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Surface coverage dictates the surface bio-activity of D-amino acid oxidase

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ABSTRACT

This work presents a systematic study on the relationship between the adsorption mechanism and the surface bio-activity of D-amino acid oxidase (*pk*DAAO). This rational approach is based on measuring the characteristic filling and relaxation times under different experimental conditions. With such a goal, real-time adsorption–desorption experiments at different degrees of surface coverage were performed tuning the electrostatic and hydrophobic interactions by changing the pH condition for the adsorption and the substrate properties (silica or gold). Surface bio-activity was measured *in situ* by amperometry using the bio-functional surface as the working electrode and *ex situ* by spectrophotometry. On both solid substrates, *pk*DAAO adsorption is a transport-controlled process, even under unfavorable electrostatic interactions (charged protein and substrate with the same sign) due to the high percentage of basic amino acids in the enzyme. On silica, the relaxation step is electrostatic in nature and occurs in the same time-scale as filling the surface when the substrate and the enzyme are oppositely charged at low surface coverage. Under unfavorable electrostatic conditions, the relaxation (if any) occurs at long time. Accordingly, the bio-activity of the native *pk*DAAO is preserved at any surface coverage. On gold, this step is driven by hydrophobic interactions (pH-independent) and the surface bio-activity is highly dependent on the degree of surface coverage. Under these conditions, the surface bio-activity is preserved only at high surfaces coverage. Our results clearly indicate that *pk*DAAO bio-functionalized surfaces cannot be coupled to amperometry because the analyte interferes the electrochemical signal. However, this simple bio-functionalized strategy can be joined to other detection methods.

1. Introduction

The adsorption of proteins on solid substrates is of primary importance in biomedical applications ranging from biosensors to tissue engineering [1]. For instance, protein–substrate interactions give a real insight into the design of highly specific bio-functional surfaces and the development of new biomaterials. The key issue behind these applications is to control the strong perturbation produced by the solid substrate on the surface bio-activity. Although this topic has been extensively explored [2–4] the relationship between the adsorption mechanism and the surface bio-activity is still not well understood. This limitation is mostly due to the extra complexity provided by the lateral interactions upon crowding the solid substrate with protein molecules [5,6].

Understanding the protein adsorption mechanism comprises the study of several stages, from the transport of the molecule toward the substrate up to the final protein conformation, which ultimately dictates the surface bio-activity [7]. After the first protein attachment step, the molecules optimize their interaction with the substrate, leading to relaxation with some degree of spreading. The extent of the protein spreading depends on the ratio between the rates of these two steps (*i.e.* filling the surface and protein relaxation). Experimentally, this ratio is evaluated from the characteristic filling (τ_f) and relaxation (τ_r) times which can be calculated from real-time adsorption profiles [8]. A limiting value of this ratio is given by $\tau_f \gg \tau_r$, where all the protein molecules have the same relaxed conformation in steady state because a quick relaxation occurs after the attachment. This relaxation step strongly affects the protein conformation of all the adsorbed molecules and the degree of surface coverage does not depend on the filling rate. In

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the other limit ($\tau_f \ll \tau_r$), the relaxation step is too slow and the adsorbed proteins do not change their conformation upon adsorption. In this case, the degree of surface coverage is also independent of the filling rate because the substrate crowding is so fast that prevents protein–substrate optimization. Finally, when both steps are competitive ($\tau_f \sim \tau_r$), the protein spreading is correlated with the surface coverage because the relaxation of the molecules strongly depends on the available surface sites [7,9].

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the conversion of D-amino acids to α-keto acids in the presence of O_2 to yield H_2O_2. DAAO is strictly stereospecific and oxidizes a variety of D-amino acids which have been recently linked to aging and pathological conditions such as schizophrenia, epilepsy, Alzheimer's disease, and renal disease [10–14]. Therefore, DAAO bio-functionalized surfaces have been used as the first step toward biosensor development to specifically detect D-amino acids [15–18]. Since H_2O_2 is one of the enzymatic products, the bio-recognition event has been mostly detected by electrochemical methods [19,20]. In fact, several articles have reported the performance of DAAO-based biosensors using this enzyme from different sources, diverse solid substrates, and immobilization strategies [13,15,17,18]. Most of these works are mainly devoted to the analytical response of the designed biosensors with little attention regarding the interactions between the enzyme and the substrates and, more importantly, the consequences of the surface perturbation on the bio-activity of adsorbed DAAO. Furthermore, DAAO has also been used for biocatalytic applications, particularly in the two-step conversion of the natural antibiotic cephalosporin C to 7-amino cephalosporanic acid [21,22]. Although this process represents the most important industrial use of DAAO, the enzyme–support interactions and their effect on the bio-activity have not been entirely described.

The purpose of this work is to systematically study the relationship between the adsorption mechanism and the surface bio-activity of *pk*DAAO (commercially available from pig kidney). This rational approach to correlate the adsorption mechanism and surface bio-activity implies measuring τ_f and τ_r under different conditions, as previously indicated. With such a goal, real-time adsorption–desorption experiments at different degrees of surface coverage were performed tuning the electrostatic and hydrophobic interactions by changing the pH of the adsorption and the substrate properties (silica or gold). Surface bio-activity was measured *in situ* by amperometry using the bio-functional surface as the working electrode and *ex situ* by spectrophotometry to evaluate the extent of the relaxation step.

2. Experimental

2.1. Chemicals and solutions

All reagents were of analytical grade and were used without further purification: *pk*DAAO (Sigma), D-alanina (Fluka), H_2O_2, $KMnO_4$ (Cicarelli), KH_2PO_4, K_2HPO_4, $K_4P_2O_7$, $HClO_4$, NaOH, $KClO_4$ and KOH (Baker), $Na_2C_2O_4$ (Riedel-de-Haën), and $KClO_4$ (Erba). Aqueous solutions were prepared by using 18 MΩ cm^{-1} resistance water (Milli-Q, Millipore, Billerica, MA). H_2O_2 concentration was determined by titration with $KMnO_4$ which was standardized against $Na_2C_2O_4$. 5 mM buffer solutions (PB) were prepared by dissolving the desired amount of KH_2PO_4, K_2HPO_4, $K_4P_2O_7$ in water or 100 mM $KClO_4$ (PBS) and adjusting the pH with either 2 M KOH or 2 M $HClO_4$ to reach pH 5.0, 7.0 or 8.5. The pH measurements were performed with a combined glass electrode and a digital pH meter (Orion 420A+, Thermo, Waltham, MA). Unless noted, all experiments were performed at room temperature (26 ± 2 °C).

2.2. Substrates

The sorbent properties were studied using two different substrates: silica (Si/SiO_2) and gold (Si/SiO_2/Au), both prepared on silicon wafers (100 mm, Silicon Valley Microelectronics, Inc., Santa Clara, CA). In order to obtain a silica layer of about 100 nm thick (essential for obtaining a high sensitivity in reflectometry experiments [23]) the wafers were oxidized at 1000 °C for 1 h (thickness was verified by ellipsometry) and cut in strips (1 cm × 4 cm) following the crystallographic plane of silicon (1 0 0). Prior to each adsorption experiment, Si/SiO_2 strips were cleaned with boiling piranha solution (2:1 H_2SO_4:H_2O_2) and rinsed thoroughly with deionized water. (*Caution! Piranha solution is a powerful oxidizing agent that reacts violently with organic compounds; it should be handled with extreme care*). Si/SiO_2/Au substrates were prepared by sputtering (SPI #12162-AB) gold on the oxidized wafer strips up to reaching a 10 nm layer (thickness was verified by ellipsometry) and used without any further treatment. This thickness did not substantially modify the optical behavior of the 100 nm SiO_2 layer [24].

2.3. pkDAAO adsorption mechanism

Real-time adsorption–desorption experiments were performed in a reflectometer (AKZO Research Laboratories, Arnhem), equipped with a stagnation point flow cell as described elsewhere [25,26] using Si/SiO_2 and Si/SiO_2/Au substrates. Briefly, from 0 to 200 s, only PB was introduced into the cell and a stable baseline was obtained. Then (from 200 to 2700 s), the flow was switched from PB to a *pk*DAAO solution (prepared in the same buffer). Next (between 2700 and 3500 s), the flow was switched back to the initial PB solution in order to analyze the desorption process by dilution. Desorption was also induced by applying a constant potential (potentiostat CHI760C; CH Instruments, Inc.). This desorption was induced on the Si/SiO_2/Au substrates (working electrode) by using a platinum wire (counter electrode) and a $Ag/AgCl/KCl_{sat}$ (reference electrode) placed directly in the reflectometer cell from 3500 to 4000 s. The adsorption–desorption experiments were conducted with *pk*DAAO at different concentrations (ranging from 0.001 to 0.100 mg mL^{-1}) and pH values (5.0, 7.0 and 8.5).

As described elsewhere [27], to calculate the sensitivity factor (Q-factor) that provides the proportionality constant between the measured signal and the adsorbed amount, the substrate was modeled as a Si substrate (refraction index of 3.80) with a 100 nm SiO_2 layer (refraction index of 1.46) and a 10 nm Au layer (refraction index of 0.10) immersed in aqueous solution (refraction index of 1.333) and the increment in the refraction index with the protein concentration (dn/dc) was considered to be 0.18 [28]. The calculated Q-factors resulted in 30 ± 5 mg m^{-2} for Si/SiO_2 and 110 ± 5 mg m^{-2} for Si/SiO_2/Au.

The protein transport toward the substrate was well controlled by the stagnation point flow of the reflectometer setup, so the supply rate depended on the geometry of the cell, the flow rate, the diffusion coefficient of the macromolecule (D), and its concentration in solution (C_p) [29]. Hence, for a particular cell setup and protein molecules, the supply rate was directly proportional to C_p. The proportionality coefficient provided the transport constant ($k_{tr} = 5 \times 10^{-6}$ m s^{-1}) of the protein ($D = 6 \times 10^{-11}$ m^2 s^{-1}) at the specified cell setup.

2.4. Enzyme bio-activity

2.4.1. pkDAAO in solution

Electrochemical experiments in solution were carried out by using two amperometric methods to determine the H_2O_2 concentration produced by the enzymatic reaction in the presence