BBA 45669

THE ELECTRON SPIN RESONANCE AND ABSORPTION SPECTRA OF MICROSOMAL CYTOCHROME P-450 AND ITS ISOCYANIDE COMPLEXES

YOSHIYUKI ICHIKAWA AND TOSHIO YAMANO

Department of Biochemistry, Osaka University, Medical School, Kitaku, Osaka (Japan)

(Received December 12th, 1967)

SUMMARY

The absorption spectra of oxidized P-450–isocyanide complexes were the same in difference spectra irrespective of the isocyanide derivative tested. However, with these reduced P-450–isocyanide complexes, absorption at 455 mμ increased, and that at 430 mμ decreased, with increasing carbon atom number of the isocyanide derivative at a definite pH. The same changes were seen with individual complexes with increasing pH.

The dissociation constants of oxidized P-450–isocyanide complexes decreased with increase in carbon atom number of the isocyanide. These results were confirmed by electron spin resonance (ESR) spectroscopy. However, the dissociation constants of reduced P-450–isocyanide complexes were essentially identical and the dissociation constants of the oxidized and reduced P-450–isocyanide complexes were little affected by pH.

The oxidized P-450–isocyanide complexes gave magnetically specific ESR signals. The orbital energy differences of d_ε orbitals of the heme iron of the complexes increased with increase in the carbon atom number of the isocyanide.

Purified P-450 and its isocyanide complexes were rapidly reduced by a ferredoxin–$NADP^+$ reductase system.

INTRODUCTION

Since WARBURG, NEGELEIN AND CHRISTIAN[1] first reported the effect of methyl isocyanide on hemoprotein, many investigations have been made on the effect of alkyl isocyanides on various hemoproteins[2–11].

The properties of the complex of the microsomal cytochrome P-450 with ethyl isocyanide were studied, and its unique spectrum possessing two pH-dependent bands in the Soret region was reported[7,10,22].

This paper reports spectrophotometric and magnetic studies on P-450 complexes with various isocyanides to obtain more information on the structure of the cytochrome around its heme iron.

Abbreviation: ESR, electron spin resonance.

MATERIALS AND METHODS

Microsomal preparations

Microsomes were prepared from the livers of rabbits which had fasted for 15 h. A modification of the method of ICHIKAWA, HAGIHARA AND YAMANO[12] was used for the preparation. All procedures were carried out at 0–5°. The microsomal pellet was stored at 0° under anaerobic conditions and used within 24 h after preparation. This microsomal preparation was practically free from adsorbed hemoglobin and serum proteins as judged by electrophoresis on acrylamide gel according to the method of TACHIBANA AND YAMAURA[13]. The absence of hemoglobin was confirmed spectrophotometrically. Contamination with mitochondria was found to be less than 1 % on a protein basis, as shown by measurement of succinic oxidase activity.

Measurements of difference spectra

Spectra were taken in a Cary model 14 recording spectrophotometer equipped with a light scattering transmission attachment, using cuvettes of 1-cm light path at room temperature.

Electron spin resonance measurements

Electron spin resonance (ESR) spectroscopy was performed with a Varian V-4500-10A spectrometer with a 100 kcycles/sec field modulation unit. Spectra were generally obtained at a sample temperature of —170°. Quartz sample tubes with 3.5 mm internal diameter were used in the Varian variable temperature attachment. The field modulation amplitude used was 15 Gauss, unless otherwise stated. An XY recorder from the Yokogawa Electric Co. was used to measure g values accurately.

Measurements of pH

The pH meter model HM-5A of Toa–Denpa Kogyo Co. was used to measure pH. The values are reported for measurements at 20°.

Analytical procedures

The protein content of the microsomes was determined by the biuret method[14] in the presence of 1 % sodium cholate. A correction for absorption of heme at 540 mμ was made by subtracting the value obtained on incubation of microsomes with 0.45 % sodium–potassium tartrate in 3.4 % NaOH. Crystalline bovine serum albumin was used as the standard.

Chemicals

Carbon monoxide was purchased from Takachiho Chemical Co. The gas was 99.7 % pure according to gas chromatographic analysis.

Methyl isocyanide and ethyl isocyanide were synthesized from silver cyanide and methyl iodide or ethyl iodide using potassium cyanide as catalyst; a modification of the method of JACKSON AND MCKUSICK[15] was used in which 2 moles of silver cyanide per mole of methyl iodide or ethyl iodide were substituted for an equimolar quantity as stipulated in the published method. *tert.*-Butyl isocyanide was synthesized by the method of UGI *et al.*[16]. Phenyl isocyanide was synthesized by the method of SCHMIDT AND STERN[17]. These isocyanides were distilled twice before use. Their purities were more than 98 % as determined by gas chromatographic analysis. The isocyanides

were stored at —70° in the dark and used within a week after distillation. The other reagents used were commercial products.

Enzyme preparation

Crystalline ferredoxin of *Clostridium pasteurianum* or spinach, and crystalline ferredoxin–$NADP^+$ reductase (EC 1.6.99.4) of spinach were prepared by the method of TAGAWA AND ARNON[18] and SHIN, TAGAWA AND ARNON[19], respectively. These enzymes were generously given by Drs. K. TAGAWA and M. SHIN in this university.

P-420, in which the ESR signal of microsomal Fe_x had decomposed, was purified by the method of OMURA AND SATO[20].

NADH and NADPH were purchased from Sigma Chemical Company, and their concentrations were determined by the method of HORECKER AND KORNBERG[21]; the value of $6.22 \cdot 10^6$ cm^2/mole was taken as the molar extinction at 340 mμ.

Measurement of isocyanide dissociation curves

The isocyanide dissociation curves of P-450 or P-420 were obtained at 20° by adding serially increasing amounts of solutions of isocyanide derivatives to oxidized P-450 and P-420, or their reduced forms, obtained by treatment with a few mg of sodium dithionite. Although the absorption peaks of the oxidized complexes were not affected by pH between 6.0 and 8.3, the absorptions of the reduced complexes varied with pH, as reported by IMAI AND SATO[22]. Therefore, the dissociation curves of oxidized P-450 were obtained from the concentration of isocyanide and the absorption of the difference spectra between 434 and 400 mμ, and the curves of reduced P-450 were obtained from the increase in absorption between 430 and 500 mμ at below pH 7.5 and at 455 and 500 mμ at higher pH values, because the peaks at 430 and 455 mμ decreased with decrease in concentration of isocyanide[10]. Similar results were obtained with all isocyanide derivatives. After spectrophotometric examination of solutions of oxidized P-450–isocyanide complexes, the solutions were centrifuged at 105000 $\times$ g for 90 min at 0°, and the precipitates of 50 mg of microsomal protein per ml were used for measurement of ESR signals. The isocyanide dissociation curves of reduced P-420 were determined from the increase in absorption of the difference spectra between 433 and 500 mμ.

Measurements of the pK of the change in the absorption of reduced P-450–isocyanide complexes

The reduced P-450–isocyanide complexes have Soret bands at 430 and 455 mμ, and the heights of these varied inversely with the pH. This may be due to deprotonation of the hydrophobic ligands around the prosthetic group of the reduced P-450–isocyanide complexes. The pH value at which the height of the two peaks was the same was tentatively defined as the pK of the complexes, since the maximum heights of peaks could not be determined outside the pH range of 6.0 to 8.3 owing to the conversion of P-450 to P-420.

Orbital energy differences of the d_ε orbitals of heme iron

The orbital energy differences of the d_ε orbital ξ, η and ζ of heme iron for P-450 and its isocyanide complexes were calculated from the ESR experiment of these hemoproteins by the method of KOTANI[28].

RESULTS

Fig. 1A shows the absorption spectra of P-450–isocyanide complexes in the oxidized form. The absorption spectra of the complexes are the same for all isocyanide derivatives and are not affected by change of pH between 6.0 and 8.3. However, the absorption spectra of the reduced P-450–isocyanide complexes of various isocyanide derivatives differ as shown in Fig. 1B. Although the absorption spectrum of the reduced P-450–ethyl isocyanide complex varies with pH (ref. 22) and induced P-450 (ref. 23), Fig. 1B shows that the absorption spectra at a definite pH vary with the species of isocyanide as that of the reduced P-450–ethyl isocyanide complex varies with pH. The Soret peak at 455 mμ increased, while that at 430 mμ decreased, with increase in the carbon number of the isocyanide derivative. Although alkyl isocyanide does not cause the conversion of P-450 to P-420, P-450 is readily converted to P-420 in the presence of excess phenyl isocyanide. Therefore, in experiments using phenyl isocyanide, care had to be taken about its concentration.

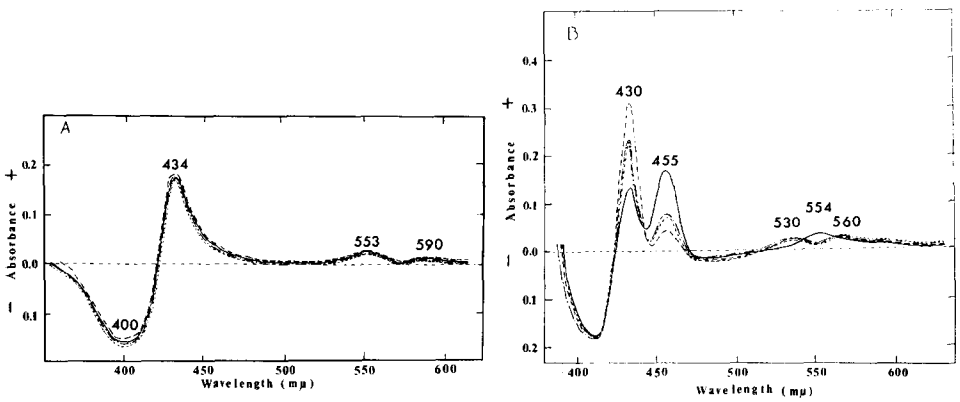

Fig. 1A. Difference spectra of oxidized P-450–isocyanide complexes of liver microsomes. Both the sample and reference cell contained a microsomal suspension (2 mg of protein per ml, 0.1 M potassium–sodium phosphate buffer, pH 7.0). After the base line had been recorded, the content of the sample cell was saturated with the isocyanide derivative, and the difference spectrum was measured aerobically 5 min later. — — —, methyl isocyanide ($5 \cdot 10^{-1}$ M); – – – – –, ethyl isocyanide ($2 \cdot 10^{-2}$ M); –·—·–, *tert.*-butyl isocyanide ($2 \cdot 10^{-2}$ M); ———, phenyl isocyanide ($1 \cdot 10^{-3}$ M).

Fig. 1B. Difference spectra of reduced P-450–isocyanide complexes of liver microsomes. The conditions were the same as for Fig. 1A, except that 2–3 mg sodium dithionite were added to both cells. — — —, methyl isocyanide; – – – – –, ethyl isocyanide; –·—·–, *tert.*-butyl isocyanide; ———, phenyl isocyanide.

The isocyanide dissociation curves of oxidized and reduced P-450 were plotted as percentages of the absorption of the saturated complexes against the isocyanide concentration. The curves for oxidized P-450 were also obtained by plotting the maximal ESR signal height of the P-450–isocyanide complexes against the concentration of isocyanide. Fig. 2 shows the isocyanide dissociation curves of oxidized and reduced P-450. Fig. 2 shows that the curves determined by spectrophotometric and magnetic measurements are essentially identical, and the dissociation constants of the oxidized P-450–isocyanide complexes decreased with increasing carbon atom number and hydrophobic character of the isocyanide derivatives. However, with

reduced P-450–isocyanide complexes, the dissociation constants were essentially identical for all isocyanide derivatives. The dissociation constants of the oxidized and reduced P-450–isocyanide complexes are summarized in Table I. According to the hypothesis of steric hindrance of ST. GEORGE AND PAULING[4] and LEIN AND PAULING[5], these results suggest that the heme group of P-450, unlike hemoglobin and myoglobin, is not buried in the apoprotein molecule, but that it may be attached to the surface. Further studies are necessary to determine the ternary structure of P-450. On introducing the data from these dissociation curves into Hill's equation[24] $Y = Kp^n/(1 + Kp^n)$, which was proposed for the reaction of reduced hemoglobin with oxygen, the sigmoid coefficients of the isocyanide dissociation curves were calculated to be apparently 1.0 for P-450 or P-420 in the oxidized and reduced forms. It does not seem that there is any heme–heme interaction in either form.

Fig. 3 shows the effect of pH on the dissociation constants of the P-450–ethyl isocyanide and P-450–*tert.*-butyl isocyanide complexes in the oxidized and reduced

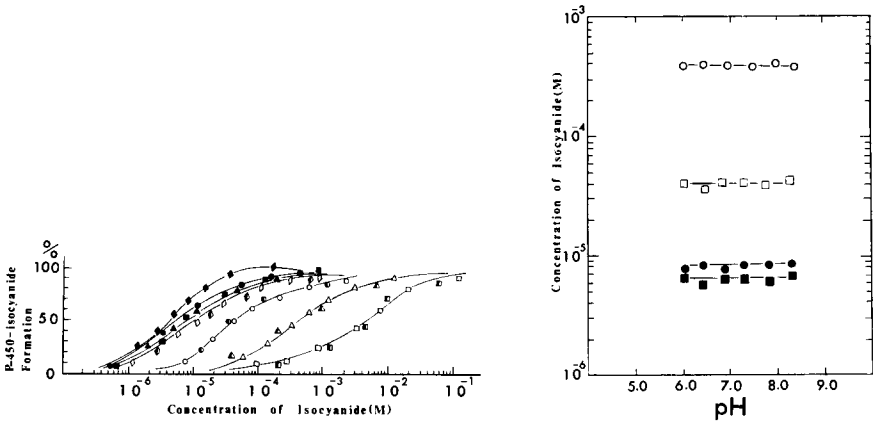

Fig. 2. Isocyanide dissociation curves of oxidized and reduced P-450 of liver microsomes. The conditions were the same as for Figs. 1A and 1B, except that the final isocyanide concentrations were varied as indicated. The isocyanide concentrations were varied by the addition of various amounts of buffer at a high concentration of isocyanide. Corrections were made for the effect of dilution due to addition of isocyanide solution. Microsomal protein 2 mg/ml, 0.1 M potassium–sodium phosphate (pH 7.0), 20°. The sample solutions at various isocyanide concentrations were centrifuged as described in the text. The precipitate (50 mg of microsomal protein per ml) was measured by ESR spectroscopy. The isocyanide dissociation curves of oxidized P-450 were plotted as a percentage of the ESR signal heights of maximal g values of the microsomal Fe_x–isocyanide complexes. Open symbols, spectra of oxidized form; filled symbols, spectra of reduced form; half-filled symbols, ESR. □, methyl isocyanide; △, ethyl isocyanide; ○, *tert.*-butyl isocyanide; ◇, phenyl isocyanide.

Fig. 3. Effect of pH on the dissociation constants of the P-450–alkyl isocyanide complex of liver microsomes. The conditions were the same as for Figs. 1A and 1B. The dissociation constants of the oxidized and reduced P-450–alkyl isocyanide complex are the concentrations of alkyl cyanide at which there is 50% association. Acidic buffer, potassium–sodium phosphate; alkaline buffer, Tris–HCl. Temperature, 20°. Open symbols, oxidized form; filled symbols, reduced form. Circles, ethyl isocyanide; squares, *tert.*-butyl isocyanide.

forms between pH 6.0 and 8.3. Although the strength of absorption of P-450–isocyanide complexes in the reduced form was influenced by pH as stated above, the dissociation constants of both forms of the complexes were not affected by change in pH, and the sigmoid coefficients were also found to be unchanged over the pH range examined.

TABLE I

DISSOCIATION CONSTANTS OF THE OXIDIZED AND REDUCED P-450–ISOCYANIDE COMPLEXES

The dissociation constants (M) of the oxidized and reduced P-450–isocyanide complexes are the concentrations at which they are 50% formed. The conditions used were the same as for Fig. 2.

	P-450–isocyanide complexes			
	Methyl isocyanide	*Ethyl isocyanide*	*tert.-Butyl isocyanide*	*Phenyl isocyanide*
Oxidized form	$4.7 \cdot 10^{-3}$	$3.9 \cdot 10^{-4}$	$4.0 \cdot 10^{-5}$	$9.8 \cdot 10^{-6}$
Reduced form	$8.2 \cdot 10^{-6}$	$8.5 \cdot 10^{-6}$	$6.9 \cdot 10^{-6}$	$4.5 \cdot 10^{-6}$

Fig. 4 shows the ethyl isocyanide dissociation curve of reduced P-420 at pH 7.0 and 20°. The P-420 used was of two types: one type had the ESR signal of microsomal Fe_x and the other was without the signal because of the decomposition of microsomal Fe_x. As shown in Fig. 4, the sigmoid coefficients are little affected by the presence of 6.0 M urea, 0.1 % snake venom[20], or 1 % desoxycholate. However, the affinity of ethyl isocyanide for reduced P-420 decreased slightly with conversion of P-450 to P-420.

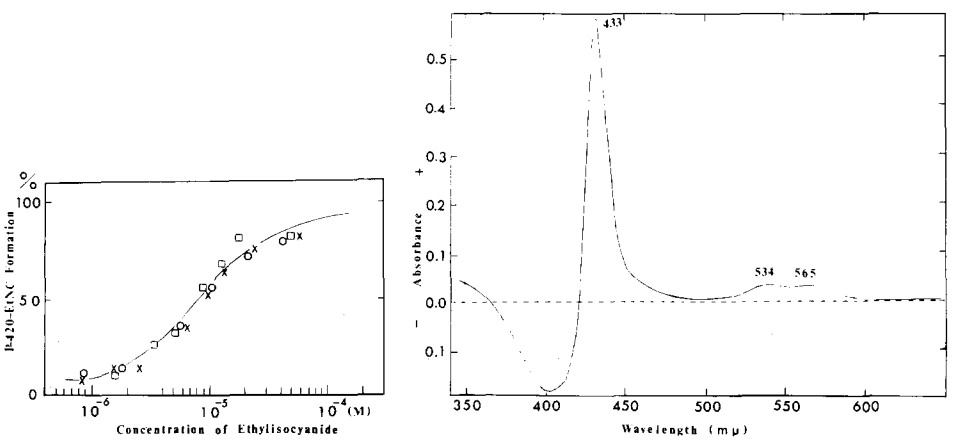

Fig. 4. Isocyanide dissociation curves of reduced P-420 of liver microsomes. P-420 was produced with 6 M urea, 1 % (w/v) desoxycholate and snake venom. The other conditions were the same as for Fig. 1B, except that the final ethyl isocyanide concentrations were varied as indicated. The isocyanide concentrations were varied by the addition of various amounts of buffer to a high concentration of isocyanide. Corrections were made for the effect of dilution caused by addition of isocyanide solution. Microsomal protein, 2 mg per ml, 0.1 M potassium–sodium phosphate (pH 7.0), 20°. △, 6 M urea; □, 1 % desoxycholate; ×, 0.1 % snake venom.

Fig. 5. Difference spectrum of purified P-420–ethyl isocyanide complex in the oxidized form. Purified P-420 was dissolved in 0.1 M potassium–sodium phosphate at pH 7.0. Ethyl isocyanide was added to the sample cell at a final concentration of 50 mM. Purified P-420, 4.0 mμmoles of protoheme per ml.

Fig. 5 shows the absorption spectra of the oxidized P-420–ethyl isocyanide complex. That of the reduced P-420–ethyl isocyanide complex has already been reported[7]. The absorption spectra of the P-420–isocyanide complexes in the reduced and oxidized forms were not affected either by the existence of the ESR signal of microsomal Fe_x or by its disappearance. However, the existence of the ESR signal of microsomal

Fe_x was responsible for the rate of combination of isocyanides with oxidized P-420. Fig. 6 represents the results.

Fig. 7 shows the ESR spectra of oxidized P-450–isocyanide complexes at −170° and pH 7.0. The signals were asymmetrical and were observed to be typical of a low-spin state of hemoproteins. As with the P-450–ethyl isocyanide complex[12], the g values of the ESR signals of the other P-450–isocyanide complexes were not affected by pH from 6.0 to 8.3. There were no ESR signals of the high-spin state or of the equilibrium between high- and low-spin states at pH 7.0 and the temperature of −170°. The alkyl isocyanide complexes yielded free P-450 by dialysis against a neutral buffer at 5° as judged spectrophotometrically and magnetically. The complexes of alkyl isocyanide, unlike those of phenyl isocyanide, were not decomposed even in the presence of oxygen for about 30 h.

The g values and the orbital energy differences of the d_ε orbitals of heme iron of P-450 and P-450–isocyanide complexes are summarized in Table II. The orbital energy differences of the d_ε orbitals of the heme iron of the complexes increased with

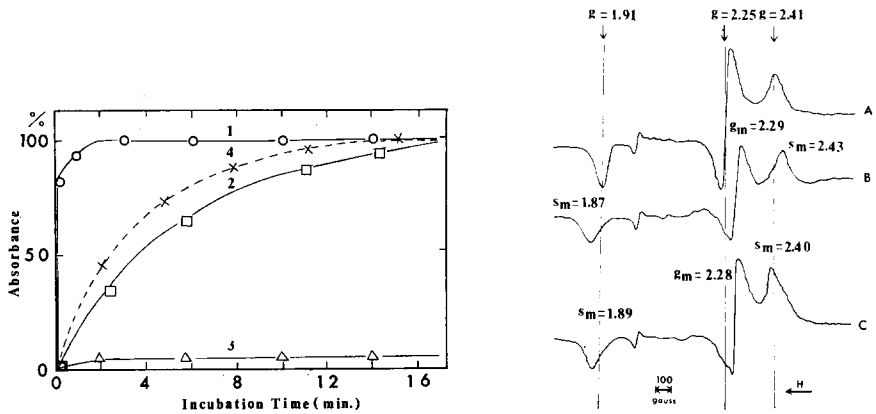

Fig. 6. Rates of combination of ethyl isocyanide with oxidized P-450 and P-420. Both the sample and reference cells contained suspension of microsomes, treated or untreated with desoxycholate. The protein concentration was 2 mg/ml in 0.1 M of potassium–sodium phosphate (pH 7.0). Ethyl isocyanide was added to the sample cell at a final concentration of 20 mM unless otherwise stated. The change in absorption at 434 mμ for P-450 or at 433 mμ for P-420 was measured. Curve 1 (○—○), P-450; Curve 2 (□—□), P-420 with ESR signal of microsomal Fe_x produced by the treatment of microsomes with 1% (w/v) desoxycholate; Curve 3 (△—△), P-420 with no ESR signal of microsomal Fe_x produced by the treatment of microsomes with 0.1% snake venom; Curve 4 (×—×), Curve 3 *plus* excess ethyl isocyanide.

Fig. 7. ESR spectra of oxidized P-450 and its isocyanide complexes. The observed g values of the ESR absorptions are indicated in the region around g = 2.0. ESR tubes contained 50 mg of microsomal protein per ml (0.1 M potassium–sodium phosphate, pH 7.0). Phenyl isocyanide and ethyl isocyanide were added to the microsomal suspension at concentrations of $1 \cdot 10^{-3}$ M and $2 \cdot 10^{-2}$ M, respectively. A, P-450; B, P-450–ethyl isocyanide complex; C, P-450–phenyl isocyanide complex.

increase in the carbon number of the isocyanides. Judging from the g values calculated for the P-450–methyl isocyanide and P-450–ethyl isocyanide complexes, further studies are necessary to determine whether the g values of these complexes are significantly different from each other. Compared with the orbital energy differences of other known hemoproteins, such as hemoglobin, myoglobin, peroxidases and cyto-

TABLE II

THE OBSERVED AND CALCULATED g VALUES AND ORBITAL ENERGY DIFFERENCES OF d_ε ORBITALS OF THE HEME IRON OF P-450 AND ITS ISOCYANIDE COMPLEXES

The orbital energy differences of d_ε orbitals of the heme iron of P-450 and its isocyanide complexes were calculated by the method of KOTANI[28], and the g values in parentheses are calculated values.

	None	*Reagents*			
		Methyl isocyanide	*Ethyl isocyanide*	*tert.-Butyl isocyanide*	*Phenyl isocyanide*
g_x	1.91 (1.90)	1.87 (1.87)	1.87 (1.87)	1.88 (1.88)	1.89 (1.89)
g_y	2.25 (2.24)	2.28 (2.29)	2.29 (2.29)	2.28 (2.28)	2.28 (2.28)
g_z	2.41 (2.40)	2.43 (2.44)	2.43 (2.43)	2.43 (2.43)	2.40 (2.40)
$E_\xi - E_\eta$	4.66a	4.21a	4.21a	4.28a	4.58a
$E_\xi - E_\zeta$	6.85a	5.74a	5.62a	5.89a	6.30a

a = orbital coupling constant.

chromes[25–28], those of P-450–isocyanide complexes are remarkably large. The temperature dependence of the ESR amplitudes of P-450–isocyanide complexes are extremely large and almost the same as that reported for free P-450 (ref. 29); EHRENBERG[25] showed that the temperature dependence of ESR signals is empirically exponential. Thus $S = S_0 e^{-T/\alpha}$, where S is the signal amplitude, T is the absolute temperature, and S_0 and α are constants characteristic of the signals. The α values of P-450–isocyanide complexes were about 50. The ESR signal heights of g_x, g_y and g_z are reciprocally parallel to the temperature. This means that the components of the signals come from the same spin entities. In addition, the ESR absorption was dependent on the changing microwave power and was not easily saturated by increase in microwave power.

Cytochrome P-450 is known to be more highly autoxidizable than other known hemoproteins[12] and it is reduced about 10–30 % by NADPH under aerobic conditions at pH 7.0. However, its autoxidizability decreased on its combination with isocyanide derivatives. This may be explained by considering that the 6th co-ordination of the heme iron of P-450 is combined with isocyanides, and this co-ordination may obstruct the combination of oxygen with heme iron. However, as described above, the dissociation constants of all reduced P-450–isocyanide complexes were essentially the same. The extent of reduction of P-450–isocyanide complexes of microsomes with NADH or NADPH varied considerably with the derivative. The extent of reduction increased with increase in the carbon atom number of the isocyanide. Similar results were obtained by magnetic measurements.

Fig. 8 shows the extent of reduction of P-450–isocyanide complexes of microsomes by NADH or NADPH at a given pH under aerobic conditions. The ordinate represents the height of the peak at 455 mμ in the difference spectrum as a percentage of that of the dithionite-treated P-450–isocyanide complex. As shown in Fig. 1B, the reduced P-450–isocyanide complexes have two Soret bands at 430 and 455 mμ. When the effect of the oxygen tension on the two peaks was studied, it was found that the peak at 430 mμ was the more autoxidizable in all isocyanide derivatives. Similar results were reported on the reduced P-450–ethyl isocyanide complex by IMAI AND SATO[30]. These results can be explained if the assumption be made that two

species are in equilibrium at a given pH. This idea is supported by the effect of pH on the partition coefficient of CO and O_2 for half formation of the P-450–CO complex[12] and on the two Soret peaks of reduced P-450–isocyanide complexes[22]. P-450–isocyanide complexes of microsomes are reduced by either NADPH or NADH, the former reduction generally being more rapid. On the contrary it was observed that the P-450–phenyl isocyanide complex was reduced at the same rate by both NADPH and NADH.

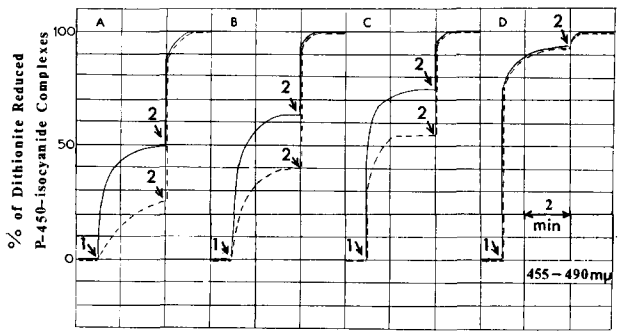

Fig. 8. Extents of the reduction of P-450–isocyanide complexes at 20% oxygen tension. The microsomal suspension contained 1 mM KCN to prevent oxygen consumption by contaminating mitochondria, if present. Both the sample and reference cells contained a microsomal suspension (2 mg of protein per ml, 0.1 M potassium–sodium phosphate, pH 7.0). Isocyanide derivatives (A, methyl isocyanide, 100 mM; B, ethyl isocyanide, 10 mM; C, *tert.*-butyl isocyanide, 10 mM; D, phenyl isocyanide, 0.5 mM) and 5 mM NADPH or 5 mM NADH were added to the sample cell. A N_2–O_2 mixture (80:20, by vol.) was bubbled through the sample cell. The absorbance change at 455 mμ was measured during this treatment. The increment of absorption is given as a percentage of that after sodium dithionite treatment at pH 7.0 and 20°. NADPH (———) or NADH (– – – –) was added at point 1; sodium dithionite, at point 2.

We found that in the presence of antioxidants of lipid such as glycerol, or chelating reagents for copper or iron[31–34], P-450 can be isolated from mammalian livers and adrenocortical microsomes by digestive enzymes such as trypsin and bacterial proteinase. The presence of these reagents stabilized P-450 under aerobic conditions and even in the presence of metal ions. The partially purified P-450 obtained in this way was not contaminated with P-420 or cytochrome b_5 and it was confirmed by ESR spectroscopy to be in a low-spin form of $g_x = 1.91$, $g_y = 2.25$ and $g_z = 2.41$ at the temperature of liquid nitrogen. The properties of purified P-450 were studied with a system of ferredoxin–$NADP^+$ reductase, ferredoxin of spinach and *C. pasteurianum* and NADPH instead of the microsomal electron transport systems. Purified P-450 and its isocyanide complexes were rapidly reduced by the system of ferredoxin–$NADP^+$ reductase, ferredoxin and NADPH. The extents of reduction of the P-450–isocyanide complexes were greater on addition of ferredoxin of spinach than that of bacteria under aerobic conditions. This may be due to a difference in the oxidation–reduction potential of the two ferredoxins. However, the activities for hydroxylation of anilines and demethylation of aminopyrine could not be restored by reduction of purified P-450 with the electron transport system of spinach. Similar results were obtained with a system of adrenodoxin, its reductase and NADPH, prepared from pig adrenocortical microsomes, though the rates of reduction of purified P-450 and its isocyanide complexes were slower. Although purified P-450 and its

isocyanide complexes were reduced by the ferredoxin–$NADP^+$ reductase system, they were not reduced in the absence of ferredoxin.

Fig. 9 shows the reduction of purified P-450 by the ferredoxin–$NADP^+$ reductase system. Ferredoxin could be replaced by safranine, methyl viologen or benzyl viologen, but not by phenosafranine, adrenodoxin nile blue, FMN or FAD. This fact suggests that P-450 may have a lower oxidation–reduction potential than the latter compounds. That P-450 may have a low oxidation–reduction potential is also pointed out by MASON (see discussion in MIYAKE *et al.*[37]).

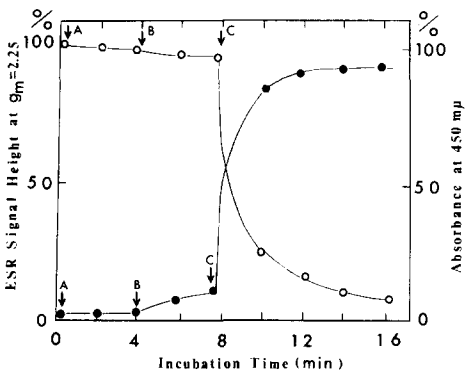

Fig. 9. The reduction of purified P-450 by the ferredoxin–$NADP^+$ reductase system. Filled circles: Absorbance change at 450 mμ in the difference spectrum. Both the sample and reference cells contained purified P-450 particles (0.5 mg of protein per ml, 0.1 M phosphate, pH 7.0, 20°). The sample cell was then saturated with CO, and the spectrum was measured after the addition of the enzymes as described below. Open circles: Change of ESR signal height at $g_m = 2.25$ of microsomal Fe_x under anaerobic conditions. ESR tubes contained 50 mg of protein per ml (0.1 M potassium–sodium phosphate, pH 7.0). The arrows indicate the times of addition of enzymes. A. NADPH, $5 \cdot 10^{-3}$ M. B. Ferredoxin–$NADP^+$ reductase, 0.01 mM. C. Ferredoxin, 0.05 mM.

DISCUSSION

The properties of cytochrome P-450 were studied by using various isocyanide derivatives. The absorption spectra of P-450–isocyanide complexes differed considerably from those of other known hemoproteins. We reported previously that the unusual properties of P-450 are possibly due to hydrophobic bondings[35,36]. If so the next problems are how the hydrophobic bondings in P-450 maintain the structure of the cytochrome and what ligands in P-450 give it its characteristic ESR signal. This signal is that of a low-spin form of microsomal Fe_x at liquid nitrogen temperature, of which the orbital energy differences of the d_ε orbitals of heme-iron are larger than those of other known hemoproteins.

Although it is desirable to use physiological substances as ligands when testing the functional action of P-450, the physiological ligand of the 6th co-ordination is still unknown. Accordingly, isocyanide derivatives were used for this purpose in the present experiments. The shape of the isocyanide dissociation curves of P-450 and P-420 conform to the theoretical curves for $n = 1.0$. The dissociation constants and the sigmoid coefficients of the oxidized and reduced P-450–isocyanide complexes were studied spectrophotometrically and magnetically between pH 6.0 and 8.3. No pH

dependence was observed for the values of the sigmoid coefficients and dissociation constants in this pH range. With reduced P-450–isocyanide complexes, when the peaks at 430 and 455 mμ were each used for studies on dissociation curves under anaerobic conditions, the dissociation constants and the sigmoid coefficients of each were essentially the same. Unexpectedly it was found that, unlike the partition coefficient of CO/O_2 for reduced P-450, the affinity for isocyanide of the oxidized and reduced P-450–isocyanide complexes did not change with pH. As described above, although the absorption spectrum of the reduced P-450–CO complex does not vary between pH 6.0 and 8.3 (ref. 36), those of the reduced P-450–isocyanide complexes do. The reason for the splitting of the Soret band of the reduced P-450–isocyanide complexes and the cause of the pH dependence of their absorption spectra are unknown. However, judging from the relationships between pH dependence of the heights of the absorption at 430 and 455 mμ of reduced P-450–isocyanide complexes and the properties of the side chains of isocyanide derivatives, the pH dependence of the absorption spectra of reduced P-450–isocyanide complexes may be affected by the hydrophobic character of isocyanide derivatives; that is, if an equilibrium be assumed between the protonation and deprotonation concerning pH dependence of the absorption of reduced P-450–isocyanide complexes, then the pK obtained from the absorption spectra of reduced P-450–isocyanide complexes may be considered to be lowered progressively with increase in the hydrophobic character of side chain of the isocyanide and with increase in the orbital energy differences of d_ε orbital of the heme iron of oxidized P-450–isocyanide complexes. Therefore, the absorption spectra of reduced P-450–isocyanide complexes at a definite pH vary with the species of isocyanide as they do with the change in pH. According to the definition as described above, the pK values of the P-450–isocyanide complexes are summarized in Table III.

TABLE III

pK's OF THE CHANGE IN THE ABSORPTION OF REDUCED P-450–ISOCYANIDE COMPLEXES

The conditions were the same as for Fig. 1B, except that the pH of the microsomal suspensions was varied. The pH value at which the heights of the peaks at 430 and 455 mμ were equal, was measured. 0.1 M potassium–sodium phosphate was used as buffer at 20°.

	Reduced P-450–isocyanide complexes			
	Methyl isocyanide	*Ethyl isocyanide*	*tert.-Butyl isocyanide*	*Phenyl isocyanide*
pK	8.4	8.2	7.9	6.7

On the other hand, in the presence of 6.0 M urea, the sigmoid coefficient of the dissociation curve of the reduced P-420 complex was calculated to be $n = 1.0$. This value coincided with that obtained for the P-450 complex. Therefore, it is considered that the heme–heme interaction, unlike that of hemoglobin, is not strong in the P-450 molecule.

The rate of combination of isocyanide derivatives with P-420 was related to the ESR signal of a low-spin form of microsomal Fe_x. This may be explained as due to decomposition of the hydrophobic 6th co-ordination of the heme group in P-450.

The 6th co-ordination seems to be hydrophobic from the following observation: CO readily combines with P-450, but CN^-, which is isoelectronic with CO, scarcely combines with it at the concentration of 10^{-3} M order level and pH 7.0. Moreover, the existence of hydrophobic substances around the 6th co-ordination of P-450 suggests that the dissociation constants of isocyanide derivatives to oxidized P-450 decreased with increasing chain length of the alkyl groups on the nitrogen atom of the isocyanide group. The isocyanide derivatives may bind to the lipid layer of the microsomes to different degrees depending on their hydrophobic character, and the isocyanide dissociation curves of oxidized P-450 might be due to local concentration of the isocyanide derivatives in the microsomes. However, this seems unlikely because experiments with purified P-450 gave essentially similar results to those with whole microsomes, and the isocyanide dissociation curves of reduced P-450 are essentially identical for all isocyanide derivatives. Although the dissociation constants of oxidized P-450–isocyanide complexes were affected by the hydrophobic character of the side chain of isocyanide derivatives, those of reduced P-450–isocyanide complexes were not so affected. These observations suggest that the hydrophobic ligand of the 6th co-ordination of heme iron of P-450 combines with the iron strongly in the oxidized form and loosely in the reduced state. Therefore, it is assumed that the dissociation constants of reduced P-450–isocyanide complexes are not affected by the character of side chain. It is presumed that oxidized P-450, unlike hemoglobin and myoglobin, is in the low-spin state even at room temperature as at liquid-nitrogen temperature[12] whereas the spin state of the reduced P-450 changes to high-spin state at room temperature.

Purified P-450 from rabbit liver microsomes is rapidly reduced with a ferredoxin–$NADP^+$ reductase system. But it is scarcely reduced directly without ferredoxin.

Although it is considered that non-heme iron may be present under physiological conditions in liver microsomes as electron transport components like adrenodoxin, the activities of hydroxylation or demethylation of liver microsomes do not parallel the ESR signal at $g = 4.3$ due to non-heme iron in the oxidized and high-spin form. Moreover, sodium dithionite-treated liver microsomes did not show an ESR signal at $g = 1.94$ at the temperature of liquid nitrogen. Accordingly, it is considered that the liver microsomal electron transport systems may not contain non-heme iron.

Further investigation is necessary to determine the components of the liver microsomal electron transport system and the structure of the P-450 molecule.

ACKNOWLEDGEMENTS

The authors are deeply indebted to Professor S. OHTUKA and Dr. K. YAMAGAMI of the School of Basic Technology, Osaka University for. their kind guidance in the syntheses of isocyanide derivatives.

REFERENCES

1 O. WARBURG, E. NEGELEIN AND W. CHRISTIAN, *Biochem. Z.*, 214 (1926) 26.
2 C. D. RUSSELL AND L. PAULING, *Proc. Natl. Acad. Sci. U.S.*, 25 (1939) 517.
3 J. KEILIN, *Biochem. J.*, 45 (1949) 440.
4 R. C. C. ST. GEORGE AND L. PAULING, *Science*, 114 (1951) 629.
5 A. LEIN AND L. PAULING, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 51.
6 T. OKAZAKI AND K. TSUSHIMA, *J. Biochem. Tokyo*, 46 (1956) 433.

7 T. OMURA AND R. SATO, *J. Biol. Chem.*, 237 (1962) PC1375.
8 K. HAMADA, T. OKAZAKI, R. SHUKUYA AND K. KAZIRO, *J. Biochem. Tokyo*, 53 (1963) 479.
9 N. SUMITA, T. OKAZAKI, R. SHUKUYA AND K. KAZIRO, *J. Biochem. Tokyo*, 55 (1964) 188.
10 T. OMURA AND R. SATO, *J. Biol. Chem.*, 239 (1964) 2370.
11 D. KEILIN, *Nature*, 171 (1953) 922.
12 Y. ICHIKAWA, B. HAGIHARA AND T. YAMANO, *Arch. Biochem. Biophys.*, 120 (1967) 204.
13 T. TACHIBANA AND K. YAMAURA, *SABCO J.*, 1 (1964) 16.
14 A. G. GORNALL AND C. S. BARDAWILL, *J. Biol. Chem.*, 177 (1949) 751.
15 H. L. JACKSON AND B. C. MCKUSICK, in T. L. CAIRNS, *Organic Syntheses*, Vol. 35, Wiley, New York, 1955, p. 62.
16 I. UGI, U. FETZER, U. EHOLZER, H. KNUPFER AND K. OFFERMANN, *Angew. Chem.*, 77 (1965) 492.
17 P. SCHMIDT AND D. STERN, *Beilstein's Handbuch der Organischen Chemie*, Band XII, Springer Verlag, Berlin, 1929, 4th ed., p. 191.
18 K. TAGAWA AND D. I. ARNON, *Nature*, 195 (1962) 537.
19 M. SHIN, K. TAGAWA AND D. I. ARNON, *Biochem. Z.*, 338 (1963) 84.
20 T. OMURA AND R. SATO, *J. Biol. Chem.*, 239 (1964) 2379.
21 B. L. HORECKER AND A. KORNBERG, *J. Biol. Chem.*, 175 (1948) 385.
22 Y. IMAI AND R. SATO, *Biochem. Biophys. Res. Commun.*, 23 (1966) 5.
23 N. E. SLADEK AND E. J. MANNERING, *Biochem. Biophys. Res. Commun.*, 24 (1966) 668.
24 A. V. HILL, *J. Physiol. London*, 40 (1910) 4.
25 A. EHRENBERG, *Arkiv Kemi*, 19 (1962) 119.
26 T. F. GIBSON, D. T. INGRAM AND D. SCHONLAND, *Discussions Faraday Soc.*, 26 (1958) 72.
27 Y. MORITA AND H. S. MASON, *J. Biol. Chem.*, 240 (1965) 2654.
28 M. KOTANI, *Progr. Theoret. Phys. Kyoto*, Suppl. 17 (1961) 4.
29 H. S. MASON, T. YAMANO, T. C. NORTH, Y. HASHIMOTO AND P. SAKAGISHI, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vol. 2, John Wiley, New York, 1964, p. 879.
30 Y. IMAI AND R. SATO, *Koso Kagaku Shimpoziumu (Symp. Enzyme Chem.)*, 18 (1966) 46.
31 Y. ICHIKAWA AND T. YAMANO, *Biochim. Biophys. Acta*, 131 (1967) 490.
32 B. R. RAY, E. O. DAUISSON AND H. L. CRESPI, *J. Phys. Chem.*, 58 (1954) 841.
33 J. L. ONCLEY, F. R. N. GURD AND M. MEILIN, *J. Am. Chem. Soc.*, 72 (1950) 458.
34 F. R. N. GURD AND P. E. WILCOX, *Advan. Protein Chem.*, 11 (1956) 311.
35 Y. ICHIKAWA AND T. YAMANO, *Biochim. Biophys. Acta*, 147 (1967) 518.
36 Y. ICHIKAWA, *Osaka Daigaku Igaku Zasshi (Med. J. Osaka Univ.)*, 19 (1967) 141.
37 Y. MIYAKE, H. S. MASON AND W. LANDGRAF, in K. BLOCH AND O. HAYAISHI, *Biological and Chemical Aspects of Oxygenases*, Maruzen, Tokyo, 1966, p. 227.