Journal of Electroanalytical Chemistry 787 (2017) 14–18

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Direct wiring of the azurin redox center to gold electrodes investigated by protein film voltammetry

Razvan C. Stan [a,*,1], Alexander Kros [b], Namik Akkilic [c], Nusrat J.M. Sanghamitra [a], Jeroen Appel [b]

[a] Leiden Institute of Physics–Huygens Laboratories, Leiden University, P.O. Box 9504, 2300 RA Leiden, The Netherlands
[b] Leiden Institute of Chemistry–Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands
[c] Mesa + Institute for Nanotechnology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

ARTICLE INFO

Article history:
Received 28 November 2016
Received in revised form 9 January 2017
Accepted 17 January 2017
Available online 18 January 2017

Keywords:
Protein active site
Cyclic voltammetry
Molecular electronics
Protein denaturation
Click chemistry

ABSTRACT

We present two surface-immobilization schemes that connect an azurin variant with mutations in the redox cavity to two oligophenylvinyl (OPV) molecular wires, along with an electrochemical study of their electron transfer (ET) properties. OPV embedded within alkanethiol films tether selectively the azurin mutants from solution and further connect them onto electrodes, as does protein reconstitution with OPV in solution followed by complex adsorption. Stable cyclic voltammograms are measured in either scenario, with highest ET values ($k0 \approx 740\ s^{-1}$) comparable to those observed for the gold-adsorbed native azurin ($k0 \approx 400\ s^{-1}$). Furthermore, subtle structural differences between the structurally-analogous OPV allow for the investigation of the optimal electronic conduit between the redox center and supporting gold electrodes (1.6–1.7 fold difference).

1. Introduction

Electron transfer reactions involving metalloproteins are central to cellular processes such as respiration or photosynthesis. While the interplay between redox events and molecular recognition is important in biological systems, this effect is also associated in vitro with electron shuttling to appropriate electrodes [1,2] Direct electron transfer from proteins onto surfaces occurs however in less than 10% of the known enzymes [3,4]. Consequently, for most adsorbed proteins, substrate access to the active site may be precluded, direct electrical communication from the redox center to electrodes is hindered, proteins are not activated solely by the applied potential and therefore fast electron transfer rates are difficult to achieve [5,6]. Furthermore, protein adsorption onto electrodes may lead to aggregation or denaturation, heterogeneously adsorbed protein populations and poor data reproducibility [7,8]. Attempts to embed proteins in conductive polymers often result in random orientations of the proteins with respect to the surface and in non-optimal electron transfer communication with the underlying electrode [9]. An alternative approach that circumvents these limitations involves wiring protein active sites directly to molecular linkers for improved electron transfer to electrodes. Such coupling may be achieved by using long molecular wires that can probe natural deep-buried protein cavities [10] or, for redox sites situated close to protein surfaces, through mechanical displacement of native axial ligands by external linkers [11,12]. For most proteins however, protein engineering is required to facilitate external access to the active site [13]. We demonstrate here this novel approach through tethering the active site of an engineered azurin from *Pseudomonas aeruginosa* to gold electrodes, by means of two structurally-analogous molecular wires. Within its surface-exposed hydrophobic patch shielding the redox center, native azurin contains a copper-coordinating histidine at position 117 that can be replaced by glycine (H117G) with minimal active site structural reorganization (transition between type-1 to type-2 copper site) [14]. Addition of exogenous ligands such as pyridine or imidazole fully recovers in the H117G azurin variant the spectroscopic properties of the wild type protein [15] and the thus-reconstituted azurin H117G can be further connected to electrodes using ligands that also contain thiol functionalities. This unique immobilization strategy of azurin H117G proceeds either directly on the gold surface via click-on chemistry with ligands from pre-formed mixed self-assembled monolayers (SAM), as presented in Fig. 1, or by a two-step process consisting of complex formation with molecular wires in solution, followed by adsorption onto unfunctionalized gold electrodes.

Functionalization of gold electrodes with the alkanethiol-embedded molecular wires used in this study has previously been characterized [16], as has the gold adsorption of reconstituted azurin H117G [17]. We extend here the work on the electrochemistry of azurin adsorbed

* Corresponding author.
E-mail address: strazvan@usp.br (R.C. Stan).
[1] Present address: Institute for Biomedical Sciences, University of São Paulo, CEP 05508-900 São Paulo, Brazil.

http://dx.doi.org/10.1016/j.jelechem.2017.01.039
1572-6657/

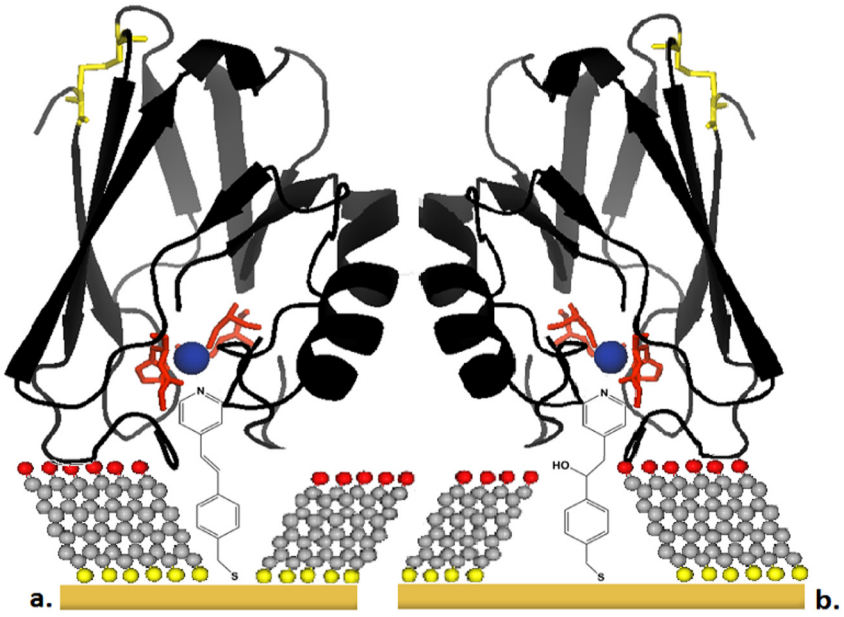

Fig. 1. Scheme (not to scale) of the immobilization of azurin H117G to pre-functionalized gold electrodes using π-conjugated molecular wires (linker 1, a.) or containing a hydroxyl group that interrupts the π-conjugation (linker 2, b.). This azurin variant can accommodate copper-coordinating pyridine head groups, while thiol end-groups further connect the complex to gold surfaces. Redox active tethering to the surface-adsorbed molecular linkers requires their prior co-assembly with 8-hydroxy-1-octanethiol to prevent unspecific protein adsorption.

onto a multitude of SAM [18,19], by using molecular ligands that directly coordinate the redox center in azurin and we observe the formation of stable protein films and fast electron transfer rates.

2. Materials and methods

2.1. Au surfaces

Commercial Arrandee (Germany) gold surfaces were annealed with a butane torch, thereafter rinsed with Milli-Q grade water and dried under N_2 flow. The surface flatness and cleanliness was checked regularly with AFM, prior to functionalization with SAM/protein complexes [16].

2.2. Preparation of molecular wires

The organic synthesis of the molecular wires (linker 1 and linker 2) used in this study has previously been presented [16]. The thiolate acetyl-protective groups used for both linkers were removed with 2 μL of saturated NH_4OH upon gold adsorption.

2.3. Mixed SAM assembly on gold surfaces

8-Hydroxy-1-octanethiol (HSC_8-OH), 99% purity, was purchased from Dojindo (Japan) and dissolved in ethanol. 100 μM of linkers 1 or 2 (dissolved in ethanol) was incubated for 1 h, followed by the addition of 1 mM HSC8-OH for 22–24 h on annealed-gold surfaces (at 20 ± 2 °C). Samples were rinsed with ethanol, dried under N_2 stream and readied for electrochemical measurements.

2.4. Reconstitution of azurin H117G-linker in solution and complex gold adsorption

One equivalent of $Cu(NO_3)_2$ was added to 50 μM of apo-azurin in 50 mM of 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0, at 20 ± 2 °C. This was followed after 1 min by the addition of linker 1 or linker 2 at 1:1 and 1:2 concentration ratios respectively, with respect to azurin H117G-Cu(II). Reconstituted H117G azurin was incubated on gold surfaces for 10 min. To remove unbound proteins, the surface was subsequently rinsed with MES buffer and Milli-Q grade (18 MΩ) water and blown-dried with Ar.

2.5. Cyclic voltammetry

A 20 μL droplet of protein solution was inserted between the working electrode and Saturated Calomel Electrode (SCE, Radiometer K-401), used as reference electrode (+244 mV vs NHE). Platinum wire served as a counter electrode, following the previously described experimental setup [5]. Cyclic voltammetry was carried out at 20 ± 2 °C with a μ-Autolab electrochemical analyzer (EcoChimie, NL), equipped with a PGSTAT 30 potentiostat and a fast analog scan generator (SCANGEN), in combination with an analog-to-digital converter (ADC750), at 2 mV potential steps. Anaerobic measurements were performed inside a home-built Faraday cage in 75 mM Na_2SO_4 and 50 mM MES buffer, at pH 6.0. Electrochemical data were analyzed using accessory software of the Autolab system and with OriginPro (OriginLab, USA).

3. Results and discussion

Cyclic voltammograms of holoazurin H117G (azurin H117G with Cu^{2+}) reconstituted in solution with either of the two structurally-related molecular wires and thereafter adsorbed on bare gold electrodes consist of single, well-delimited quasi-reversible signals, due to single electron redox activity of its type-1 copper center (Fig. 2.a). Assuming a one-electron transfer process, sub-monolayer coverages of reconstituted azurins on gold were calculated (Table 1). These values are consistent with AFM and ellipsometrical measurements [17] and with other electrochemical data [20].

In order to further investigate whether the surface packing on gold surfaces of the protein complexes influences their redox potential, as observed with other systems [21] we have embedded the molecular wires into supporting alkanethiol films, as shown in Fig. 2.b.

Electrochemical responses were stable, with 10% signal loss after scanning for 1 h (the reconstituted complexes in solution are stable for 4 h until copper is reduced and linkers uncouple from the active site) [17]. Values for the surface coverage for either of the protein complexes tethered to the protruding pyridine rings are at least an order of magnitude lower compared to direct adsorption of reconstituted azurin