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Short communication

Bioelectrochemistry as a tool for the study of aromatization of steroids by human aromatase

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ABSTRACT

Bioelectrochemical conversion of androgens into estrogens was achieved using human aromatase immobilized on glassy carbon electrodes. According to substrate concentration used in the electrochemical cell, it was possible to accumulate the intermediates or to proceed toward the formation of the final estrogen product confirming the distributive nature of catalytic reaction. Furthermore, the catalytic rates showed that the first step of reaction is the limiting one. The results demonstrate that bioelectrochemistry can be employed for understanding complex enzymatic reactions, such as aromatization of steroids.

1. Introduction

Aromatase is a cytochrome P450 that catalyzes a three step reaction leading to the conversion of androgens into estrogens [1–3]. Since this enzyme is overexpressed in estrogen-dependent tumors, it is an important pharmacological target and aromatase inhibitors are currently used to treat breast cancer in post-menopausal women [4]. Furthermore, aromatase plays a role in the development of neurodegenerative disorders such as Alzheimer's disease [5].

Aromatase reaction proceeds through the formation of two stable intermediates (19-hydroxy- and 19-oxo-) deriving from 2 consecutive hydroxylation reactions at C19. In the third and last step, the removal of C19 as formic acid and the abstraction of protons from the A-ring cause the aromatization of the steroid molecule with the generation of the final estrogen product (Scheme 1) [6–8].

The conversion of androgen substrates into estrogen products is mediated by cytochrome P450 reductase (CPR) and requires 6 electrons donated from 3 NADPH molecules [9]. Furthermore, pulse-chase experiments suggest that the enzyme is distributive rather than processive [10]. This means that intermediates of the reactions are free to be accepted in, and can dissociate from, the protein active site according to their affinities for the enzyme.

Here, recombinant human aromatase (rArom), sharing the same structural features as full-length enzyme [11,12], was immobilized on glassy carbon electrodes. Direct electrochemistry and bioelectrocatalysis experiments were performed for the study of its catalytic reaction and comparison to the data for the protein in solution.

2. Material and methods

2.1. Aromatase preparation

Recombinant aromatase (rArom) was purified as described before [13]. For the preparation of rArom in complex with substrate or intermediates, saturating amounts of androstenedione (AD, 10 μM), or 19-hydroxyandrostenedione (19-OHAD, 150 μM) or 19-oxoandrostendione (19-OXOAD, 150 μM) were added in the purification buffers.

2.2. Immobilization procedure

Substrate-free and rArom in complex with AD, 19-OHAD and 19-OXOAD were immobilized on poly-diallyldimethylammonium chloride (PDDA) [14] modified glassy carbon (GC) electrodes (area of 0.07 cm^2, BASi) [15–19]. A 1:1 (5 μl + 5 μl) mixture of pure PDDA and protein solution (85 μM) was cast onto clean GC electrodes and allowed to set overnight at 4 °C.

2.3. Electrochemical measurements

Electrochemical experiments were performed at room temperature in 50 mM phosphate buffer pH 7.4, containing 100 mM KCl as supporting electrolyte, using an Autolab PGSTAT12 potentiostat (Ecochemie). A cell equipped with a platinum counter electrode, an Ag/AgCl (3 M NaCl) reference electrode and a GC working electrode (BASi) was used.

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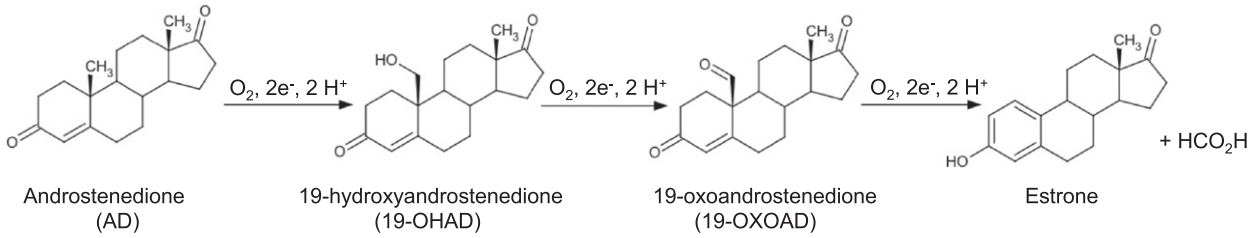

Scheme 1. The three step reaction catalyzed by aromatase.

The detectability of rArom on PDDA electrodes was investigated using cyclic voltammetry (CV) by scanning the potential between 200 and −600 mV at increasing scan rate values (from 20 to 120 mV s^{-1}). CV experiments were performed in a glove box to ensure anaerobic conditions.

Redox properties of rArom were also investigated using square wave voltammetry (SWV) between 0 and −600 mV (step potential: 5 mV; amplitude: 50 mV; scan frequency: 50 Hz).

Electrocatalysis was performed using chronoamperometry with an applied potential of −600 mV for 30 min. To minimize mass transport influence, rotating disk electrodes were used on a BASi RDE-2 rotator system. Chronoamperometry was applied on freshly prepared electrodes and the product obtained after 30 min was immediately quantified by high performance liquid chromatography (HPLC).

2.4. HPLC analysis of the reaction products

Identification and quantification of electrocatalysis products were achieved by HPLC coupled with a diode array UV–vis detector equipped with an Eclipse Plus-C18 column (Agilent Technologies). The mobile phase was a mixture of water and acetonitrile. Standards of AD, 19-OHAD, 19-OXOAD and estrone (Sigma Aldrich, purity ≥ 98%) were used to establish retention times, UV–vis spectra and to construct calibration curves by injecting known amounts of each compound. Mixtures of standards were also injected to exclude potential interferences between the different steroids. AD, 19-OHAD and 19-OXOAD were detected and quantified at 237 nm, whereas estrone was followed at 280 nm (Fig. 2).

3. Results and discussion

3.1. Direct electrochemistry of human aromatase

Direct electrochemistry of rArom (55 kDa) immobilized on PDDA-modified glassy carbon electrodes both in the AD-free and -bound form was performed by CV and SWV (Fig. 1) in anaerobic conditions (<10 ppm oxygen). In both cases, a single redox couple corresponding to heme $Fe^{III/II}$ transition was detected. Reduction potentials measured from SWV were −284 mV and −247 mV versus Ag/AgCl for substrate-free and -bound rArom, respectively (Table 1), consistent with published data on direct electrochemistry of CYP enzymes [16,20–23]. CV applied to protein previously denatured by 6 M guanidinium hydrochloride resulted in a midpoint potential of −42 mV (versus Ag/AgCl). Using CV, for both rArom immobilized in the presence and absence of the substrate AD, a linear dependency was found between peak currents and scan rate (Fig. 1B). This is characteristic of thin film confined electroactive species that are not under diffusion control [24]. Moreover, the calculated surface coverage of rArom was 2.9 ± 0.2 pmol cm^{-2}.

Reduction potentials of rArom in complex with the intermediates 19-OHAD and 19-OXOAD are similar to those obtained for AD complex and positively shifted with respect to the substrate-free enzyme (Table 1). These results are consistent with the substrate-induced positive shift of reduction potential essential for the electron transfer from cytochrome P450-reductase (CPR) to the catalytic heme in cytochromes P450 [25,26].

3.2. Electrocatalysis experiments

Experiments were initially performed with immobilized substrate-free rArom. In the presence of saturating amounts of AD (up to 100 μM) no reaction products were detected by HPLC analysis.

On the other hand, when rArom already in complex with AD was immobilized on the electrode surface, the product of the first step of the reaction, 19-OHAD (0.15 ± 0.05 μM), was detected (Fig. 2). This

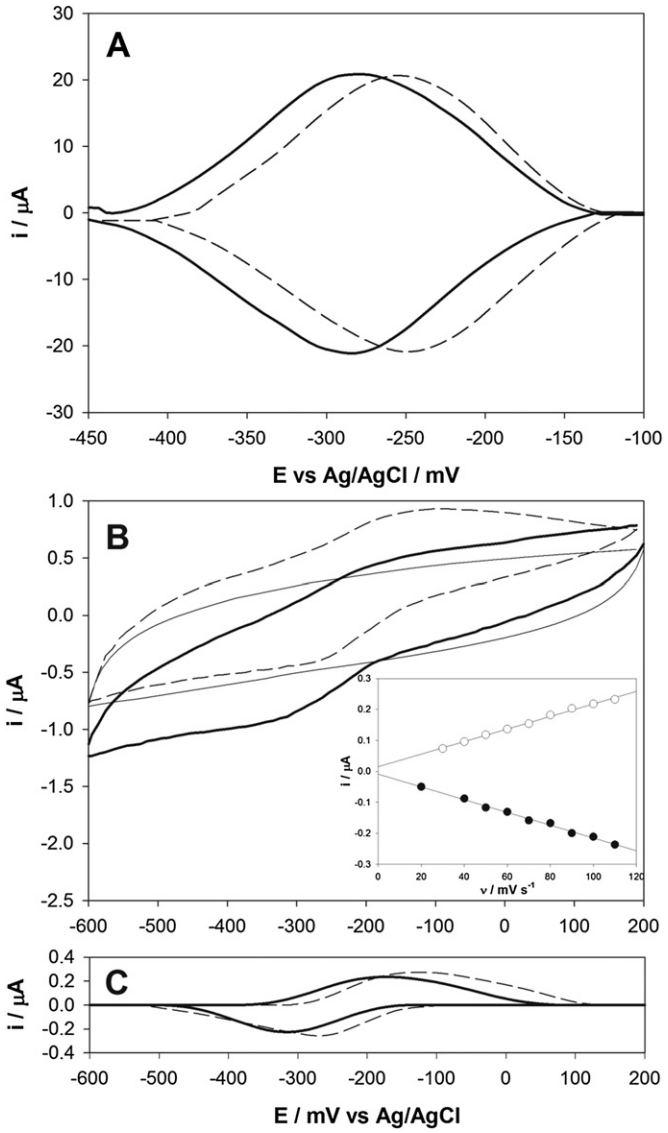

Fig. 1. A) Square wave, B) original and C) buffer (thin solid trace)-corrected cyclic voltammograms of substrate-free (thick solid line) and AD-bound (dashed line) immobilized rArom. AD concentration was 10 μM, the scan rate for CV was 120 mV s^{-1}. Inset: plot of cathodic (filled circles) and anodic (open circles) peak currents versus scan rate for substrate-free-rArom on PDDA GC electrodes.