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Voltammetry and in situ scanning tunnelling microscopy of de novo designed heme protein monolayers on Au(111)-electrode surfaces

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Abstract

In the present work, we report the electrochemical characterization and in situ scanning tunnelling microscopy (STM) studies of monolayers of an artificial de novo designed heme protein MOP-C, covalently immobilized on modified Au(111) surfaces.

The protein forms closely packed monolayers, which remain electroactive upon immobilization. In situ STM images show circular structures indicating that MOP-C stands upright on the surface in accordance with the molecular design. Despite the large spatial extension of MOP-C, about 5 nm in height, conditions could be found where tip/sample interaction is minimal and proteins could be imaged without detectable tip interference.

The results indicate further that the structural sensitivity of (in situ) STM depends to a significant extent on associated electron transfer kinetics. In the present case, the heme group does not contribute significantly to the tunnelling current, apparently due to slow electron transfer kinetics. As a consequence, STM images of heme-containing and heme-free MOP-C did not reveal any notable differences in apparent height or physical extension. The apparent height of heme-containing MOP-C did not show any dependence on the substrate potential being varied around the redox potential of the protein. The mere presence of an accessible molecular energy level is not sufficient to result in detectable tunnelling current modulation.

Keywords: De novo design; Artificial protein; Electrochemical STM; Single-crystal electrochemistry

1. Introduction

Structural organization and functional properties of two-dimensional films of proteins, DNA-based molecules and other biomolecules adsorbed on solid supports are of broad scientific and technological interest. Focus areas include: two-dimensional adsorption patterns [1a–1c], electron transfer (ET) mechanisms of immobilized redox metalloproteins and -enzymes [1d], construction of artificial biological ET chains [1e–1g], protein/membrane interactions and drug delivery [1h,i], biologically induced corrosion and biofilms [1j,k], biocompatibility of metallic implants [1m] and analytical applications involving immobilized enzymes, antibodies and DNA-based molecular probes [1o–1q]. Most approaches to these areas have relied on macroscopic concepts and ensemble-averaged observables such as adsorption isotherms. Methods include classical surface spectroscopies, including infrared and Raman spectroscopy, ellipsometry, surface plasmon resonance spectroscopy, quartz crystal microbalance and bioelectrochemistry. The latter is here understood as interfacial electrochemical ET between metallic electrodes and adsorbed redox metalloproteins or other biological redox molecules [2,3].

Novel microscopic techniques approaching the single-molecular level of surface organization and function of biological molecules have, however, been introduced. These include, first, single-molecule fluorescence spectroscopy, single-molecule surface-enhanced resonance Raman spectroscopy (SERRS) [4] and, not in the least, the scanning probe microscopies such as atomic force (AFM) and scanning tunnelling microscopy (STM) directly in the aqueous biological media (in situ AFM and STM) [5]. Combination of in situ STM with other state-of-the-art physical electrochemistry, particularly single-crystal, well-defined electrode surfaces [6], has opened

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new approaches to interfacial bioelectrochemistry of redox metalloproteins and metalloenzymes at the monolayer and single-molecule levels [5].

In this report, we combine voltammetry on "ultra-clean" single-crystal Au(111)-electrodes with in situ STM for the investigation of a de novo designed synthetic 4-α-helix bundle heme protein, MOP-C, immobilized on Au(111) surfaces (MOP: modular organized protein, "C" for cysteine), shown schematically in Fig. 1. The protein is composed of two different types of helices, A and B, bound alternatingly to the cysteines of the template T via maleimidopropionyl (mp) linker groups. Each helix A contains a histidine (his) residue that provides a binding site for a heme group bis-histidine coordinated in the holo MOP-C. The shielding helices B each possess a cysteine residue at the binding site opposite to the template T, which may be used to immobilize MOP-C directly on a Au(111)-surface (see, however, below) [7].

Such artificial metalloproteins or maquettes bridge the gap between natural proteins and artificial nanoscopic objects. Their secondary and tertiary structural elements can be varied systematically [8]. This facilitates approaches to new levels of metalloprotein structure-functional detail and functions have in fact been assigned to de novo designed proteins [9]. At the same time, this paves the way for efficient protein and solid surface functionalization, for example in novel types of biosensors.

The results presented extend and support previous investigations based on cyclic voltammetry, resonance Raman and surface-enhanced resonance Raman spectroscopy of MOP-C and other related MOPs [10]. They substantiate in situ STM as a powerful tool to image large (on the STM scale) protein structures, up to several nanometers, such as reported previously for natural redox metalloproteins and DNA-based molecules [11]. These observations also hold implications for intriguing and so far unsettled issues regarding the mechanism of electron tunnelling through the immobilized proteins, via the metal redox centers [11].

2. Experimental

2.1. Materials

All chemicals for electrochemistry and STM were of highest purity grade available. 9-Fluorenyl-methyloxycarbonyl (Fmoc)-protected amino acids, (2(1-*H*-benzotriazol-1-yl-1.1.3.3-tetramethyluronium)-tetrafluoroborate (TBTU), diisopropylamine (DIEA), piperidine and 5-(4-(aminomethyl)-3, 5-bis(methyloxy)phenoxy)valeric acid/polyethylene glycol/polystyrene (PAL-PEG-PS) resin were from Perseptive Biosystems. Pre-loaded Fmoc-Gly-NovaSyn TGT resin was from Nova Biochem. HPLC grade acetonitrile was from SDS. All other chemicals, including DMSO, KH_2PO_4, K_2HPO_4, K_2SO_4, cysteamine and *N*-succinimidyl-3-maleimidopropionic acid, were highest purity grade from Aldrich/Sigma or Merck.

2.2. Synthesis

The synthesis of the cyclic decapeptide serving as the template for the 4-α-helix-bundle proteins is described in detail elsewhere [12]. The Fmoc/*t*-butyl protecting group strategy was used except for the ε-NH_2 group of the C-terminal lysine and the SH group of the N-terminal cysteine which were protected by allyloxycarbonyl (alloc) and acetamidomethyl (Acm) groups, respectively. Before cleaving the peptide from the resin, the N-terminus of helix A was modified by 3-maleimidopropionic (Mp) acid for attachment to cysteines of the template [13]. The N-terminus of helix B was acetylated, the alloc group of the C-terminal lysine selectively cleaved and 3-maleimidopropionic acid coupled to the ε-NH_2 group of this lysine. The amino acid sequences for the template with alternating protecting groups of trityl (Trt) and Acm at the cysteines and the two helices with amidated C-terminus are:

Template: cyclo[C(Trt)-A-C(Acm)-P-G-C(Acm)-A-C(Trt)-P-G-]
Helix A: Mp-G-N-A-R-E-L-H-E-K-A-L-K-Q-L-E-E-L-L-K-K-W-A-NH_2
Helix B: Ac-C(Acm)-G-G-N-L-E-E-F-L-K-K-F-Q-E-A-L-E-K-A-Q-K-L-L-K(Mp)-NH_2

The helical peptides A and B were linked to the template for protein assembly [12b,13]. After selective deprotection of the Acm groups of the N-terminal cysteine, the modular protein with two free cysteines (MOP-C) was available. The helices of MOP-C are oriented in an antiparallel way such that the individual dipole moments cancel each other.

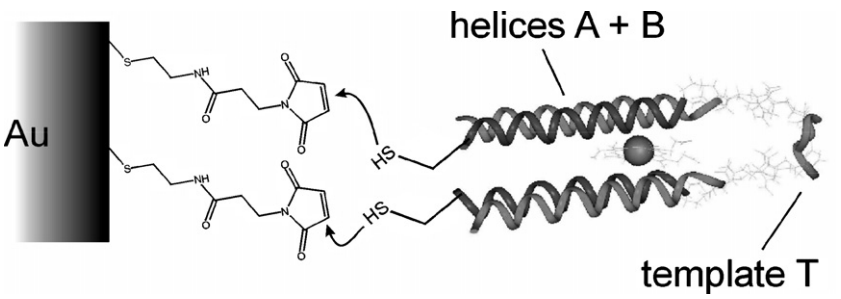

Fig. 1. Immobilization of MOP-C on a modified gold substrate; the MOP-C protein is shown to the right, with its template T and the helices A and B bound to the template in an alternating way. Only helix B contains cysteine for surface attachment and helix A histidine for heme ligation.