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Commentary by

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on 'Crystallization of Michaelis complex of D-amino acid oxidase'
by K. Yagi and T. Ozawa
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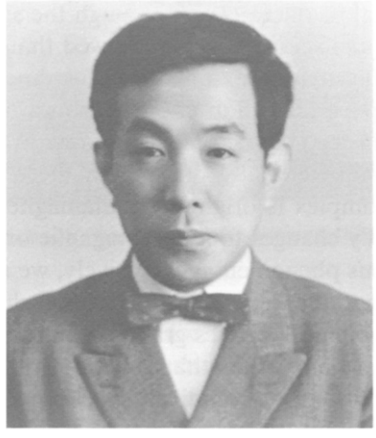
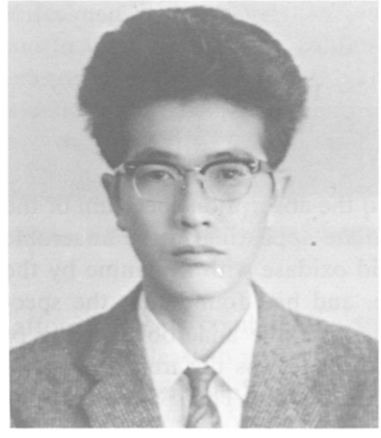


Kunio Yagi (in 1962)



Takayuki Ozawa (in 1962)

This paper described the first ever crystallization of an enzyme-substrate complex, which definitely confirmed the Michaelis-Menten theory by demonstrating chemically the existence of an equimolar complex of an enzyme and its substrate. More detailed information was later published in the same journal [1], and related data were also reported in the same as a series of papers [2–4].

The reason for my study of this subject was closely related to Professor Leonor Michaelis. Professor Michaelis is famous for the Michaelis-Menten theory, in addition to his many pioneering works, but little is known about his contribution to the Japanese biochemical community in its early days. In 1922, he came to Japan from Germany at the invitation of Aichi Medical College, now the University of Nagoya, and was asked to found the Department of Medical Chemistry, now the Institute of Biochemistry, Faculty of Medicine, University of Nagoya. In 1923, he began his lectures for medical students, and in addition he conducted highly inspiring and stimulating special courses for young Japanese biochemists in this new department (see photograph). After many contributions to the development of biochemistry in Japan, he left for New York in 1926.

When I was studying as a medical student in the Department of Biochemistry, School of Medicine, Nagoya University, during the period 1939–42, some years had passed since Professor Michaelis had left Nagoya, but many things he brought from Germany still remained and strongly influenced me. In addition, when I started my research work just after the Second World War, the mechanism of enzyme action was one of the most challenging targets for young biochemists. For these reasons, I decided to study the enzyme-substrate complex and I thought that an enzyme which has characteristic properties such as color, as is the case for flavin enzymes, would be advantageous for the demonstration of an enzyme-substrate complex. I finally selected D-amino acid oxidase, a dissociable flavin enzyme, as a tool. As a working hypothesis, I reasoned that if an enzyme once forms an enzyme-substrate complex and gives rise to the enzyme and product, and

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if the enzymatic reaction is reversible, a certain amount of the enzyme-substrate complex should exist in the presence of adequate amounts of the substrate and the product. I predicted that the complex might be stabilized under reaction conditions other than maximal and that the complex might be crystallized under appropriate conditions. With this idea in mind, I conducted extensive research, and after a longer time than I had expected, I succeeded, in collaboration with Dr. T. Ozawa, in crystallizing a beautiful purple-colored enzyme-substrate complex.

Through the publication of this paper, which was followed by a series of papers related to this problem, I was recognized by many specialists and was given several opportunities to present this work at various symposia. When I presented my paper at the symposium 'Mechanism of Enzyme Action' at the 5th Congress of the International Union of Biochemistry held in New York in 1964, my presentation was reported by Chemical & Engineering News. This aided wider recognition of our results. When I was invited to talk at the 1st symposium on 'Flavins and Flavoproteins' organized by Professor E.C. Slater in Amsterdam in 1965, however, I was opposed very seriously by Dr. V. Massey. He and his colleagues had measured the absorption spectrum of the purple-colored intermediate appearing in the anaerobic reaction of D-amino acid oxidase with D-alanine by the stopped-flow technique, and had found that the species measured at 150 ms after the anaerobic mixing of the enzyme with the substrate was different from that measured at 1.8 s after the mixing. The spectrum of our crystals was different from that measured at 150 ms but identical to that measured at 1.8 s, and they argued that the crystallized species was catalytically meaningless, since a 1.8 s species could not explain the high molecular activity of this enzyme [5]. We carefully examined their results and found that their experimental data were quite reproducible. Naturally, their findings had a strong impact on me. Therefore, we examined this problem extensively, and analyzed the absorption spectrum of the 'rapidly appearing intermediate' and that of the 'slowly appearing intermediate', and found that the difference spectrum between the two agreed with the difference spectrum between the oxidized enzyme and the 'slowly appearing intermediate'. This phenomenon can be interpreted as indicating that the entity of the 'rapidly appearing intermediate' is not homogeneous but is composed of a major purple intermediate and, to a minor extent, the oxidized enzyme. This means that a major portion of the enzyme changes rapidly to the purple entity, while a minor portion does so only slowly. Since we had already discovered the monomer-dimer equilibrium of this enzyme, we interpreted the data to indicate that these two entities could be ascribed to the dimer and monomer; that is, the dimer reacts with the substrate more rapidly to form the purple intermediate than does the monomer [6]. Thus, we concluded that the crystalline purple complex is identical to the intermediate appearing in the catalytic process. Such results prompted additional interest in the monomer–dimer equilibrium of enzymes in general. Further, they provided a crucial warning for the adjustment of the parameters obtained with a low concentration of the enzyme with those obtained with a high concentration of the enzyme, since the former contains a larger amount of monomer than the latter.

Another question arising from the crystallization of this enzyme-substrate complex was the nature of the complex. When we obtained the crystal, I thought at first from its purple color that the entity of the enzyme moiety would be in the semiquinoid form, since it was known that the oxidized enzyme has a yellow color and the fully reduced enzyme is colorless. To examine this possibility, we studied the electron spin resonance signal of the crystal. Although the signal was detected, the quantitative analysis showed that only a minute part of the coenzyme, flavin adenine dinucleotide, was a radical species. In addition, the amount of radicals contained in the crystals varied with time. We investigated further, and eventually found that the entity of the crystalline complex is originally a diamagnetic species, but gradually changes to a paramagnetic one [2]. After examining this phenomenon extensively, we reached the conclusion that the complex is a strong charge-transfer complex which dissociates gradually into radical cation and radical anion, and that substrate radicals disproportionate to yield non-radical species, whereas flavin radicals remain unchanged due to stabilization by the apoprotein. Starting from the purple complex, we were able to crystallize the semiquinoid enzyme of D-amino acid oxidase [3]. This was the first instance of the crystallization of a semiquinoid flavin enzyme.

Since the purple intermediate complex was found to be the very complex that reacts with oxygen [7], it appeared that the slow dissociation of the complex, in other words the long lifetime of this strong charge-transfer complex, is important in giving the complex enough time to react with oxygen. Thus, the reason why this strong charge-transfer complex is to some extent stabilized became the next problem to be solved. We predicted that the environment surrounding the charge-transfer interaction might be hydrophobic. To examine this prediction, we synthesized several hydrophobic probes which fluoresce upon combination with a hydrophobic locus of the protein. Among them, the novel compound 4,4′-diaminostilbene 2,2′-disulfonate [8] was found to be a suitable probe, since this dye can be exchanged for the coenzyme, flavin adenine dinucleotide, to combine with the apoenzyme of D-amino acid oxidase. We demonstrated that when the dye combines with the apoenzyme it fluoresces and that the fluorescence decreases upon the addition of flavin

Professor Leonor Michaelis (fourth from left) demonstrating a physicochemical experiment to young Japanese biochemists in 1923.

adenine dinucleotide; the fluorescence spectrum finally becomes identical to that of the D-amino acid oxidase holoenzyme. This means that the coenzyme is in a hydrophobic pocket of the apoprotein, and that the environment surrounding the charge-transfer interaction will be hydrophobic.

I presented this result at the 2nd International Symposium on Oxidases and Related Redox Systems held in Memphis in 1971. I still remember the comment made by Dr. W.E. Blumberg after my presentation. He said, "In case anybody missed the point of Dr. Yagi's talk in relevance to the discussion earlier today, let me reiterate that one could paraphrase and say that anybody who is talking about flavin water chemistry in this enzyme is all wet" [9].

From another point of view, such a hydrophobic environment surrounding the coenzyme was found to be important for the catalytic activity of the enzyme. Thus, we demonstrated later the importance of hydrogen bonding at particular heteroatom(s) of the isoalloxazine nucleus of flavin for its reactivity [10,11]. If the environment of the coenzyme were aqueous, all the heteroatoms would form hydrogen bonds with water molecules, and such hydrogen bonding at particular heteroatom(s) would be impossible. However, hydrogen bonding between heteroatom(s) of flavin and amino-acid residues in a hydrophobic region of the apoenzyme would explain the increased reactivity of the coenzyme afforded by its combination with its apoenzyme. This answers, at least in part, the question raised by Professor Hugo Theorell, who invited me to collaborate with him in his laboratory in 1957. In his Nobel Lecture in 1955 he said, "We still do not quite understand how through its linkage to the coenzyme the enzyme-protein 'activates' the latter to a rapid absorption and giving off of hydrogen".

Skipping other developments originating from this work, I want to mention some of the human aspects related to it. Professor Slater, who accepted this paper as an Editor of BBA, came to Nagoya in 1972 at my invitation, and attended the symposium dedicated to the late Professor Michaelis on the occasion of the 50th anniversary of the inauguration of his lectures in Nagoya [12]. In the meeting he delivered a warm address which gave considerable pleasure to those co-workers of Professor Michaelis who were still alive at that time. Professor Massey, who had argued initially about the purple crystals, became a very good friend of mine thereafter, and in 1985 he even stayed in my new laboratory for 8 months to collaborate with me. Dr. Ozawa, the co-author of this paper, was a post-graduate student at the time of its publication, and is now a full Professor of Biochemistry at the University of Nagoya. We still interact intensively.

In conclusion, this paper is the most memorable in my life so far.

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Crystallization of Michaelis complex of D-amino acid oxidase

To elucidate the mechanism of flavin enzyme catalysis, the artificial Michaelis complexes of D-amino acid oxidase (D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3) were isolated as crystals[1-3]. They were found to be composed of equimolar amounts of apo-enzyme, prosthetic group and substrate-substitute, and their physicochemical properties were reported[4-6]. We now wish to report the isolation of a real Michaelis complex of the oxidase, *viz.* an intermediate product formed during its catalytic process.

Since the Michaelis complex is rapidly converted into free enzyme and the product of the reaction under optimum conditions, it would be quite difficult to isolate Michaelis complex under these conditions. However, we found that the Michaelis complex of the oxidase was relatively stable at low temperature in the absence of molecular oxygen at a lower pH than the optimum for the enzyme action. Under these conditions, we succeeded in crystallizing the Michaelis complex of this enzyme. The results are briefly reported here.

1 g of the artificial Michaelis complex (apo-enzyme–FAD–benzoate) crystal[3] was dissolved in 50 ml of oxygen-free pyrophosphate buffer (0.0167 *M*, pH 8.3), and 1 g of crystalline D-alanine was added with stirring. The pH of the solution was brought to 6.1 by adding 1 *N* acetic acid, and 6 g of $(NH_4)_2SO_4$ were then added to the solution. The precipitate was collected by centrifugation and dissolved in 50 ml of the buffer. Alanine was again added and the above procedure repeated. The precipitate was dissolved in 3 ml of the pyrophosphate buffer, and the solution was transferred into a vessel, which was filled with O_2-free N_2 and kept at 5° overnight. A crop of faint-purple-coloured crystals, hexagonal prisms with bipyramids (40 × 16 μ), was obtained, as shown in Fig. 1. The mother liquid of the crystal was transparent and purple-coloured. When

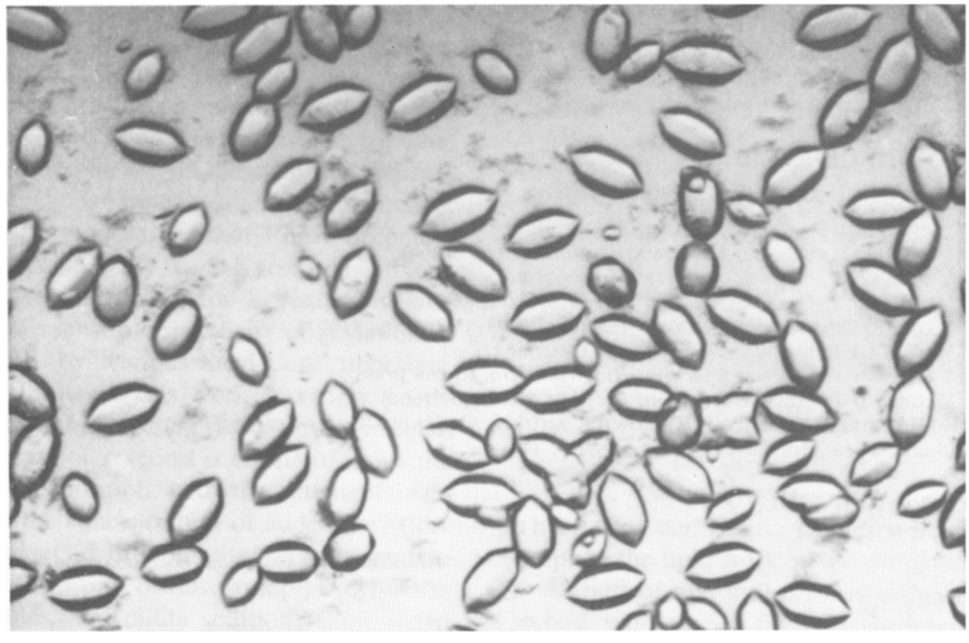

Fig. 1. Crystals of the Michaelis complex of D-amino acid oxidase (× 250).

an excess D-alanine was dissolved in the mother liquid, fine needle-shaped crystals gradually appeared.

The hexagonal crystals were washed twice with cold O_2-free distilled water, and dissolved in the pyrophosphate buffer. On aeration, the colour of the solution turned to yellow, with absorption peaks at 275 mμ, 375 mμ and 453 mμ. Spectrophotometric analysis[7] of the yellow solution showed that it contained the holoenzyme, consisting of equimolar proportions of the apo-enzyme and FAD.

After aeration in the presence of a trace of crystalline catalase, 1 mole pyruvate per mole enzyme was found by hydrazone formation with 2,4-dinitrophenylhydrazine[8] (Table I). By aeration in the absence of catalase, formation of H_2O_2 was demonstrated iodometrically.

TABLE I

NUMBER OF MOLES OF THE REACTION PRODUCT OF THE SUBSTRATE AND THE ENZYME AFTER AERATION OF THE CRYSTAL

Expt. No.	*Apo-enzyme (moles × 10⁷)*	*FAD (moles × 10⁷)*	*Pyruvate (moles × 10⁷)*
1	1.4	1.2	1.3
2	0.84	0.90	1.0

When the crystal was incubated with excess D-alanine and FAD in the pyrophosphate buffer, the oxygen uptake was observed by routine Warburg technique. The catalytic activity calculated from the rate of oxygen uptake was the same with that measured with the purest sample of the oxidase[3].

The same results were obtained with the needle-shaped crystals.

On the basis of these results as well as those of the artificial Michaelis complex, it is considered that the crystal is an intermediate product, the Michaelis complex, of the enzyme.

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