Signal-Transfer Models

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Communication in a Protein Stack: Electron Transfer between Cytochrome *c* and Bilirubin Oxidase within a Polyelectrolyte Multilayer**

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Communication between proteins in living organisms plays a vital role in many physiological systems, such as respiration or photosynthesis.[1] For this purpose, the proteins are arranged in permanent or temporary complexes, thus leading to sophisticated redox cascades by exchanging electrons directly or through mediators. The efficiency and selectivity of signal transfer in these complexes has stimulated much interest in constructing artificial signal chains. Protein assemblies with the ability to perform selective signal transduction are of fundamental importance and have application potential in biosensors, bioelectronics, and biofuel cells.[2–6] Implementation of direct electron transfer (DET) in protein assemblies was a breakthrough in bioelectronics, by providing an efficient method for coupling biological recognition events to a signal transducer.[7,8] DET avoids the use of redox mediators, thus reducing potential interferences and side reactions, as well as being more compatible with in vivo conditions. However, only a few heme proteins, including cytochrome *c* (cyt.*c*), and blue copper enzymes show efficient DET on different kinds of electrodes.[7,9,10]

Previous investigations with cyt.*c* have mainly focused on heterogeneous electron transfer of monolayers of this protein on gold.[11] An important advance was the fabrication of cyt.*c* multilayers by electrostatic layer-by-layer self-assembly. The layer-by-layer approach is known for the precise tuning of the protein concentration on the electrode, while the polyelectrolyte provides a biocompatible matrix that maintains the activity of the biomolecule.[3,12] However, with cyt.*c* and poly(aniline sulfonic acid) (PASA) it was possible for the first time to prepare fully electroactive multilayers of the redox protein.[13] This approach has recently been extended to design an analytical signal chain based on multilayers of cyt.*c* and xanthine oxidase. The system does not need an external mediator but relies on the in situ generation of a mediating radical, and thus allows signal transfer from hypoxanthine via the substrate-converting enzyme and cyt.*c* to the electrode.[14]

Herein, we introduce a new type of signal chain by assembling proteins in complexes on electrodes in such a way that a direct protein–protein electron transfer becomes feasible. Our design does not need a mediator, in analogy to natural protein communication. For this purpose, cyt.*c* and the enzyme bilirubin oxidase (BOD, EC 1.3.3.5) are co-immobilized in a polyelectrolyte multilayer on gold electrodes. Although these two proteins are not natural reaction partners, the protein architecture facilitates an electron transfer from the electrode through multiple protein layers to molecular oxygen, which results in a significant catalytic reduction current.

BOD belongs to the class of blue copper enzymes responsible for the four-electron reduction of molecular oxygen to water without formation of reactive intermediates and, therefore, it is of great interest for bioenergetics.[5,7,10] Recently, it was found that BOD can accept cyt.*c* as a reaction partner to deliver electrons for oxygen reduction.[15] Herein, we confine this protein–protein reaction to a surface and couple it with the DET of cyt.*c* to the transducing electrode.

Multilayers with embedded cyt.*c*, BOD, and PASA as counter polyelectrolyte are assembled on a cyt.*c* monolayer electrode by alternating incubation in solutions of PASA and a cyt.*c*/BOD mixture. The monolayer electrode consists of cyt.*c* adsorbed on a thiol layer of mercaptoundecanoic acid (MUA) and mercaptoundecanol (MU). As reported previously, this monolayer electrode shows an efficient protein–electrode communication.[16] The design of the resulting multilayer is shown in Figure 1. The formation of the assembly with cyt.*c*, BOD, and PASA on the gold surface was confirmed by surface plasmon resonance experiments, which indicate a fast binding of PASA to the surface, whereas the protein mixture shows slower binding kinetics (data not shown).

When the cyt.*c*/BOD multilayer electrode is exposed to an air-saturated solution and a cathodic potential sweep is performed, a substantial reduction current can be observed at neutral pH (Figure 2). The catalytic current results from reduction of cyt.*c*, most of which is in its ferric form as a result of oxidation by neighboring BOD molecules. Catalytic reduction of oxygen occurs at the BOD trinuclear cluster,

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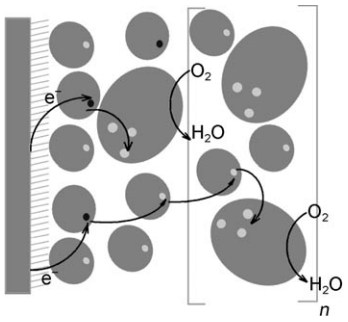

Figure 1. The redox chain in cyt.*c*/BOD multilayer electrodes. Dark gray circles: cyt.*c*; ellipses: BOD; arrows indicate electron-transfer pathways between cyt.*c* and BOD within the polyelectrolyte network or the four-electron oxygen reduction process.

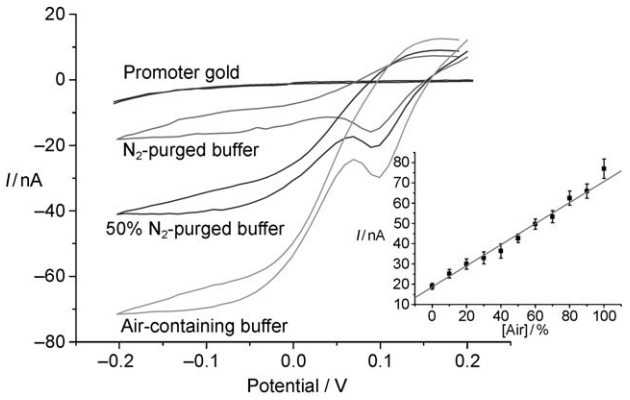

Figure 2. Cyclic voltammograms of an Au–MUA/MU–cyt.*c*–(PASA–cyt.*c*/BOD)$_3$ multilayer electrode measured at different oxygen concentrations. Inset: dependence of the catalytic current *I* at −0.2 V on oxygen concentration in N_2/air-saturated buffers.

where electrons are transferred to molecular O_2, to produce water. Thus, a catalytic current is produced. This voltammetric observation confirms the functioning of the signal chain from the electrode via cyt.*c* to BOD and finally to O_2. Control experiments in which only one protein, either BOD or cyt.*c*, is immobilized in the multilayer show no catalytic behavior.

The electron-transfer kinetics of the protein interaction in the immobilized state differs from the behavior in solution; therefore, the kinetic analysis cannot be carried out on the basis of the rate constants for the corresponding reactions in solution (see Supporting Information). Thus, to evaluate the rate-limiting step in the electron-transfer chain, the assembly is analyzed by cyclic voltammetry (CV) at different oxygen concentrations (Figure 2). The experiments show a linear dependence of the reduction current on the oxygen content in solution (5 mV s^{-1}). As the system can follow the enhanced oxygen conversion at the BOD, it suggests that the overall current is limited by the catalytic reduction at the enzyme rather than by the reactions cyt.*c*–BOD, cyt.*c*–cyt.*c*, or cyt.*c* and the electrode (at least up to the oxygen concentrations of about 250 μM found in air-saturated buffer). The additional peak visible in Figure 2 ($E_f \approx 130$ mV) results from the conversion of surface-bound PASA. This reaction occurs in parallel to the conversions of the protein molecules, but with much slower kinetics. It does not interfere with the electron transfer towards BOD.

As the reduction of O_2 at BOD is the rate-limiting step in the electron-transfer chain under nearly stationary conditions, more insight into the kinetics of the system can be obtained by a systematic variation of the scan rate (see Figure 3). At low

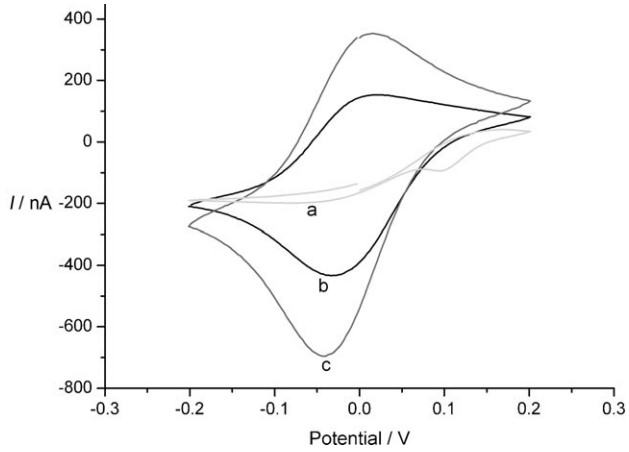

Figure 3. Cyclic voltammograms of an Au–MUA/MU–cyt.*c*–(PASA–cyt.*c*/BOD)$_6$ multilayer electrode at scan rates of a) 5, b) 100, and c) 200 mV s^{-1}.

scan rates, a pronounced catalytic current is observed, which disappears at higher scan rates and the electrochemical signal of cyt.*c* becomes visible. In analogy to the reaction in solution, the electrochemical driving force above a certain scan rate changes more quickly than the catalytic reaction at the BOD can follow. So electrons are only exchanged with cyt.*c*, and no electron transfer to the enzyme occurs. The arrangement shows a quasi-reversible electron transfer with a formal potential of -13 ± 8 mV, which is in good agreement with data found for multilayers embedding only cyt.*c*.

If the scan rate is further enhanced, the peak current is still increased although the amount of addressable cyt.*c* decreases. This means that a scan rate is reached which is in the range of the cyt.*c*–cyt.*c* electron-transfer rate, so not all cyt.*c* molecules immobilized in the multilayers can be oxidized/reduced in the time period of a voltammetric sweep. Hence, the system behaves similarly to a multilayer assembly with only cyt.*c*, as protein–protein electron transfer is limiting. Rate constants of about 1 s^{-1} can be evaluated for the heterogeneous electron transfer of the outer cyt.*c* layers in the network. Notably, the multilayer assembly is stable during these investigations: by decreasing the scan rate after the variation the same catalytic oxygen current is detected again.

Within the assembly the proteins are arranged in stacked layers on the electrode—thus, rather thick layers can be prepared. To gain more insight into the electron-transfer process we studied the dependence of the catalytic efficiency on the number of layers. Electrodes with different numbers of cyt.*c*/BOD layers were prepared, and the CV data were measured in air-saturated buffer at low scan rate (Figure 4). A proportional increase of the catalytic current with the number of layers can be clearly seen. This means that by increasing the

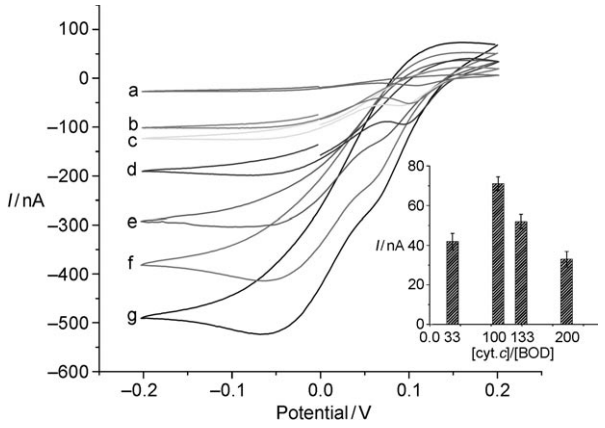

Figure 4. Cyclic voltammograms of cyt.*c*/BOD multilayer electrodes in air-saturated buffer as a function of the number *n* of (PASA–cyt.*c*/BOD)$_n$ layers: a) 1; b) 2; c) 4; d) 6; e) 8; f) 10; g) 12. Inset: catalytic current *I* as a function of [cyt.*c*]/[BOD] ratio for a three-layer electrode.

number of catalytic sites (amount of BOD), the bioelectrocatalytic conversion can be enhanced. This, however, also implies that all deposited BOD molecules are in electrical contact with the electrode. As a large proportion of the enzyme molecules are immobilized rather far from the electrode surface, we conclude that cyt.*c* provides the electrons for the enzymatic activity. This notion is supported by the fact that assemblies of BOD and PASA alone do not show a catalytic oxygen reduction.

For applications of such protein/enzyme multilayer systems, the sensitivity towards the enzyme substrate (here oxygen) can be tuned to a large extent by the number of deposited layers without being limited by mediator diffusion or leakage. In addition, the electrode shows significant activity at neutral pH; however, at pH 5 the oxygen reduction is further enhanced by a factor of about 3. This finding illustrates again the efficient electron supply for the BOD-catalyzed reaction within the multilayer.

As discussed above, the two proteins have a different function within the layer assembly. Therefore, the relative amounts of BOD and cyt.*c* in each layer should have a profound effect on oxygen reduction if this hypothesis is correct. When the [cyt.*c*]/[BOD] ratio is decreased from 100 to 33, we note a 40% decrease in the catalytic response (Figure 3, inset). Therefore, although the number of recognition sites for oxygen is increased (larger amount of BOD), the delivery of electrons to the enzyme becomes limited and thus the activity is diminished. As electrons are transferred from cyt.*c* to the enzyme, the proposed role of cyt.*c* as an immobilized electron carrier is again supported. When high concentrations of BOD are present, the electron pathway from cyt.*c* to cyt.*c* is hindered.

In conclusion, DET has been established for a two-protein system in a multilayer architecture for the first time. For this purpose, a redox protein was combined with the enzyme BOD. The results may have importance for the development of electrodes for biofuel cells,[17] although the electrode potential is fixed to the potential of the redox protein. The approach is expected to have a considerable impact on the development of biosensors, and also represents a significant advance in modeling of biological signal transfer. With cyt.*c* as the electron carrier, other potential reaction partners might be used in the system, for example, sulfite oxidase, laccase, or nitrate reductase. These combinations will be the direction of further research. We anticipate that this concept will stimulate progress in the multilayer design of even more complex biomimetic signal cascades that take advantage of direct communication between proteins.

Experimental Section

Cyt.*c* and BOD were obtained from Sigma (Steinheim, Germany) and used as received. MUA, MU, and PASA (number-average molecular weight $M_n = 10000$) were supplied by Aldrich (Taufkirchen, Germany). Gold wire with a diameter of 0.5 mm was purchased from Goodfellow (Bad Nauheim, Germany). All solutions were prepared in 18 MΩ Millipore water (Eschborn, Germany).

A cyt.*c* monolayer was produced by adsorption of a solution of cyt.*c* (20 μM) in potassium phosphate buffer (5 mM, pH 7.0) on gold-wire electrodes previously modified with a solution of MUA/MU (1:3) in ethanol (5 mM). A premixed protein solution was made in potassium phosphate buffer (0.5 mM, pH 5.0) and contained cyt.*c* (20 μM) and BOD (200 nM). Each of the 10-min adsorption steps of PASA (0.2 mg mL^{-1}) and a premixed protein solution was followed by rinsing in potassium phosphate buffer (0.5 mM, pH 5.0).

All electrochemical measurements were performed in 1-mL cells using Ag/AgCl/1M KCl as reference (Biometra, Germany) and a Pt-wire counter electrode. The working electrodes were modified gold wires. CV experiments were performed with the Autolab system (Metrohm, Netherlands). A Biacore 2000 (Biacore AB, Sweden) was used for surface plasmon resonance experiments.

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