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Electrochemical detection of the 2-isobutyl-3-methoxypyrazine model odorant based on odorant-binding proteins: The proof of concept

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ABSTRACT

We developed an electrochemical assay for the detection of odorant molecules based on a rat odorant-binding protein (rOBP3). We demonstrated that rOBP3 cavity binds 2-methyl-1,4-naphtoquinone (MNQ), an electrochemical probe, as depicted from the decrease of its electrochemical signal, and deduced the dissociation constant, $Kd_{MNQ} = 0.5(\pm 0.2)$ μM. The amount of MNQ displaced from rOBP3 by 2-isobutyl-3-methoxypyrazine (IBMP), a model odorant molecule, was measured using square-wave voltammetry. The release of MNQ by competition led to an increase of the electrochemical response. In addition, this method allowed determination of the dissociation constant of rOBP3 for IBMP, $Kd_{IBMP} = 0.5(\pm 0.1)$ μM. A negative control was performed with a non-binding species, caffeic acid (CA). The determined binding affinity values were confirmed using a fluorescent competitive binding assay and isothermal titration microcalorimetry. This electrochemical assay opens the way for designing robust, reliable and inexpensive odorant biosensors.

1. Introduction

The demand for versatile tools able to detect odorant molecules is rapidly increasing with potential applications on many different fields, such as assessment of various foodstuffs and beverages [1], environmental contamination monitoring [2], medical diagnostics [3,4] or detection of explosives [5]. The olfactory system has the ability to detect and discriminate myriads of odorant molecules at a very low concentration. This olfactory function is mediated by the combinatorial activation of olfactory receptors expressed by olfactory neurons located in the olfactory epithelium [6]. The understanding of the olfactory system offers the promise of insight into volatile compound detection, especially to design biosensors. The use of biological molecules as sensing material has been demonstrated to be efficient to improve selectivity of the sensing material-target analyte interaction [7].

Vertebrate odorant-binding proteins (OBPs) are small (around 18 kDa) and highly soluble proteins secreted in large amount in the nasal mucus covering the olfactory epithelium. OBPs have been demonstrated to reversibly bind odorants with affinities in the micromolar range including 2-isobutyl-3-methoxypyrazine (IBMP) [8,9]. This smelling compound generates an extremely intense odor of bell-pepper and is one of the odorants with the lowest detection threshold for humans. Although there is no absolute evidence to support their functional roles in vertebrates, OBPs are good candidates for the transport of odorants towards olfactory receptors through the nasal mucus [10]. OBPs have been described in a variety of vertebrate species including cow, pig, rabbit, mouse, rat, elephant and human beings [11–13]. In rat, three OBPs (rOBP1, rOBP2 and rOBP3) have been described with quite different amino acid sequences and binding properties [8,9,14]. rOBP1 and rOBP3 bind a large number of odorants whereas rOBP2 appears to be specific for long-chain aliphatic compounds [14]. Vertebrate OBPs belong to the lipocalin family whose members share a common folding pattern of an 8-stranded antiparallel β-barrel linked together by seven loops, and connected to an α-helix. The β-barrel creates a central apolar cavity, called calyx, whose role is to bind generally hydrophobic odorant molecules [15]. An interesting property of OBPs is their exceptional thermal stability. They can withstand temperatures up to 80 °C–and even 100 °C for some of them–for several minutes without suffering irreversible denaturation and recovering their full binding activity when returned to room temperature [16]. Moreover, the compact folding of OBPs makes them resistant to proteolytic degradation. Recombinant OBPs have been expressed in bacteria as well as in yeast in high yields [12,14,17], making their production simple and economical. Consequently, these proteins have a great potential to be used as sensing elements in biosensors [18].

Electrochemical methods are versatile tools to probe biochemical interactions and quantify small biologically relevant molecules [19,20]. These methods are characterized by their sensitivity, low cost and instrumental simplicity and portability. In addition, the development of electroanalytical techniques [21] resulted in a better understanding of electrode processes and in improvement in measurements. The use

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of pulse techniques such as differential pulse voltammetry or square wave voltammetry (SWV) [22] has helped to increase the resolution and the sensitivity. The discrimination between the target and other compounds present in a biological matrix is now possible thanks to the control of the electrode surfaces. Lastly, the use of protective and/or selective barriers and recourse to catalytic species prevent adsorption of contaminants and perturbation of the signal.

To date, only a few studies employed OBPs as biosensing elements to detect odorant molecules combined with different monitoring techniques [23–26]. Di Pietrantonio et al. [23] employed surface acoustic wave biosensor array. The other groups [24–26] monitored the presence of odorants by electrochemical impedance spectroscopy. This technique is able to detect the changes in electrochemical properties of the electrode surface upon ligand binding, i.e. not the odorant molecule itself but rather the changes due to the ligand binding, namely conformational changes of protein, alterations in charge or hydrophobicity.

Here, we report a novel electroanalytical binding assay for the detection of odorant molecules based on OBP-odorant interactions. The chosen OBP was rOBP3 because this protein was shown to be highly expressed in bacteria [9] and is therefore easily produced. Using competitive fluorescent binding assays with a fluorescent probe and isothermal titration calorimetry, we investigated the ability of rOBP3 to bind redox molecules. We selected 2-methyl-1,4-naphtoquinone (MNQ) that exhibits a high affinity for rOBP3. We chose IBMP as a model odorant because it is the reference molecule to study the binding properties of vertebrate OBPs [8,9]. We monitored MNQ released from rOBP3 using square wave voltammetry and confirmed our results using competitive fluorescent binding assay and isothermal titration calorimetry.

2. Materials and methods

2.1. Materials

2-methyl-1,4-naphtoquinone (MNQ, Sigma-Aldrich) and caffeic acid (CA, Sigma Aldrich) were prepared in phosphate buffer (PB, 50 mM, pH 7.5). 2-isobutyl-3-methoxypyrazine (IBMP, Sigma Aldrich) and N-phenyl-1-naphtylamine (NPN, Acros Organics) were prepared in MeOH and then diluted in PB. The final MeOH concentration never exceeded 0.1% (v/v). Potassium ferricyanide ($K_3Fe(CN)_6$, Sigma-Aldrich) was used as 2 mM PB solution. 11-mercaptoundecanoic acid (MUA, Sigma-Aldrich) and 6-mercapto-1-hexanol (MH, Sigma-Aldrich) were used as 0.5 mM PB/MeOH (50/50 v/v) solutions. The Ni^{2+}-chelating Sepharose™ High Performance resin (Ni^{2+} resin) was purchased from GE Healthcare.

2.2. rOBP3 production and purification

The recombinant His-tagged rOBP3 protein was produced using *Escherichia coli* and purified as described [14] with a few modifications. Briefly, after purification on a HisTrap™ Ni^{2+}-chelating column (GE Healthcare), the protein was extensively dialyzed 24 h against a buffer (100 mM sodium phosphate, 300 mM NaCl, pH 7.5) containing 5% (v/v) acetonitrile (to remove potentially bound contaminants) at 4 °C. After an additional 24 h dialysis against the same buffer without acetonitrile, rOBP3 was aliquoted and stored at −20 °C. rOBP3 purity was assessed by SDS-PAGE. rOBP3 concentration was determined by spectrophotometry using its extinction coefficient measured at 280 nm (ε_{280} = 13,075 $M^{-1}\cdot cm^{-1}$ [27]).

2.3. Fluorescent-based binding assay

Fluorescent ligand binding assays were performed with 2 μM of rOBP3 solutions in PB buffer, as reported previously [13,17] using a Cary Eclipse fluorometer (Agilent Technologies). Briefly, NPN probe was dissolved in MeOH/PB solution (50/50) (v/v) as a 1 mM solution. One μL successive NPN probe aliquots were added to 1 mL of rOBP3 solutions. After 3 min incubation time, spectra were recorded at 20 °C between 350 and 500 nm using an excitation wavelength of 337 nm (peak emission in presence of rOBP3 is 405 nm). For each addition, the fluorescence was measured at 405 nm and the normalized fluorescence was calculated. The hyperbolic titration curves were fitted with a nonlinear regression method [28] using Origine®Pro8 Software. Competitive binding assays were carried out with 2 μM of rOBP3 incubated using 2 μM of NPN. After addition of competitor compound, the fluorescence spectrum was recorded after stabilization of the signal (3–4 min). For each addition, the relative fluorescence was calculated.

2.4. Isothermal titration microcalorimetry (ITC)

ITC experiments were carried out at 25 °C with a VP-ITC microcalorimeter (GE Healthcare) using MilliQ water as a reference. For comprehensive description of ITC applications and data analysis, refer to the Leavitt & Freire [29]. Before initiating the titration run, rOBP3 solutions (25–30 μM in PB) were thoroughly degassed under vacuum. Ligands solutions (250 μM in PB) were injected in 25 successive 10 μL aliquots at 3.5 min interval. Heats of dilution determined in the absence of rOBP3 were subtracted from the titration data prior to curve fitting. Thermodynamic parameters were obtained by fitting the values to a single site binding model through nonlinear regression using Microcal Origin® software as described in the Supplementary data.

2.5. Electrochemical measurements

All the electrochemical experiments were performed with a PGSTAT302 N (Metrohm) potentiostat. Data were collected and analyzed using Nova® 1.7 Software. Cyclic voltammetry and square wave voltammetry (SWV) measurements were carried out by means of a three-electrode configuration consisting of the modified gold disk (1.6 mm diameter, Bioanalytical Systems) as working electrode, a platinum wire as counter electrode and an Ag/AgCl, 3 M NaCl electrode as reference. Potentials are reported versus the Ag/AgCl, 3 M NaCl electrode ($E_{Ag/AgCl,\ 3\ M}$ = −0.040 V vs. SCE). Square Wave voltammograms were recorded between 0 and −0.6 V (step potential 1 mV, frequency 25 Hz, amplitude 20 mV). All solutions were deoxygenated with argon prior to introduction of the modified working electrode, and a positive overpressure of argon was maintained above the electrolyte during the entire measurement performed at room temperature. The gold electrode was cleaned and the SAM prepared by passive immobilization of thiol compounds as previously described [30]. The newly cleaned gold electrode was immersed in a freshly prepared MUA solution overnight. Then, the modified gold electrode was rinsed with PB.

MNQ calibration curve experiments were performed by SWV in a solution of 100 μM of $K_3Fe(CN)_6$ by adding aliquots of 0.2 mM of MNQ solution to get a final concentrations of 0–4 μM. MNQ binding experiments were carried out with 1 μM of rOBP3 solutions with an excess of Ni^{2+} resin by adding increasing quantities of 0.2 mM MNQ solution in distinct OBP solutions. After being incubated for 5 min, the solutions were centrifuged for 20 s and the concentration of MNQ in the supernatants was determined by SWV with 100 μM of $K_3Fe(CN)_6$. Competitive binding assays were performed by adding increasing amounts of ligands to obtain final concentrations of 0–10 μM for IBMP and 0−40 μM for CA into distinct 1 μM of rOBP3 solutions incubated with an excess of Ni^{2+} resin and 1 μM of MNQ. The free MNQ concentration was measured by SWV as described above.

3. Results and discussion

3.1. Production, purification and functional characterization of rOBP3

Recombinant rOBP3 was expressed as a His-tag N-terminal fusion using *Escherichia coli* as previously described [14]. The His-tag allows