Review

Recollection of the early years of the research on cytochrome P450

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Abstract: Since the publication of the first paper on "cytochrome P450" in 1962, the biochemical research on this novel hemoprotein expanded rapidly in the 1960s and the 1970s as its principal roles in various important metabolic processes including steroid hormone biosynthesis in the steroidogenic organs and drug metabolism in the liver were elucidated. Establishment of the purification procedures of microsomal and mitochondrial P450s in the middle of the 1970s together with the introduction of molecular biological techniques accelerated the remarkable expansion of the research on P450 in the following years. This review paper summarizes the important developments in the research on P450 in the early years, for about two decades from the beginning, together with my personal recollections.

Keyword: cytochrome P450

Introduction

The first paper on "cytochrome P450" (P450) was published in 1962.[1] It reported the hemoprotein nature of "microsomal carbon monoxide-binding pigment" described in 1958.[2] In the next year, 1963, the monooxygenase activity of the P450 of adrenal cortex microsomes was found.[3),4] Basic properties of P450 and its solubilized inactive form, P420, were reported in 1963[5] and in 1964.[6),7] Based on these findings, rapid expansion of the research on P450 ensued in the following years of the 1960s, and the research expanded further in the 1970s. Major subjects of study on the physiological functions of P450 in the first decade of the P450 research were the biosynthesis of steroid hormones in the adrenal cortex and the oxidative metabolism of drugs in the liver.

Purification of microsomal and mitochondrial P450s was achieved in the middle of the 1970s, which resulted in the identification and characterization of many forms of P450 purified from animal tissues. Wide distribution of P450 among various organisms including plants and microbes also suggested the presence of many different forms of P450 and their diversified physiological functions. Studies on the mechanism of P450-catalyzed oxygenation reactions indicated the formation of chemically reactive intermediates that can react with proteins and nucleic acids in the cells, and the principal role of P450 in the chemical carcinogenesis was recognized by the beginning of the 1970s. Research on P450 expanded into various fields of biological and medical sciences in the 1970s, and continued the expansion in the following years. It has developed into a big research field. About 2,000 papers on P450 are now published annually.

The opening of a new research field is always exciting and full of interesting events. I had a fortunate opportunity to participate in the research on P450 from the beginning, and have acquainted with many of the pioneers in this research field. This paper describes important historical events in the early years of research on P450, for about two decades from the beginning. Contributions by the pioneering scientists to the elucidation of various problems on P450 are highlighted with my personal recollections.

Heralding events in the 1950s

Two important events in the 1950s, one in cell biology and another in enzymology, were the preludes to the discovery of P450. They were the isolation of microsomes from animal tissues and the discovery of oxygenase reactions, respectively.

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Successful fractionation of animal tissues into various distinct subcellular fractions was one of the most important achievements in the beginning of cell biology. A novel subcellular fraction named "microsomes" by A. Claude in 1943[8] was isolated from the homogenates of animal tissues by fractional centrifugation, and was identified to be the fragmented membrane vesicles of endoplasmic reticulum by G. E. Palade and P. Siekevitz in 1956.[9] Characterization of microsomes led to the discovery of the presence of several electron-transport enzymes in this novel subcellular fraction.

NADH-cytochrome *c* reductase,[10),11)] NADPH-cytochrome *c* reductase,[12)] and cytochrome b_5[13),14)] were found to be associated with the microsomal fraction prepared from animal liver. Since the mitochondrial electron-transport system and oxidative phosphorylation were the hot research subjects in biochemistry in the 1950s, possible contribution of these microsmal electron-transport activities to mitochondrial respiratory system was searched, but the results were all negative. The physiological significance of the "microsomal electron-transport system" remained unknown.[15)] Another redox component "carbon monoxide-binding pigment" in liver microsomes was reported by M. Klingenberg in 1958,[2)] but the nature of this novel microsomal component was not clarified.

The oxygenase reaction was confirmed for mushroom phenolase by H. S. Mason, W. L. Fowlks, and E. Peterson[16)] and for *Pseudomonas* pyrocatechase by O. Hayaishi, M. Katagiri, and S. Rothberg[17)] in 1955. The incorporation of atmospheric oxygen into the substrate molecule in the hydroxylation of steroids catalyzed by the homogenates of beef adrenal was also reported in the same year by M. Hayano *et al.*[18)] Introduction of oxygen atom from atmospheric oxygen into organic compounds was a remarkable novel enzymatic reaction, and was soon found to be involved in various important metabolic activities in animal tissues; metabolism of tryptophan,[19)] biosynthesis of steroid hormones from cholesterol,[18),20)] oxidative metabolism of drugs in the liver,[21)] etc. The oxygenases involved in the metabolism of tryptophan were soluble. They were purified and characterized. However, the oxygenases involved in the biosynthesis of steroid hormones and the oxidative metabolism of drugs were both associated with membrane fractions, and resisted to solubilization and purification. When the membrane fractions were treated with detergents to solubilize these oxygenases, the enzyme activities were always lost. The nature of these important oxygenases remained unknown until the discovery of P450.

Discovery and characterization of cytochrome P450

Presence of a novel carbon monoxide-binding pigment in liver microsomes was found by M. Klingenberg in 1954, when he was a postdoctoral fellow in the laboratory of B. Chance at the Johnson Research Foundation of the University of Pennsylvania, Philadelphia. He was a physical chemist graduated from the University of Heidelberg, Germany, and came to the Chance's laboratory in 1954 to study biochemistry. He was instructed by Chance to examine the oxidation-reduction kinetics of cytochrome b_5 in rat liver microsomes. He reduced a suspension of rat liver microsomes with sodium dithionite and bubbled carbon monoxide through the suspension, and unexpectedly observed a prominent optical absorption peak appearing at 450 nm. This strange carbon monoxide difference spectrum of the liver microsomes (Fig. 1) showed no resemblance to any of the known colored proteins including hemoproteins and copper proteins that could bind carbon monoxide. He tried to examine the properties of the novel pigment by solubilizing it from microsomes, but various solubilization treatments always resulted in complete disappearance of the 450 nm spectrum. Since the nature of the "microsomal carbon monoxide-binding pigment" was not clear, the publication of his novel spectral observation was much delayed. Klingenberg left the Chance's laboratory in 1956, and returned to Germany to work on a different research subject in the laboratory of T. Bücher at the University of München. Klingenberg's work was published in 1958[2)] accompanied by a paper by D. Garfinkel,[22)] who was also a postdoctoral fellow in the Chance's laboratory and confirmed the Klingenberg's observations using pig liver microsomes. A short paper written by Klingenberg describes the story of his work in the Johnson Research Foundation during 1954–1956.[23)]

When Klingenberg was in the Johnson Research Foundation, a visitor from Japan, R. Sato, and an American postdoctoral fellow, R. W. Estabrook, were also in the Chance's laboratory. The three young biochemists enjoyed friendly relation during their stay in the same laboratory, and their acquaintance happened to pave a way to the discovery of the hemoprotein nature of "microsomal carbon monoxide-binding pigment" and its physiological function as the oxygenase. Estabrook wrote a paper of his

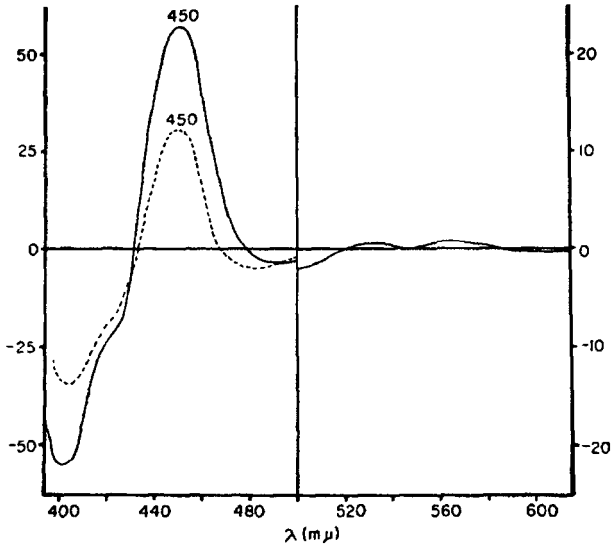

Fig. 1. Carbon monoxide difference spectra of rat liver microsomes (Ref. 2). ----- Carbon monoxide with NADH reduction. —— Carbon monoxide with dithionite reduction.

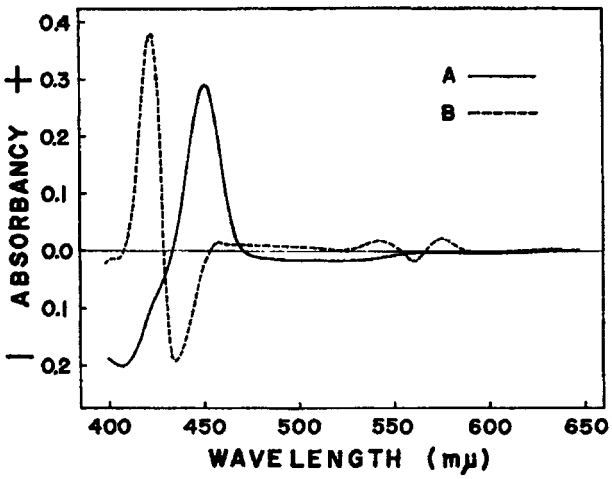

Fig. 2. Carbon monoxide difference spectra of dithionite-reduced rabbit liver microsomes (A: P450) and dithionite-reduced solubilized microsomes (B: P420) (Ref. 6).

remembrances of the early history of research on P450, in which he describes the work of Klingenberg at the Johnson Research Foundation.[24)]

Sato came back to Japan in 1954, and became a professor of the newly established Institute for Protein Research of the Osaka University, Osaka, in 1959. As I heard from him, he was very much interested in the mechanism of mitochondrial oxidative phosphorylation, but he decided not to work on mitochondria at his new laboratory because the research on mitochondrial electron transport system and oxidative phosphorylation was too keen in competition by many laboratories worldwide. He decided to work on a less competitive subject, non-mitochondrial electron transport enzymes.

Sato invited me to join his new laboratory as an assistant professor. I was studying plant laccase in the Department of Chemistry of the Shizuoka University, Shizuoka, at that time. Laccase is a beautiful blue-colored protein purified from the latex of Japanese lacquer tree (*Rhus vernicifera*). It was an enjoyable subject of study for me, and I hesitated to change my research subject. However, I finally decided to accept Sato's invitation to come to the Institute for Protein Research to work on a new research subject with him. I moved to Osaka in the spring of 1960, and had intensive discussion with Sato concerning my new research subject. He suggested to me several possible research subjects, among which was Klingenberg's "microsomal carbon monoxide-binding pigment". He said, "Nothing is known about the nature of this curious pigment in liver microsomes, and, as far as I know, nobody is currently working on it. Since it is reducible by NADH and NADPH, it may have some function in cellular respiration." I read the papers by Klingenberg and Garfinkel, and decided to work on this curious pigment in liver microsomes.

I started my new work by repeating the experiments described in Klingenberg's paper[2)] using rabbit liver microsomes instead of rat liver microsomes used by Klingenberg, and fortunately I could soon make a breakthrough observation on the spectral properties of the "carbon monoxide-binding pigment". I added deoxycholate to the suspension of rabbit liver microsomes reduced with dithionite in the presence of carbon monoxide. The 450 nm peak of the carbon monoxide compound of the microsomal pigment quickly disappeared as described by Klingenberg, but, unexpectedly, a prominent new peak appeared at 420 nm in the carbon monoxide difference spectrum of the solubilized microsomes (Fig. 2). The new 420 nm spectrum was apparently the spectrum of a hemoprotein with α- and β-bands in the longer wavelength region, and was very similar to the carbon monoxide difference spectrum of hemoglobin, but I could rule out the contamination of hemoglobin in the microsomal preparation used in the spectral study. Since the 420 nm peak was as high as the original 450 nm peak, the most likely possibility was the conversion of the 450 nm form of the microsomal pigment to a new 420 nm form. To distinguish the

two forms, I named the former "P-450" and the latter "P-420". P is the abbreviation of "pigment".

I tried to confirm the conversion of P-450 to P-420 by following the time course of the spectral change. Since the conversion of P-450 to P-420 by the solubilization of microsomes with detergents was too rapid to follow the time course, I had recourse to the use of phospholipase C of a snake venom. Digestion of biomembranes with phospholipase C releases lysophospholipids from the membrane phospholipids, and causes gradual solubilization of the membranes. The venom of the Japanese snake Habu (*Trimeresurus flavoviridis*) was used, and it gave a perfect result. The disappearance of the 450 nm peak of the carbon monoxide compound of P-450 by digesting the microsomes with the snake venom was quantitatively paralleled the appearance of the 420 nm peak of P-420,[6] which strongly suggested the hemoprotein nature of the original "microsomal carbon monoxide-binding pigment". It was also found that the microsomal carbon monoxide-binding pigment could bind ethyl isocyanide to give a difference spectrum suggestive of the hemoprotein nature of the microsomal pigment.[1] Sato was very happy about these breakthrough observations, but he requested me to provide more decisive evidence for the hemoprotein nature of P-450.

Since P-420 was already solubilized from the membrane, it was possible to purify it by conventional purification procedures. Although the purification was still partial, I could clearly separate P-420 from cytochrome b_5 that could interfere with the spectral observation of P-420. As shown in Fig. 3, the absolute spectra of the partially purified P-420 were characteristic of a *b*-type cytochrome, confirming the hemoprotein nature of P-450.[1),5)–7)] Determination of the content of protoheme in the P-420 preparation gave the molar extinction coefficients of the spectra of P-420, and the comparison of the carbon monoxide difference spectra of P-450 and P-420 allowed the calculation of the molar extinction coefficient of the 450 nm peak of P-450, 91 $mM^{-1}\,cm^{-1}$, which made it possible to quantitate P-450 from its carbon monoxide-difference spectrum.[5),6)] This value is still used today in determining the amounts of P450 in biological samples. It was also found that the amounts of protoheme in the microsomal preparations could be accounted for by P450 and cytochrome b_5.[6)]

I talked with Sato concerning the publication of these novel observations, and we wrote a short preliminary paper reporting the hemoprotein nature of the microsomal carbon monoxide-binding pigment

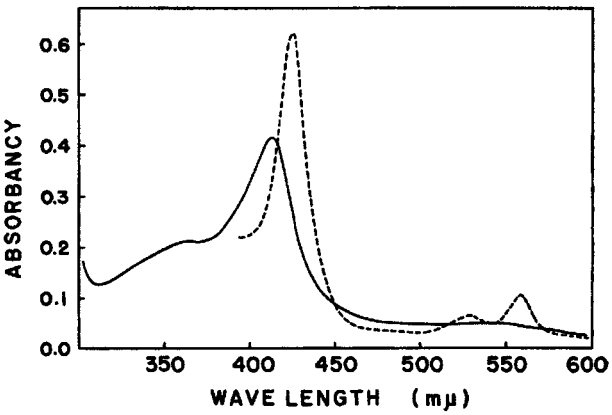

Fig. 3. Absorption spectra of P420 partially purified from solubilized rabbit liver microsomes (Ref. 7). —— oxidized form. ----- dithionite-reduced form.

and sent it to *Journal of Biological Chemistry*. To our great pleasure, the paper was smoothly accepted for publication, and it appeared on the April issue of the journal in 1962.[1)] We also wrote another preliminary report describing the properties of the solubilized form, P-420, and sent it to *Biochimica et Biophysica Acta*. It was accepted in December, 1962.[5)] We were naturally very happy, and began writing two full papers describing the details of our study on P450. Then we were startled to find a short paper by Y. Hashimoto, T. Yamano, and H. S. Mason[25)] appeared in the December issue of *Journal of Biological Chemistry*. The paper reported the discovery of a novel iron protein in rabbit liver microsomes that gave a unique electron spin resonance signal in the oxidized form. The signal disappeared upon the reduction of the microsomes by NADH or NADPH, and the reduction was stimulated by carbon monoxide indicating the carbon monoxide-binding property of the protein. They gave a tentative name "microsomal Fex" to this novel iron-containing protein. It was apparent for us that microsmal Fex was the same with P450. Hashimoto and Yamano were visitors to the Mason's laboratory from Japan.

The paper on "microsomal Fex" was a big surprise for me, but I was happy to find the name H. S. Mason among the authors of the paper. Mason was famous for his studies on copper enzymes including mushroom laccase and phenolase. I contacted him while I was studying plant laccase at the Shizuoka University, and he gave me a kind response. I had great respect to him as a leading scientist in the same field of research, biochemistry of copper enzymes. I unexpectedly came across his path again.

We wrote two full papers on microsomal P450, and sent them to *Journal of Biological Chemistry* in the end of 1962, but the response from the journal was disappointing. The two manuscripts were not accepted, and were returned to us with many critical comments. Some more experiments were apparently needed to answer the comments. I spent several months to prepare new experimental data to revise the papers, and we sent the revised manuscripts to the journal. They were again returned to us with some more comments, and we had to revise the papers once more spending a few months. They were finally accepted, and appeared on the April issue of the journal in 1964.[6),7)]

Mason and his collaborators continued their study on "microsomal Fex". They showed that the spectral conversion of P450 to P420 by detergent treatment was accompanied by the change of Fex from its low spin state to high spin state.[26)] The ESR measurement of microsomes could determine the amount of Fex in the oxidized form, whereas the spectrophotometry of the carbon monoxide compound of P450 measured its reduced form. Yamano came back to Japan from Mason's laboratory in 1964 to become professor of the Osaka University, Osaka, and continued his ESR study on microsomal Fex. The identity of microsomal Fex with P450 was confirmed by Y. Ichikawa and T. Yamano in 1967.[27)]

Monooxgenase activity of cytochrome P450

It was fortunate that the physiological function of P450 was soon found after its hemoprotein nature had been elucidated. The breakthrough discovery was reported in 1963 by R. W. Estabrook, D. Y. Cooper, and O. Rosenthal of the University of Pennsylvania, Philadelphia.[3),4)] Cooper was a surgeon. He was working with Rosenthal, who was Professor of Biochemistry in Research Surgery at the Harrison Department of Surgical Research, to investigate the relation between hypertension and the regulation of steroid hormone biosynthesis in the adrenal gland. His study was difficult, however, because the nature of the enzymes involved in the biosynthesis of steroid hormones was unknown at that time. Cooper asked Estabrook of the Johnson Research Foundation in the same university to support his study by giving advice on spectrophotometric experiments.

In 1961, the 5th Congress of the International Union of Biochemistry was held in Moscow. Sato attended the Congress, and presented our novel observation, the hemoprotein nature of "microsomal carbon monoxide-binding pigment" at the meeting. Estabrook and Klingenberg were also attending the Congress. They met together and were happy to renew their old friendship. Sato came back to Osaka after the Congress, and told me "To my great pleasure, Ron (Estabrook) and Martin (Klingenberg) evaluated our work highly. In particular, Ron was very much interested in the hemoprotein nature of P450. It is understandable. He is an expert in peroxidase." The information that the Klingenberg's "microsomal carbon monoxide-binding pigment" is a hemoprotein might have given Estabrook some idea regarding the nature of the steroid hydroxylase of adrenal cortex microsomes. Strong inhibition of the steroid hydroxylase activity of adrenal cortex microsomes by carbon monoxide had already been reported by K. J. Ryan and L. L. Engel in 1957.[28)] They also noticed that the inhibition of the hydroxylase activity by carbon monoxide was partially reversed by the irradiation with white light, and suggested possible involvement of some hemoprotein in the hydroxylation reaction.[28)]

Estabrook and Cooper measured the carbon monoxide difference spectrum of adrenal cortex microsomes, and found P450 in the microsomes. They then constructed a simple homemade optical instrument to measure the photochemical action spectrum of the light reversal of the carbon monoxide inhibition of the steroid hydroxylation by bovine adrenal cortex microsomes. The light reversal of the carbon monoxide inhibition of the microsomes-catalyzed C-21 hydroxylation of 17-hydroxyprogesterone was analyzed.[3)] As shown in Fig. 4, the photochemical action spectrum showed a broad peak at around 450 nm, indicating the involvement of P450 in the hydroxylation reaction. They determined the effect of the carbon monoxide to oxygen ratio on the hydroxylation reaction, and confirmed the competition of carbon monoxide with oxygen for the hydroxylation reaction. Based on these observations they concluded that P450 is involved in the steroid hydroxylation reaction catalyzed by adrenal cortex microsomes, and that P450 is the oxygenase that activates molecular oxygen for the hydroxylation reaction.[3),4)] They also confirmed the participation of P450 in the oxidative metabolism of various drugs by liver microsomes using the same photochemical action spectrum method, and reported it in 1965.[29)] A story of their study is described in a paper written by Cooper in 1973.[30)] Estabrook also wrote a chapter for a book in 1996 describing the discovery of the function of P450.[31)]

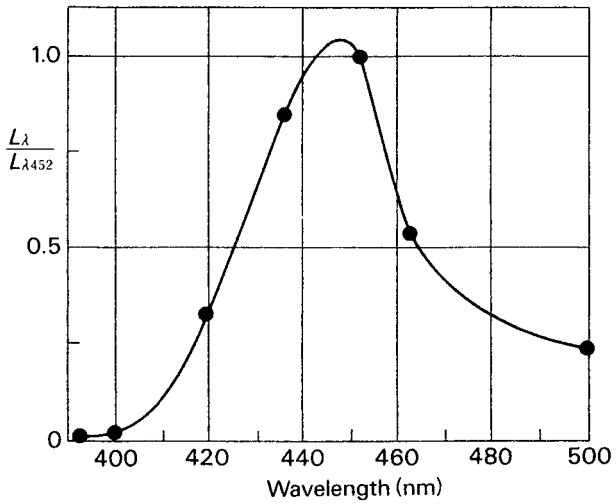

Fig. 4. Photochemical action spectrum for the light reversal of carbon monoxide inhibition of steroid C21 hydroxylase activity of bovine adrenal cortex microsomes (Ref. 3).

The findings that the biosynthesis of steroid hormones in the adrenal gland and the oxidative metabolism of drugs in the liver are both catalyzed by P450 attracted much attention of the biochemists working on these physiologically important metabolic reactions. Many of them rushed into the research on P450, and the photochemical action spectrum method was widely utilized to confirm the participation of P450 in various metabolic reactions.

Wide distribution of cytochrome P450 among various organisms

P450 was originally found in the microsomes from animal livers,[1),2)] and its presence in mitochondria was soon discovered by B. W. Harding, *et al.* in 1964.[32)] They were studying the synthesis of steroid hormones in the adrenal gland, and detected the presence of P450 in the mitochondrial fraction prepared from rat adrenal glands. Later studies by other groups confirmed the presence of P450 in the mitochondria of various animal tissues including liver and kidney.

P450 was found in the yeast *Saccharomyces cerevisiae* by A. Lindenmayer and L. Smith in 1964.[33)] They detected P450 in the cell-free extracts of the yeast cells from its carbon monoxide-difference spectrum, but the physiological function of P450 in the yeast was not clear at that time. Presence of P450 in the fungus *Cunninghamella bainieri* was suggested by J. P. Ferris, *et al.* in 1973.[34)] The fungus mycelia showed NADPH-dependent oxygenase activities to several chemical compounds including aniline, naphthalene, etc., and the activities were inhibited by carbon monoxide, although the spectral confirmation of P450 in the mycelial extract was not successful. Later studies by other groups confirmed the presence of P450 in various filamentous fungi.

The first bacterial P450 was found in the bacteroids of nitrogen-fixing *Rhizobium* by C. A. Appleby in 1967.[35)] The *Rhizobium* P450 was apparently soluble, and was partially purified from the supernatant of the homogenates of the bacteroids. However, the function of the P450 in the metabolism of *Rhizobium* was not elucidated. In the next year, 1968, another bacterial P450 was reported by M. Katagiri, B. N. Ganguli, and I. C. Gunsalus.[36)] They were studying the hydroxylation of camphor by *Pseudomonas putida* grown in a medium containing camphor, and obtained a soluble extract from the bacterial cells that catalyzed the NADH-dependent hydroxylation of camphor. The extract was separated into three fractions, one of which was P450. In the same year, one more bacterial P450 was reported by G. Cardini and P. Jurtshuk.[37)] The sonic extract of *Corynebacterium sp. Strain 7E1C* grown in a medium containing n-octane as the sole source of carbon and energy catalyzed NADH-dependent oxidation of n-octane to 1-octanol and octanoic acid. The extract contained a soluble P450, which was converted to P420 by the treatment with deoxycholate. The *P. putida* P450 was purified to homogeneity and crystallized in 1970.[38),39)] This was the first purified preparation of P450, which was widely utilized in the following years for studying the molecular properties of P450 and the mechanism of P450-catalyzed oxygenation reactions.

The first report on the presence of P450 in plants was published by P. J. Murphy and C. A. West in 1969.[40)] They studied the NADPH-dependent hydroxylation of kaurene by the microsomal fraction from the endosperms of *Echinocystis macrocarpa*, and found P450 in the microsomes from its characteristic carbon monoxide difference spectrum. The hydroxylation reaction was inhibited by carbon monoxide, and the photochemical action spectrum of the light reversal of the carbon monoxide inhibition confirmed the participation of P450 in the hydroxylation reaction. Presence of P450 in the microsomes from various plant tissues was reported a few years later; the seedlings of *Vinca rosea*,[41)] the endosperms of caster bean,[42)] the etiolated seedlings of sorghum, etc.[43)]

By the end of the 1960s, wide distribution of P450 among animals, plants, fungi, and bacteria was

thus recognized, and, three decades later, the first report describing the presence of P450 in the archaeon *Sulfolobus solfataricus* by R. L. Wright *et al.* in 1996[44] finally confirmed the universal distribution of P450 among all of the five kingdoms of life.

Induction of drug-metabolizing cytochrome P450 in animal liver by drug administration

Since the confirmation of the NADPH-dependent oxidative metabolism of various drugs by liver microsomes in 1955,[21] the drug metabolism in animal liver was actively studied in the latter half of the 1950s, and an interesting finding that the hepatic drug metabolizing activity was induced by the administration of certain chemical compounds was reported by A. H. Conney *et al.* in 1956.[45] They found that the N-demethylation and the azo linkage reduction of 3-methyl-4-monomethylaminoazobenzene by rat liver homogenate were greatly stimulated by the intraperitoneal injection of certain polycyclic aromatic hydrocarbons including 3-methylcholanthrene to the animals. They reported in the next year, 1957, the induction of liver microsomal NADPH-dependent benzopyrene hydroxylase activity by the administration of benzopyrene or some other polycyclic aromatic hydrocarbons to rats.[46] Various drugs including barbiturates were then found to induce the microsomal drug-metabolizing activities in the livers of the treated animals by H. Remmer in 1959,[47] by A. H. Conney, *et al.* in 1960,[48] and also by R. Kato in 1960.[49] H. Remmer and H. J. Merker reported in 1963 significant proliferation of endoplasmic reticulum in the hepatocytes of phenobarbital-treated rats in addition to an increase of the drug metabolizing activity in the liver.[50] Discovery of the principal role of P450 in the oxidative metabolism of drugs by liver microsomes in 1965[29] prompted the investigation of the induction of hepatic microsomal P450 by drugs. S. Orrenius, *et al.* reported in 1965[51] that the phenobarbital-induced increase of aminopyrine N-demethylation activity in rat liver was accompanied by parallel increases of microsomal P450 and NADPH-cytochrome *c* reductase activity. They also noticed remarkable proliferation of smooth endoplasmic reticulum in the hepatocytes of the phenobarbital-treated animals.

I was in the laboratory of P. Siekevitz at the Rockefeller University in the middle of the 1960s studying the biosynthesis of microsomal membrane proteins. Siekevitz and I were interested in the induction of specific microsomal proteins accompanied by remarkable proliferation of smooth endoplasmic reticulum in the hepatocytes by drug administration, and we examined the effects of phenobarbital on the synthesis and turnover of cytochrome b_5 and NADPH-cytochrome *c* reductase in the livers of the treated animals. I continued this study after returning to the Institute for Protein Research, Osaka University, in 1966 together with a young collaborator, Y. Kuriyama. We found that the rates of synthesis of these two microsomal proteins were stimulated, and the turnover of NADPH-cytochrome *c* reductase was prevented by the administration of phenobarbital, resulting in a significant increase of the content of the reductase in the microsomes (Y. Kuriyama, *et al.*, 1969).[52] H. Jick and L. Shuster[53] also reported the effect of phenobarbital on the turnover of microsomal NADPH-cytochrome *c* reductase in mouse liver.

Although the purification and characterization of microsomal P450 was not yet possible in the latter half of the 1960s, studies on the induction of hepatic metabolism of various drugs by the administration of several chemical inducers indicated possible presence of multiple forms of P450 in the liver microsomes of the treated animals. N. E. Sladek and G. J. Mannering reported in 1966 that the ethylisocyanide difference spectra of microsomal P450 in the livers of 3-methylcholanthrene-treated rats were different from those of normal and phenobarbital-treated animals.[54] They concluded that the P450 induced by 3-methylcholanthrene was different from the P450 of normal and phenobarbital-treated rats. In the next year, 1967, A. P. Alvares, *et al.* reported that the induction by 3-methylcholanthrene resulted in a shift of the maximum absorption peak of the carbon monoxide compound of liver microsomal P450 from 450 nm to 448 nm, and proposed the presence of more than one P450 in rat liver microsomes.[55] However, some other groups did not agree with such a view, and proposed that liver microsomal P450 was interconvertible between two spectrally different forms due to the binding of the chemical compounds used in the induction (A. Hildebrandt, *et al.*, 1968),[56] (J. B. Schenkman, *et al.*, 1969).[57] Y. Imai and P. Siekevitz proposed that the hydrophobicity around the heme of P450 was different between the microsomes of normal and 3-methylcholanthrene-treated rats, which caused the observed difference in the spectral properties of the same P450.[58] The former groups postulated the presence of multiple molecular species of P450 in liver microsomes, whereas the latter groups claimed the existence of only one drug-metabolizing P450.

Intense controversy on this problem that had lasted for several years in the latter half of the 1960s came to an end when successful solubilization and purification of P450 from liver microsomes was achieved in the middle of the 1970s. The P450 purified from the liver microsomes of phenobarbital-treated rats was clearly different from its counterpart purified from the 3-methylcholanthrene-treated animals (D. Ryan, *et al.*, 1975).[59] Specific induction of each of these two molecular species of P450 by corresponding inducers was confirmed by the use of specific antibodies (P. E. Thomas, *et al.*, 1979),[60] (N. Harada and T. Omura, 1981).[61]

Selective induction of different forms of P450 by various chemical compounds attracted much attention. Regulation of steroidogenic P450s in the steroidogenic organs by pituitary hormones was also a hot subject of study. Successful cloning of the genes of various forms of P450 in the 1980s enabled the analysis of the structures of the genes and their regulatory regions. Discovery of the participation of several novel nuclear receptors including AhR in the regulatiohn of drug-metabolizing P450s in 1976[62] and Ad4BP in the regulation of steroidogenic P450s in 1992[63] was the highlight of the intensive research on the molecular mechanism of the regulation of P450 genes in the following years.

Constitution of cytochrome P450 enzyme systems

The monooxygenase activity of microsomal P450 required the supply of reducing equivalents from NADPH in addition to molecular oxygen. It was therefore likely that some enzyme or enzyme system is associated with P450 in the microsomes to supply electrons from NADPH to P450. It was known that the synthesis of steroid hormones from cholesterol in the adrenal gland requires the monooxygenase activities in both microsomes and mitochondria. Since the presence of P450 in the mitochondria of the adrenal gland was found by B. W. Harding, *et al.* in 1964,[32] participation of P450 in the steroid hydroxylation reactions in mitochondria as well as in microsomes was anticipated. It was also reasonable to assume that the monooxygenase activity of mitochondrial P450 was also dependent on the supply of reducing equivalents from NADPH by some electron transport enzyme in the mitochondria. After the confirmation of the monooxygenase activity of P450, the constitutions of microsomal and mitochondrial P450 enzyme systems were imminent problems to be clarified.

1) Mitochondrial P450 enzyme system. After confirming the role of P450 in the steroid hydroxylation reactions of adrenal cortex microsomes, D. Y. Cooper and R. W. Estabrook wanted to extend their study to mitochondrial steroid hydroxylation reactions. However, the photochemical action spectrum method, which worked nicely with adrenal cortex microsomes,[3] did not give satisfactory results with the mitochondrial steroid hydroxylation system. Meanwhile Estabrook invited me to spend a year in his laboratory as a visiting scientist to work with him on P450. I gladly accepted his invitation, took a leave of absence from the Osaka University, and came to Philadelphia in the summer of 1964. Estabrook suggested to me working on the microsomal and mitochondrial P450s of the adrenal gland, which was his collaborative research project with Cooper at that time. I met Cooper, and he told me that he recently prepared a "soluble" steroid hydroxylase preparation from adrenal cortex mitochondria by sonication. He gave me his solubilized mitochondrial preparation, and suggested to me trying the purification of the solubilized mitochondrial P450 to confirm its role in the steroid hydroxylation reactions. I accepted his proposal, but I soon found that the P450 in his preparation was not soluble. It was associated with small membrane fragments that could be sedimented completely by extensive ultracentrifugation. I was then surprised to find that the sedimented P450 was no longer reducible by NADPH, whereas the P450 in the "solubilized" mitochondrial preparation was rapidly reduced by NADPH. The addition of the supernatant to the sedimented P450 restored its reduction by NADPH. It was apparent that the supernatant contained an enzyme that transfers electrons from NADPH to the membrane-bound P450.

Since the "P450-reducing enzyme" in the supernatant was soluble, it was possible for me to purify it by routine methods. Unexpectedly, it was clearly separated into two fractions, a red and a yellow fraction, by DEAE-cellulose chromatography. Neither of them alone was able to catalyze the reduction of P450 by NADPH, but the P450 reducing activity was restored when they were combined. The oxidized and reduced spectra of the red fraction indicated it a ferredoxin-type iron-sulfur protein. The yellow fraction showed typical flavoprotein spectra. Estabrook and Cooper were highly excited by this novel observation. Cooper quickly measured the deoxycortisol 11β-hydroxylase activity of the separated fractions, and confirmed that both of the red and

yellow fractions were needed for the reconstitution of the hydroxylase activity with the membrane-bound P450. We concluded that the electrons for the P450-catalyzed steroid hydroxylation were transferred from NADPH to mitochondrial P450 *via* a flavoprotein and a ferredoxin-type iron sulfur protein. This was the first elucidation of the constitution of the P450 enzyme systems.

We reported these novel findings at the symposium "Electron Transport Systems in Microsomes" of the 49th Annual Meeting of the Federation of American Societies for Experimental Biology held in April 1965, in Atlantic City.[64] The symposium was organized by P. Siekevitz of the Rockefeller Institute, New York. He gave an introductory talk titled "Origin and functional nature of microsomes"[65] followed by P. Strittmatter and H. Kamin talking about microsomal NADH-cytochrome b_5 reductase and NADPH-cytochrome c reductase, respectively. H. S. Mason gave a talk on the metabolism of "xenobiotics" by microsomal mixed-function oxidase. L. Ernster reported the phenobarbital-induced increase of microsomal drug oxidation activity in rat liver. It was the first meeting on the microsomal electron transport system, and announced the start of this new research field. The full account of our study on the P450 reductase system in adrenal cortex mitochondria was published in 1966.[66]

At about the same time, K. Suzuki and T. Kimura at the College of Science, St Paul's University, Tokyo, were also studying the steroid hydroxylase of bovine adrenal cortex mitochondria. They reported the isolation of an iron-sulfur protein from the supernatant of the sonicated mitochondria in 1965, and named it "adrenodoxin"[67]. They also reported the reduction of adrenodoxin by NADPH catalyzed by a flavoprotein in the supernatant.[68] Although they did not describe the role of adrenodoxin in the mitochondrial P450-catalyzed steroid hydroxylation reaction, the identity of adrenodoxin with our ferredoxin-type iron-sulfur protein purified from adrenal cortex mitochondria was apparent. We were willing to accept their proposal to call the mitochondrial iron-sulfur protein "adrenodoxin". Suzuki came to the Johnson Research Foundation later to work with Estabrook after I left Philadelphia in the summer of 1965 for New York to work with P. Siekevitz in the Cell Biology Department of the Rockefeller University.

I enjoyed my stay in Estabrook's laboratory, which made me interested in the biosynthesis of steroid hormones. My later studies on the regulation of steroidogenic P450s and the biogenesis of mitochondrial P450s were based on my experience during my work with Estabrook and Cooper. I recently wrote a short paper[69] describing my research in the Estabrook's laboratory during 1964–1965.

2) Bacterial P450 enzyme systems. Unexpectedly, a P450 reductase system similar to the mitochondrial counterpart in its constitution was found in a bacterium by M. Katagiri, B. N. Ganguli, and I. C. Gunsalus in 1968.[36] The NADH-dependent camphor-hydroxylating system of *Pseudomonas putida* was separated into three soluble components, a soluble P450, an iron-sulfur protein, and a flavoprotein. The three components constitution of the *P. putida* P450 system was apparently very similar to the adrenal mitochondrial P450 system, although the bacterial P450 was soluble and the P450-catalyzed oxygenase reaction required NADH in contrast to the NADPH-dependent animal mitochondrial P450 system. Katagiri was a visitor to the Gunsalus' laboratory from Japan. He returned to Japan later to become professor of the Kanazawa University, Kanazawa, and studied the steroid-hydroxylating P450s of adrenal cortex in the following years.

P. putida P450 system had remained the only bacterial P450 system whose constitution had been studied in detail until a few other bacterial P450 systems were found and characterized in the 1980s.[70–72] All of them were soluble systems similar to the camphor hydroxylating P450 system of *Pseudomonas putida*, and constituted of P450, ferredoxin, and NADH-ferredoxin reductase.

3) Microsomal P450 enzyme system. Elucidation of the constitution of the P450 enzyme system in microsomes was difficult because solubilization treatments with detergents caused the conversion of microsomal P450 to P420 resulting in the complete loss of its oxygenase activity. The breakthrough discovery to overcome this difficulty was made by Y. Ichikawa and T. Yamano in 1967.[73] They found the stabilization of microsomal P450 against detergent treatments by polyols including glycerol. Presence of 20–30% of glycerol in the suspension of liver microsomes allowed the solubilization of P450 by sodium cholate without its conversion to P420. Reversal of the PCMB-induced conversion of P450 to P420 by GSH was also found. In the next year, 1968, A. Y. H. Lu and M. J. Coon reported the successful solubilization of the P450-catalyzed fatty acid ω-hydroxylase activity of rabbit liver microsmes by deoxycholate in the presence of glycerol and DTT.[74] They could

separate the solubilized hydroxylase into three fractions, a P450-containing fraction, an NADPH-cytochrome *c* reductase-containing fraction, and an unknown factor. The three fractions were necessary to reconstitute the NADPH-dependent lauric acid ω-hydroxylase activity. Their finding suggested the role of NADPH-cytochrome *c* reductase in the NADPH-dependent P450-catalyzed oxygenation reactions of microsomes. The unknown factor was later proved to be phosphatidylcholine.[75)]

Solubilization and purification of NADPH-cytochrome *c* reductase from liver microsomes was first reported by C. H. Williams and H. Kamin,[76)] and independently by A. H. Phillips and R. G. Langdon[77)] in 1962. Williams and Kamin solubilized the reductase from microsomes by the treatment with pancreatic lipase, whereas Phillips and Langdon used trypsin to solubilize the reductase. The solubilized and purified reductase preparations contained FAD, and retained the enzymatic activity of reducing cytochrome *c* by NADPH. However, Y. Miyake, J. L. Gaylor, and H. S. Mason reported in 1968 that the reductase purified by the method of Williams and Kamin was unable to reduce P450 by NADPH.[78)] They prepared a particle fraction containing P450 from rabbit liver microsomes by treating the microsomes with Lubrol WX in the presence of glycerol. NADPH-cytochrome *c* reductase and cytochrome b_5 were removed from the particle fraction by the Lubrol treatment, and the particle-bound P450 was no longer reducible with NADPH. The addition of partially purified NADPH-cytochrome *c* reductase to the particle fraction did not restore the reducibility of P450 by NADPH. A. Y. H. Lu *et al.* also reported in 1969 the inability of the NADPH-cytochrome *c* reductase purified by the method of Williams and Kamin to reconstitute the ω-hydroxylation activity when added to the P450-containing fraction solubilized from rabbit liver microsomes by cholate.[79)] More study was apparently needed to confirm the role of microsomal NADPH-cytochrome *c* reductase in the transfer of electrons from NADPH to P450.

An important clue to solve this problem was provided by A. Ito and R. Sato who reported the solubilization and purification of cytochrome b_5 from liver microsomes by detergent treatment in 1968.[80)] Purification of microsomal cytochrome b_5 had already been reported by P. Strittmatter and S. F. Velick in 1956.[81)] They solubilized the cytochrome from rabbit liver microsomes by the digestion with pancreatic lipase, and purified it to homogeneity. They also reported the purification of NADH-cytochrome b_5 reductase solubilized from rabbit liver microsomes by lipase treatment in 1956,[82)] and also by the treatment with the venom of the snake *Naja naja* in 1957.[83)] Pancreatic lipase and the snake venom were supposed to decompose the phospholipids of the microsomal membrane by lipase and phospholipase activity, respectively, to release cytochrome b_5 and NADH-cytochrome b_5 reductase from the membrane. However, the enzymes responsible for the solubilization of these membrane-bound proteins were not clearly identified.

Ito was a graduate student studying in the Sato's laboratory at the Institute for Protein Research, Osaka University, and the aim of his study was to elucidate the molecular mechanism of release of cytochrome b_5 from microsomal membrane by various solubilization treatments. Ito started his graduate study in 1963. I came back to the Sato's laboratory in 1966 from two years' stay in America. I studied the biosynthesis and turnover of microsomal NADPH-cytochrome *c* reductase and cytochrome b_5 in rat liver[84)] when I spent one year in the laboratory of P. Siekevitz, who was intensively studying the mechanism of biogenesis of membrane structures in the cells. Since the state of binding of protein molecules to the membrane is essential information for the elucidation of the molecular mechanism of formation of biomembranes, I was very much interested in Ito's study on the solubilization of cytochrome b_5 from microsomes by various treatments. At the same time, Masahiko Negishi, who was also a graduate student in the Sato's laboratory, was working with me on the biosynthesis of cytochrome b_5.[85)] Yoshiaki Kuriyama, who was a visitor from a pharmaceutical company, was also working with me in the Sato's laboratory on the biosynthesis of NADPH-cytochrome *c* reductase.[52),86)] We were all interested in the structure of microsomal membrane, and we often discussed together the molecular mechanism of binding of microsomal proteins to the membrane structure.

Ito worked hard and purified cytochrome b_5 solubilized from rabbit liver microsomes by the treatment with a neutral detergent, Triton X-100, and examined the properties of the detergent-solubilized cytochrome b_5. Interestingly, the molecular weight of the detergent-solubilized cytochrome b_5 determined by gel filtration was larger than that of the trypsin-solubilized counterpart. He concluded that the detergent-solubilized cytochrome b_5, whose molecular weight was 25,000, was the native membrane-bound form, whereas the trypsin-solubilized

cytochrome b_5 with a molecular weight of 12,000 was a proteolytic fragment of the native form. The former was named detergent b_5 or d-b_5 to distinguish it from the trypsin-solubilized form, trypsin-b_5 or t-b_5. It was also found that d-b_5 formed an oligomer in aqueous solutions. Ito proposed that the native cytochrome b_5 molecule is amphipathic, and anchors to the membrane by the hydrophobic portion of the molecule, which is split off from the hydrophilic heme-containing portion of the native molecule when the microsomes are treated with trypsin, forming water-soluble t-b_5.[80] It was therefore highly likely that the solubilization of NADPH-cytochrome c reductase and cytochrome b_5 by "lipase" reported previously[76),81] was actually due to some proteolytic activity, possibly the activity of trypsin and/or chymotrypsin, in the lipase preparation. S. Takesue and T. Omura re-examined the solubilization of NADH-cytochome b_5 reductase from rat liver microsomes by *Naja naja* venom reported before by P. Strittmatter and S. F. Velick,[83] and found that the lysosomal proteases contaminated in the microsomal preparation, not the phospholipases in the venom, were mainly responsible for the solubilization of the reductase.[87] Confirmation of the amphipathic nature of membrane proteins was one of the important bases for the formulation of the "fluid-mosaic model" of biomembranes by S. J. Singer and G. L. Nicolson in 1972.[88]

After Ito's study of the purification of detergent-solubilized cytochrome b_5, solubilization and purification of other microsomal proteins including NADPH-cytochrome c reductase by the use of detergents were attempted by many other researchers studying the microsomal P450 enzyme system. K. Ichihara, E. Kusunose, and M. Kusunose reported successful purification of NADPH-cytochrome c reductase solubilized from pig liver microsomes by Triton X-100 in 1973.[89] The purified reductase could reconstitute fatty acid ω-hydroxylase activity when added to a partially purified detergent-solubilized P450. Although the purification of microsomal P450 was yet to be achieved at that time, similar reconstitution experiments by various groups in the beginning of the 1970's confirmed the role of NADPH-cytochrome c reductase in the supply of electrons from NADPH to microsomal P450 for the P450-catalyzed oxygenase reactions.[90)–92]

Another approach to confirm the role of NADPH-cytochrome c reductase in the transfer of electrons from NADPH to microsomal P450 was the use of the inhibitory antibodies raised to the trypsin-solubilized and purified reductase. When I came back to Osaka in 1966, I started the preparation of rabbit antibodies to NADPH-cytochrome c reductase and cytochrome b_5. My original purpose was to use the antibodies to study the biosynthesis of those enzymes by immunoprecipitation, but I soon found that the antibody to NADPH-cytochrome c reductase effectively inhibited the NADPH-dependent P450-catalyzed oxygenation reactions of microsomes. I reported the observation at the "Microsomes and Drug Oxidations Symposium" held in 1968 in Bethesda.[93] Similar studies using the antibodies to purified NADPH-cytochrome c reductase were carried out in the following years to elucidate the role of the reductase in the transfer of electrons from NADPH to P450 in microsomes (B. S. S. Masters, *et al.*, in 1971).[94] Since the function of NADPH-cytochrome c reductase in the reduction of P450 by NADPH was thus confirmed, it became customary to call it NADPH-cytochrome P450 reductase instead of NADPH-cytochrome c reductase.

One more important development in the study of NADPH-cytochrome c reductase in the beginning of the 1970s was the finding by T. Iyanagi and H. S. Mason in 1973 that the reductase contains both FMN and FAD in equimolar amounts.[95] Since previous papers by other groups[76),77] reported the presence of only FAD in the purified reductase preparations, the presence of FMN in NADPH-cytochrome c reductase was an unexpected and highly important finding. The oxidation-reduction of the FAD and FMN prosthetic groups in the reductase molecule was studied in detail, and provided important information for the molecular mechanism of the transfer of electrons from NADPH to P450.[95),96] The FAD receives electrons from NADPH, and then transfers them to the FMN in the reductase molecule. Cytochrome c receives electrons from FAD, whereas P450 receives them from FMN. Iyanagi was visiting the Mason's laboratory from Japan, and came back later to the Tsukuba University, Tsukuba, to continue his study on NADPH-P450 reductase.

Although NADPH-P450 reductase, an NADPH-specific flavoprotein, was thus proved to be an essential component of the microsomal P450 system, stimulation of microsomal NADPH-dependent oxygenase activity by NADH was found by B. S. Cohen and R. W. Estabrook in 1971.[97] This phenomenon was called "NADH-synergism", and suggested the contribution of some NADH-linked component to the microsomal P450-dependent oxygenase activity. A. Hildebrandt and R. W. Estabrook proposed the contribution of cytochrome b_5 to the electron transfer

from NADH to microsomal P450 in the same year.[98] Their proposal was based on the observation that a drug substrate lowered the steady state of reduction of cytochrome b_5 when NADH was included in the NADPH-containing reaction medium. Later studies by other groups confirmed the NADH-synergism. The NADH-synergism was observed not only with microsomes but also with the reconstituted systems containing d-b_5 in addition to P450 and NADPH-P450 reductase, and the extent of stimulation of the NADPH-supported oxygenation activities of liver microsomes by NADH was found to be significantly depending on the P450 species used (A. Y. H. Lu, *et al.*, 1974).[99] Some P450 showed almost obligatory requirement for cytochrome b_5 for the drug oxidation in the presence of NADPH and NADH (T. Sugiyama, *et al.*, 1979).[100] Y. Imai and R. Sato studied the role of cytochrome b_5 in the NADPH-dependent drug oxidation reaction using a reconstituted system in 1977, and found that cytochrome b_5 supplied the second electron to P450 and improved the coupling of NADPH oxidation with the drug oxidation.[101]

In addition to the researches with reconstituted systems, use of inhibitory antibodies to cytochrome b_5 contributed to the elucidation of the role of cytochrome b_5 in the electron transfer from NADH to P450 in intact microsomes. G. J. Mannering, S. Kuwahara, and T. Omura reported in 1974 strong inhibition of the NADH-synergism in the N-demethylation of ethylmorphine catalyzed by rat liver microsomes by rabbit antibodies raised to trypsin-solubilized rat cytochrome b_5.[102] Mannering was very much interested in the use of inhibitory antibodies in studying the mechanism of electron transfer from NADPH and NADH to P450 in the microsomal drug oxidation reactions. He visited my laboratory at the Kyushu University, Fukuoka, to spend several months in 1973. Kuwahara was a graduate student in my laboratory, and worked with Mannering.

M. Noshiro, V. Ullrich, and T. Omura analyzed the NAD(P)H-linked reduction of microsomal P450 in 1981 by using the rabbit antibodies to cytochrome b_5, and concluded that the first electron for the reduction of ferric iron of the heme of P450 comes from NADPH *via* NADPH-P450 reductase whereas the second electron for the reduction of the oxygenated form of ferrous P450 may come either from NADPH *via* NADPH-P450 reductase or from NADH *via* cytochrome b_5.[103] Noshiro was a graduate student in my laboratory at the Kyushu University. He studied the pathways of electron transfer from NADH and NADPH to cytochrome P450 in microsomes by the use of antibodies to cytochrome b_5 and NADPH-cytochrome *c* reductase (M. Noshiro, *et al.*, 1980),[104] and then visited the Ullrich's laboratory at the University of Saarland, Germany, to study the role of cytochrome b_5 in the electron transfer from NADH and NADPH to P450 in microsomes.

Some P450s seem to be highly dependent on cytochrome b_5 for the catalysis of NADPH-dependent oxygenation of substrates, whereas some others showed only slight dependence on cytochrome b_5 (S. Kuwahara, and T. Omura, 1980).[105] However, some of more recent studies with reconstituted P450 systems showed that apo-cytochrome b_5 is as effective as its holo-form in stimulating the NADPH-dependent drug oxidation reactions, which seemed to deny the role of cytochrome b_5 in the electron transfer to P450.[106),107] It is generally agreed that cytochrome b_5 contributes to the NADPH-dependent oxygenation reactions catalyzed by microsomal P450s, in particular to the stimulation of the reactions by NADH, but the molecular mechanism of its contribution to the reactions are not yet clearly understood.

4) P450-reductase fusion proteins. In addition to the soluble P450 enzyme systems discovered in the 1960s, soluble P450-reductase fusion proteins were also found in bacteria. The first of such catalytically self-sufficient P450 enzyme was reported by L. O. Narhi and A. J. Fulco in 1986, and named P450 BM-3.[108] It was a fusion protein consisting of a P450 domain and an NADPH-linked P450 reductase domain, and catalyzed NADPH-dependent ω-2 hydroxylation of fatty acids.[109] The reductase domain contained both FAD and FMN, and was very similar to animal NADPH-P450 reductase. Similar P450-reductase fusion proteins were later found in some other bacteria and fungi, and other P450-reductase fusion proteins of different types have recently been found in bacteria.

The four types of P450 enzyme systems were thus found and characterized in the early years of P450 research. Both microsomal and mitochondrial P450 enzyme systems are present in animals, whereas plants and fungi have only microsomal P450 enzyme system. Soluble P450 enzyme system including P450-reductase fusion proteins is found in prokaryotic organisms. I recently wrote a review paper on the structural diversity of P450 enzyme system.[110]

Coordination state of the heme of cytochrome P450

When the hemoprotein nature of the carbon monoxide-binding pigment in liver microsomes was

reported in 1962,[1] its unique spectral properties provoked intensive discussion about the coordination state of the heme of P450. A remarkably big red shift of the Soret peak of the carbon monoxide compound of reduced P450 compared with known protoheme hemoproteins, which had an imidazole group of histidine as the axial ligand of the heme and showed their Soret peaks at around 420 nm when reduced and ligated to carbon monoxide, suggested some novel ligand coordinated to the axial position of the heme of P450.

Contribution of sulfhydryl group to the "abnormal" spectra of P450 was suggested by several lines of evidence. K. Murakami and H. S. Mason reported the conversion of low spin microsomal Fex (P450) to its high spin form (P420) by sulfhydryl reagents in 1967.[26] Stabilization of microsomal P450 against its detergent-induced conversion to P420 by mercaptoethanol described by Y. Ichikawa and T. Yamano in 1967[73] also suggested the role of sulfhydryl group in the unique spectral properties of P450. C. R. E. Jefcoate and J. L. Gaylor reported in 1969 that the addition of n-propylmercaptan to metmyoglobin converted the high-spin EPR spectrum of metmyoglobin to a low spin spectrum that was almost superimposable with the EPR spectrum of the low-spin form of P450.[111] However, the problem of the axial ligand that is responsible for the unique 450 nm optical absorption peak of the carbon monoxide compound of ferrous P450 was not clarified.

Strong evidence for the mercaptide anion (thiolate anion), possibly donated by a cysteine residue of the P450 protein, as the ligand trans to carbon monoxide in the carbon monoxide-compound of reduced P450 was presented by J. O. Stern and J. Peisach in 1974.[112] They could prepare a model compound that had the same optical peoperties in the Soret and visible regions as the carbon monoxide adduct of ferrous P450 by dissolving hemin chloride and 2-mercaptoethanol in dimethylsulfoxide-ethanol solvent in the presence of tetramethylammonium hydroxide and carbon monoxide. Since the presence of a thiol compound and a strong base was essential for the appearance of the P450-type spectra, they concluded that the axial ligand in the model compound and also in the carbon monoxide adduct of ferrous P450 is a mercaptide anion. This was the first successful preparation of a model compound that reproduced the unique 450 nm optical absorption peak of the carbon monoxide compound of reduced P450.

Concerning the cysteine residue that donates the thiolate anion ligand to the heme, O. Gotoh *et al.* compared the primary sequences of various cysteine-containing portions in the primary sequences of several microsomal and bacterial P450s, and suggested in 1983 that a cysteine residue in the middle of a short conserved segment near to the carboxy terminus of those P450 molecules is the heme-binding cysteine residue.[113] Their prediction was confirmed in the next year, 1984, when the primary sequence of a bovine mitochondrial P450, P450scc, was elucidated by K. Morohashi, *et al.*.[114] Bovine P450scc was found to contain only one cysteine residue in the molecule, and the position of the cysteine residue corresponded with the suggestion by O. Gotoh, *et al.*.[113] Presence of a thiolate anion donated by the cysteine residue at the 5th coordination position of the heme of P450 was later confirmed when the X-ray crystallographic analysis of P450cam by T. L. Poulos, *et al.* elucidated its tertiary structure in 1985.[115]

I happened to be intimate with Peisach long before he published his first paper on P450, the paper of the model compound, in 1974. He was interested in the optical and EPR spectra of blue-colored copper proteins in the beginning of the 1960s, and wanted to study laccase. He noticed my papers on Japanese lacquer tree (*Rhus vernicifera*) laccase published in 1961,[116] and sent a letter to me asking me for the supply of Japanese lacquer latex that was difficult to obtain in US. Since there were only a few biochemists interested in plant laccase, I was very much happy to hear of his interest in laccase, and supplied him with the acetone powder of fresh lacquer latex in 1962. We had an opportunity to meet together first at a symposium on copper proteins held at the Arden House in Harriman State Park, New York, in September 1965, when I was in the laboratory of P. Siekevitz of the Rockefeller University. The symposium was organized by H. S. Mason, who kindly invited me to come to the symposium. I was happy to meet Peisach at the meeting, and we talked a lot about laccase. When I saw his paper on the P450 model compound in 1974, I was amused to find him again interested in the same subject of research with me, this time P450.

Reaction mechanism of cytochrome P450-catalyzed oxygenation reactions

The monooxygenase reactions catalyzed by P450 require one mole each of NADPH and molecular oxygen to oxygenate one mole of substrate.

It means two electrons are supplied to P450 to introduce one atom of oxygen to one molecule of the substrate. Since the reduction of the ferric form of P450 to the ferrous form, which can bind and activate molecular oxygen, requires only one electron (J. A. Peterson, *et al.*, 1977),[117] some reasonable reaction mechanism had to be designed to define the roles of the two electrons in the oxygenation reaction.

First clue for the elucidation of the reaction mechanism was a spectral evidence for the specific binding of the substrate to the oxidized form of P450. S. Narasimhulu, D. Y. Cooper and O. Rosenthal reported in 1965 that the addition of a steroid substrate, 17-hydroxyprogesterone, to Triton-solubilized adrenal cortex microsomes, which had P450-catalyzed C-21 hydroxylase activity, gave a novel difference spectrum with an absorption minimum at 420 nm and a broad maximum at 390 nm.[118] The difference spectrum disappeared when the substrate was hydroxylated by the addition of NADPH. Reduction of P450 by sodium dithionite also resulted in the disappearance of the difference spectrum. Non-substrate steroids did not show the difference spectrum when added to the adrenal cortex microsomal P450 preparation. The same substrate-induced difference spectrum was also observed when a substrate, cortexone, was added to an adrenal cortex mitochondrial preparation having P450-catalyzed 11β-hydroxylase activity (D. Y. Cooper, *et al.*, 1965).[119] These observations suggested specific binding of substrates to the oxidized form of P450.

Similar substrate-induced spectral change was observed with liver microsomal P450 by H. Remmer, *et al.*.[120] and also by Y. Imai and R. Sato[121] in the next year, 1966. Several drug substrates of liver microsomal P450, hexobarbital, aminopyrine, *et al.*, induced the spectral change of P450 when added to liver microsomes. J. B. Schenkman, H. Remmer, and R. W. Estabrook analyzed the drug-induced spectral change of liver microsomal P450 in 1967, and identified three types of spectral change, Type I, Type II and modified Type II spectral changes, which seemed to be due to slight blue or red shift of the Soret peak of the heme prosthetic group of P450, depending on the drug substrates employed.[122] Since the concentration of a substrate necessary for the half-maximal enzyme activity was similar to the concentration of the same substrate necessary for half-maximal spectral change, they concluded that the binding of the substrate to the oxidized form of P450 is an essential step of the oxygenation reaction.

When Schenkman was studying the substrate-induced spectral change of liver microsomal P450, I was also in Estabrook's laboratory. Schenkman was a very pleasant person, and I enjoyed friendly relation with him. He later visited Japan to spend one year in Ryo Sato's laboratory at the Institute for Protein Research, Osaka University, in 1968–1969.[123] I was also in Sato's laboratory at that time, and we were happy to be together again.

Second important information for the mechanism of the P450-catalyzed oxygenation reactions was the spectral confirmation of the existence of an oxygenated form of reduced P450. R. W. Estabrook, *et al.*.[124] and Y. Ishimura, *et al.*.[125] reported the formation of a new spectral intermediate in the oxygenation reaction catalyzed by liver microsomal P450 and bacterial soluble P450cam, respectively, in 1971, and they concluded that the spectral intermediate was the oxygenated form of reduced P450s. Ishimura was visiting Estabrook's laboratory from Japan. He later came back to Japan to become professor of the Medical School of the Keio University, Tokyo, and continued his study on P450.

Based on these lines of information, Estabrook *et al.* proposed a scheme of electron transport reactions associated with the oxygenation reaction catalyzed by microsomal P450 in 1971.[124] As shown in Fig. 5, the scheme postulates the binding of a substrate to the oxidized form of P450, and then the reduction of the heme of P450 from ferric to ferrous form by the supply of "first" electron from NADPH-P450 reductase. The reduced P450 binds oxygen to form oxygenated compound, and the introduction of "second" electron from cytochrome b_5 or NADPH-P450 reductase to the oxygenated P450 activates the bound oxygen molecule resulting in the introduction of one atom of oxygen to the substrate. Another atom of oxygen is reduced to water, and P450 returns to the original oxidized form to start another cycle of the reaction. This scheme, which was originally proposed in 1968,[126] nicely explained the supply of two electrons from NADPH to P450 in the P450-catalyzed oxygenation of one molecule of the substrate, and was widely accepted to explain various observations on P450-catalyzed reactions in the following years.

The oxidation-reduction potential of microsomal P450 was first measured by M. R. Waterman and H. S. Mason in 1970.[127] It was very low, -410 mV. The low redox potential of P450 was confirmed by later studies for liver microsomal P450 (M. R. Waterman and H. S. Mason, 1972),[128] (J. Ingledew,

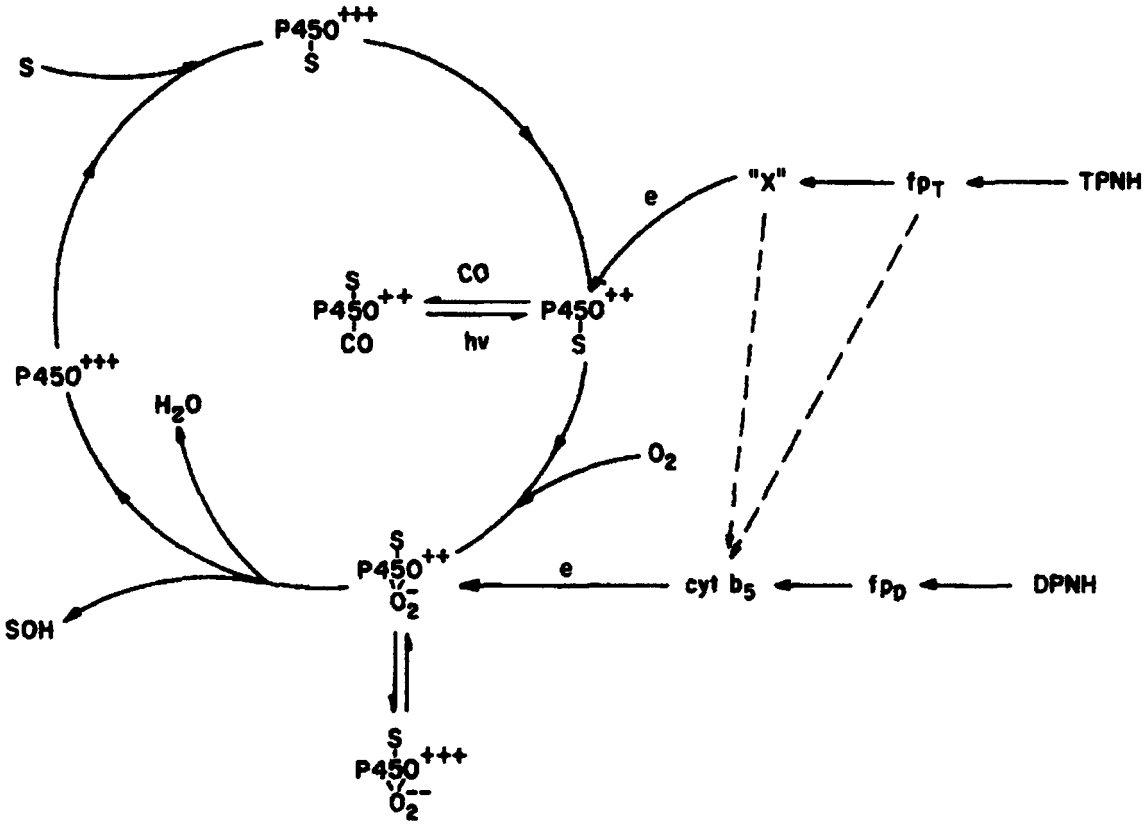

Fig. 5. A scheme of microsomal electron transport reactions associated with cytochrome P450 function during hydroxylation reactions (Ref. 119). fp_T: NADPH-cytochrome *c* reductase, fp_D: NADH-cytochrome b_5 reductase.

et al., 1976),[129] and for adrenal cortex mitochondrial P450 (D. L. Williams-Smith and R. Cammack, 1977).[130] It was also found that P450 exists in two forms, low redox potential low spin form and high redox potential high spin form. The former is a substrate-free form, whereas the latter is substrate-bound form.[130] The elevation of the low redox potential of P450 by substrate binding should make the transfer of electrons from NADPH-P450 reductase easier. In the absence of a suitable substrate, the reduction of P450 will be slower owing to its low redox potential. The binding of a substrate to oxidized form of P450 initiates the oxygenation reaction, whereas the absence of a suitable substrate will prevent the futile formation of the oxygenated form of reduced P450 that will decay fruitlessly forming noxious activated oxygen species.

An interesting novel type of the P450-catalyzed oxygenation reaction, the peroxide-dependent oxygenation of various substrates, was found in 1975. A. D. Rahintula and P. J. O'Brien reported P450-catalyzed dealkylation of various drug substrates when cumene hydroperoxide was added to liver microsomes.[131] Neither NADPH nor oxygen was required, and the reaction was not inhibited by carbon monoxide. E. G. Hrycay, *et al.* also reported in the same year peroxide-dependent hydroxylation of steroids by partially purified P450.[132] Sodium periodate, sodium chlorite, and hydrogen peroxide as well as organic hydroperoxides could support the P450-catalyzed hydroxylation reaction. The mechanism of peroxide-supported oxygenation reactions catalyzed by P450 was studied by several groups in the following years.

Formation of reactive intermediates in the P450-catalyzed oxygenation reactions was another important subject of research on the mechanism of the oxygenation reactions in the 1970s. It was already known since the 1950s that various carcinogenic hydrocarbons bind covalently to DNA, RNA, and proteins in the animal tissues. Since the carcinogenic hydrocarbons, polycyclic aromatic hydrocarbons, are not chemically reactive, they must be metabolically activated in the cells to electrophilic compounds to

react with the cellular macromolecules and exert their carcinogenic effects.[133] Various lines of evidence including the migration of aryl ring substituents (NIH shift) indicated that the epoxides, the arene oxides, were the intermediates of the P450-catalyzed hydroxylation of polycyclic aromatic hydrocarbons. The formation of 1,2-naphthalene oxide as an intermediate in the oxidative metabolism of naphthalene by liver microsomes to 1-naphthol was confirmed by D. M. Jerina, *et al.* in 1970.[134] J. K. Selkirk, *et al.* also reported in 1971 the formation of an epoxide of a carcinogenic polycyclic aromatic hydrocarbon, dibenzanthracene, by the metabolism by liver microsomes.[135] Metabolic activation of carcinogenic polycyclic aromatic hydrocarbons by liver microsomes was actively studied in the following years, and the principal contribution of microsomal P450 to chemical carcinogenesis was firmly established.

Purification of cytochrome P450

Purification of P450 from microsomes and mitochondria was naturally a major target of biochemical research on P450 from the beginning, but the conversion of P450 to the denatured inactive form P420 by the treatment with detergents[1),5)] had been a deadly obstacle to various trials to solubilize and purify membrane-bound P450 in the 1960s. In contrast, a soluble P450, P450cam, of the bacterium *Pseudomonas putida* was purified to homogeneity in 1968.[38]

The breakthrough discovery by Y. Ichikawa and T. Yamano in 1967 that polyols like glycerol stabilize membrane-bound P450 against detergent treatments[73] opened the way to successful solubilization and purification of P450s from microsomes and mitochondria. Several papers reported partial purification of P450 from liver microsomes and adrenal cortex mitochondria in the beginning of the 1970s,[136)–139)] followed by successful purification of several forms of microsomal and mitochondrial P450s to homogeneity in the middle of the 1970s; from rabbit liver microsomes by T. A. van der Hoeven, *et al.*,[140] by D. A. Haugen and M. J. Coon,[141] and also by Y. Imai and R. Sato,[142] from rat liver microsomes by D. Ryan, *et al.*,[143] from mouse liver microsomes by M. T. Huang, *et al.*,[144] and by M. Negishi and D. W. Nebert,[145] from bovine adrenal cortex mitochondria by S. Takemori, *et al.*,[146),147)] from bovine adrenal cortex microsomes by S. Kominami, *et al.*[148] Since the induction of liver microsomal P450 by chemical inducers greatly increased the contents of specific forms of P450 in the microsomes, phenobarbital-treated or 3-methylcholanthrene-treated animals were used in the purification of P450s from liver microsomes. Polychlorinated biphenyls (D. E. Ryan, *et al.*)[149] and tetrachlorodibenzo-*p*-dioxin (E. F. Johnson and U. Muller-Eberhard)[150] were also used to induce P450 in animal livers for purification. Although the purification studies were mainly focused on mammalian sources, purification of P450 from yeast microsomes was achieved by Y. Yoshida *et al.* in 1977.[151] The homogeneity of the purified preparations was usually assessed by SDS-polyacrylamide gel electrophoresis.

Purification of distinct phenobarbital-inducible and 3-methylcholanthrene-inducible forms of P450 from liver microsomes confirmed the presence of multiple forms of drug-metabolizing P450 in the microsomes. They were different in the molecular weight determined from the mobility on the SDS-polyacrylamide gel, and also in the substrate specificity of their catalytic activities measured by the use of reconstituted systems. The antibodies prepared to each of phenobarbital-induced and 3-methylcholanthrene-induced P450s could clearly distinguish the two forms.

Presence of several other forms of P450 in liver microsomes was found in the following years by examining the induction of characteristic drug-metabolizing activities by various inducers. Chronic administration of ethanol to rats was found to induce a novel form of P450 in the liver microsomes (J. P. Villeneuve, *et al.*, 1976).[152] The effect of pregnenolone-16α-carbonitrile administration to rats on the drug-metabolizing activities of the liver microsomes was explained by an induced increase of a new form of P450 (N. A. Elshourbagy and P. S. Guzelian, 1980).[153] The microsomal P450 induced by the administration of isosafrole to rats was a new form different from either of 3-methylcholanthrene-inducible and phenobarbital-inducible P450s (D. E. Ryan, *et al.*, 1980).[154] Clofibrate was found to induce a new form of P450 in the liver of the treated rats (G. G. Gibson, *et al.*, 1982).[155] Since the metabolism of drugs in the liver was one of the major subjects of research on P450 in the 1970s, purification and characterization of P450s from the liver microsomes of drug-treated animals were intensively studied.

In addition to the difference in the molecular weight determined by SDS-polyacrylamide gel electrophoresis and in the substrate specificity of the oxygenase activity measured with reconstituted

systems, difference in the amino-terminal amino acid sequences determined by chemical sequencing of the purified samples confirmed the molecular distinction of various P450 species (D. A. Haugen, *et al.*, in 1977),[156] (L. H. Botelho, *et al.*, in 1979).[157] Ouchterlony double immunodiffusion test using the antibodies raised to the purified P450 samples was also utilized to confirm the distinction of the purified P450 species.

Availability of the antibodies specific to each of the multiple forms of P450 enabled the detection of these P450s in various animal tissues by immunohistochemical staining of the tissue slices using optical microscopes and electron microscopes, and confirmed the presence of P450s in almost all tissues examined including brain. Expression of steroid hormone-synthesizing P450s in particular neurons in various parts of animal brains attracted special attention. Local production of steroid hormones, in particular estrogens, in the brain suggested paracrine or autocrine functions of the "neurosteroids", which has been actively studied in the following years.

Primary structures of cytochrome P450

When the purification of microsomal and mitochondrial P450s was achieved, next target of research was the determination of the complete amino acid sequences of purified P450s. First successful elucidation of the primary sequences of two P450s, a soluble bacterial P450 and a liver microsomal P450, was reported in 1982. The former, P450cam of *Pseudomonas putida*, was sequenced by chemical sequencing by M. Haniu, *et al.*,[158] whereas the primary sequence of the latter, a phenobarbital-induced rat liver microsomal P450, was deduced from the nucleotide sequence of a cloned cDNA by Y. Fujii-Kuriyama *et al.*.[159]

The use of a molecular biological technique, cDNA cloning, greatly accelerated the elucidation of the complete amino acid sequences of various P450s. The primary sequences of many P450s were reported in the 1980s, and the accumulation of the primary sequence data necessitated a systematic classification of these 450s. Based on the similarity of the amino acid sequences, P450s were classified into many families and subfamilies, and the symbol "*CYP*" for P450 genes was proposed by D. Nebert *et al.* in 1987.[160] Comparison and analysis of the available amino acid sequences of animal, plant, and bacterial P450s led O. Gotoh and Y. Fujii-Kuriyama in 1989 to the conclusion that all of their genes have evolved from a single ancestral P450 gene,[161] and the P450 genes of various organisms form one super gene family.

In the following years, the genes of many mammalian P450s were cloned and characterized, and the molecular mechanisms of the regulation of the expression of various P450 genes in the liver and the adrenal cortex were intensively studied in the 1980s and the 1990s. Drug-induced increase of particular P450s in the liver was the major focus of the studies. The research on P450 shifted from biochemical studies to molecular biological studies, and expanded further.

Epilogue

Fifty years have passed since the first paper on "cytochrome P450" was published in 1962. Major events in the research on P450 during the initial two decades, the 1960s and the 1970s, are reviewed in this paper. It was the first stage of the research on P450 in the times of traditional biochemistry, starting from the characterization of P450 and P450-catalyzed reactions followed by the purification of many forms of P450 from animal tissues, and finally the elucidation of their primary structures. P450-catalyzed metabolism of drugs in the liver and the role of P450 in the biosynthesis of steroid hormones in steroidogenic organs were actively studied. The role of P450 in chemical carcinogenesis by polycyclic aromatic hydrocarbons was also a major subject of study.

Cloning of the cDNAs and the genes of various P450s in the beginning of the 1980s opened the second stage of P450 research. Expression of many forms of P450 in various non-hepatic animal tissues including brain, which had been difficult to detect by spectral observations, was elucidated by Northern blotting and immunohistochemical staining, and tissue-specific expression of various P450s and their physiological functions were studied. Characterization of P450 genes enabled the analysis of the molecular mechanism of the regulation of expression of the genes, which was the major focus of P450 research in the 1980s and the 1990s. These two decades were the times of molecular biology of animal P450s.

Elucidation of the total nucleotide sequence of human genome in 2001 heralded a new age of biology research. It was soon followed by the elucidation of the genomes of various eukaryotic and prokaryotic organisms, and the presence of many P450 genes in animals, plants, and microorganisms was confirmed by the analysis of their genomes. It has now become

possible to study and compare the physiological functions of P450s in various organisms of different kingdoms and phyla to elucidate the roles of P450s in the evolution and differentiation of eukaryotic organisms. We are now in the third stage of P450 research brighter than the previous two stages.

I wrote a short historical review titled "Forty Years of Cytochrome P450" in 1999.[162] I am happy to see remarkable expansion of P450 research in the past 10 years, and expect further progress in coming years.

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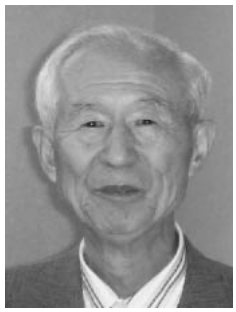
Profile



Tsuneo Omura was born in 1930. He was graduated from the Department of Chemistry, Faculty of Science of the University of Tokyo in 1953, and started his research career studying the enzymatic and molecular properties of the laccase of Japanese lacquer tree at the Department of Chemistry, Faculty of Liberal Arts and Science of the Shizuoka University. He then moved to the Protein Research Institute of the Osaka University in 1960 to become Assistant Professor in Professor Ryo Sato's laboratory, and changed his research subject to the electron transport enzymes in liver microsomes. He published the first paper on "cytochrome P450" in 1962, and contributed further to this new research area in the following years. He moved to the Kyushu University in 1970 to become Professor of the Department of Biology, Faculty of Science, and continued the research on cytochrome P450 with his collaborators. He also studied the biogenesis of mitochondria in animal cells. He was Professor of the Department of Molecular Biology, Graduate School of Medical Science since 1986, and retired from the Kyushu University in 1994 to become Emeritus Professor. He was Visiting Professor at the Medical School of the Vanderbilt University, Nashville, USA, from 1965 to 1999. He is an honorary member of Japanese Biochemical Society, Japanese Society for the Study of Xenobiotics, American Society of Biochemistry and Molecular Biology, and International Society for the Study of Xenobiotics.