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Nanotechnology-based sensors for monitoring single molecules of cytochrome P450 BM3

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Nanotechnology-based sensors – an electrical nanopore detector (END) and an atomic force microscope (AFM) – were used to monitor the behaviour of single molecules of cytochrome P450 BM3 enzyme. An END, based on a solid-state nanopore formed in silicon nitride, has been used to monitor the functional activity of P450 BM3 in the reaction of laurate hydroxylation in the presence of NADPH. The functional activity of the studied enzyme has been confirmed by spectrophotometry. Using high-speed AFM, the dynamics of the enzyme molecules on the surface of an inorganic substrate has been visualized. The obtained results clearly demonstrate the potential of using nanotechnology-based detectors to determine the physicochemical properties of enzymes at the single-molecule level, thus gaining a deeper understanding of the fundamental principles of functioning of enzyme systems.

Keywords: cytochrome P450, solid-state nanopore, enzymatic activity, atomic force microscopy, spectrophotometry.

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Introduction

Devices with nanoscale sensing elements that are comparable in geometric dimensions to single macromolecules of proteins and nucleic acids are increasingly used in fundamental research. Thus, an atomic force microscope (AFM) enables visualization of single protein macromolecules, including enzymes [1, 2], adsorbed on a solid surface; in turn, electrical nanopore detectors (ENDs) based on solid-state

nanopores allow the detection of single enzyme macromolecules [3, 4] and the monitoring of enzymatic processes [5, 6].

Processes in living organisms are catalyzed by enzymes [7]; therefore, understanding any process requires a detailed insight into the mechanism and kinetics of the functioning of the enzymes that regulate it. Currently, macroscopic methods are overwhelmingly used to study the kinetics of enzymatic processes; these methods record an averaged signal coming from an

ensemble of a large number of molecules of the enzyme under study [8]. Analysis of the kinetics of an enzymatic catalytic process at the single-molecule level is more profound and informative, and it is exceptionally useful for understanding processes occurring in living cells [8]. In this regard, the advantages of using ENDs to study enzymatic processes at the single-molecule level are evident. The operating principle of an END consists in recording the ionic current flowing through a nanopore formed in an inorganic membrane that separates two chambers filled with an electrolyte solution [4–6]. The macromolecule of the enzyme under study, when inserted into the nanopore, partially blocks the nanopore, modulating the ionic current flowing thereby. During enzymatic catalysis, changes occur in the structure of the enzyme macromolecule inside the nanopore. These changes, accordingly, causes fluctuations in the ionic current that are then recorded.

It is also worth noting the importance of using AFM to study the physicochemical properties of single enzyme macromolecules – such as their adsorption [2], diffusion properties [9], and enzymatic activity [1]. For instance, the successful use of AFM to determine the activity of single molecules of the enzyme cytochrome P450 BM3 was previously demonstrated [1].

In the present work, we propose the use of an END based on a solid-state nanopore for recording the enzymatic activity of a single molecule of cytochrome P450 BM3 in the hydroxylation reaction of laurate in the presence of NADPH. The solid-state nanopore was formed in a silicon nitride (Si_3N_4 , hereinafter referred to as SiN) membrane. The interaction of the enzyme molecule with the surface of the inorganic membrane can affect the process of insertion of this molecule into the nanopore. In this regard, we proposed the use of AFM, in parallel with END and spectrophotometry (hereinafter referred to as SP), to study the behavior of the enzyme under study on the solid surface of an inorganic silicon-containing substrate. Considering the positive experience of the combined use of nanotechnological (atomic force microscopy) and macroscopic (SP) methods for studying the physicochemical properties of enzymes [2], in the present work we propose the application of this approach to cytochrome P450 BM3. The use of the classical SP method [10] allows the functional

activity of the enzyme under study to be confirmed [2].

The object of study in our experiments was cytochrome P450 BM3, which belongs to the superfamily of heme-containing monooxygenases (cytochromes P450) and catalyzes the monooxygenation of fatty acids [11]. This enzyme is catalytically self-sufficient [11], meaning that it does not require partner proteins for its functioning. This fact makes cytochrome P450 BM3 a very convenient model object for studying the cytochrome P450 system, which plays a key role in the metabolism of xenobiotics [12].

The aim of the present work is to investigate the dynamics of the behavior of a single cytochrome P450 BM3 molecule on an inorganic substrate both before and during the enzymatic reaction.

Materials and methods

Experimental design. To study the functioning of a single cytochrome P450 BM3 molecule in the reaction of laurate hydroxylation in the presence of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), we used an END based on a solid-state nanopore formed in a SiN membrane. In parallel, the functional activity of the enzyme in this reaction was confirmed by the classical SP method [10]. To observe the behavior of the molecules of the enzyme under study on the surface of an inorganic silicon-containing substrate, they were visualized by AFM in liquid.

Reagents and enzyme. Wild-type cytochrome CYP102A1, which was kindly provided by Professor A. W. Munro (University of Manchester, UK), was used in this work; the enzyme was expressed and purified as described by Neeli et al. [10]. The enzyme was also expressed by A. V. Grudo (Institute of Bioorganic Chemistry, NAS of Belarus, Minsk, Republic of Belarus). Sodium laurate (purity $\geq 99\%$) and NADPH were purchased from Sigma (USA). Potassium hydroxide (analytical grade) and monobasic sodium phosphate (Na_2HPO_4 , analytical grade) were purchased from Reakhim (Moscow). Potassium chloride (KCl) was purchased from Fluka (Germany). Dulbecco's phosphate-buffered saline (DPBS buffer, pH 7.4; 8 mmol/L Na_2HPO_4 , 2 mmol/L

KH_2PO_4 , 140 mmol/L NaCl, 10 mmol/L KCl) was prepared from a mixture of salts purchased from Pierce (USA). All solutions used in the experiments were prepared using deionized ultrapure water (18.2 M Ω -cm) obtained with a Simplicity UV system (Millipore, France).

Measurements using END. The END comprised a liquid measurement cell with two chambers of 700 μL volume each, separated from each other by a 40-nm-thick SiN membrane. A nanopore with a diameter of ~ 5 nm was formed in this membrane by electron beam drilling using a JEM-2000F transmission electron microscope (TEM) (JEOL, Japan). Prior to the experiments, both chambers were filled with ultrapure water, which was replaced during the experiments with pure 2 mmol/L DPBS containing 0.6 mol/L KCl. Solutions of cytochrome P450 BM3, sodium laurate, and NADPH were added to one of the chambers to final concentrations of 10 nmol/L, 500 $\mu\text{mol/L}$, and 200 $\mu\text{mol/L}$, respectively. After each measurement, the chip was rinsed with ultrapure water. During measurements, a constant voltage of -400 mV was applied between the chamber volumes using Ag/AgCl electrodes. The ionic current was measured in the 1000 Hz frequency range using an amplifier with an internal noise level of ~ 0.3 pA and recorded with a 16-bit analog-to-digital converter (ADC). The signal was processed using a 1 kHz Butterworth filter. The detector was shielded with a Faraday cage to prevent exposure to external electromagnetic interference.

Measurements using AFM. To investigate the behavior of cytochrome P450 BM3 on the surface of an inorganic substrate, the enzyme was adsorbed onto the surface of a freshly cleaved mica substrate from 2 μL droplet of a 0.25 $\mu\text{mol/L}$ enzyme solution in 10 mmol/L DPBS, pH 7.4, which was incubated on the substrate surface for 3 min and then rinsed off with ultrapure water. The substrate thus prepared was placed into the measurement cell of a RIBM high-speed AFM (manufactured by Prof. T. Ando, Japan) containing 2.5 mmol/L DPBS, pH 7.4. AFM scanning was performed in liquid using NanoWorld probes with a tip radius of less than 10 nm and gold coating (cantilever length 2.5 μm ; cantilever resonant frequency in air and in liquid ~ 1.5 MHz and 0.9 MHz, respectively; spring constant ~ 0.6 N/m).

Spectrophotometric measurements. The activity of cytochrome P450 BM3 in the laurate hydroxylation reaction was determined by the method developed by Neeli et al. [10] using a model 8453 spectrophotometer (Agilent Deutschland GmbH, FRG). In the experiments, 2 μL of a 50 $\mu\text{mol/L}$ enzyme stock solution was pipetted into a 3-mL quartz cuvette with a 1-cm optical path length containing 500 $\mu\text{mol/L}$ sodium laurate in 50 mmol/L phosphate buffer solution, pH 7.0, and mixed thoroughly. After that, an NADPH solution in the same buffer solution was pipetted into the cuvette to adjust the final NADPH concentration in the cuvette to 200 $\mu\text{mol/L}$ [10], and the recording of the time dependence of the absorbance of the solution in the cuvette was started immediately. Measurements were carried out at a wavelength of 340 nm for 600 s at 25 $^\circ\text{C}$.

Results and Their Discussion

Let us consider the behavior of the enzyme during the reaction it catalyzes. Figure 1 shows typical time (t) dependences of the absorbance of the reaction solution at 340 nm (A_{340}) (left) and the ionic current (I) (right), obtained by monitoring the enzymatic reaction process using the SP method and with the END, respectively.

The $A_{340}(t)$ dependence, which illustrates the kinetics of NADPH consumption during the enzymatic reaction [10] and is shown in Fig. 1, confirms that under the experimental conditions the functional activity of the enzyme under study was quite sufficient for the enzymatic process to occur. Indeed, the activity value of cytochrome P450 BM3 determined from the results of our experiments was 20 s^{-1} . This value correlates well with that obtained by Neeli et al. under the same experimental conditions ($50 \pm 2 \text{ s}^{-1}$ [10]). It should be emphasized that the enzymatic hydroxylation reaction of laurate in the presence of cytochrome P450 BM3 proceeds only in the presence of the electron donor, NADPH [10]. Characteristic peaks with an amplitude of 2–3 pA, indicative of fluctuations of the nanopore-confined enzyme macromolecule during into the nanopore during catalysis [6], are observed on the $I(t)$ curve only when all components of the reaction mixture are present in the measurement cell chamber, and are not observed in the absence of NADPH. It should be noted that characteristic peaks are observed on

the $I(t)$ curve for at least 1200 s, indicating that the molecule retains activity during this period of time.

As noted in the introduction, AFM makes it possible to observe the behavior of biological macromolecules on the surface of solid inorganic

substrates [13]. In our AFM experiments, when scanning in liquid (buffer solution with pH 7.4), it was found that the enzyme macromolecules move along the substrate surface, i.e., a surface diffusion process was observed [13]. The AFM images presented in Fig. 2 illustrate this fact.

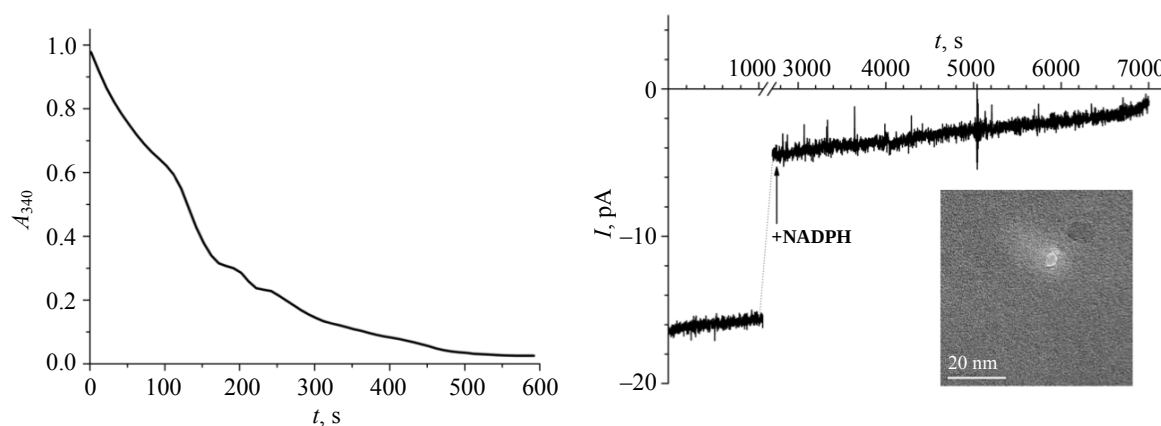


Fig. 1. $A_{340}(t)$ (left) and $I(t)$ (right) dependencies obtained by monitoring the enzymatic reaction process using the SP method and with the END, respectively. The inset shows a TEM image of the END nanopore



Fig. 2. AFM images of cytochrome P450 BM3 macromolecules in liquid, sequentially acquired with a high-speed AFM. Time per frame 1 s. Scan area 200×200 nm. Height scale from 0 to 4.5 nm. Frames were acquired at time points 0 s (left), 23 s (center), and 60 s (right)

It should be noted that silanol (Si-OH) groups are exposed on the mica surface [14]. The same groups are present on the surface of the membrane material (Si_3N_4) [15]. Consequently, it can be expected that the behavior of the molecules of the enzyme under study on the surface of the nanopore membrane is similar to their behavior on the mica surface, visualized by AFM in a solution with the same pH value at which the END experiments were conducted. From this, it can be concluded that the process of surface diffusion of the enzyme molecules can influence their transport toward the nanopore and their insertion into it.

Conclusion

Thus, the possibility of using an END to observe the behavior of a single cytochrome P450 BM3 molecule during the laurate hydroxylation reaction catalyzed by this molecule has been experimentally demonstrated. The activity of the enzyme in the reaction under study was confirmed by the SP method. The process of surface diffusion of the enzyme molecules on an inorganic substrate, which can influence their transport toward the nanopore and their insertion into it, was visualized by the AFM method.

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