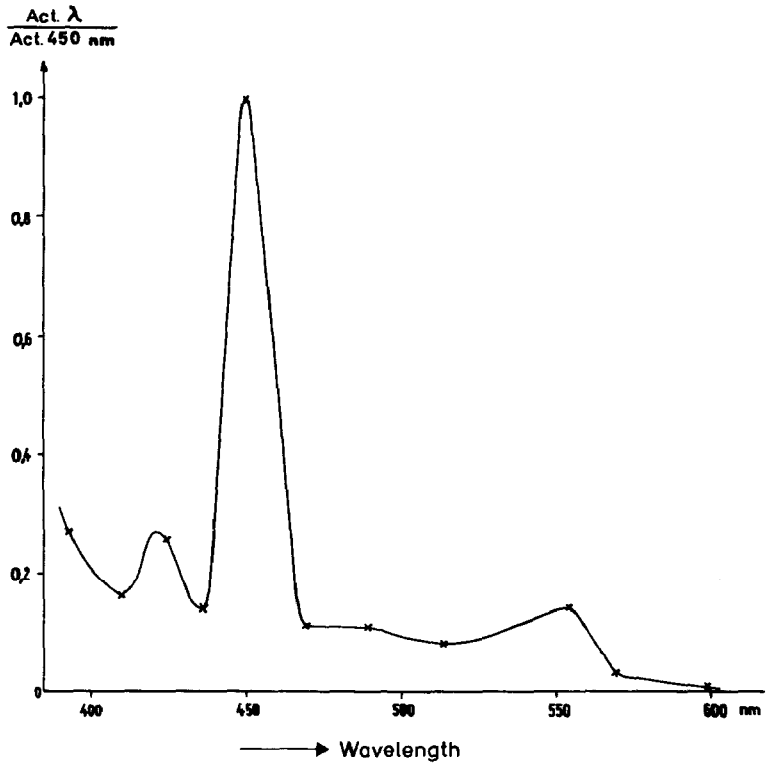

Fig. 2. Photochemical action spectrum of the reversibility of the CO-inhibited cyclohexane hydroxylation. Each cuvette contained a suspension of 10^{-2} M C_6H_{12}, 10^{-3} M NADPH, in 0.05 M phosphate buffer. 5 min incubation at 25°C was started with addition of 0.1 ml rat liver microsomes containing 29.5 mg protein/ml. Sample cuvettes were gassed with a mixture of 23% CO and 52% O_2 in N_2. (CO/O_2 = 0.44), reference cuvettes were gassed with air. Results are plotted in terms of relative light sensitivity $L_\lambda/L_{450\,nm}$,

$$\frac{L_\lambda}{L_{450\ nm}} = \frac{K_\lambda - K_{dark}}{K_{450\,nm} - K_{dark}}, \text{ where } L = \frac{1}{i}\frac{K_{light}\,K_{dark}}{K_{dark}}.$$

K = the partition constant and i the quantum density of light flux. The uninhibited specific activity of this preparation of rat liver microsomes at 25°C was 18 nmol (mg protein)$^{-1}$ min^{-1}.

as described recently [3]. Protein was determined according to the method of Gornall et al. [4].

Cyclohexanol was analyzed by gas chromatography by an adaptation of our method described previously [1]. After stopping the reaction by addition of 20 µl of a saturated solution of uranium acetate the mixture was heated for 5 min on a boiling water bath.

The reaction mixture was centrifuged and 1 µl aliquots of the supernatant were assayed directly for cyclohexanol on a Varian Aerograph (model 20) gas chromatograph. A 6 feet stainless steel column (5% tetracyanoethylated pentaerythritol on Aeropak-30, 80-100 mesh) was used for the separation at 105°C together with a FID detector. The amount of cyclohexanol formed during the incubation was calculated by means of calibration curves obtained from adding cyclohexanol to complete assay mixtures from which NADPH was omitted.

The incubations and irradiations were performed in the experimental set-up shown in fig. 1. A xenon high pressure lamp (Osram XBO, 1600 watts) served as light source. The light beam passed a quartz con-

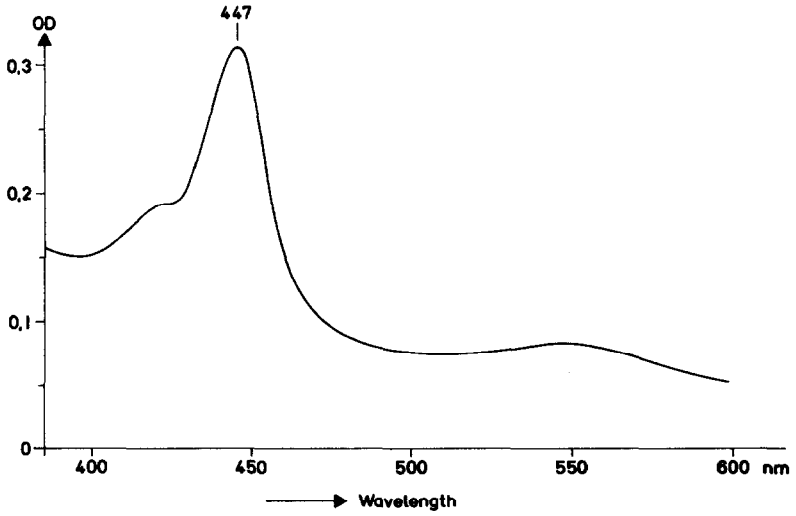

Fig. 3. "Absolute" spectrum of cytochrome P 450 – CO complex. The spectrum was obtained by measuring the absorption of a microsomal preparation from phenobarbital-induced rats (containing 3.38 nMoles/3 ml of cytochrome P 450) versus a preparation from control animals 1.25 nMoles/3 ml cytochrome P 450 with equal amounts of cytochrome b_5 [6]. Both cuvettes were gassed with CO and reduced by 2 mg of sodium dithionite. The difference in absorption between the two cuvettes was recorded on an Aminco – Chance Dual Wavelength Split Beam Spectrophotometer and corrected for the base line.

densor lens and entered the quartz window which contained two heat filters (Schott KG $\frac{1}{2}$ mm and Schott KG $\frac{4}{3}$ mm) in front of the interference filter (Schott, half width of transmission = 12 nm, 30% maximal transmission). The filters were cooled by streaming the box with tap water. The experimental cuvette is fixed behind the exit quartz window in the diffuse focus of the light beam. The cuvette holder is chromium coated where it holds the back wall of the cuvette and is cooled to 25°C.

The complete incubation medium without microsomes was equilibrated in the cuvette for 3 min by bubbling with a cyclohexane-saturated gas mixture from two outlets at the side walls near the bottom of the cuvette.

Control experiments as reference values were run in parallel with air as the gas phase. The relative energy distribution of light at the various wavelengths was measured by a compensated thermopile (Kipp and Zonen, CA 1).

3. Results and discussion

In the presence of 23% carbon monoxide and 52% oxygen (CO/O_2 ratio = 0.44) the hydroxylation of cyclohexane by rat liver microsomes and NADPH in the dark was inhibited to about 75%.

Irradiation with light of 450 nm wavelength reversed the inhibition almost completely; about 95% of the original specific activity in air was recovered. Following the theory of Warburg [5] the results are presented in units of light sensitivity related to that at 450 nm and plotted against wavelength of acting light. As the transmission characteristics of the interference filters were slightly different from each other integral quantum transmission was normalized mathematically to equal values.

The action spectrum shown in fig. 2 closely resembles the absolute spectrum of cytochrome P 450-CO complex which is shown in fig. 3.

It can be seen that the visible absorption peak of the carbon monoxide complex contributes to the action spectrum. A third peak in the action spectrum at about 420 nm was also observed. Its significance,

however, is not obvious since the corresponding peak in the CO-spectrum of isolated reduced cytochrome P 450 probably is caused by a degradation product of cytochrome P 450 called cytochrome P 420, which also may be present in microsomes although it is believed to be inactive in the enzymatic hydroxylation mechanism [7].

Our results establish a role for microsomal cytochrome P 450 in the oxygen activation for cyclohexane hydroxylation in the liver. Together with our observation that cytochrome P 450 forms an enzyme-substrate complex with cyclohexane [1], this indicates strongly that this enzymatic hydroxylation involves a ternary complex of reduced cytochrome P 450, oxygen and cyclohexane.

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References

[1] V.Ullrich, Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 357.
[2] D.Y.Cooper, S.Levine, S.Narasimhulu, O.Rosenthal and R.W.Estabrook, Science 147 (1965) 400.
[3] W.C.Schneider, J. Biol. Chemistry 176 (1948) 259.
[4] A.G.Gornall, C.J.Bardawill and M.M.David, J. Biol. Chemistry 177 (1949) 751.
[5] O.Warburg and E.Negelein, Biochem. Z. 189 (1927) 354.
[6] T.Kinoshita and S.Horie, J. Biochem. (Japan) 61 (1967) 26.
[7] T.Omura and R.Sato, J. Biol. Chemistry 239 (1964) 7.