Probing redox reaction of azurin protein immobilized on hydroxyl-terminated self-assembled monolayers with different lengths

Ali Hamzehloei [a,*], S. Zahra Bathaie [b], Mir Fazlollah Mousavi [a]

[a] Department of Chemistry, Faculty of Sciences, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran
[b] Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

ARTICLE INFO

ABSTRACT

Immobilization of proteins with controlled electron transfer properties is an important task for future biomedical applications. In this work, immobilization of azurin on gold electrodes modified with 6-mercaptohexanol (MCH), and 11-mercaptoundecanol (MUD) self-assembled monolayers (SAMs) was studied by electrochemical techniques. The immobilized azurin showed a quasi-reversible redox response on MCH/Au and MUD/Au electrodes, with $E_{1/2} = 0.11$ V vs. Ag/AgCl and $\Delta E_p = 10$ mV and 40 mV, respectively. Cyclic voltammograms (CVs) of azurin at different pHs revealed that proton-coupled redox reaction of azurin is a one-electron, three-proton process. The tunneling electron transfer rate constant (k^0) of immobilized azurin on MCH/Au electrode was estimated 1.35 (±0.05) $\times 10^3$ s^{-1} using a fast scan CV method, and that on MUD/Au electrode was measured as 120 ± 10 s^{-1} by scanning electrochemical microscopy (SECM). In SECM studies, the approach curves were recorded at different substrate overpotentials with different surface coverages of azurin. For extraction of kinetic parameters, azurin was reduced on MUD/Au electrode via tunneling electron transfer across the SAM, and oxidized back via solution-phase tip-generated ferrocyanide. The bimolecular electron transfer rate constant (k_{BI}) between immobilized azurin and [Fe(CN)$_6$]$^{4-}$ mediator was estimated as 3×10^8 mol^{-1} cm^3 s^{-1}. Based on distribution of hydrophilic and hydrophobic amino acids on azurin structure, and the distinct voltammetric behavior of azurin on MCH and MUD SAMs as hydroxyl-terminated SAMs vs. methyl- and thiol-terminated SAMs, possible orientations of azurin on different SAMs were also assessed.

1. Introduction

Azurin is a small bacterial protein with 128 amino acids, and one of the simplest redox-active proteins that a single copper ion, Cu (II), in its structure acts as a redox-active center. The copper ion is coordinated by Cys112, Gly45, His46, His117 and Met121 residues, and azurin's properties stem from geometric structure of the copper binding site [1]. Electrochemical and kinetic properties of azurin have extensively been studied. Although azurin can be immobilized on an Au electrode surface through its exposed disulfide moiety (Fig. S1), previous studies have shown that direct immobilization of azurin on a bare Au electrode makes the protein electrochemically inactive [2]. To circumvent this problem, one can use solid electrodes that are modified with a self-assembled monolayer (SAM), at the nanometer scale.

SAM-modified electrodes with high degrees of molecular orientation are usually used for electrochemical investigations of immobilized proteins vs. studies of proteins in a solution phase. The reasons behind the development of SAM-modified electrodes for proteins' electrochemical studies are, first, some proteins are being denatured on metallic bare electrodes, while SAM modified electrodes, as a biocompatible surface, prevent proteins from denaturation [2]. Second, SAM-modified electrodes provide an ability to study fast electron transfer (ET) of the proteins by removing their sluggish diffusion [3]. Third, SAM-modified electrodes pave the way for protein investigation with high sensitivity in trace amounts [4]. Lastly, it is possible to have a control-over-orientation of immobilized protein, by using different functional groups on the SAM [5].

Different research groups have attempted to study the electrochemical behavior of azurin on modified electrodes. It has been already shown that wild type of azurin (*Pseudomonas aeruginosa*) can be adsorbed through exposed hydrophobic residues on a methyl-terminated alkanthiol SAM [3,4,6–8]. The effect of SAM length on the ET kinetics of azurin has been studied [9,10]. In addition, adsorption possibility of azurin on a pyrolytic graphite electrode (PGE) has also been reported [11]. Electrochemical properties of immobilized azurin on SH-/CH$_3$-terminated alkylsilane SAM modified indium tin oxide electrode has also been investigated [12]. In another study, hybrid of azurin-gold nanoparticles has been investigated using scanning tunneling microscopy and CV techniques [13]. Previous studies on electrochemical behavior of azurin, immobilized on SAM-modified electrodes, have shown that Faradaic signal of azurin on a 1:1 mixture of OH- and CH$_3$-terminated SAM is higher than that on a pure CH$_3$-terminated SAM

* Corresponding author.
 E-mail address: hamzehloei@gmail.com (A. Hamzehloei).

[14]. However, the electrochemical behavior of azurin, immobilized on a pure OH-terminated SAM, as well as the mechanism of azurin conjugation on OH- and CH$_3$-terminated SAMs have not yet been studied.

One of the important applications of SECM technique is the study of ET kinetics of biomolecules on SAM-modified electrodes, developed by Liu and coworkers [15]. The main advantages of SECM over CV technique in measurement of ET kinetics of proteins is the possibility of simultaneous measurement of the k_{BI} (bimolecular ET rate constant between solution-phase redox probe and immobilized redox protein), and k^0 (tunneling ET rate constant between the immobilized redox protein and underlying electrode). Based on this methodology, ET kinetic studies of cytochrome c protein immobilized on COOH-terminated [16,17], and phenolic-terminated SAMs have been reported [18]. In addition the ET kinetics of some porphyrins immobilized on SH-terminated SAMs have been reported [19,20]. In this study, we have investigated the immobilization of azurin on a 6-mercaptohexanol (MCH) SAM-modified gold electrode (MCH/Au) with fast scan CV method, and that on an 11-mercaptoundecanol (MUD) SAM-modified electrode (MUD/Au) with SECM technique. It is considerable to mention that there is no report on ET kinetics study of azurin using SECM until now. Azurin electron transfer kinetic parameters were extracted and the redox behaviors of azurin on OH-terminated SAMs were fully compared with that on the other SAM-modified electrodes. In addition, the possible orientations of azurin on OH- and CH$_3$-terminated SAMs and the binding modes that are involved in azurin conjugation to the SAMs were investigated. This is an interesting contribution to understand ET on an electrode/metalloprotein interface, a topic with impact on the future design of bioelectronic devices where the interface engineering is of vital importance.

2. Material and methods

2.1. Chemicals and reagents

Azurin, potassium ferrocyanide tri-hydrate (K$_4$[Fe(CN)$_6$]·3H$_2$O), potassium ferricyanide (K$_3$[Fe(CN)$_6$]), 6-mercaptohexanol, and 11-mercaptoundecanol were purchased from Sigma-Aldrich and used without further purification. Other chemical agents, used in this study, were purchased from Merck. Phosphate buffer solutions were freshly prepared using KH$_2$PO$_4$ and were adjusted to different pHs with diluted KOH and HCl solutions. The experiments were carried out at room temperature. Doubly-distilled water was used to prepare the solutions. Before each experiment the solutions were degassed by 10 min nitrogen purging.

2.2. Instrumentation

Cyclic voltammetry studies were carried out in a three-electrode cell, involving an Au (bare or SAM-modified) working electrode (2 mm diameter, CHI), a platinum auxiliary electrode, and an Ag| AgCl|saturated KCl reference electrode. Electrochemical investigations were accomplished using an Autolab electrochemical analyzer (PGSTAT30 potentiostat, Eco Chemie, B. V. Utrecht, Netherlands) equipped with a fast analog scan generator (SCANGEN) and a fast analog-to-digital convertor (ADC750). To remove double layer charging current from CVs of azurin on an MCH/Au electrode, which is considerably higher at high scan rates, all the voltammograms were background-subtracted. In this process the voltammogram of MCH/Au electrode (in the absence of azurin) was subtracted from that of azurin/MCH/Au electrode in corresponding scan rate. SECM experiments were carried out using a CHI 900b electrochemical analyzer (CH Instruments, Austin, TX). For SECM studies we used a 25-µm-diameter Pt electrode (RG = 10) as a tip electrode, bare or modified 2-mm-diameter gold electrode as a substrate, a Pt wire as the counter electrode, and an Ag|AgCl|saturated KCl as a reference electrode. A homemade Teflon cell with a quartz window which allows visual positioning of the tip

near the substrate surface by a video microscope (Watec, Japan), was used in this study. The gold substrate was fixed in an upright orientation on the bottom of the SECM cell with a thin layer of parafilm. Approach curves were recorded by moving the tip toward the bare or modified Au substrate at a constant speed of 3 µm s^{-1}. While approaching the substrate, the tip potential was held at a constant potential to guarantee diffusion-limited current of solution-phase mediator at the tip. For kinetic measurements, the experimental approach curves were fitted to theory to yield k^0 values. All the solutions were purged with nitrogen before measurements.

2.3. Substrate preparation and SAM formation

First, the gold disk electrode was extensively polished to a smooth and mirror-like finish at a polishing pad and then sonicated in a de-ionized water. The electrode was further cleaned by consecutive potential cycling from −0.5 to +1.3 V vs. Ag/AgCl at a scan rate of 50 mV s^{-1} in 0.1 M H$_2$SO$_4$ until the characteristic cyclic voltammogram for a clean gold electrode was obtained [21]. Uniformity of the electrode surface was also checked under an optical microscope.

For preparation of MCH and MUD SAMs on an Au electrode, the pretreated electrode was rinsed with de-ionized water, and incubated in a 1.0 m methanolic solution of either MCH or MUD for 24 h. The SAM modified electrode was washed thoroughly with doubly-distilled water to remove physically adsorbed materials, and then the electrode was immediately used for electrochemical experiments.

2.4. Azurin protein immobilization

In this study, the azurin solutions, were freshly prepared before use (in PBS buffer, 10 mM, pH 7) and their concentrations were checked spectrophotometrically (ε 5100 M^{-1} cm^{-1} at 628 nm). For immobilization of azurin on a SAM-modified electrode, the electrode was immersed into a 100–200 pM protein solution for 12–24 h at 4 °C.

2.5. Ultramicroelectrodefabrication

The ultra-microelectrode (UME) tip was prepared by heat sealing a platinum wire (25 µm diameter, CHI) in a glass capillary under vacuum according to the procedure reported previously [22]. The tip was connected to a larger copper wire through the use of silver epoxy. Then the tip of the UME was exposed by polishing the head of the glass capillary. The RG values of UMEs were adjusted while polishing and continually checked by an optical microscope. The prepared UMEs, were characterized by CV in a 1.0 mM ferrocyanide solution and prepared in a 0.1 M PBS buffer (pH 7.0). The CV of fabricated tip is shown in Fig. S2, which displays a well-defined sigmoidal CV, characteristic of a good-quality UME tip.

3. Theory/calculation

Scheme 1 illustrates the principle of the method that was used to study the kinetics of ET between azurin, immobilized on MUD/Au electrode, and the underlying gold substrate. A suitable potential (0.5 V) was applied to the UME tip to oxidize the ferrocyanide to ferricyanide. The applied potential is more positive than E^0 of the dissolved ferro/ferricyanide redox mediator, so the mediator is oxidized at a diffusion-limited rate at the UME tip electrode (Eq. (1)).

$$\text{Reaction on tip}: \quad \text{Fe(CN)}_6{}^{4-} - \text{e}^- \rightarrow \text{Fe(CN)}_6{}^{3-} \tag{1}$$
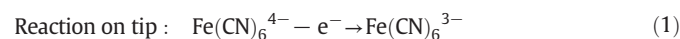
The steady-state limiting current at the tip, $i_{T,\infty}$, is given by Eq. (2).

$$i_{T,\infty} = 4nFDc_0a \tag{2}$$