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Review

Early years of oxygenase research in Bethesda, Osaka, Urbana, and Kanazawa

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Abstract

In this brief review, I recollect my experiences of how the studies of pyrocatechase and salicylate hydroxylase led to the isolation and revelation of $P450_{cam}$. Those experiences were instrumental in the separation, purification, and characterization of the two forms of adrenal cortex mitochondrial P450.

Keywords: CYP101; Flavoprotein; Hydroxylation; Oxygenase; P450; $P450_{cam}$; $P450_{scc}$; $P450_{11\beta}$; Pyrocatechase; Salicylate hydroxylase

My interest in the metabolism of aromatic amino acids began with my first academic experience at the Department of Bacteriology, Osaka City Medical School, Osaka, Japan, in collaboration with the late Dr. R. Sato who, at that time, was working at Kanazawa University. Later, he became famous for coining the term P450. Our discovery of a phenylalanine precursor, now known as prephenic acid, was published in *Science* in 1953 [1]. That year also marked the birth of modern molecular biology, i.e., it was the year that the Watson–Crick model of DNA was published and the complete determination of the amino acid sequence of "insulin" was accomplished by Fredrick Sanger.

Bethesda, the discovery of oxygenase

After continuing work on the aromatic pathway in the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, I was transferred through arrangements made by Dr. Sato to the National Institutes of Health (NIH) in Bethesda to join Dr. O. Hayaishi's laboratory. I knew his name, having read his papers on pyrocatechase, an enzyme that catalyzes the cleavage of the aromatic ring of catechol, one of the microbial metabolites of tryptophan, to form *cis*,*cis*-muconic acid. For me, this was virtually an extension of my previous work on the intermediates of aromatic amino acid metabolism: the only difference was that one dealt with biosynthesis, and the other, biodegradation. I was probably the first visiting fellow from Japan in his laboratory and am proud to have been there as one of the three members who pioneered oxygenase research during this exciting time of 1955 [2–5]. The photograph in Fig. 1, which was taken in the summer of 1955, shows Dr. Hayaishi in his office at Building 4 (Fig. 2) of the NIH, where the first enzymatic incorporation of both atoms of molecular oxygen into catechol to form *cis*,*cis*-muconic acid was discovered. This was also the year that McDonald's, the world's largest fast food chain, opened its first restaurant in a Chicago suburb with nine employees. P450 and the Jet Age were unknown entities then.

The strategies and techniques for studying the mechanisms of enzyme reactions and enzymatic incorporation of molecular oxygen greatly influenced me throughout my research career in the field of

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Fig. 1. Photograph of Dr. Hayaishi in his office during the summer of 1955. Photographed by M. Katagiri.

Fig. 2. Building 4 of the NIH, where the first enzymatic incorporation of both atoms of molecular oxygen into catechol to form *cis*,*cis*-muconic acid was discovered. Photographed by M. Katagiri in 1955.

biochemistry. Initially, Dr. Hayaishi would emphasize the importance of critical examination of experiments, particularly those with isotopes, either stable or radioactive. He also introduced very basic methodology for the study of enzyme reactions: the importance of directly monitoring the initial rate of change in a substrate or a product under metabolic, e.g., zero-order, conditions, preferably by using a spectrophotometer instead of a manometer or chemical analysis: the product of pyrocatechase reaction, *cis*,*cis*-muconic acid, has a large absorption peak at 260 nm. As will be described later in this review, such practical experience led me to the discoveries of not only salicylate hydroxylase, a flavoprotein monooxygenase, in Osaka but also $P450_{cam}$, a heme monooxygenase in Urbana [6,7].

Osaka, FAD-containing oxygenase

In 1961, Dr. Hayaishi, at that time a professor of the Faculty of Medicine, Kyoto University, had held a post concurrently in the Department of Biochemistry, Osaka University School of Medicine. This department was founded in 1905, exactly 100 years ago, by Professor Y. Kotake, who was famous for his discovery of kynurenine, a major metabolite of tryptophan. Fortunately, I was offered a post as an assistant professor under Dr. Hayaishi in Osaka. After an initial discussion of the possible projects suitable for this laboratory in Osaka, we selected a way to study the mechanism of monooxygenase reactions. Then in collaboration with Dr. S. Yamamoto, at that time a graduate student and now one of the organizers of this special issue of BBRC, we started to isolate necessary soil bacteria by enrichment culture techniques [8]. Among the various strains obtained, we chose a strain that was tentatively identified as *Pseudomonad*, which utilized salicylate as the sole carbon and energy source. The cells were grown for 30 h with constant shaking at 30 °C in a medium containing salicylate with occasional supplementation to maintain its concentration at ~0.1%. The cells were then harvested in a continuous-flow centrifuge and the cell-free extracts were prepared by subjecting the wet cells to sonication followed by centrifugation.

The first important finding on the catalytic properties of this enzyme was that the rate of consumption of NADH (then known as DPNH), which was monitored with a spectrophotometer in terms of the decrease in absorbance at 340 nm, was dependent on the presence of salicylate, the substrate. In addition, the conversion of NADH into NAD was proportional to the concomitant consumption of salicylate to form catechol and CO_2. This technique provided us with a specific, accurate, and convenient assay for the reaction. The second important finding was that salicylate hydroxylase lost its activity after ammonium sulfate fractionation and dialysis, and its full activity was restored by the addition of a heat-stable factor of rat liver. Soon, it was found that a catalytic amount of FAD was able to replace the responsible co-factor. FMN, ferrous sulfate, riboflavin, or tetrahydrofolate was not a suitable replacement for FAD. These results provided the first experimental evidence of the novel capacity of FAD as a co-oxygenase that functions in O_2 binding and activation [6,9]. Salicylate hydroxylase was purified to homogeneity and its molecular weight was estimated to be approximately 57,000 [9]. Equilibrium dialysis indicated the number of molecules of FAD bound to one molecule of enzyme protein to be approximately 0.9. $^{18}O_2$ experiments proved that one atom of molecular oxygen was incorporated into catechol, the product, whereas another oxygen atom was presumed to be reduced to water. These lines of evidence suggested that the reduction of FAD with NADH and the hydroxylation of salicylate were catalyzed by only a single protein enzyme and that the two reactions were tightly coupled [10].

Many of the classic oxygenases, including the first two types of oxygenases, contain transition metals, i.e., copper or non-heme iron [2,3]. It has therefore been believed that in an oxygenase reaction, the oxygen

molecule is enzymatically activated by the formation of a metal complex at the active center of the enzyme molecule. However, in the case of salicylate hydroxylase in the purified form, all attempts, including inhibition studies with chelators, failed to provide definitive evidence for the participation of metals in the reaction [11]. Following the characterization of salicylate hydroxylase, a diverse group of FAD-containing oxygenases was found and characterized extensively. Most of them were monooxygenases that required NAD(P)H as an external electron donor [12,13]. As will be described later, this type of hydroxylase served as a model for other NAD(P)H requiring oxygenase systems such as those catalyzed by P450.

Kanazawa 1, interaction of substrate

In 1964, I assumed the position of professor at the Department of Chemistry, Faculty of Science, Kanazawa University, Kanazawa. There, I continued work on salicylate hydroxylase and other related enzymes in collaboration with Drs. S. Takemori, K. Suzuki, and K. Suhara, and the students in our laboratory. We first found that, when the substrate salicylate was added to the salicylate hydroxylase holo-enzyme, the peaks at 375 and 450 nm on its absorption spectrum were equally shifted to the red and a new marked shoulder appeared around 480 nm. The magnitude of this spectral change was dependent on the concentration of salicylate and spectral titration of the enzyme with salicylate indicated that the enzyme combined specifically with salicylate to form a statistically stable 1:1 complex [14]. Evidence that the complex is an actual intermediate involved in the overall reaction was provided by experiments under anaerobic conditions. When a limited amount of NADH in the side arm of an anaerobic cuvette was added to a solution into the main vessel containing salicylate hydroxylase and salicylate, the decrease in absorbance at 450 nm indicated that 1 mol of FAD moiety in the complex was reduced by the addition of 1 mol of NADH. On admitting air to the cuvette, the reduced complex was rapidly re-oxidized and the absorption in the visible region returned to the same level as that of the untreated preparation. Under these conditions, 1 mol of catechol was shown to be produced from 1 mol of the reduced complex. These results suggest that salicylate hydroxylase undergoes sequential reactions of substrate binding and reduction of the bound FAD to $FADH_2$ with NADH. The subsequent binding of oxygen to the complex yielded the products with concomitant reversion of the bound $FADH_2$ to FAD. As these findings may offer a simple and excellent model mechanism for many other hydroxylase reactions, we presented them at the Symposium on the Biological and Chemical Aspects of Oxygenases held on May 16–19, 1966 in Kyoto, Japan, organized by Drs. Konrad Bloch and Hayaishi under the auspices of the US-Japan Committee on Scientific Cooperation [15].

At the same congress, I was attracted to the camphor 5-*exo*hydroxylase system presented by Dr. I.C. Gunsalus et al. [16] because while their system appeared to be basically similar to that of ours, theirs seemed to be more complicated and more closely resembled mammalian steroid hydroxylase system.

Urbana, $P450_{cam}$ (CYP101)

Through the arrangements made by Dr. Hayaishi, I was able to join Dr. Gunsalus at the Department of Biochemistry, University of Illinois, Urbana, in the September of 1967 for my 6-month sabbatical. There, I began working on the ongoing project to elucidate the mechanism of the camphor 5-*exo*hydroxylase reaction of *Pseudomonas putida*. At the time, the camphor hydroxylating system had already been separated into two fractions: a non-heme iron–sulfur protein, called putidaredoxin, and a crude brown component, tentatively called fraction A [17]. Thus, armed with sufficient knowledge and a steady supply of materials, I was able to start my study immediately. The first thing I noted was that the camphor-5-*exo*hydroxylating activity determined by the conventional assay system was too weak to indicate that the cells metabolize camphor as the sole carbon and energy source. Astonishingly, however, fraction A closely resembled salicylate hydroxylase in that the hydroxylase substrate, in this case camphor, definitely accelerated the rate of putidaredoxin-mediated NADH oxidizing activity. I took advantage of this and monitored the activity within minutes in a recording spectrophotometer. NADPH, initially presumed to be the electron donor for this system, was far less active than NADH. What I also found was that the addition of the substrate camphor to the preparation of fraction A, once again similar to the case of salicylate hydroxylase, caused a marked change in its absorption spectrum. This clearly indicates that fraction A contained a substrate-binding colored component. Its P450 nature was soon confirmed by the change in absorption spectrum of the CO-bubbled reduced sample. Subsequently, fraction A was separated on a DEAE-cellulose column into the presumed two components, a P450 protein and an FAD-containing flavoprotein, to which I proposed the names $P450_{cam}$ and NADH-putidaredoxin reductase, respectively [7]. This separation led to the revelation that, in addition to the known putidaredoxin, an NADH-dependent flavoprotein and a P450 hemoprotein are involved in the camphor 5-*exo*hydroxylase reaction. In this system, NADH is regarded as the electron donor through a short electron transport chain composed of NADH-putidaredoxin reductase and

putidaredoxin to the substrate-bound $P450_{cam}$. Unlike many of the hitherto known P450-catalyzed hydroxylase systems, all components, flavoprotein, iron–sulfur protein, and P450, are soluble and the substrate specificity of the system is restricted to camphor.

This demonstration of the characteristics of purified $P450_{cam}$ provides the first direct evidence for the current common knowledge that P450 is a type of porphyrin-containing protein that binds oxygen and substrate. Large-scale purification was undertaken via the collaboration between Urbana and Kanazawa, and crystallization was accomplished by Dr. C.-A. Yu [18]. After its first crystallization, $P450_{cam}$ has, to this day, served as the paradigm in P450 structure–function studies, although it is no longer alone [19–21].

Kanazawa 2, $P450_{scc}$, and $P450_{11\beta}$

After I came back to Kanazawa, our group began work on bovine adrenal cortex mitochondrial P450, because a striking similarity existed between the *Pseudomonas* camphor-5-*exo*hydroxylating system and the adrenal cortex mitochondrial steroid-metabolizing system. Both catalyze the substrate-specific monooxygenase reactions of isoprenoids and both, unlike the microsomal systems, are composed of an NAD(P)H-dependent non-heme iron protein reductase, a non-heme iron protein, and a hemoprotein P450 [22]. However, at this time, the individual forms of P450 have not yet been isolated in their purified forms and thus their actual properties have not been characterized extensively. We chose two typical forms of adrenal cortex mitochondrial P450, $P450_{scc}$, which catalyzed the side-chain cleavage of cholesterol to form pregnenolone, i.e., a C–C lyase reaction, and $P450_{11\beta}$, which catalyzed the hydroxylations of deoxycorticosterone at the 11β- and 18-positions, i.e., methylene and methyl hydroxylations. We developed a new method for separating the ammonium sulfate fractions of the solubilized mitochondrial preparation, which involved the use of a hydrophobic affinity column called "aniline–Sepharose" [23,24]. It was also found that the stability of $P450_{11\beta}$ was specifically dependent on the presence of its own substrate, deoxycorticosterone, during purification and storage; on the other hand, cholesterol present endogenously in the preparation was usually sufficient to stabilize $P450_{scc}$. On SDS–polyacrylamide gel electrophoresis, only one protein band for each purified preparation was observed, indicating that the two P450 preparations had been satisfactorily separated in purified forms for further characterization. When the preparations were carboxymethylated in the presence of 6 M guanidine HCl and 100 mM 2-mercaptoethanol, the sedimentation equilibrium data gave molecular weights of 46,000 and 43,000 for $P450_{scc}$ and $P450_{11\beta}$, respectively [25,26]. Under a newly developed sensitive assay system, the purified $P450_{scc}$ preparation specifically catalyzed the side-chain cleavage of cholesterol to form pregnenolone with a turnover number of 16 min^{-1} in the presence of adrenodoxin and NADPH-adrenodoxin reductase, but did not catalyze 11β- or 18-hydroxylase activity for deoxycorticosterone. In contrast, $P450_{11\beta}$ catalyzed 11β- and 18-hydroxylase activities for deoxycorticosterone with turnover numbers of 110 and 18 min^{-1}, respectively [27,28]. Each rabbit antibody produced against P450 interacted with respective P450, but not with alternative adrenal cortex mitochondrial P450 or $P450_{cam}$. Each antibody inhibited specifically the corresponding enzyme reaction [29].

To summarize, although much of the above is now common knowledge, our work is the first to provide clear-cut evidence of the existing of at least two typical forms of P450 in adrenal cortex mitochondria. Each P450 exhibited a specific difference with respect to both spectral and molecular properties, and substrate-specific monooxygenase activity indicating its central role as the substrate- and oxygen-reactive component in steroid hormone biogenesis in the adrenal cortex. Many reviews are available on the recent advances in the structure–function relationship of a variety of P450, in both prokaryotic and eukaryotic organisms [30–33].

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