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Electrochemical Reduction of Sterol-14α-demethylase from *Mycobacterium tuberculosis* (CYP51b1)

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Abstract—The electrochemical reduction of the heme protein sterol-14α-demethylase from *Mycobacterium tuberculosis* (CYP51b1, or further CYP51) was investigated. Direct electron transfer was demonstrated between CYP51 and graphite screen-printed electrodes modified with gold nanoparticles and with the membrane-like synthetic surfactant didodecyl dimethylammonium bromide. The formal potential of the Fe^{3+}/Fe^{2+} pair, $E_{1/2}$, is equal to −273 mV (vs. Ag/AgCl). The cathodic current corresponding to the reduction of oxygen by immobilized heme protein was registered in the presence of oxygen. Addition of lanosterol, one of the substrates of the CYP51 family, to the oxygenated solution caused a concentration-dependent increase in the reduction current in voltammetric and amperometric experiments. Ketoconazole, an inhibitor of CYP51, inhibited the catalytic cathodic current in the presence of lanosterol. Electrochemical reduction of CYP51 may serve as an adequate alternative to the reconstituted system, which requires additional redox partners for the exhibition of catalytic activity of heme proteins of the cytochrome P450 superfamily.

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Cytochromes P450 are heme-thiolate containing monooxygenases, enzymes occurring in all living organisms and oxidizing both endogenous and exogenous low-molecular-weight organic substances. Cytochromes P450, catalyzing the splitting of the 14α-methyl group of steroid substrates, are classified with the CYP51 family [1]. So far, this system includes 100 enzymes. The role of sterol-14α-demethylase in eukaryotes (participation in the biosynthesis of cholesterol) as well as in plants (phytosterol synthesis) and fungi (ergosterol synthesis) is well known. Unfortunately, nothing is yet known about the actual role of CYP51 in the vital activity of mycobacteria. The structure of the bacterial enzyme CYP51b1 has been determined by X-ray analysis [2]. For the first time the substrate-recognizing sites of sterol-14α-demethylases were identified [3] and the structural–functional motifs of this class of heme proteins were studied [4].

Recently, the ability of various reconstituted systems to reduce CYP51 in the presence of the natural substrate of sterol-14α-demethylases, lanosterol [5], has been the subject of intensive research, with special emphasis on elucidation of the mechanism of their functioning. However, various electron-transfer redox partners used for this purpose (ferredoxin/ferredoxin reductase from spinach, NADPH-dependent reductase isolated from the recombinant form of *Mycobacterium tuberculosis*) did not possess the ability to reduce CYP51. Flavodoxin and flavodoxin reductase from *E. coli* reduced CYP51 at about 20% of the full reduction by sodium hydrosulfite [5]. In view of this, to reveal the catalytic activity of CYP51 in the presence of lanosterol, the direct electrochemical reduction of the enzyme with the aid of nanostructurized with gold nanoparticles electrodes was carried out. It was presumed that in studying the catalytic properties of CYP51 the electrochemical reduction may well be used to replace the reconstituted system, which utilized for its functioning electron donors and specific redox-partner proteins. Being an interesting subject for fundamental P450 studies, CYP51 is also of great practical importance as a drug target. The electrochemical catalytic system provides the universal basis for creation of chips to be used for screening of prototypes of new anti-fungal drugs, herbicides, and cholesterol lowering drugs.

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MATERIALS AND METHODS

Spectral studies were conducted using a Cary 100 Scan UV-Vis spectrophotometer (Varian). All electrochemical measurements were carried out using a computer-controlled Autolab PSTAT10 (Eco Chemie, The Netherlands) with GPES software. Screen-printed graphite electrodes were used as working electrodes, and screen-printed Ag/AgCl as reference electrodes (Elkom, Russia). The diameter of the working electrode was 2 mm. The working buffer contained 100 mM potassium phosphate and 50 mM NaCl, pH 7.4. All electrochemical experiments were carried out at room temperature. The potentials are all referred to the Ag/AgCl screen-printed reference electrode. Cyclic voltammetric (CV) responses were recorded from an initial potential of –700 mV to the end-point potential of 100 mV, scan rate being varied between 10 and 100 mV/sec. The following experimental square wave voltammetry (SWV) parameters were used: initial potential 100 mV, end potential –600 mV (for reduction processes), square wave amplitude 20 mV, step height 5 mV, the frequency being varied between 10 and 100 Hz. Parameters used upon differential impulse voltammetry (DPV) were: pulse amplitude 25 mV, initial potential 100 mV, end potential 600 mV, potential step 1 mV, pulse duration 50 msec.

Reagents. Didodecyl dimethylammonium bromide (DDAB), $HAuCl_4 \cdot 3H_2O$, sodium borohydride, lanosterol, ketoconazole, and Triton X-100 were from Sigma-Aldrich (USA).

In electrochemical experiments, freshly prepared 8 mM lanosterol in isopropanol and 3.6 mM ketoconazole in acetone–dimethylformamide mixture (1 : 1) were used.

Synthesis of colloidal solution of Au stabilized by DDAB. To 1 ml of 0.1 M DDAB in chloroform, 0.5 ml of 10 mM $HAuCl_4 \cdot 3H_2O$ (aqueous solution) was added under vigorous stirring. Then, with continuing stirring, freshly prepared aqueous solution of sodium borohydride $NaBH_4$ (0.2 ml, 0.4 M) was slowly added. After stirring for 2 h, the colored organic layer was separated. Colloidal solution of Au nanoparticles stabilized by DDAB in chloroform was characterized by UV-Vis spectrophotometry (λ_{max} = 520 nm) [6]. The concentration of gold nanoparticles in 0.1 M DDAB was calculated according to stoichiometry of the reaction (5 mM).

CYP51 (20 μM) was kindly provided by Prof. V. M. Govorun (Institute of Physico-Chemical Medicine, Moscow, Russia) [7]. Concentration of CYP51 was determined using the extinction coefficient ε_{450} = 91 $mM^{-1} \cdot cm^{-1}$ of the reduced P450 with carbon monoxide.

Preparation of electrodes. Chloroform solution (2 μl) of 5 mM colloidal solution of gold nanoparticles in 0.1 M DDAB were deposited onto the surface of working carbon electrode and allowed to dry for 10 min. Then 2 μl of heme protein was deposited and electrodes were kept overnight at 4°C in a humid chamber, preventing total drying.

When the experiments were carried out in anaerobic conditions, argon was passed through the electrolyte and through the analyzed enzyme solution for 30 min.

Absorption spectra of CYP51 on DDAB films were recorded after casting of these proteins (4 μl, 20 μM) onto transparent plastic 4 × 2.5 mm sheets with 0.1 M DDAB (5 μl). Plastic sheets were placed in a standard 1.5 ml cuvette. As a control, DDAB sheets were used (5 μl of 0.1 M DDAB in chloroform).

Electrolysis was performed at 400 mV in the presence of 10-80 μM lanosterol. For formaldehyde determination, 0.5 ml of Nash reagent consisting of 4 M ammonium acetate, 0.1 M glacial acetic acid, and 0.04 M acetylacetone were added to 0.5 ml of analyzed solution and incubated at 37°C for 30 min. The extinction coefficient of the colored product at 412 nm was 4 $mM^{-1} \cdot cm^{-1}$ [8, 9].

LC/MS analysis was used to determine high-molecular-weight products (lanosterol derivatives). LC-MS/MS method was used with atmospheric pressure chemical ionization for relative quantification of products of lanosterol electrochemical modification. An Agilent 1100 Series HPLC system was used at a flow rate 0.4 ml/min to supply the solution into the Bruker Esquire Ion Trap mass-spectrometer. HPLC was operated with an RP-18 column (Hewlett Packard, HYPERSIL BOS-C18, 5 μm, 250 × 2 mm) using water/0.1% formic acid (solution A) and acetonitrile/0.1% formic acid (solution B). After an initial time of 2 min at 50% of solution B, the gradient was linearly increased over 8 min up to 100% of solution B, and then decreased back to 50% B in the next 10 min.

The relative amounts of products were assessed by integral peak area of the selected ion chromatogram.

RESULTS AND DISCUSSION

Investigation of catalytic activity of isolated cytochromes from the P450 superfamily requires the presence of redox partners and electron donors (NADPH) [5, 10, 11]. Upon electrochemical reduction of heme proteins of the cytochrome P450 family, the presence of redox partners is not a requirement, which substantially simplifies the catalytic system. The electrochemical approach is especially important when physiological partners of cytochromes P450 are unknown, as is the case with sterol-14α-demethylase from *M. tuberculosis* CYP51b1 (CYP51) [5, 12, 13]. Electrochemical systems execute a dual function: they substitute partner proteins and provide electrons for redox enzymes [14-16].

In the course of electrochemical experiments on CYP51 reduction, screen-printed electrodes were used. The advantages of screen-printed electrodes are the

miniaturization of electroanalysis, the possibility of modification of the electrode surface, low basic current and wide range of working potentials [17]. Usage of liquid-crystal matrices of synthetic membrane-like surfactant films on the electrode surface enables fixing enzymes onto this surface and thus bringing about direct electron transfer [18]. Such membrane-like films contain sufficient water to maintain structure and activity of proteins. The enhancement of sensitivity and the lowering of the limit for determination of analyzed substances of electrochemical sensors are possible with the use of electrodes whose sizes are small enough to pass from the planar diffusion of the substance under study to the semi-spherical one. With diminishing electrode size, the signal/noise ratio is increased [19]. To obtain nano- and microelectrode systems, a variety of methods have been used, such as microelectronic, electronic beam, and ion-beam techniques, X-ray lithography, high-temperature lithography, and photolithography [20]. Nanostructuring of electrodes provides a convenient method for obtaining the nanoelectrode system on the microelectrode surface [21, 22]. To obtain the nanoelectrode system on the basis of gold nanoparticles, we used colloidal DDAB-stabilized nanoparticles [6]. The combination of membrane-like liquid-crystal films and metal nanoparticles provides stable films with high capacity for electron transfer between electrode and heme protein. Inclusion of gold nanoparticles in the films of membrane-like substances enhances the sensitivity of electrodes and stability of their electrochemical parameters. This is explainable in view of the fact that metal nanoparticles on standard metallic or graphite electrode behave as nanoelectrode systems [22-26].

Figure 1 shows a cyclic voltammogram (CV) of DDAB/Au/CYP51 electrode under anaerobic conditions (argon). The midpoint potential $E_{1/2}$ for the Fe^{3+}/Fe^{2+} pair is equal to −273 mV (vs. Ag/AgCl), with the separation of anodic and cathodic peak potentials $\Delta E = 159$ mV. In the potential scan rate in the range from 10 to 100 mV/sec, linear dependence of current amplitude on scan rate was observed, which is typical for surface-controlled process [27] (Fig. 2 and inset). The integration of reductive peaks under anaerobic conditions makes in possible to calculate the number of electroactive particles on the electrode. Upon depositing on the electrode 40 pmol CYP51 (2 μl of 20 μM solution), 1.7-2.0 pmol of the heme protein (4%) appeared to be electroactive. These data are consistent with the results on electrochemical reduction of cytochrome P450cam [18] and of cytochromes P450 from the 2C family [28, 29] in DDAB films.

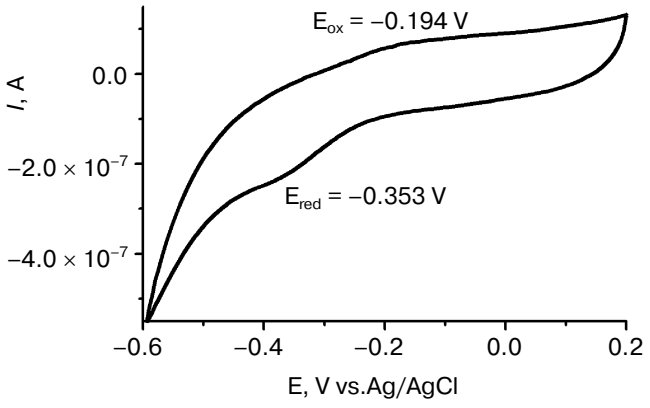

Fig. 1. Cyclic voltammograms of a screen-printed DDAB/Au/CYP51 electrode in anaerobic buffer at the scan rate 50 mV/sec. Electrolyte volume is 1 ml (100 mM potassium phosphate buffer containing 50 mM NaCl and 0.04% Triton X-100, pH 7.4).

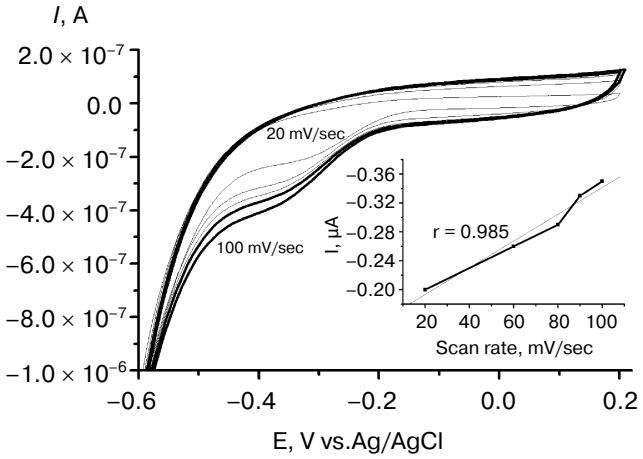

Fig. 2. Cyclic voltammograms of a screen-printed DDAB/Au/CYP51 electrode in aerobic buffer at scan rates 20-100 mV/sec. Electrolyte volume is 1 ml (100 mM potassium phosphate buffer containing 50 mM NaCl and 0.04% Triton X-100, pH 7.4). Inset, dependence of the cathodic peak current on the scan rate (data were taken from Fig. 2).

The electrocatalytic properties of CYP51 are revealed in the presence of oxygen. The ratio between the catalytic current (in the presence of oxygen) and diffusion current (in argon), $I(O_2)/I(Ar)$, is estimated as 1.1-1.2 at the scan rate 50 mV/sec (Fig. 3). The electron transfer rate constant k_s (calculated by Laviron equation [30]) is equal to 1.3 sec^{-1} for the scan rate of 100 mV/sec, which is comparable with the reduction rates in NADPH-dependent reactions. For instance, the reduction rate of cytochrome P450 produced by the first electron in microsomes constitutes 0.51 sec^{-1} (fast phase) and 0.1 sec^{-1} (slower phase) [31].

Since CYP51 immobilization on the electrode surface leads to the incorporation of the enzyme into the membrane-like DDAB film, it seemed to us worthwhile to study the influence of DDAB on the spectral characteristics of the heme protein. The absorption spectrum of the enzyme upon its incorporation into DDAB deposited

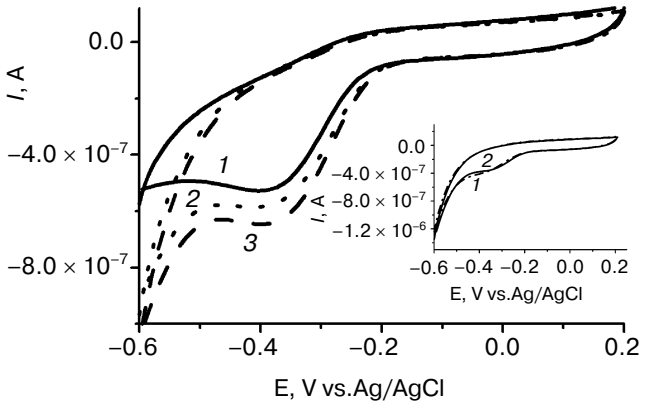

Fig. 3. Cyclic voltammograms of a screen-printed DDAB/Au/CYP51 electrode in anaerobic buffer (argon) (*1*), in aerobic buffer (*2*), and in the presence of 10 μM lanosterol (*3*) at the scan rate 50 mV/sec. Electrolyte volume is 1 ml (100 mM potassium phosphate buffer containing 50 mM NaCl and 0.04% Triton X-100, pH 7.4). Inset, cyclic voltammograms of a screen-printed DDAB/Au/CYP51 electrode before (*1*) and after (*2*) the addition of 36 μM ketoconazole at scan rate 50 mV/sec. Electrolyte volume is 1 ml (100 mM potassium phosphate buffer containing 50 mM NaCl and 0.04% Triton X-100, pH 7.4).

on plastic plates (the basis for electrode printing) has an absorption maximum at 412-413 nm. The absorption spectrum of CYP51 in solution has λ_{max} = 417 nm. This was interpreted as indicating that upon CYP51 incorporation into DDAB, there were no significant conformational changes in the protein structure and that DDAB did not cause its denaturation. It is necessary to point out that hemin in a DDAB film has λ_{max} = 395 nm.

Lanosterol is a substrate of CYP51 with binding constant $K_s = 1 \pm 0.5$ μM [5, 12, 13]. In the reconstituted system where CYP51, flavodoxin, and flavodoxin reductase are used as redox partner, only 1% of lanosterol is subjected to C14α-demethylation [5]. Demethylation of the substrate occurs in three stages, with formation (at the final step) of formaldehyde and a sterol derivative. In the enzymatic electrochemical system, the electrocatalytic reaction in the presence of substrate is possible, which is registered by the availability of the catalytic cathodic current [14-16, 27]. In the case of cytochromes P450, for which the enzymatic reaction proceeds in the presence of two substrates, oxygen and an organic molecule, the increase in catalytic current both in the presence of oxygen and in the presence of substrate (the indication of monooxygenase reaction) is highly indicative. The cyclic voltammogram of DDAB/Au/CYP51 in the presence of lanosterol (Fig. 3) under aerobic conditions confirms the enzyme–substrate interaction. The efficiency of electrocatalysis to lanosterol (L), $I(O_2)/I(L)$, is estimated to be 1.15 at the scan rate of 50 mV/sec. These data are comparable with the earlier reported results on the efficiency of electrocatalysis: CYP2B4/aminopyrine – 1.18 [32] and CYP3A4/verapamil – 1.38 [33].

From the data of square wave voltammetry (SWV), a technique possessing much higher sensitivity and better signal/noise ratio [34, 35], the reduction potential of the DDAB/Au/CYP51 electrode, E_{red}, is equal to −274 mV (vs. Ag/AgCl); in the presence of lanosterol, this potential is even higher, i.e. −293 mV (Fig. 4). The maximal current of SW voltammograms was proportional to frequency in the range of 10-50 Hz. The ratio of the amplitudes of maximal current of SW voltammograms of appropriate reductive processes $I_{sw}(L)/I_{sw}(O_2)$, with baseline correction [35], was found to be 3.3.

Amperometry at controlled potential can be used as a direct method for detection of specific P450 substrates in analyzed media. Figure 5 presents the current response of electrolysis at applied potential −400 mV for the

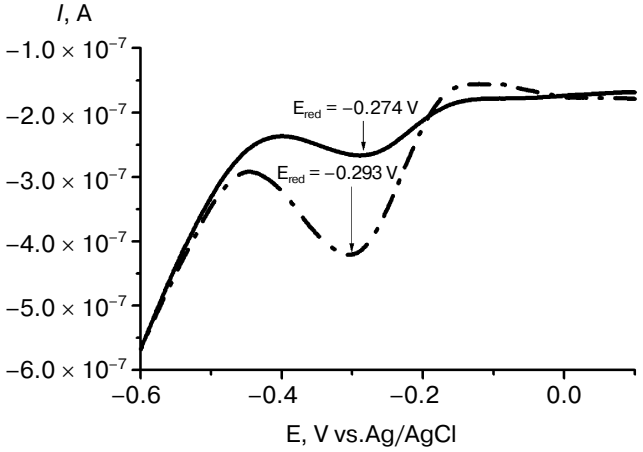

Fig. 4. Reduction SWV of a screen-printed DDAB/Au/CYP51 electrode before (solid line) and after (dot-dashed line) the addition of 10 μM lanosterol. Electrolyte volume is 1 ml (100 mM potassium phosphate buffer containing 50 mM NaCl and 0.04% Triton X-100, pH 7.4). The frequency is 10 Hz.

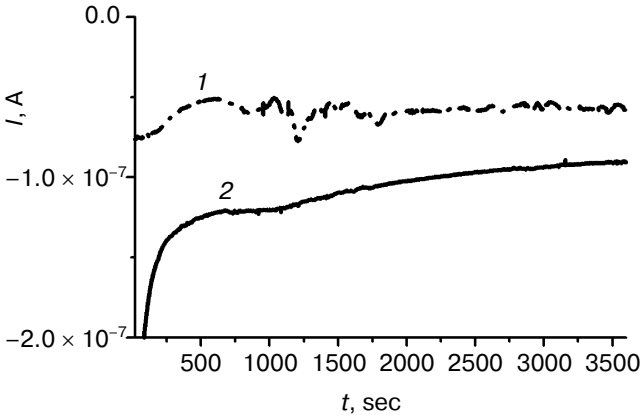

Fig. 5. Amperometric response at controlled potential −400 mV of a screen-printed DDAB/Au electrode (*1*) and DDAB/Au/CYP51 electrode (*2*) in the presence of 80 μM lanosterol.

DDAB/Au (*1*) and for DDAB/Au/CYP51 (*2*) electrode in the presence of 80 μM lanosterol. The sensitivity of the DDAB/Au/CYP51 electrode to lanosterol is 0.22 nA/μM. The electrochemical Lineweaver–Burk plots ($1/I$ vs. $1/[L]$, where I is the steady-state current and $[L]$ is the lanosterol concentration) permit calculation of the electrochemical Michaelis constant $K_m = 30 \pm 2$ μM.

As a result of the CYP51-catalyzed C14α-demethylation, the following reaction products are formed: formaldehyde and the sterol derivative [5]. The analysis of low-molecular-weight product of electrolysis for 1 h at controlled potential –400 mV was carried out. Acetylacetone forms in acid media a colored complex ($\lambda_{max} = 412$ nm) with formaldehyde [8, 9]. The maximal rate of electrocatalytic reaction of C-demethylation determined as formaldehyde formation was equal to $4 \cdot 10^{-4}$ mM/min, and activity calculated per nmol of immobilized enzyme was 10 nmol/nmol CYP51 per min.

As appears from the analysis of mass-spectrometric data after 30-min electrolysis, an increase in 24,25-dihydrolanosterol (24,25-DHL, $M^+ = 429$) is observed if compared to the control sample. Within the next hour the content of 24,25-DHL falls, being substituted by products of 14-demethylation of sterols, namely 24,25-dihydro-4,4-dimethylcholesta-8,14-dien-3β-ol ($M^+ = 413$) and 4,4-dimethylcholesta-8,14,24-trien-3β-ol ($M^+ = 411$). The intensity of the 14-DML related peaks achieves its maximum after 2 h of electrochemical reaction. These data comply with the specific catalytic activity of CYP51 as described in [5]. Therefore, we conclude that basically electrochemical catalysis has a common mechanism to the reconstituted system, which includes redox partners and NAD(P)H.

Ketoconazole is an inhibitor of CYP51 with $K_s = 5$ μM [5]. The cyclic voltammogram of the DDAB/Au/CYP51 electrode before and after the addition of 36 μM ketoconazole is presented in Fig. 3 (inset). Ketoconazole does not cause a significant change in the current amplitude for the DDAB/Au/CYP51 electrode, which is typical for the behavior of inhibitors in electrochemical systems [29, 32]. Square wave voltammetry (SWV) analysis also confirmed the inhibitory influence of ketoconazole on the electrochemical activity of CYP51. Upon addition of lanosterol in the presence of 36 μM ketoconazole, the amplitude of the catalytic current of the SW voltammogram does not reach the maximal value (Fig. 6) and constitutes only 70-73% of the maximal level. Thus, the azole inhibitor ketoconazole ($IC_{50} = 27 \pm 2$ μM), by blocking the active center of the enzyme, lowers the maximal value of the catalytic current in the presence of the substrate. Ketoconazole inhibited the NADPH-dependent sterol-14α-demethylase activity of CYP51 at 20 μM concentration with flavodoxin/flavodoxin reductase as redox partner [5]. The results of amperometric response of DDAB/Au/CYP51 electrode on the addition of lanosterol and on the addition of lanosterol in the presence of 72 μM ketoconazole are shown in Fig. 7 and in axes lanosterol concentration/current change in Fig. 7 inset.

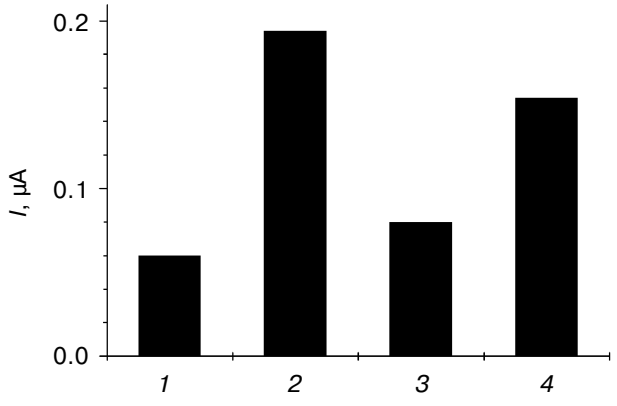

Fig. 6. Peak intensity of reduction SWV of a screen-printed DDAB/Au/CYP51 electrode in aerobic buffer (*1*), in the presence of 10 μM lanosterol (*2*), in the presence of 36 μM ketoconazole (*3*), and in the presence of 10 μM lanosterol and 36 μM ketoconazole (*4*).

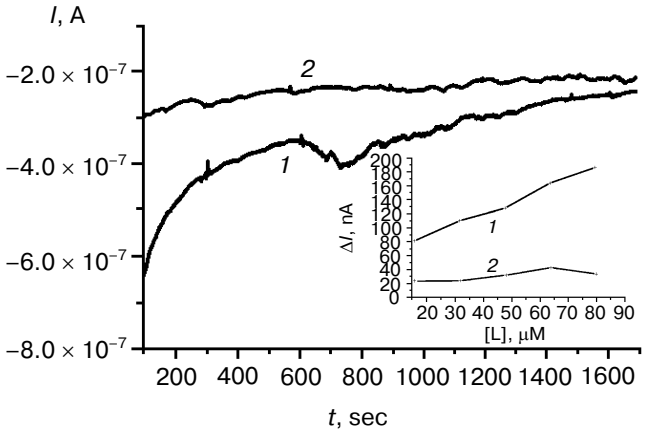

Fig. 7. Amperometric response of a screen-printed DDAB/Au/CYP51 electrode on the successive addition of 2 μl of 80 μM lanosterol at controlled potential –400 mV without ketoconazole (*1*) and in the presence of 72 μM ketoconazole (*2*). Electrolyte volume is 1 ml (100 mM potassium phosphate buffer containing 50 mM NaCl and 0.04% Triton X-100, pH 7.4). Inset, dependence of current increment on the lanosterol (L) concentration at –400 mV (data were obtained from Fig. 7).

In conclusion, the direct electrochemical reduction of CYP51 was carried out with the aid of electrodes nanostructured with gold nanoparticles. The applicability of electrochemical methods to the analysis of enzyme–substrate and/or enzyme–inhibitor interactions was demonstrated.

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