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Detection of microwave radiation of cytochrome CYP102 A1 solution during the enzyme reaction

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ABSTRACT

Microwave radiation at 3.4–4.2 GHz frequency of the cytochrome P450 CYP102 A1 (BM3) solution was registered during the lauric acid hydroxylation reaction. The microwave radiation generation was shown to occur following the addition of electron donor NADPH to a system containing an enzyme and a substrate. The radiation occurs for the enzyme solutions with enzyme concentrations of 10^{-8} and 10^{-9} M. The microwave radiation effect elicited by the aqueous enzyme solution was observed for the first time. The results obtained can be used to elaborate a new approach to enzyme systems research, including studying of the mechanism of interaction of a functioning enzyme system with microenvironment.

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1. Introduction

Cytochromes P450 are a superfamily of heme-containing monooxygenase enzymes are playing an important role in the oxidation of both endogenous (steroids, fatty acids, etc.) and exogenous molecular entities (drugs, toxins, pesticides, etc.) [1]. Monooxygenases catalyze the incorporation of one oxygen atom into different substrates, while the other oxygen atom is reduced to water. The catalytic mechanism can be described as follows [2]:

$$RH + O_2 + 2\bar{e} + 2H^+ \rightarrow ROH + H_2O,$$

where RH is the substrate, $(2\bar{e}+2H^+)$ is the electron donor, either NADPH or NADH.

Flavocytochrome CYP102 A1, isolated from the soil bacterium *Bacillus megaterium* in 1974 by Miura and Fulco [3], holds a particular place in the superfamily of cytochromes P450 as the first bacterial cytochrome (P450) to be discovered fused with its redox partner [1,4]. This is why CYP102 A1 is an important model system to study and understand the structural and functional mechanisms of the cytochrome P450 superfamily.

CYP102 A1 catalyzes hydroxylation (mainly (ω-1), (ω-2) and (ω-3)-hydroxylation) of saturated and unsaturated fatty acids with different chain lengths [5], as well as of alcohols and amides [1]. The physiological function of CYP102 A1, as of 2002, has not yet been defined, though the study suggests that its primary function is to eliminate toxic fatty acids from the environment, in other words to protect the *B. megaterium* bacterium [6]. The study of the catalytic activity of cytochrome P450 was conducted earlier using optical spectrometry [7], electrochemical [8] and nanomechanical researches [9–11], and other methods. The study showed that CYP102 A1 catalytic activity is the result of the transfer of electrons from the NADPH cofactor through the flavinic domain to the enzyme heme [7].

CYP102 A1 has the highest monooxygenase activity among P450 cytochromes; the rate of arachidonic acid oxidation is $\approx 250\ s^{-1}$ [12], whereas that of lauric acid is $\approx 50\ s^{-1}$ [13]. Atomic force microscopy investigation [10] reveals that the catalytic activity of CYP102A1 manifests itself through the fluctuating state of the protein globule. The fluctuations were detected in Hertz frequency range, the average time between them being several seconds. The maximum fluctuation amplitude was observed at 22 °C. Such low-frequency fluctuations coincide with the vibration frequency of big water clusters [14]. As shown in [15], molecules in the water not only vibrate, but also rotate, similar to gas molecules. The rotation of water molecules occurs at GHz frequencies. It was inferred therefore that fluctuations of CYP102 A1 enzyme globule occurring within seconds during the catalytic cycle [10], should have not only a vibrating, but also a rotating

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structure, while rotating motion can be accompanied by GHz-frequency radiation (microwave radiation). Our study aims to reveal the generation of microwave radiation of cytochrome CYP102 A1-containing system during lauric acid hydroxylation. The study showed that in the course of CYP102 A1 functioning, a microwave radiation of 3.4–4.2 GHz frequency range can be observed.

In our present study 10^{-8} M and 10^{-9} M enzyme solutions were used. The justification for selection of these concentrations for the experiments are the following factors. First, the activity of cytochrome P450 depends on its concentration: it was shown that the enzyme is active in the dimeric form, but is virtually inactive in monomeric form [13]. As was shown in [13], at concentration of 10^{-9} M and higher, the enzyme is mainly presented in solution in dimeric form; in this concentration range the enzyme activity is of about the same level. At concentrations below 10^{-10} M its activity sharply decreases with concentration. Authors [13] explain this fact by the increase in the fraction of the monomeric form of the enzyme, which is less active. Therefore, to provide the constant activity of the enzyme, its minimal enzyme concentration used in our study was chosen at the level of 10^{-9} M. The use of 10^{-8} M level as the maximal concentration is conditioned by the need to decrease the mutual influence of the protein molecules, i. e. to use sufficiently dilute solution to avoid the quenching effect. Another factor determining the choice of the investigated concentration range, is the concentration of the enzyme in the cell. So, according to [16], the size of *B. megaterium* cell is 10 µm, while the total number of protein in the cell is $\sim 10^9$ molecules [17]. This corresponds to the total protein concentration at the level of 10^{-3} M. At the same time, it is known that the fraction of cytochrome P450 makes up less than 10^{-4} of the total amount of protein [4]. Thus, the concentration of Cytochrome P450 makes up less than 10^{-7} M.

The program of the present study also included control measurements with use of hexane as substrate or, alternatively, mutant form of CYP102A1 as enzyme. It was shown [18] that in the case of hexane as substrate, the turnover number of CYP102A1 is an order of magnitude lower ($\sim 1\ s^{-1}$) than in the case of lauric acid. Moreover, mutant CYP102A1 enzyme containing amino acid substitutions (A264H mutant) has 5 times reduced activity towards lauric acid as compared to the native form of the protein [13].

2. Materials and methods

2.1. Reagents

2.5 mM phosphate saline buffer containing 30 mM NaCl (PBSD, pH 7.4) was purchased from Pierce (USA). Lauric acid sodium salt and NADPH were purchased from Sigma (USA). Hexane was obtained from Reakhim (Russia). Deionized ultrapure water was obtained using Simplicity UV system (Millipore, USA).

2.2. Proteins

Cytochrome CYP102 A1 was expressed according to [13,19] and kindly provided by Prof. S.A. Usanov.

Mutant of cytochrome CYP102 A1 was expressed according to [13] and kindly provided by Prof. V.G. Zgoda.

Protein solutions (10^{-8} M and 10^{-9} M) were prepared from stock solution (54 μM in 25 mM KP buffer) through a consecutive ten-fold dilution in the working buffer solution.

2.3. Analytical measurement

Protein concentration was measured using a spectrophotometric method. CYP102 A1 absorption spectrums were measured using Agilent Model 8453 spectrophotometer (USA) at 25 °C. The concentration of purified CYP102 A1 was determined by the difference in absorbance of carboxyl complex in its reduced form. We used the extinction coefficient of 91 $mM^{-1}\ cm^{-1}$ for the difference of absorption on 450 nm and 490 nm according to the method described by Omura and Sato [20].

2.4. Procedure for monitoring the microwave radiation of BM3 solution

2.4.1. Catalytic reaction in CYP102 A1 system

A catalytic reaction in the enzyme system was carried out in a reconstituted CYP102 A1 system containing cytochrome CYP102 A1 and its substrate, lauric acid (0.5 mM) in PBSD, pH 7.4. The reaction was initiated by the addition of NADPH water solution (0.2 mM) to the incubation medium. Measurement conditions for detection of microwave radiation were as follows: 20 µl sample solution volume, temperatures of 18 °C and 23 °C. Measurements of microwave radiation at the temperature of 39 °C, when the enzyme is not active, were taken as well [21]. The control measurements were carried out using 2 types of solutions: solutions containing no protein and solutions without the NADPH electron donor.

The measurements were conducted for at least 400 s.

2.4.2. Measurement of CYP102 A1 solution microwave radiation

A broadband radiometer RTM-01 RES operating in 3.4–4.2 GHz range was used as a microwave detector. For measurements, the antenna of the microwave analyzer (buggy-whip antenna) was entirely immersed in the sample solution.

The microwave radiation measurement data obtained is presented through brightness temperature units T_b, in which the radiometer RTM-01 RES is calibrated. The measurement error is ± 0.1 °C. In the measurements, T_b is determined as a function of time.

3. Results

3.1. Results of microwave radiation control measurements

Control tests were carried out in three variants:

1) In a solution containing protein and its substrate, lauric acid, but without NADPH electron donor. These tests were conducted in order to determine the base level of microwave radiation noises in an inactive CYP102 A1 enzyme system.
2) In a solution containing a substrate but without protein. For control, NADPH (electron donor) was added to the substrate-containing solution. The tests were conducted in order to elucidate how NADPH, added to the buffer solution, affects microwave radiation level.
3) In a solution containing protein CYP102 A1 without substrate and NADPH.

Fig. 1 shows the examples of registered microwave radiation signal as a function of time. As shown in Fig. 1, the baseline level of noise in microwave range is ± 0.1 °C, thus being within the experimental error.

Control measurements' results show that the level of microwave radiation signal of the solution containing protein and substrate but without NADPH (variant 1) equals that of the solution without protein (variant 2). Also, in the control experiments carried out according to variant 3 no microwave radiation was registered after the addition of water to the protein solution. Solutions containing only protein (variant 3), or protein and substrate without NADPH (variant 1) are corresponding to conditions when cytochrome CYP102 A1 was represented in the inactive state.

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