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

Yu.D. Ivanov [a,*], K.A. Malsagova [a], A.A. Izotov [a], T.O. Pleshakova [a], V.Yu. Tatur [b], S.G. Vesnin [c], N.D. Ivanova [d], S.A. Usanov [e], A.I. Archakov [a]

[a] Institute of Biomedical Chemistry, ul. Pogodinskaya 10, Moscow 119121, Russia
[b] Foundation of Perspective Technologies and Novations, Moscow, Russia
[c] RES LTD., Moscow, Russia
[d] Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology, Moscow, Russia
[e] Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Minsk, Belarus

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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.



## 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

* Corresponding author.
*E-mail address:* yurii.ivanov@rambler.ru (Yu.D. Ivanov).

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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 $\pm 0.1$ °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 $\pm 0.1$ °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.

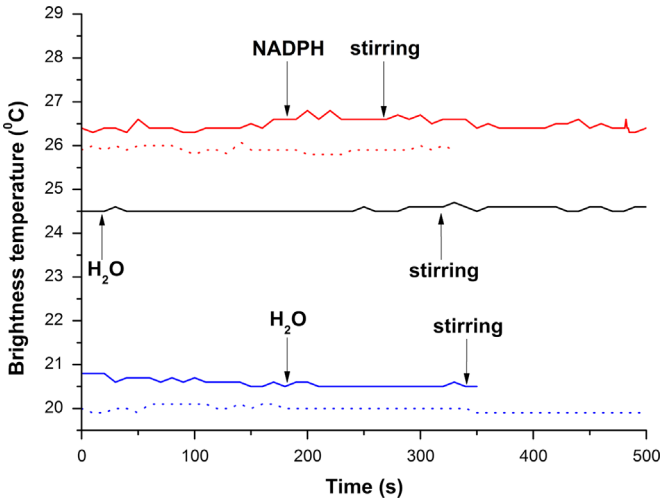


**Fig. 1.** Results of control experiments: brightness temperature as a function of time $T_b(t)$.
Experimental conditions of the control experiments:

- Temperature of measurements 18 °C (dotted lines) and 23 °C (solid lines);
- (blue lines) the solution under analysis contains protein CYP102 A1 ($10^{-9}$ M) and substrate (lauric acid, 0.5 mM) (variant 1);
- (red lines) the solution under analysis contains substrate (lauric acid 0.5 mM) upon addition of electron donor (NADPH, 0.2 mM) (variant 2).
- (black line) the solution under analysis contains protein CYP102 A1 ($10^{-9}$ M) (variant 3);
  Arrows indicate the time points of addition of electron donor NADPH (variants 1 and 2) and $H_2O$ (variant 3) to the system containing protein and substrate, and time points of stirring the solution in the cell.

### 3.2. Results of the measurement of microwave radiation of CYP102 A1 solution in the process of lauric acid hydroxylation

Microwave radiation of enzyme solution during the catalytic cycle was measured at 23 °C, 18 °C and 39 °C by the procedure described above in the Section 2.

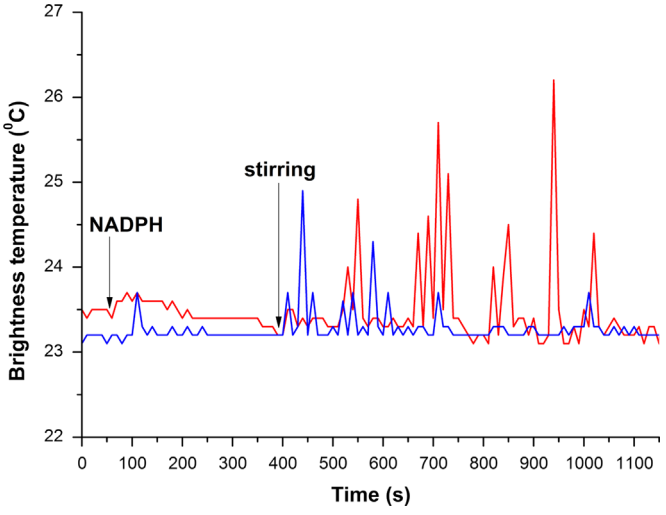


**Fig. 2.** Results represent the function of $T_b(t)$ for cytochrome CYP102 A1 solution at 23 °C.
Experimental conditions:

- cytochrome CYP102 A1 concentration $10^{-8}$ M (blue line) and $10^{-9}$ M (red line);
- the temperature of solution under analysis is 23 °C;
  Arrows indicate the time points of the addition of electron donor (NADPH, 0.2 mM) to the system containing protein and substrate (lauric acid, 0.5 mM), and the time points of stirring the solution in the cell.

Brightness temperature ($T_b$) of the solution was measured for the reconstituted cytochrome CYP102 A1 system containing protein at $10^{-8}$ M and $10^{-9}$ M concentrations. The examples of data obtained at 23 °C are shown in Fig. 2. As seen from this figure, the addition of NADPH to the solution, containing the protein at $10^{-9}$ M and $10^{-8}$ M concentration, and its substrate causes major changes in the form of impulses to the $T_b(t)$ function. Short-term changes in $\Delta T_b$ are equal to $0.5 \div 2$ °C ($S/N$ $2 \div 10$) within 10–20 s. Impulses in the $T_b(t)$ function determine the generation of microwave radiation in the solution.

Fig. 3 shows an example of these measurements obtained at 18 °C for $10^{-9}$ M and $10^{-8}$ M CYP102 A1 solution. As shown in the figure, low impulses of the $T_b(t)$ function occur after the addition of NADPH to protein solution ($S/N \sim 2$) with the $10^{-9}$ M protein concentration only.

At 39 °C no increase in levels of microwave radiation was registered either (data not shown).

To elucidate the influence of the rate of substrate oxidation by cytochrome CYP102 A1 on the generation of microwave radiation, two additional comparative experiments were carried out. In these experiments the enzyme exhibited two orders of magnitude lower activity than in above-described CYP102 A1-containing system. The first comparative experiment (1) consisted in monitoring of the microwave radiation in the enzyme system containing CYP102 A1 (wild type), but with use of hexane as substrate instead of lauric acid. This experiment was carried out with use of minimal ($10^{-9}$ M) protein concentration, for which the radiation was observed in the system containing lauric acid (Fig. 2). The second comparative experiment (2) consisted in monitoring of the microwave radiation in the enzyme system containing mutant form of the enzyme – CYP102 A1 (mutant) – and lauric acid as substrate. In the conditions of experiments (1) and (2) the activity (turnover number) of CYP102 A1 makes up 0.2–1 $s^{-1}$, and this is two orders of magnitude lower than the enzyme activity in the system containing CYP102 A1 (wild type) and lauric acid as substrate (shown in Figs. 2 and 3) [13,18]. Fig. 4 displays the results of the comparative experiments. These results have indicated that no microwave radiation is observed in the case of decrease in the enzyme activity.

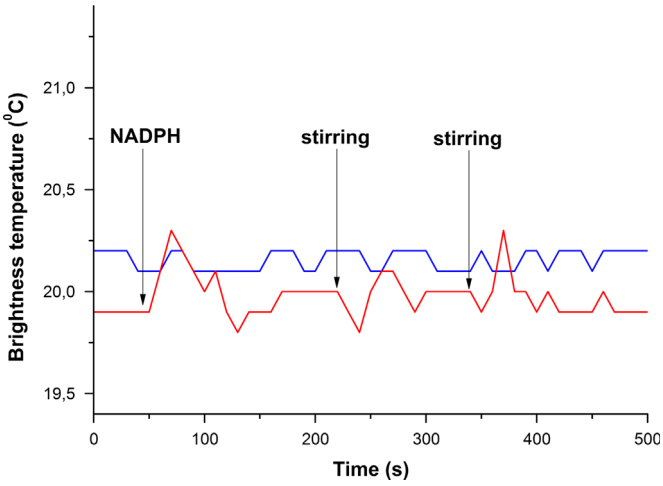


**Fig. 3.** Results represent the function of $T_b(t)$ for cytochrome CYP102 A1 solution at 18 °C.
Experimental conditions:

- cytochrome CYP102 A1 concentration $10^{-8}$ M (blue line) and $10^{-9}$ M (red line);
- the temperature of solution under analysis is 18 °C;
  Arrows indicate time points of addition of electron donor NADPH to the system containing protein and substrate, as well as time points of stirring the solution in the cell.

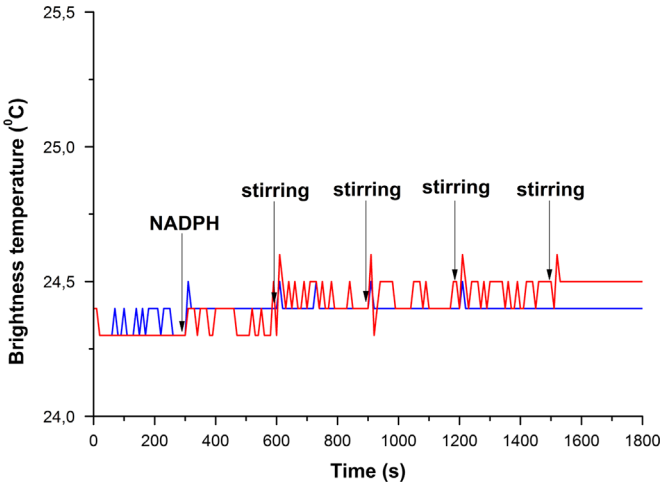


**Fig. 4.** The results of the comparative experiments.
Experimental conditions:

- the temperature of solution under analysis is 23 °C;
- (blue line) the solution under analysis contains protein CYP102 A1 (wild type, $10^{-9}$ M) and substrate (hexane, 1% v/v);
- (red line) the solution under analysis contains protein CYP102 A1 (mutant form, $10^{-9}$ M) and substrate (lauric acid, 0.5 mM);

Arrows indicate time points of addition of electron donor NADPH to the system containing protein and substrate, as well as time points of stirring the solution in the cell.

## 4. Discussion

As we mentioned in the Introduction, CYP102A1 enzyme activity was described in several works [12,13,22,23], whereas a detailed description of methods to determine its activity is only given in [13,24]. Thus, in the study by Munro [13] the enzyme activity was obtained from the characteristic rate of specific oxidation of NADPH in the course of stationary kinetics at 25 °C in 50 mM potassium-phosphate buffer (pH 7.0). The initial oxidation rate was relatively permanent with enzyme concentration $\geq$ 10 nM and amounted to 50 $s^{-1}$. At lower concentrations, when CYP102A1 dimers were supposed to dissociate into monomers, the initial oxidation rate was 10 $s^{-1}$. In the study by Peterson [24], the enzyme activity was estimated from the obtained time function of the reaction product yield as was obtained by reverse-phase HPLC. In this case, the reaction was conducted in 50 mM MOPS, pH 7.4, and the enzyme concentration was 0.8 μM. The enzyme reaction rate determined in this study was 2 $s^{-1}$. Thus, according to the data published, the time of the catalytic cycle stays within the millisecond range.

The results of our study show that a new approach can be proposed for enzyme system researches, based on the detection of the microwave radiation of enzyme-containing solution. Now, let us analyze the data obtained.

It was supposed therefore, that the microwave radiation we observe is due to the change of protein's microenvironment during its functioning. Thus, among the causes of microwave radiation there are:

(1) radiation corresponding to the spinning motion of molecules and ions (OH, $H_2O$ and $H_2O_2$) activated during the catalysis;
(2) increased quantity of ortho isomers resulting from the hydroxylation (reaction) and their interaction with protein;
(3) increased mobility of protein globule during the catalytic reaction.

Factors (2) and (3) affect the structure of water directly, water being a group of nanoclusters rather than a homogenous environment [25,26]. Besides that, these factors influence the structure of an ice-like enzyme hydration shell in the solution, the existence of which at 20 °C is under discussion [15].

Hydroxylation reaction in our test was carried out in conditions of excess of both substrate and electron donor NADPH (initiator of the reaction). Microwave radiation generation was registered after the addition of the reaction initiator, i.e., the electron donor NADPH at 23 °C. We know that at this temperature cytochrome CYP102 A1 exhibits an activity level close to its maximum [21]. The increase in the microwave radiation signal at 23 °C was 2–10 times higher than the noise level both for $10^{-8}$ M and $10^{-9}$ M concentrations (see Section 3). The number of impulses depending on $T_b(t)$ for $10^{-8}$ solution was similarly as for $10^{-9}$ M. The intensity of impulses was comparable at both concentration rates. It is worth noting that the main pool of impulses appears for $10^{-9}$ M solution later than for $10^{-8}$ M solution (see Fig. 2). As seen from Fig. 2, where the effect of generation of microwave radiation is well pronounced, a difference in time of the appearance of a pool of radiation peaks is observed: the pool of radiation peaks appears earlier at lower enzyme concentration. This is possibly due to the fact that the liquid represents a nonequilibrium environment in terms of the ratio of ortho and para isomers of water [15]. The energy imparted to the liquid by the mechanical excitation, connected with fluctuations of the protein globule during the enzyme reaction, is accumulated and, at the same time, initiates loosening of the protein hydration shell and increase in content of ortho-isomers of water upon such conversion of ice clusters. A part of this liberated energy is supposed to be released in the form of microwave radiation. At that, the effect of mechanical excitation of the liquid increases with increase in enzyme concentration, and this leads to the faster accumulation of the energy and, accordingly, to the earlier de-excitation of the medium, i.e., to the reduction in the time of occurrence of pool of radiation impulses.

It is curious to note that at 18 °C the activity of the enzyme decreases, though to a small extent [18], but the microwave radiation intensity under our test conditions is very low. Decrease in the radiation intensity in this case can be connected with such a property of water as phase change at 20 °C [15]. Phase change is accompanied by the change in superficial tension of the water. Thus, phase change during the transition from 23 to 18 °C probably encourages the microwave radiation quenching, connected with the enzyme activity.

At the 39 °C temperature of the medium, when the enzyme is not active [18], no microwave radiation generation was detected. Thus, microwave radiation appears following the activity of the enzyme.

The results of our control and comparative experiments have shown that in all cases when the enzyme activity is reduced, no microwave radiation is observed. So, no microwave radiation was registered from the following systems:

- the solution containing CYP102 A1 (wild type) and hexane as substrate (Fig. 4);
- the solution containing only CYP102 A1 without substrate (Fig. 1);
- the solution containing CYP102 A1 (mutant) and lauric acid as substrate (Fig. 4).

Thus, possibility of generation of microwave radiation from the enzyme solution is related to the activity of the system forming enzyme.

It is to be noted that there is a correlation between the results of the current study and our previous studies. Earlier, by use of AFM monitoring [10] we have demonstrated that CYP102 A1 system functioning is also accompanied by protein globule's impulse fluctuations in second-long intervals. Such turnover rate of the enzyme (~10 ms) can be interpreted as the result of both

microwave radiation generation and globule height fluctuations.

Taking into account that the activity of many enzyme systems is accompanied by protein globule fluctuations we can infer that, in the case of other enzymes, the microwave radiation generation is to be expected. Since microwave radiation can penetrate rather deeply into water (at a depth of several centimeters) and, hence, affect the proteins, it can also exert influence on the activity of other enzyme systems.

## 5. Conclusion

It was found that in the process of hydroxylation of heme-containing enzyme CYP102 A1, the radiation generation of microwave range (3.4–4.2 GHz) can be observed. The discovered effect can be used in enzyme systems studies, particularly as it is associated with the appearance of the interaction of the enzyme system with a fine structure of the microenvironment (which is a non-equilibrium liquid in terms of the ratio of ortho and para isomers of water), resulting in a change of state of this liquid. Thus, the study of this phenomenon is important to clarify the mechanism of interaction of the functioning enzyme system with the microenvironment.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.12.013.

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