The recognition of a soluble cytochrome P450 in rat liver

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The presence of hepatic soluble cytochrome P450 was investigated in the rats that were pretreated with CCl_4, CS_2 and DENA + PCBs. The dithionite-reduced CO difference spectrum of the hepatic 204000 × *g* supernatant of these rats showed its maximum at 455 nm. Aniline and hexobarbital could react with the hepatic 204000 × *g* supernatant to give the substrate binding spectra of P450; differences were observed between the substrate-induced difference spectra of the 204000 × *g* supernatant and that of the microsomal fraction. These results indicate the existence of a hepatic soluble P450.

Cytochrome P450, soluble *(Rat liver)* *Difference spectrum*

1. INTRODUCTION

Cytochrome P450 (P450), the major hemoprotein present in mammalian endoplasmic reticulum functions as a terminal component of the microsomal mixed-function oxidase system [1]. Soluble P450 is known to be present in microorganisms [2–7], but a soluble form of mammalian P450 has not been reported.

A soluble protein, extracted from liver cytoplasm of male rats which were intoxicated with CCl_4, stimulated microsomal NADPH oxidase [8]. This stimulation was inhibited by carbon monoxide, suggesting that the soluble protein extract contained a carbon monoxide-sensitive component, such as hemoprotein [8]. Here, I show that the 204000 × *g* supernatant of the rat liver homogenate contains a soluble P450 which results from the impairment of microsomal P450.

2. MATERIALS AND METHODS

Male Wistar rats (300–350 g body wt) were divided into 5 groups. Each group was composed of 6 rats. Group 1 was the control while other groups were experimental. CCl_4, CS_2 (carbon disulfide) and DENA (diethylnitrosamine), known to impair microsomal P450 [9–11], were administered to group 2–5. Group 2 was given CCl_4, group 3 was given CS_2, group 4 was given DENA, and group 5 was given DENA and PCBs. CCl_4 (1 ml/kg body wt) was injected into the rats intraperitoneally; 7 days after the CCl_4 injection, the rats were decapitated. Livers were immediately perfused in situ with ice-cold 0.9% NaCl. A 20% liver homogenate was made in ice-cold 0.15 M KCl, 10 mM Tris–HCl buffer (pH 7.5). This was centrifuged at 10000 × *g* for 20 min, and a soluble fraction was prepared from the 10000 × *g* supernatant by centrifuging it further at 204000 × *g* for 240 min to avoid contamination of free ribosomes. The resulting 204000 × *g* supernatant was saved for a soluble fraction and the sedimented microsomes were resuspended in 0.15 M KCl, 10 mM Tris–HCl buffer (pH 7.5). Group 3 received CS_2 (200 mg/kg body wt) intraperitoneally; 10 days after the CS_2 injection, group 3 was sacrificed. DENA (Tokyo Kasei Chemical Co.) was offered to groups 4, 5 in the drinking water at 50 ppm for 5 weeks. Group 5 received thereafter PCBs (Kanechlor 500, Kanegafuchi Chemical Co., Osaka) for 5 weeks; 0.2 ml 5% PCBs was administered in olive oil to group 5 by gastric intubation twice a week. Ten weeks after the DENA administration, groups 4, 5 were then sacrificed; the 204000 × *g* supernatant fraction and microsomal

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fraction were then prepared. The normal 204000 × *g* supernatant and the normal microsomal fraction were prepared from normal rats.

P450 was determined from the carbon monoxide-induced difference spectrum of dithionite-reduced 204000 × *g* supernatant or microsomes assuming a molar extinction coefficient of 91 $mM^{-1}.cm^{-1}$ between 450 and 490 nm [12].

Difference spectra of hexobarbital and aniline with the 204000 × *g* supernatant and with the microsomes were measured as follows. The 204000 × *g* supernatant (10.8 mg protein/ml) was divided equally into sample and reference cuvettes and scanned between 500 and 350 nm to produce a baseline using Shimazu UV-190 double beam spectrophotometer. Hexobarbital and aniline were added at 5 mM to the sample cuvette, while the reference cuvette received solvent in appropriate amounts. Microsomes were suspended in 0.15 M KCl, 10 mM Tris–HCl buffer (pH 7.5) to 1.5–2.2 mg protein/ml. Reaction temperature was 22°C.

3. RESULTS AND DISCUSSION

3.1. *Dithionite-reduced carbon monoxide difference spectrum of the hepatic 204000 × g supernatant (fig.1)*

The hepatic 204000 × *g* supernatant was extracted at 7 days after the CCl_4 injection. The difference in absorbance at 460 nm was low at 2 min after the addition of dithionite (4 mg), but gradually reached its maximum value after 14 min; its absorbance change represented a blue shift from 460–455 nm. The time which is needed for the change to develop maximally shortened when 10 mg dithionite was added; in which case 4 min were needed to reach its maximum. In [13], slow reduction of yeast P450 was observed on addition of Triton X-100. These results suggested that the time dependency of the carbon monoxide difference spectrum was due to the slow reduction of the heme. The dithionite-reduced CO difference spectrum of the 204000 × *g* supernatant of the groups 3, 4 and 5 also showed its maximum at 455 nm. However, the dithionite-reduced CO difference spectrum of the normal 204000 × *g* supernatant did not show its absorbance maximum at 450–500 nm; that is, control group 1 did not possess the heme compound evident in the 204000 × *g* supernatant. Thus it is suggested that the hepatic 204000 × *g* supernatant of treated rats might contain a P450-like hemoprotein.

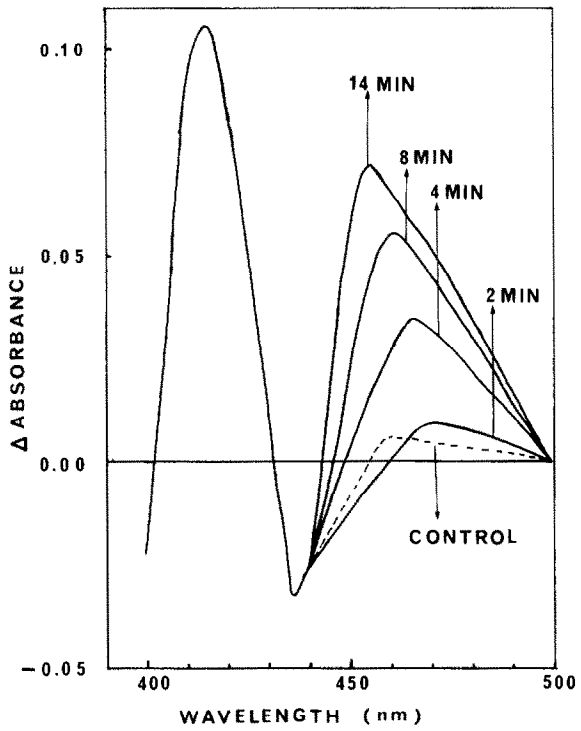

Fig.1. Time dependency of carbon monoxide-induced difference spectrum of dithionite-reduced hepatic soluble fraction extracted from CCl_4-treated rats. A 3 ml sample of soluble fraction was placed in each of two spectrophotometer cuvettes and the baseline between 400 and 500 nm was recorded using Shimazu UV-190 double-beam spectrophotometer. After that, carbon monoxide was bubbled (at 1 bubble/s) through the sample cuvette for 1 min and 4 mg sodium dithionite was added to both cuvettes. The scanning was repeated. Soluble fraction from CCl_4-treated rats had 10.8 mg protein/ml; a control spectrum (– – –) obtained from normal rats and measured after 14 min contained 11.4 mg protein/ml. Spectrophotometry was carried out at 22°C.

3.2. *Aniline and hexobarbital-induced difference spectra of the hepatic 204000 × g supernatant*

Aniline and hexobarbital substrates were then tested for reaction with the hepatic 204000 × *g* supernatant to give the characteristic substrate-binding spectra of P450. According to [14–17], the spectral changes induced by substrate are due to

the interaction between the P450 molecule and the substrate. In fig.2, the hepatic 204000 × *g* supernatant was extracted from rats at 7 days after the CCl_4 injection. When 5 mM hexobarbital was added to the sample cuvette, it induced an anomalous spectral change; absorption maxima and minima occurred at 410 nm and 430 nm. Such anomalous spectral change induced by hexobarbital has been reported in [18]. P450 extracted once with 1-butanol and twice with acetone from hepatic microsomes of 3-methylcholanthrene-treated rats exhibited an anomalous spectral change (410 nm peak, 430 nm trough) on addition of hexobarbital in the absence of 25% glycerol [18]. However, 5 mM aniline induced a typical type II spectral change (390 nm trough, 420–425 nm peak). Normal hepatic 204000 × *g* supernatant from the group 1 rats, however, did not show the substrate-induced spectral change.

In the event that the hepatic 204000 × *g* supernatant was simply contaminated by microsomal fragments, the same substrate-induced difference spectra would then be expected for that observed with the microsomal fraction. However, in certain cases some differences between the substrate-induced difference spectra of the 204000 × *g* supernatant and that of the microsomal fraction were evident (fig.3). The 204000 × *g* supernatant–substrate binding spectra were not the same as that

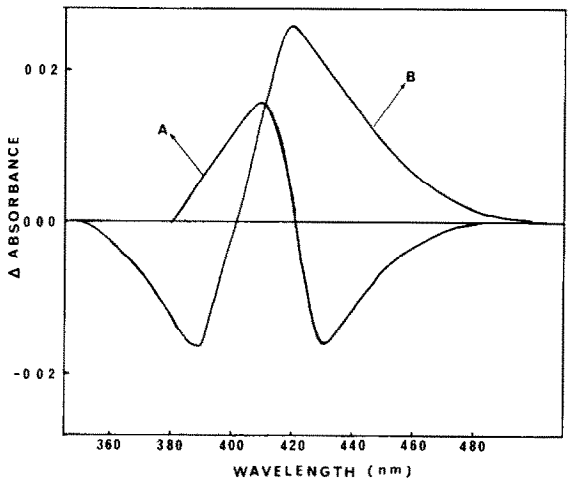

Fig.2. Difference spectra due to the presence of 5 mM hexobarbital (A) and 5 mM aniline (B) in a soluble fraction from CCl_4-treated rats. Soluble fraction had 9.8 mg protein/ml.

of the microsomal fraction (fig.3). In group 4, the aniline-binding spectrum was not observed in the 204000 × *g* supernatant from 1–10 mM; however, it was observed in microsomes, where the magnitude of the spectral change was dependent on the concentration of aniline. Further, the positions of the peak and trough of the hexobarbital-induced difference spectrum in the supernatant were not the same as that in the microsomes. In group 5, 1–10 mM hexobarbital induced a type II-like spectral change in the supernatant, but it induced a typical type I spectral change in microsomes. This type I spectral change did not change over 1–10 mM hexobarbital, while the magnitude of spectral change was dependent on the concentration of hexobarbital in this range. This hexobarbital-induced type II-like spectral change was reported in [19]. When rat liver microsomes were extracted with isooctane, the resultant preparation was essentially devoid of the ability to produce a type I difference spectrum upon addition of hexobarbital [19]; a type II spectral change was obtained at high concentrations of hexobarbital [19]. The effect of cytosol on the microsomal spectral change was almost negligible. These results suggested that P450 in the 204000 × *g* supernatant was not the result of contamination by microsomes.

Since the hepatic 204000 × *g* supernatant contained comparatively low amounts of oxyhemoglobin and P420, the substrate-binding spectra of oxyhemoglobin and P420 was investigated. In the case of oxyhemoglobin, hexobarbital and aniline induced a spectral change which included an absorption minimum at 405 nm, but no absorption maximum. In the case of P420 which was made by treatment of mercuric chloride (100 μM) with normal microsomes for 30 min at 37°C (70% of P450 was converted to P420), anomalous spectral changes were observed; hexobarbital yielded a peak at 410 nm, while aniline yielded a trough at 410 nm. Soluble P420 did not show the substrate-binding spectral changes in [20]. These results indicated that oxyhemoglobin and P420 which were present in the 204000 × *g* supernatant did not show the same substrate-induced spectral change as the supernatant showed.

Purification of the soluble P450 in the 204000 × *g* supernatant by *n*-octylamine affinity chromatography and the difference between the soluble

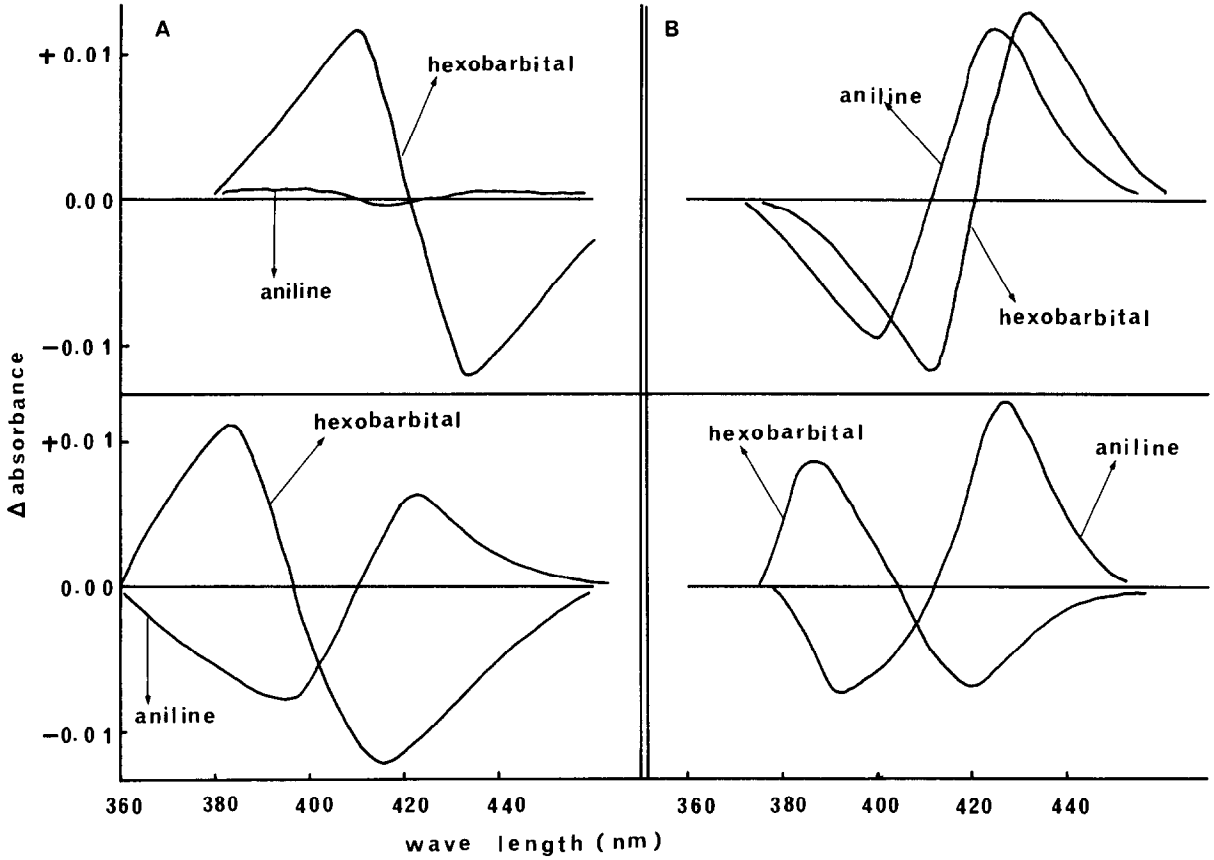

Fig.3. Differences between the substrate-induced difference spectra of the soluble fraction and that of the microsomal fraction. The upper part of each figure represents the soluble fraction–substrate binding spectra. The lower part represents the microsomal substrate binding spectra for (A) rats treated with DENA, (B) rats treated with DENA and PCBs. Experimental conditions were as in fig.1. Microsomes were suspended in 0.15 M KCl, 10 mM Tris–HCl buffer (pH 7.5) to 1.5–2.2 mg protein/ml. Reaction temperature was 22°C.

Table 1

The relationship between a soluble and a microsomal P450 (nmol/mg protein)

	Microsomal P450	P420	Supernatant
Control	1.000 ± 0.33	(n.d.)	n.d. (462–464 nm)[a]
Days after the CCl_4 injection			
1 Day	0.301 ± 0.13	0.663 ± 0.43	n.d. (462–464 nm)[a]
3 Days	0.685 ± 0.10	0.164 ± 0.02	0.0018 ± 0.00001 (452–454 nm)[a]
7 Days	1.08 ± 0.01	(n.d.)	0.092 ± 0.0060 (455 nm)[a]

[a] Peak of CO difference spectrum

n.d., not detected

All values are expressed as the average of 5 experiments ± SD

Table 2

The aniline hydroxylase activity of normal and CCl_4 microsomes in the presence of hepatic 204000 × *g* supernatant extracted at 7 days after CCl_4 injection or extracted from normal rat

Added (2.5 ml)	Normal microsomes		CCl_4 microsomes	
	Aniline hydroxylase	P450	Aniline hydroxylase	P450
0.15 M KCl, 10 mM buffer Tris–HCl (pH 7.5)	0.73 ± 0.15	0.943	0.12 ± 0.059	0.204
Normal S	0.58 ± 0.18	0.825	0.15 ± 0.088	0.246
7 Days S	1.08 ± 0.03	1.040	0.67 ± 0.088	0.810

Experimental conditions were described in the text, except that the reaction was for 12 min at 37°C, aniline was 1 mM, and the total volume was 4.5 ml. Microsomal protein was 1.5 mg/ml. Normal supernatant protein was 10.1 mg and 7 days (after the CCl_4 injection) supernatant protein was 9.75 mg. All values were the mean of 5 expt ± SD. P450 value is expressed as the average of 3 expt. Aniline hydroxylase activity: nmol *p*-aminophenol formed . min^{-1} . mg $protein^{-1}$. Activity of aniline hydroxylase was measured as in [21]

P450 and microsomal P450 are being studied; the presence of P450 in the 204000 × *g* supernatant has been confirmed (in preparation). The 204000 × *g* supernatant shows neither glucose 6-phosphatase activity nor the spectral changes associated with cytochrome b_5, providing further support to the conclusion that microsomal fragments do not contaminate the supernatant.

The above phenomena were not observed at the first stage of drug administration, but at the recovery stage of microsomal P450 (table 1).

Would addition of this P450-like hemoprotein to the microsomes next result in an increase in the activity of the microsomal aniline hydroxylase? Normal and CCl_4-treated microsomes, which were extracted 1 day after the CCl_4 injection, were added to the 204000 × *g* supernatant extracted at 7 days after the CCl_4 injection. The mixture was incubated at 25°C for 10 min, then centrifuged at 204000 × *g* for 90 min. The content of the sedimented microsomal P450 and the activity of aniline hydroxylase were measured (table 2). The normal and CCl_4 microsomes added to the 7 day supernatant had the highest activity of aniline hydroxylase and showed the highest P450 content. These results indicated that the increase in aniline hydroxylase activity and P450 content was due to the incorporation of P450 present in the 204000 × *g* supernatant.

Hepatic soluble P450 is present in the rats pretreated with CCl_4, CS_2, DENA and DENA plus PCBs. This hepatic soluble P450 was not contaminated by microsomal fragments.

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