SPECIFIC QUENCHING OF THE FLUORESCENCE OF BENZO(A)PYRENE BY HEPATIC MICROSOMES FROM 3-METHYLCHOLANTHRENE-TREATED RATS

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Summary The fluorescence of benzo(a)pyrene is markedly quenched by 3-methylcholanthrene-induced microsomes, but not by control microsomes. From the quantitative analysis of the quenching curve it is concluded that the quenching is due to the preferential association of benzo(a)-pyrene with cytochrome P-448.

Introduction The activity of aryl hydrocarbon hydroxylase of 3-methyl-cholanthrene-induced microsomes is higher than that of control microsomes(1). From the analysis of reaction velocity, Alvares et al.(1) suggested that 3-methylcholanthrene induces the formation of a hydroxylase with a greater affinity for benzo(a)pyrene. Here, we obtained direct evidence for the much greater binding affinity of cytochrome P-448 for benzo(a)pyrene than that of cytochrome P-450.

Materials and Methods Benzo(a)pyrene was purchased from Sigma. It was passed through an activated alumina column and then recrystallized from benzene. Dimethylsulfoxide was spectroscopical grade from E. Merck. Male Sprague-Dawley rats weighing 110~120g were used. The rats were injected intraperitoneally once with 3-methylcholanthrene(9mg/kg) in 0.4ml of corn oil 24hr before being killed. Throughout the text induced microsomes refer to those obtained with this treatment. Untreated rats received corn oil only. Hepatic microsomes were prepared according to the method of Ernster et al.(2). A fluorescence cell with the light path of 3mm and a specially designed cell holder were used(Fig. 1). Microsomes were suspended in 0.25M sucrose, 10mM Tris-HCl, pH7.0. Three milliliters of the microsomal suspension were preincubated at 26±0.1° C for 5 min, and benzo(a)pyrene in 10μl of dimethylsulfoxide was added, and then the mixture was shaken well by use of a Vortex mixer. After 20 min incubation small aliquots were transferred into the microcell kept at 26±0.1° C. Fluorescence spectra were obtained with an Aminco Bowman spectrofluorometer, equipped with an ellipsoidal mirror condensing system.

Results The quantitative analysis of the fluorescence of a turbid sample such as the microsomal suspension is difficult because of the

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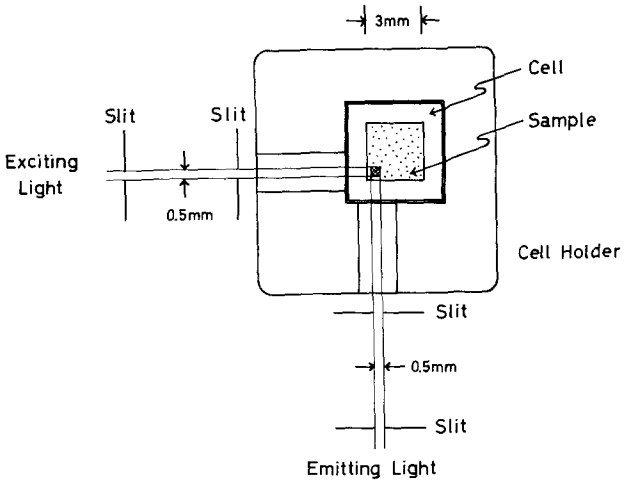

Fig. 1 Illustration of the cell and cell holder used. The cell is made of quartz glass of 1mm in thickness. Fluorescence only from the hatched area is observed under the present conditions.

scattering of exciting and emitting lights. In order to overcome such a difficulty we used the microcell and the specially designed cell holder. Over the wide range of the concentration of benzo(a)pyrene from $3.0X10^{-7}$M to $2.0X10^{-5}$M we found the linear relationship between the fluorescence intensity of benzo(a)pyrene at 431nm and the concentration of benzo(a)pyrene in the suspension of hepatic microsomes(1.70mg protein/ml) from the control rats. Furthermore, the intrinsic fluorescence intensity of microsomes, which is very weak under the present experimental conditions, increases linearly with an increase in concentration of microsomal proteins up to 4.5mg/ml. These findings indicate that the present method is applicable to the quantitative analysis of the fluorescence of benzo(a)pyrene in the microsomal suspension.

Benzo(a)pyrene is gradually incorporated into microsomes when added to the microsomal suspension. The details of this phenomenon will be published elsewhere. Figure 2 shows the fluorescence spectra of benzo(a)pyrene in dimethylsulfoxide, in control and induced microsomes,

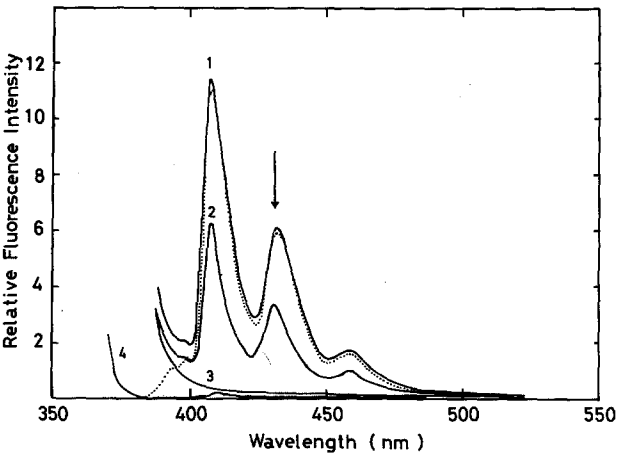

Fig. 2 Fluorescence spectra of benzo(a)pyrene in control microsomes(1) and in induced microsomes(2). Intrinsic fluorescence spectra of control and induced microsomes(3), and the fluorescence spectrum of benzo(a)pyrene in the buffer solution, 0.25M sucrose, 10mM Tris-HCl, pH7.0(4). The dotted line represents the fluorescence spectrum of benzo(a)pyrene in dimethylsulfoxide. The arrow indicates the wavelength at which the fluorescence intensity was measured in the following experiments. Concentrations of benzo(a)pyrene and microsomes are 1.93×10^{-6}M and 1.55mg/ml, respectively. Excitation wavelength, 360nm. Temperature, 26±0.1° C.

and in the buffer solution. The fluorescence spectrum of benzo(a)pyrene in control microsomes is almost identical with that in the organic solvent, and benzo(a)pyrene is practically insoluble in the buffer solution. These facts indicate that almost all the molecules of benzo(a)pyrene are incorporated into microsomes. It should be noted that induced microsomes reduce the fluorescence intensity of benzo(a)pyrene by 50% or more. Changes in fluorescence intensities of benzo(a)pyrene at 431nm on varying the concentration of microsomal proteins are shown in Fig. 3. It is clear that the fluorescence intensity of benzo(a)pyrene in control microsomes remains constant up to the protein concentration of 2.5mg/ml, but in induced microsomes it decreases markedly with an increase in protein concentration. Since the intrinsic fluorescence intensity of microsomes is less than 4% of that of benzo(a)pyrene at 431nm and there is no difference in fluorescence intensity between con-

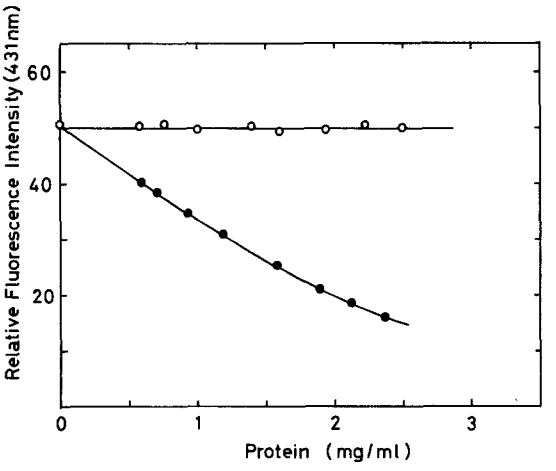

Fig. 3 Changes in fluorescence intensity of benzo(a)pyrene at 431nm on varying the protein concentrations of control(○) and induced(●) microsomes. The intrinsic fluorescence intensity of microsomes is subtracted from the observed intensity, and net changes in intensity are plotted against microsomal proteins. The fluorescence intensity at the protein concentration of 0 indicates the intensity in dimethylsulfoxide. Each point is the average of 5~6 experiments. Concentration of benzo(a)-pyrene, 1.93×10^{-6} M. Excitation wavelength, 360nm. Temperature, 26±0.1°C.

trol and induced microsomes, the observed fluorescence quenching must be due to protein(s) induced by the 3-methylcholanthrene-treatment. Of the proteins induced, cytochrome P-448 is the most probable candidate of the quencher. Our preliminary experiments indicate that phenobarbital-induced microsomes do not quench the fluorescence of benzo(a)pyrene as control microsomes. Furthermore, Jakcobsson et al. (3) reported that 3-methylcholanthrene-induced microsomes revealed the type I difference spectrum on association with benzo(a)pyrene, but control microsomes did not. These facts are additional evidence for the preferential association of benzo(a)pyrene with cytochrome P-448. However, the quantitative analysis of the difference spectrum is difficult because the wavelength region of the absorption of benzo(a)-pyrene overlaps with that of the difference spectrum.

An attempt to analyze the quenching curve by use of the Stern-Volmer equation was not achieved, indicating that the quenching is not

due to collisions. Therefore, we assumed the following mechanism; benzo(a)pyrene in the ground state is associated with cytochrome P-448, the complex is completely nonfluorescent and the equilibrium in the ground state is maintained in the excited state.

$$BP + Q \underset{}{\overset{K}{\rightleftharpoons}} BPQ \qquad K = \frac{[BPQ]}{[BP][Q]} \tag{1}$$

where K is the association constant, and [BP], [Q] and [BPQ] indicate the concentrations of benzo(a)pyrene, the quencher and the complex, respectively. The initial concentrations of BP and Q can be expressed as follows;

$$[BP_o] = [BP] + [BPQ] \tag{2}$$

$$[Q_o] = [Q] + [BPQ] \tag{3}$$

Eliminating [BP] and [BPQ] from (1), (2) and (3), we obtain

$$K[Q]^2 + \{K[BP_o] - K[Q_o] + 1\}[Q] - [Q_o] = 0 \tag{4}$$

The fluorescence intensities in the absence and presence of the quencher are given by

$$F_o = \phi_{BP}\varepsilon_{BP}[BP_o]\ d\ I_o \tag{5}$$

$$F = \phi_{BP}\varepsilon_{BP}[BP]\ d\ I_o + \phi_{BPQ}\varepsilon_{BPQ}[BPQ]\ d\ I_o \tag{6}$$

where ϕ is the fluorescence quantum yield, ε is the extinction coefficient, d is the pathlength of the cell, and I_o is the intensity of the exciting light. If we assume that benzo(a)pyrene becomes to be completely nonfluorescent on association with the quencher ($\phi_{BPQ} = 0$), we obtain

$$F_o / F = [BP_o] / [BP] \tag{7}$$

Substituting (1) and (2) into (7) yields

$$[Q] = \frac{1}{K}\left(\frac{F_o}{F} - 1\right) \tag{8}$$

From (4) and (8) we obtain finally

$$\frac{F_o}{F} = K\left\{[Q_o]\ \frac{F_o}{F_o - F} - [BP_o]\right\} \tag{9}$$

Thus, a plot of F_o/F vs. $\left\{[Q_o]\ \frac{F_o}{F_o - F} - [BP_o]\right\}$ gives a straight line passing through the origin, and the association constant can be de-

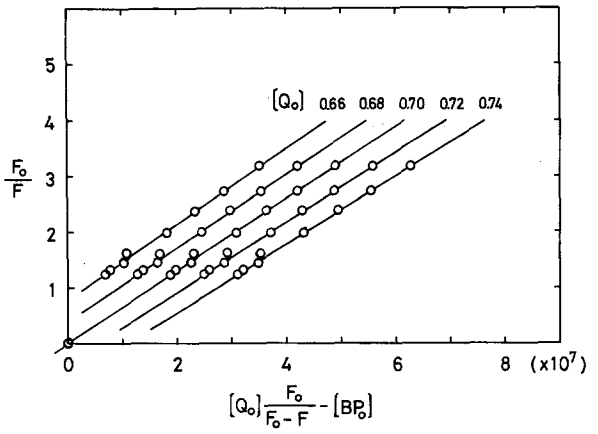

Fig. 4 Plots of F_0/F vs. $[Q_0]\frac{F_0}{F_0 - F} - [BP_0]$. The values of $[Q_0]$ are tentatively assumed as indicated. The unit of $[Q_0]$, 10^{-6}M/mg of protein.

termined from the slope. In equation (9), $[Q_0]$ is unknown because it is the concentration of cytochrome P-448 available for the association with benzo(a)pyrene, and not always equal to that of total enzymes. However, $[Q_0]$ can be graphically determined without difficulty. As shown in Fig. 4, a proper assumption of the value of $[Q_0]$ gives a straight line though it does not always pass through the origin. Accordingly, by means of adjustment of the value of $[Q_0]$, the straight line passing through the origin can be easily obtained. In this manner the values of $[Q_0]$ and K were determined to be 0.70×10^{-6}M/mg of protein and 6.48×10^{6}1/M, respectively. The value of the association constant is very high. This is indicative of the great affinity of benzo(a)-pyrene for cytochrome P-448. The successful analysis of the experimental data by use of equation (9) clearly indicates the validity of the assumptions that the quenching is due to the association of benzo(a)pyrene with cytochrome P-448 and the equilibrium in the ground state is maintained during the lifetime of the singlet-excited state of benzo(a)pyrene.

Discussion We observed that the fluorescence of benzo(a)pyrene is quench-

ed by induced microsomes, but not by control microsomes. From the quantitative analysis of the quenching curve we concluded that the quenching is due to the association of benzo(a)pyrene with cytochrome P-448. The recent reports (4, 5) indicate that a part of purified cytochrome P-448 is associated with 3-methylcholanthrene. However, even though some molecules of 3-methylcholanthrene remain in microsomes, the observed quenching is not due to 3-methylcholanthrene because our separate experiments indicate that more than 5000 times concentration of 3-methylcholanthrene is necessary to quench the fluorescence of benzo(a)pyrene by 10%.

Equation (8) is similar to the Stern-Volmer equation. Therefore, when $[Q]$ can be approximated by $[Q_o]$ because of the low value of the association constant, the Stern-Volmer plot must be linear even in the present case. However, this was not the case. This fact shows that the value of the association constant is very high and $[Q]$ cannot be approximated by $[Q_o]$. Accordingly the explicit equation (9) must be employed.

As shown in Fig. 3, control microsomes did not quench the fluorescence of benzo(a)pyrene, but at the lower concentration of benzo(a)-pyrene such as $2X10^{-7}M$ we observed a slight decrease in the fluorescence intensity of benzo(a)pyrene (not shown). In this case quantitative analysis is difficult because the intrinsic fluorescence of microsomes is not negligible. The association constant, however, could be roughly estimated to be the order of 10^5. Therefore the binding affinity of cytochrome P-448 for benzo(a)pyrene is about ten times stronger than that of cytochrome P-450.

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