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p-Aminothiophenol modification on gold surface improves stability for electrochemically driven cytochrome P450 microsome activity

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ABSTRACT

The electrochemically driven cytochrome P450 (CYP) reaction of a human microsome is expected to increase efficiency of drug metabolism assays and as well as prove useful for drug research. We previously reported that a nanostructured gold electrode modified with thiophenol (SPh) enabled the electrocatalytic CYP microsome reaction. However, repeated measurements resulted in a significant decrease in the activity. In the present study, we examined the immobilization and electrochemical measurements of the recombinant CYP2C9 microsome on gold electrodes modified with 4-aminothiophenol (SPh–NH_2), 4-hydroxythiophenol, or 4-carboxythiophenol as the promoter. A clear pair of peaks in the voltammogram, assigned to the electron transfer between the electrode and CYP microsome, was observed at the SPh–NH_2 modified surface. In the presence of oxygen and the well-known substrate, tolbutamide, the electrocatalytic current by the CYP reaction was observed. Interestingly, the responses were stable and were maintained compared with those at the SPh modified surface. It was suggested that this stable activity was related to less reactive oxygen species being produced at the SPh–NH_2 modified surface. We also measured the tolbutamide metabolism reactions by the allelic variants of the CYP2C9 microsome on SPh–NH_2 modified electrode. The estimated K_m and k_{cat} values were comparable to those obtained from the solution system. Therefore, SPh–NH_2 modification gave an exquisite surface for electrochemically analyzing the recombinant CYP microsome reaction, indicating the usefulness for rapid assay of the enzyme.

1. Introduction

Electrochemical analysis of a redox-enzyme with direct electron transfer between the enzyme and the electrode is useful not only to gain insight into the catalytic mechanism but also to achieve an efficient assay system for the catalysis. This system can activate the enzyme at an electrode by controlling the electrode potential, instead of using oxidizing and reducing reagents. The reaction kinetics generally can be analyzed by the observed currents [1–4].

Cytochrome P450 (CYP) forms one class of monooxygenases mainly responsible for oxidative transformation of xenobiotics in humans, animals, and plants. The human CYP family plays a crucial role in the oxidative metabolism of most currently available drugs [5]. Single nucleotide polymorphisms of CYPs are shown to be partially responsible for the differences in drug effects among individuals [6]. In addition, drug metabolism via CYPs can cause drug–drug/drug–food interactions that result in toxicities [6,7]. Hence, measurement of CYP activity for drugs and drug candidates is very important [5]. A reaction catalyzed by CYP involves the use of two reducing equivalents supplied by NADPH. In the past decade, electrodes have been shown to be an alternative source of electrons in place of NADPH, where an electrochemical current is measured with an intensity that is proportional to the rate of substrate turnover by the CYPs, for a cost-effective and rapid analysis of the enzymes [8–13]. However, the focus is mostly on isolated CYPs. On the other hand, the membrane (microsome) containing human CYP is frequently used in conventional assays for drug research because of its easy preparation and mimic to the biological system. Unfortunately, the electrochemical reports for the microsome are very limited [14,15]. Fairly recently, we found that the gold nanostructured surface modified with thio-2-naphthol or thiophenol is effective for the direct electron supply from the electrode to the CYP microsome, and electrochemically driven drug metabolism reactions can be observed using voltammetry [16,17]. However, CYP activity drastically decreased at the surface by repetition of the electrochemical measurements in the presence of oxygen. Since the hydrophobic electrode surface is reported to produce the reactive oxygen species [18], it may attack and decompose the active site of the enzyme in the system.

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In the present study, we studied several aromatic thiol derivatives with hydrophilic substituents as the modifier of the gold surface to ascertain the favorable surface for stable observation of the electrochemical CYP microsome reaction. Next, the electrochemical response of CYP2C9 microsome was analyzed using the suitable surface. CYP2C9 is involved in the metabolism of ~16% of therapeutically important drugs such as the anticoagulant warfarin, hypoglycemic tolbutamide and glipzide, and numerous nonsteroidal anti-inflammatory drugs [19]. Several reports have shown that CYP2C9*2 and CYP2C9*3, the two main allelic variants of this isoform, exhibited a reduced catalytic activity with increased K_m values and/or decreased catalytic rate constant (k_{cat}), resulting in decreased intrinsic clearance [20–24]. We report herein our findings that (1) 4-aminothiophenol (SPh–NH_2) modification of the nanostructured gold electrode was very useful for the stable observation of the electrochemical CYP microsome reaction and (2) the drug (tolbutamide) metabolism reactions by the microsome of CYP2C9 and its allelic variants (CYP2C9*2 and CYP2C9*3) could be electrochemically analyzed.

2. Experimental

2.1. Materials

Microsomes CYP2C9 and its allelic variants (CYP2C9*2 and CYP2C9*3) were obtained from Cypex (Dundee, UK). *p*-Aminothiophenol (SPh–NH_2), *p*-hydroxythiophenol (SPh–OH), *p*-carboxythiophenol (SPh–COOH), thiophenol (SPh), and cytochrome *c* (cyt.*c*) were obtained from Sigma–Aldrich. Tolbutamide and 4-hydroxytolbutamide were purchased from Wako (Osaka, Japan) and Cypex, respectively. All other reagents used in this study were of analytical grade.

2.2. Preparation of nanostructured electrode surface

Nanostructured gold surfaces were prepared by sputtering gold onto a gold disk electrode (ø = 3 or 8 mm) using a JEOL (Tokyo, Japan) sputter system at ambient temperature and 0.7 Torr or oxidation reduction cycle (ORC) treatment of the disk electrode in a 0.1 M HCl solution according to the previous method [13]. By estimating the actual surface area from the voltammogram in H_2SO_4 [25], we calculated the surface roughness of the nanostructured gold electrode [13].

2.3. Preparation of CYP immobilized electrode

The surfaces of the gold electrodes were modified with thiol derivatives by overnight dip treatments in ethanol solutions containing ca. 0.1 mM of each thiol. The surface hydrophilicity/hydrophobicity of the modified surfaces was evaluated by contact angle measurements of a water droplet on the surface using a CA-A model contact angle gauge (Kyowa Interface Science, Corp., Japan). After modification, the surfaces were thoroughly washed by ethanol and pure water, and then a 4 μL solution of microsome CYP was placed on the electrode surface for 10–30 min at 25 °C. The electrode was washed with a 0.1 M phosphate buffer containing 20% glycerol. The modification was confirmed (Fig. S2) by FTIR reflection adsorption spectroscopy with a model 4100 Fourier transform infrared spectrophotometer with RAS equipment (JASCO Corp., Japan). Quartz crystal microbalance (QCM) measurements were carried out using an ALS model 400A quartz crystal microbalance analyzer (CH Instruments Inc.). A gold resonator (8 MHz; ø = 5.1 mm) was coated with a monolayer of thiolates and soaked in 600 μL of the buffer solution. Then, 6 μL of the microsome sample was added at 25 °C, and the frequency changes were monitored. The immobilization of the microsome was further confirmed (Fig. S8) by atomic force microscopy (AFM). Imaging was performed using a scanning probe microscopy system (Nano Navi II, Seiko Instruments, Tokyo) under ambient conditions in the dynamic force mode with a silicon cantilever (Seiko Instruments). The cantilever had a nominal spring constant of 2 N/m. Films were prepared on a gold/mica surface (Phasis Ltd., Geneva).

2.4. Electrochemical measurements and product analysis

Voltammetry was performed with an electrochemical analyzer (CH Instruments Inc., USA) with a normal three-electrode configuration comprising an Ag|AgCl|sat. KCl reference electrode, a Pt auxiliary electrode, and a microsome CYP immobilized working electrode. Baseline subtraction for the obtained voltammogram was conducted according to a reported method using SOSA program [26]. Voltammetric data (trumpet plot: potential scan rate vs. peak position) was analyzed using the JellyFit program (courtesy of Dr Lars Jeuken, University of Leeds, Leeds, UK) to estimate the interfacial electron transfer rate constant (k_s), assuming a Butler–Volmer model [27]. Electrolysis was conducted at–0.45 V vs Ag/AgCl for 2 h using a microsome-modified gold electrode ($A = 0.7\ cm^2$) with 400 μL of pH 7.3 buffer containing 500 μM tolbutamide and 0.5% EtOH. Data for three electrolysis experiments were averaged. Product analyses were conducted by high-performance liquid chromatography (HPLC). Electrolyzed solutions were diluted with 0.1 M triethyl ammonium acetate (pH 7). Chromatographic separation was achieved using a HPLC system (Gilson Inc.) using a μBondashpere 250 × 39 mm of C18 column (Waters Inc.) with a linear gradient of 0–60% of CH_3CN at a flow rate of 1 mL/min. Retention times monitored at 240 nm for 4-hydroxytolbutamide and tolbutamide were 12.24 min and 19.01 min, respectively. The K_m values were calculated using a Hanes–Woolf plot from the Michaelis–Menten plot (electrocatalytic current vs. substrate concentration). The k_{cat} value of the immobilized CYP was determined from the amount of product formed in the electrochemical cell during electrolysis estimated from the HPLC analysis [28] using the surface coverage of electrochemically active CYP estimated from the voltammogram without drug and oxygen (non-turnover condition). We avoided a method using the bioelectrocatalytic current to determine the k_{cat} value, because this is affected by the uncoupling intrinsic to the CYP turnover [28].

3. Results and discussion

3.1. SPh–NH_2 modified surface enables direct electron transfer of CYP microsome

We compared the electrochemical responses of CYP microsome using the gold electrodes modified with the aromatic thiol derivatives, i.e., 4-aminothiophenol (SPh–NH_2), 4-hydroxythiophenol (SPh–OH), and 4-carboxythiophenol (SPh–COOH) with a reference of thiophenol (SPh) to ascertain the effective surface for increase in stability of electrochemically driven CYP activity. Fig. 1 shows the cyclic voltammograms of the modified gold electrodes at pH 7.3 under an Ar atmosphere after a casting treatment of microsome CYP2C9. For both SPh–OH and SPh–COOH surfaces, no faradaic currents were observed (Fig. 1(a) and (b)), indicating that these surfaces were not suitable for direct electron transfer of the CYP microsome. On the other hand, a clear pair of peaks were observed in the voltammogram at SPh–NH_2 and SPh modified electrodes. Since no such faradaic currents were observed using control microsome without CYP (Fig. S3), the voltammetric peaks should correspond to the direct electron transfer between the CYP and the electrode. The formal potential ($E^{0\prime}$) at the SPh–NH_2 electrode was estimated to be–0.399 V from the voltammogram and this