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Reconstitution of peroxidase onto hemin-terminated alkanethiol self-assembled monolayers on gold

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ABSTRACT

Reconstitution of enzymes onto surface tethered cofactors may be considered as a powerful tool both for design of biorecognition interfaces with a molecular-level control over spatial distribution and orientation of biosensing elements and for fundamental studies of their structure–function relationships. Here, reconstitution of horseradish peroxidase (HRP) on the heme cofactor covalently attached to the mixed self-assembled monolayers (SAM) on gold electrodes was studied. The efficiency of the holoenzyme formation was assessed by analysis of the hemin electrode reaction and bioelectrocatalytic activity of HRP. The formal potential, $E^{0\prime}$, of the electrode-tethered hemin approached −340 mV vs. Ag/AgCl, while the heterogeneous electron transfer (ET) rate constant, k_s, for its redox transformation ranged between 245 and 340 s^{-1}, depending on the SAM composition. After enzyme reconstitution, both the $E^{0\prime}$ and k_s values for the hemin ET reaction showed a very little variation, while the onset of electrocatalytic reduction of H_2O_2 shifted by ca. 500 mV more positive by this approaching the potentials of compound I of native HRP. Both the onset potential and bioelectrocatalytic currents were lower than observed with native HRP adsorbed on gold (Ferapontova and Gorton, 2001), being consistent with a lower surface population of the holoenzyme and/or less efficient ET pathway.

1. Introduction

Proteins and enzymes, containing iron protoporphyrin IX (heme b) cofactor are involved in a variety of biochemical processes, such as oxygen transport and storage, lipid peroxidation, and metabolism of NO and other reactive nitrogen and oxygen species accomplished by hemoglobins [1,2], catalytic oxidations of a range of substrates by peroxidases using peroxides as electron acceptors [3,4]; shuttling electrons in electron transport chains by cytochromes [5], and living organisms metabolic transformations by complex heme-containing proteins such as flavohemoglobins [6] or theophylline oxidase [7]. These diverse functions make heme proteins very attractive for bioelectronic and bioelectrochemical sensors applications [8–14], in which an appropriate orientation of protein at the electrode surface may be critical to provide a direct and efficient path for the electrons between the redox cofactor and the electrode, important for the construction of reagentless (i.e. direct electron transfer based) biosensors [15,16].

Redox cofactors can be electrically addressed by rationally designed molecular wires [17,18] and redox relays comprised of the redox mediator covalently attached either to the enzyme polypeptide chain [19–21] or to the polymer matrix the enzyme is entrapped in [22–25]. Specific adsorption of the enzyme onto promoter- and alkanethiol self-assembled monolayer (SAM)-modified electrodes can also provide the protein orientation favouring direct electronic communication between the cofactor and the electrode, via protein matrix, that allows efficient bioelectrocatalytic function of complex enzymes [26–29].

For enzymes with non-covalently bound redox cofactors,their reconstitution onto cofactors tethered to electrodes through different types of linkers allows the molecular-level controlled spatial distribution and orientation of the bioelectrocatalytically active enzyme monolayers at electrodes [30]. Reconstitution of the apoenzyme onto the electrode-wired cofactor then assures the minimal electron transfer (ET) distance to the redox centre of oriented protein and bioelectrocatalytic function of the reconstituted holoenzyme. This approach was successfully applied to study a number of flavin- [31–33], PQQ- [34,35] and heme-containing proteins [36–41].

In the latter case, a non-covalently bound heme active site enables exploitation of the reconstitution approach to produce

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heme protein-modified electrodes. Myoglobin was reconstituted onto synthetic dodecanethiol-conjugated hemin immobilised onto the gold electrodes [38]. Similarly, modification of the hemin propionate groups by thiol terminated alkanes, followed by reconstitution of cytochrome b_{562} and its further chemisorption and studies on gold, was reported [42]. Upon reconstitution, a small increase in hemin peak separations, from 128 to 162 mV, has been observed [38,42]. Alternatively, horseradish peroxidase (HRP) was reconstituted onto hemin covalently attached to the aliphatic spacers coupled to the activated fraction of the SAM of mixed alkanethiols formed on gold [41]. Holoenzyme formation was associated with the enhanced electrocatalytic currents of H_2O_2 reduction (chronoamperometry at −0.1 V) in the presence of ET mediator phenylenediamine, which is not supposed to mediate the reaction of H_2O_2 with free hemin. The hemin-SAM surface modification was also used to reconstitute a range of *de novo* proteins [36] exhibiting a peroxidise activity at −0.5 V in the presence of hydroxyquinone as a redox mediator. Alternatively, HRP was reconstituted onto the hemin-terminated DNA strand hybridised to the electrode-tethered complementary strand [37]. Although no appreciable direct electrocatalysis of H_2O_2 reduction was detected, the authors observed oxidation of phenylenediamine in the presence of H_2O_2. Reconstitution was also used to form photoactive supramolecular architectures of cytochrome b_{562}, generating photocurrents at −0.2 V in the presence of methyl viologen [39].

Estimations of the reconstitution efficiency by surface characterisation techniques, e.g., surface plasmon resonance [30,42], suffer from the dependence of the signal on non-specific protein adsorption, though atomic force microscopy was shown to be useful for analysis of large supramolecular structures [39]. The most common approach is electrochemical assessment of non-turnover electrochemistry of hemin and the electrocatalytic activity of the reconstituted enzyme [36–38,41,43]. The bioelectrocatalytic activity of the reconstituted enzyme is probably the most important evidence of successful reconstitution since the free redox cofactor is often either incapable of catalysis or the electrocatalysis onset potential significantly differs from that of the enzyme. The latter is a typical case of HRP, which bioelectrocatalysis of H_2O_2 reduction is observed at potentials much more positive than that characteristic for hemin alone [9,13] as a result of the formation of higher oxidation states of HRP (Compound I) upon reaction of ferric HRP with H_2O_2 [3]. The redox potential of Compound I of ca. 0.72 V vs. Ag/AgCl at pH 7 [44] is consistent with the data on bioelectrocatalytic reduction of H_2O_2 by HRP immobilised at electrodes [9], starting from potentials exceeding 0.6 V at HRP-modified heat-treated graphite [45], graphite [46], graphite modified with carbon microfibers/carbon nanotubes [47] and gold electrodes with directly adsorbed native and recombinant forms of HRP [48–50].

Though HRP reconstitution onto hemin-modified electrodes was first reported as far as in 2000 [41], its bioelectrocatalytic activity has never been interrogated under conditions of its natural catalytic cycle, namely, at 0.6–0.7 V corresponding to the Compound I formation. In the present work hemin was covalently attached to the mixed SAMs composed of long amine-terminated aliphatic thiol and shorter diluting hydroxyl-terminated alkanethiols. The surface concentration and chemical environment of the tethered hemin were varied as a function of the composition of the underlying SAM. HRP was then reconstituted onto the hemin-modified electrodes (Fig. 1) and the efficiency of reconstitution was evaluated electrochemically by following changes in hemin electrochemistry, ET kinetics and bioelectrocatalytic activity of the reconstituted HRP-modified electrodes in the reaction of H_2O_2 reduction at potentials corresponding to the Compound I formation in the native enzyme.

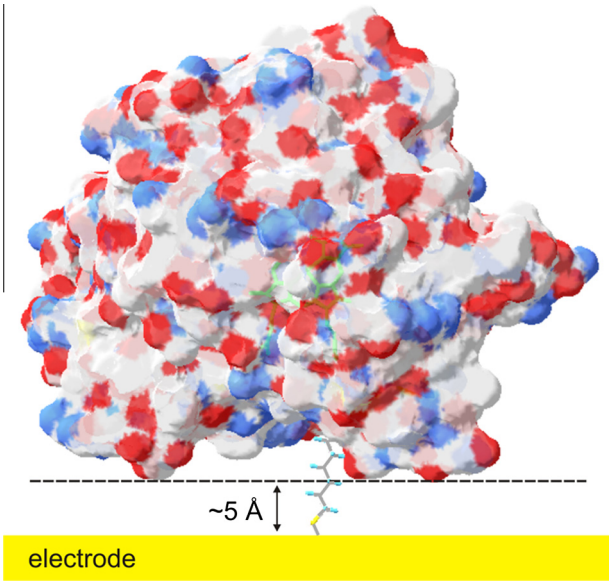

Fig. 1. Schematic representation of horseradish peroxidase reconstituted onto hemin covalently attached to 11-aminoundecanethiol linker tethered to the gold electrode and thus correspondingly orientated at the electrode. The HRP molecular structure was from PDB, PDB ID: 1ATJ [51].

2. Experimental

2.1. Electrodes and instrumentation

All electrochemical experiments were performed in a standard single compartment three-electrode configuration glass cell with a 2 mm diameter gold working electrode (CH Instruments Inc., USA), a platinum wire counter electrode and an Ag/AgCl (3 M KCl) reference electrode (Metrohm) using PGSTAT302 N potentiostat running Nova 1.8 software (Metrohm Autolab, The Netherlands). All potentials are quoted with respect to Ag/AgCl (3 M KCl). The working electrodes were pre-treated by cycling in 0.5 M KOH between −0.5 and −1.4 V to desorb any surface bound thiols, polished on polishing cloths (Buehler, Germany) with a 1 m diamond paste and 0.1 m alumina slurry (Struers, Denmark), ultrasonicated in ethanol, followed by electrochemical cleaning in 1 M H_2SO_4 and 1 M H_2SO_4 in the presence of 10 mM KCl in the potential range of 0–1.7 V. The electrode roughness factor derived from the charge of the gold oxide stripping peak was 2.5 ± 0.3. The hemin and HRP surface coverage was related to the geometric surface area of electrodes (0.031 cm^2).

2.2. Materials and methods

Reagents: All solutions were prepared using reagent grade water (18.2 MΩ cm) purified with Milli-Q Reference system (Merck Millipore, Germany). When required the solutions were deoxygenated with a stream of argon (AGA, Denmark). Mono- and di-basic sodium phosphate, hemin (chloroprotoporphyrin IX iron(III)), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), 6-mercapto-1-hexanol (MH), 2-mercaptoethanol (ME), dimethyl sulfoxide (DMSO), ethanol, hydrogen peroxide (30%), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Denmark) and used without further purification. 11-amino-1-undecanethiol (AUT) was purchased from Dojindo EU GmbH, Germany.