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Dihemic c_4-type cytochrome acting as a surrogate electron conduit: Artificially interconnecting a photosystem I supercomplex with electrodes

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ABSTRACT

Connection of photosystem I (PSI) with electrodes has been shown to create artificial photosynthetic systems that hold promise for the synthesis of solar fuels. The high quantum yields of PSI require efficient electron transfer from the electrode to the reaction center of PSI in order to restock the light-induced holes, a task which in nature is performed by small redox proteins. Here, we have investigated the potential "wiring" properties of a dihemic *c*-type cytochrome (cyt c_4), in order to efficiently connect PSI with electrodes. Cyt c_4 has shown direct electron transfer (DET) with both hemes in electrical communication with two different electrode materials (ITO and Au) and on the basis of cyt c_4-multilayer electrodes "self-exchange" properties can also be deduced. Investigation of cyt c_4 in combination with PSI within an inverse opal ITO electrode has shown the dihemic protein to be a valuable molecular electron conduit, able to interconnect the photoenzymatic reaction with the 3D electrode. The properties have been compared with those of electrodes based on monohemic cyt *c* derived from horse heart.

1. Introduction

Redox reactions play a vital role in all biological processes, including respiration and photosynthesis, the essential energy processes that sustain life [1,2]. These reactions have also been exploited in the development of bioelectronic devices such as electrochemical biosensors [3,4], biofuel cells [5,6] and photobiohybrid systems [7,8]. Small redox proteins have attracted considerable attention in this regard; their usefulness depends to a large extent on their ability to achieve direct heterogeneous electron transfer (DET) at interfaces. This is in contrast to enzymes and other large catalytic protein complexes like photosystem I and II with redox centers that are buried within the structure [9,10]. The insuperable electron tunneling distance and the often suboptimal orientation of these biocatalysts at interfaces are major drawbacks preventing electron transfer (ET) between the catalytic center and the electrode, and therefore their efficient use in bioelectronic devices. For this reason small redox proteins have been utilized as molecular electron conduits, wiring electrons between biocatalysts and interfaces [11–14]. However, not only enzymes, but also photosynthetic supercomplexes need to be connected for the development of photobiocatalytic electrodes. Up to now a range of different strategies has been applied for the efficient coupling of photosynthetic complexes with electrodes [15–24]. Molecular wiring has been accomplished by reconstitution of a photosynthetic complex with vitamin K1 derivatives [25], by cross-linking of platinum-nanoparticle photosystem composites with ferredoxin [26], via a linkage of pyrroloquinoline-quinone to a photosynthetic complex [27], or by embedding in redox polymer-modified electrodes [28–30]. In this regard protein-based approaches are rather powerful, as it has been shown that the redox protein cytochrome *c* derived from horse heart (cyt c_{hh}) is a valuable electron conduit for wiring photosystem I to electrodes [14,31,32]. Photosystem I (PSI) from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (*T. elongatus*) has predominantly been used for the construction of such electrodes on account of its high quantum efficiency (~100%), accompanied by fast charge separation and high stability [33,34]. PSI is a trimeric supercomplex consisting of 12 different subunits, and harboring 96 chlorophylls *a* (Chl *a*) and 22 carotenoids (per monomer) [35]. Charge separation occurs at the luminal pigment dimer, Chl *a*/Chl *a'* (P700), resulting in the reduction of the terminal iron sulfur cluster (F_B) [36,37]. In nature the light-induced electron-holes pairs are restocked via cytochrome c_6 (cyt c_6) reducing $P700^+$, while oxidation occurs at the F_B-cluster by ferredoxin (Fdx) [38].

So far a limited number of redox proteins have been reported to

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show wiring properties, allowing interconnection of ET reactions [39,40]. But monohemic cytochrome *c* (cyt c_{hh}, from horse heart)-based systems are virtually the only ones displaying efficient wiring in an immobilized state, connecting photosynthetic complexes with electrodes [14,31,32].

Being aware of this limitation, and in search of new "redox protein-based wires" for the development of photoelectrochemical cells, we have been investigating the potential wiring properties of a dihemic *c*-type cytochrome (cyt c_4) derived from the acidophilic bacterium *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*) [41]. The physiological function of this protein is to act as part of a high potential respiratory chain [42]. This chain couples Fe^{2+}/Fe^{3+} oxidation with the reduction of oxygen to water. It is composed of an outer membrane monoheme *c*-type cyt c_2 where Fe^{2+} oxidation occurs, the periplasmic copper protein rusticyanin, the diheme cyt c_4, and an integral inner membrane aa_3-type cytochrome *c* oxidase (CcO) that catalyzes O_2 reduction [42]. Cyt c_4 from *A. ferrooxidans* has a molecular mass of 26.5 kDa, a pI value of 8, and two distinguishable heme centers with redox potentials of +385 mV (heme-I) and +480 mV (heme-II) vs. SHE [43].

2. Materials and methods

Details of the preparation and characterization of inverse opal indium tin oxide electrodes (IO-ITO) have been published previously [44]. A six-layer deposition procedure was used in the study. Preparation of IO-ITO-PSI-cyt *c* electrodes: 4 μL of a 20 μM PSI solution (10 mM tricine, 50 mM $MgSO_4$, 0.02% β-DM, pH 8) was drop cast onto the IO-ITO electrode and incubated for 2 min. The unbound protein was removed by repeated washing in a phosphate buffer (5 mM, pH 7). 10 μL of 300 μM cyt c_4 or cyt c_{hh} in phosphate buffer (5 mM, pH 7) was then drop cast onto the resulting electrode and incubated for 2 min.

Protein isolation, preparation, and characterization were performed according to previously established protocols for PSI from *T. elongatus* [45,46] and cyt c_4 from *A. ferrooxidans* [47].

Cyt c_4 multilayer electrodes and SiNPs were prepared according a published protocol [13,48]. 11-Mercaptoundecanoic acid (MUA) and 11-mercapto-1-undecanol (MU) were used as gold modifiers.

2.1. Electrochemical & photoelectrochemical investigations

Electrochemical measurements were performed with a CHI-660E potentiostat (CHI Instruments, Austin, TX). A custom-made cell (1 mL) was used with a platinum electrode as counter electrode, and an Ag/AgCl (1 M KCl) with a potential of +0.237 V versus NHE (Biometra, Göttingen, Germany) as reference electrode. Cyclic voltammetry (CV) was recorded between −300 and + 500 mV vs Ag/AgCl (scan rate = 100 mV s^{-1}). Photoelectrochemical measurements were performed using an integrated system (CIMPS, Zahner) containing a white LED light source (4300 K, Zahner) with adjustable intensity (max. 100 mW cm^{-2}), an electrochemical cell and a photodiode with feedback control to the light source via a potentiostat (PP211, Zahner). Photoamperometric experiments (PA) were performed at potentials of +200, +100, 0, −100, and −200 mV vs Ag/AgCl. In all experiments a buffer containing dioxygen (acting as electron acceptor) was used. Linear sweep voltammetry (LSV) measurements were performed with a potential window of +0.5 to −0.3 V, at a scan rate of 5 mV s^{-1}. In all experiments a Pt counter electrode and an Ag/AgCl (3 M KCl) reference electrode were used.

3. Results and discussion

In previous investigations we have shown that cyt c_{hh}, albeit not its physiological reaction partner, is a valuable electron conduit allowing ET between different electrode materials (gold and ITO) and PSI from *T. elongatus* [14,44]. In order to assess the wiring properties of dihemic cyt c_4, we have addressed the issue in three stages: Firstly, we investigate the electrochemical properties of the dihemic protein, harboring two redox active groups. Secondly, arguments for the reaction capabilities among cyt c_4 molecules are collected. Thirdly, we address the feasibility of a cyt c_4 reaction with PSI and analyse the potential-dependent behavior of photocurrent generation. The latter is particularly relevant since the two heme groups have different redox potentials and thus a different potential difference to the oxidized reaction center of PSI.

To study its electrochemical properties, the protein has been incorporated in custom-made IO-ITO electrodes [44]. Cyt c_4 has been immobilized by drop casting 10 μL of a 300 μM solution for 2 min onto a macroporous IO-ITO electrode, followed by a rinsing step. The DET properties of cyt c_4 in a surface-fixed state within an IO-ITO/cyt c_4 electrode have been assessed via CV, recorded at different scan rates (10–100 mV s^{-1}). Cyt c_4 exhibits a quasi-reversible electrochemistry, with well-separated redox waves for both hemes (Fig. 1a). Scan rate variation at small rates between 2 and 60 mV s^{-1} results in stable redox waves with formal potentials (E_f) of +82 and + 197 mV vs Ag/AgCl, respectively. The peak currents show a linear dependence on the scan rate, confirming the surface-controlled reaction of cyt c_4. By integration of the redox waves and taking into account the real surface area (38 ± 3 cm^{-2}), the geometrical surface coverage has been calculated as 11 ± 1 pmol cm^{-2}.

Considering the reaction capabilities of cyt c_4 molecules, a possible interprotein electron transfer between the cyt c_4 molecules has been investigated in the light of the proven self-exchange properties of cyt c_{hh}. An established modular multilayer concept employing silica nanoparticles (SiNPs) [48,49] as artificial matrix is therefore used to construct cyt c_4-SiNPs multilayer electrodes. The formation of multiple layers is based on electrostatic interactions between the negatively charged SiNPs and the positively charged cyt c_4 (pI 8) (Fig. 1b). For these experiments, a gold wire electrode modified with a thiol layer has been used. Representative CVs for a cyt c_4 monolayer electrode and for electrodes with 1, 2, 3, or 4 cyt c_4/SiNPs bilayers are shown in Fig. 1c.

The adsorption of cyt c_4 on a SAM-modified gold electrode has been productive, as well-defined oxidation and reduction waves of both heme groups are displayed. Also here the peak currents show a linear dependence on the scan rate (0.02–1 V s^{-1}), confirming the immobilization of cyt c_4 on the electrode. The geometrical surface coverage for the cyt c_4-layer has been calculated by integration of the redox waves, and corresponds to 4 ± 0.3 pmol cm^{-2}. If we compare this coverage with the value of a theoretical perfect monolayer (7 pmol cm^{-2}) it can be stated that rather good surface coverage has been achieved with cyt c_4.

Subsequently, cyt c_4-multilayer electrodes with 1, 2, 3, or 4-bilayers have been investigated (Fig. 1c). The multilayers display well-defined oxidation and reduction waves, showing an increase in the peak current with the number of layers, indicating that not only the cyt c_4 molecules in front of the electrode, but also those in the external layers are electrically communicating with the electrode. Integration of the redox waves for each multilayer electrode (1×, 2×, 3×, 4×) results in surface coverage of 7.5 ± 0.5, 10.4 ± 0.3, 12.3 ± 0.2, 14.3 ± 0.2 pmol cm^{-2}, respectively, reflecting an increase in electro-active cyt c_4 with the increasing number of layers. Comparing these findings with cyt c_{hh} indicates that, although multilayers can be constructed with cyt c_4, the electro-active amount does not increase in the same linear fashion as for cyt c_{hh}. This might be caused by the different adsorption behavior of the proteins or by the different modes of interaction between dihemic and monohemic proteins. More investigations of the mechanism are necessary. Nevertheless, multiple layers of cyt c_4 have been constructed and are electrochemically connected to the electrode, which is most likely attributed to cyt c_4–cyt c_4 self-exchange reactions, revealing that cyt c_{hh} is not the only redox protein possessing self-exchange properties. This view is supported by the rather high amount of electro-active cyt c_4 found in the IO-ITO electrodes, clearly exceeding the perfect monolayer coverage (see above).

So far the focus has been on elucidating the DET of cyt c_4 with