Published in final edited form as:

Electrochem commun. 2013 January 1; 26: 105–108. doi:10.1016/j.elecom.2012.10.024.

Direct Electrochemistry of Cytochrome *bo*₃ Oxidase at a series of Gold Nanoparticles-Modified Electrodes

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Abstract

New membrane-protein based electrodes were prepared incorporating cytochrome *bo*₃ from *E. coli* and gold nanoparticles. Direct electron transfer between the electrode and the immobilized enzymes was achieved, resulting in an electrocatalytic activity in presence of O₂. The size of the gold nanoparticles was shown to be important and smaller particles were shown to reduce the overpotential of the process.

Keywords

membrane protein; gold nanoparticles; direct electron transfer; electrocatalysis

1. Introduction

Protein film voltammetry has become a crucial technique for the study of redox proteins. [1] When immobilizing proteins on electrode surfaces, three main criteria have to be fulfilled. [2] The immobilization method must provide a suitable environment for the enzymes in order to prevent their denaturation. It must also allow a high loading of enzymes on the surface. It must finally promote an efficient electron transfer between the electrode and the immobilized enzymes. Among redox enzymes, integral membrane proteins are critically important for multiple central functions in living cells, like for example terminal oxidases that are known to catalyze efficiently the reduction of O₂. [3, 4] However, due to their hydrophobic and amphiphilic nature, membrane proteins are extremely difficult to study. Direct electron transfer (DET) between these large enzymes and the electrode is often impaired since the cofactors are usually buried inside the polypeptide backbone and achieving satisfying electron transfer rates between electrodes and these proteins for the catalytic reduction of oxygen, have often turned out to be quite challenging. The most favored procedure has been the adsorption within lipid membranes reconstituted on the surface of electrodes, [5–9] which have helped maintaining the structural integrity of the

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enzyme and at the same time its redox activity. In these studies, smaller molecules have often been required to mediate the transfer of electrons between the electrode and the enzyme.

Willner et al. have shown that gold nanoparticles (NPs) can enhance the interfacial electron transfer rates by several orders of magnitude [10] and this approach seems promising for the study of membrane proteins. In addition, the high surface to volume ratio of these materials is an asset for immobilizing a large amount of proteins. Nakamura et al. have described a simple elaboration method of three dimensional gold NPs-modified electrodes, and reported the direct electrochemistry of cytochrome *c*. [11] We have later shown that these electrodes constitute an efficient support of immobilization of soluble membrane proteins fragments from the respiratory chain of *T. thermophilus*. [12] High protein loading, and high electron transfer rates have been achieved. Here we report on the immobilization and electrochemical characterization of cytochrome *bo*₃, a membrane protein from the *E. coli* respiratory chain. Given the size of this enzyme (4 subunits, 144 kDa), [13] gold nanoparticles of various mean diameters: 15, 38 and 56 nm have been considered. The membrane-protein based electrodes are also characterized by Surface-Enhanced IR Absorption Spectroscopy (SEIRAS). This is to the best of our knowledge the first study of immobilization of respiratory membrane proteins on gold NPs.

2. Experimental

2.1 Chemicals

Sodium citrate, potassium cyanide, chlorauric acid (HAuCl₄, 3H₂O), 6-mercaptohexan-1-ol, 1-hexanethiol, KH₂PO₄, EtOH were purchased from Sigma-Aldrich and used without further purifications.

2.2 Production and purification of the enzymes

The cytochrome *bo*₃ from *E. coli* was purified as previously reported. [14]

2.3 Preparation of gold nanoparticles

Gold nanoparticles were prepared following published procedures [15] by addition of sodium citrate to a boiling solution of HAuCl₄ (125 mL, 1mM) under vigorous stirring. Various amounts of citrate have been used (4eq, 1 eq, 0.8 eq) to prepare particles of different size (15, 38 and 56 nm respectively). These solutions were further concentrated by centrifugation at 10,000 rpm and removal of 95 % of the supernatant. The TEM pictures were acquired at 200 kV with a Tecnai G2 (FEI) microscope and an Eagle 2k (FEI) ssCCD camera.

2.4 Electrochemistry

Polycrystalline gold disk electrodes were polished with diamond paste and activated in a 0.1 M H₂SO₄ solution by maintaining the potential at 2.20 V vs SHE for 5 s, -0.14 V for 10 s and then cycling 100 times between 0 V and 1.7 V at 4 V/s. Then, three drops of gold colloid were deposited successively on the electrode surface and allowed to evaporate under air. The modified electrode was immersed overnight in a 1/1 ethanolic solution mixture of 6-mercaptohexan-1-ol and 1-hexanethiol, rinsed with fresh solvent and dried under argon. The stock solution of detergent-solubilized protein was washed with 50 mM phosphate pH 7 buffer without detergent in a 100 kDa centrifugal filter unit. Subsequently, 3 µL of the solution were deposited on the electrode surface and left at 4°C overnight. Finally, the electrode was rinsed with fresh buffer to remove the excess of protein. CV measurements were performed in a standard three electrode cell connected to a Princeton Applied Research VERSASTAT 4 potentiostat. An aqueous AgCl/Ag 3M NaCl electrode was used as

reference electrode and a platinum wire as counter electrode. The zero oxygen measurements were obtained by immersing the electrode in phosphate pH 7 buffer solution flushed for 1 hour with argon and a positive pressure of argon was maintained over the solution during the experiment. For inhibition studies, sodium cyanide was added to the buffer solution, and the electrode was allowed to equilibrate for 10 minutes before measurement.

2.5 SEIRAS studies

A 20 nm thick gold substrate was sputtered on the surface of a silicon crystal from a Harrick ATR cell unit. The gold surface was then successively modified with 3 μ l of a concentrated gold colloid solution, 3 μ l of the thiols solution mixture, and finally 3 μ l of the cytochrome *bo*₃ solution. SEIRAS spectra were measured for each modification step using a Bruker Vertex 70 Spectrometer. 256 spectra with 4 cm^{-1} resolution were averaged.

3. Results and discussion

3.1 electrode preparation

Drop-casting of 15 nm gold NPs (3 times) on a gold disk electrode (0.1 cm^2) increases the gold specific surface to c.a. 15 cm^2 , as estimated by integration of the Au-O reduction peak at 1.1 V. This surface should allow adsorption of a sufficient amount of membrane proteins for electrochemical studies. Since the surface of cytochrome *bo*₃ is largely hydrophobic [13] we have favored a mixture of 6-mercaptohexan-1-ol and 1-hexanethiol to modify the gold surface. We have observed that cytochrome *bo*₃ can be immobilized on these modified gold nanoparticles, provided the concentration of detergent in the protein sample is below 0.1 %, as obtained by washing the stock solution with buffer devoid of detergent.

The whole modification procedure was followed by SEIRAS, which is a very convenient technique to study biomolecules adsorbed on nanostructured electrodes. [16] After deposition of the gold colloid, the spectrum shows the large bands characteristic for citrates at 1597 and 1401 cm^{-1} [17] which stabilize the particles. These bands disappear after addition of the thiols, whereas C-H stretching signals at 2930 and 2858 cm^{-1} can be seen. Upon addition of the protein, amide I and amide II bands centered at 1657 and 1546 cm^{-1} respectively are obtained. The amide I band is specific for the secondary structure of a protein. [18] The signal identified here corresponds to the predominantly alpha helical structure of cytochrome *bo*₃ [13] and confirms that the protein retains its secondary structure when adsorbed on these modified gold nanoparticles.

3.2 Electrochemical behavior of cytochrome *bo*₃ on 15 nm gold nanoparticles

Figure 1 shows the cyclic voltammograms (CVs) of cytochrome *bo*₃ immobilized on 15 nm gold NPs-modified electrodes under air (solid black trace) or argon (solid grey trace) at pH 7. In absence of oxygen, a broad cathodic peak and a weaker anodic peak are observed at -0.13 V. These signals exhibit a -60 mV/pH dependence (see inset figure 1). The cathodic signal transforms into an intense wave at -0.22 V under air whereas the anodic signal is no longer present. This behavior is typical for the electrocatalytic reduction of O₂. In the absence of gold nanoparticles, in contrast, no signal is observed (dotted grey curve). Gold nanoparticles are known to be active in the electrocatalysis of O₂ reduction. [19–22] The background measured in the presence of air before adsorption of the protein (dash curve), however, shows that the NPs are not contributing significantly to the reduction of oxygen above -0.4 V when modified with the thiols mixture. Cytochrome *bo*₃ is thus at the origin of the electrocatalytic activity observed here, which confirms that a DET has been achieved between the electrode and immobilized enzymes. Average coverage values of 3 $\text{pmol}/\text{cm}^{-2}$ were estimated by integration of the cathodic signal in absence of O₂.

Potassium cyanide is a known inhibitor of the bimetallic active site of heme-copper terminal oxidases. [23] Upon addition of KCN in the buffer solution (figure 2), the catalytic wave is indeed shifted to more negative potentials and decreases continuously. This behavior confirms again that cytochrome *bo*₃ is responsible for the reduction of oxygen here. These curves allow determining a cyanide half maximal inhibitory concentration (IC₅₀) of 15 μ M. Jeuken et al. reported a lower value of 8 μ M for cytochrome *bo*₃ immobilized in a tethered bilayer lipid membrane on a gold surface, [24] with lower enzyme coverages.

Interestingly, the signal in absence of oxygen is not affected upon cyanide addition, which probably rules out a DET to the bimetallic heme *o*₃/Cu_B active site. The signal seems to correspond to an overlap of the contribution of the first electron acceptor, a tightly bound ubiquinol-8 molecule and the low spin heme *b*, both being independent from the CN binding site. Quinones typically show a low reversibility and broadness of the signal, and the pH-dependency found here (−60 mV/pH) is consistent with a Q/QH₂ reaction. The redox potential of the free Q/QH₂ couple is 70 mV at pH 7. The discrepancy between our value and this thermodynamic value may arise from kinetic complications or electrostatic interactions with other residues or cofactors within the protein.

3.3 Influence of nanoparticle size

Given the size of cytochrome *bo*₃, we have also considered larger gold nanoparticles: 38 and 56 nm, as estimated by TEM (inset figure 3). These nanoparticles are obtained at lower amounts of sodium citrate. [15, 25] Similarly to the smaller NPs, drop-casting of these larger nanoparticles on a gold disk electrode (0.1 cm²) increases the gold surface area, albeit to a lesser extent: to c.a. 5.5 and 2.5 cm² respectively.

The cathodic peak potential under O₂ is shifted towards more negative values when the nanoparticles size is increased (figure 3), which means that catalysis occurs at higher overpotentials for enzymes immobilized on larger NPs. Smaller NPs apparently allow faster electron exchange rates with this enzyme. Due to the low reversibility of the signal in absence of oxygen, it was not possible to determine accurately the electron transfer rates. Coverages of 3–4 pmol/cm^{−2} were obtained, regardless of the NPs size.

4. Conclusion

We have successfully immobilized cytochrome *bo*₃ on gold nanoparticles modified with a mixture of 6-mercaptohexan-1-ol and 1-hexanethiol and achieved DET, possibly via the tightly-bound quinone. We have also observed that overpotentials are reduced on smaller nanoparticles, probably due to faster electron transfer rates between the electrode and the protein. Comparison with other terminal oxidases is underway in our laboratory.

Acknowledgments

The “Conseil Scientifique” of the University of Strasbourg and the ANR chaire d'excellence is acknowledged for its financial support.

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Figure 1.

CVs of cytochrome *b*₃ immobilized on 15 nm gold NPs in air-equilibrated phosphate pH 7 buffer (solid black curve), or argon-flushed buffer (solid grey curve); electrode background before adsorption of the protein in air-equilibrated KPI pH 7 buffer (dash curve); cytochrome *b*₃ adsorbed on the gold disk electrode without gold nanoparticles (dotted grey curve); scan rate 0.02 V.s⁻¹, 20°C; inset: pH dependency of the cathodic signal in absence of O₂.

Figure 2.

CVs of cytochrome *bo*₃ immobilized on 15 nm gold NPs in air-equilibrated phosphate pH 7 buffer with increasing cyanide concentration at 0.02 V.s⁻¹; for clarity only the forward scan is shown; inset: peak current versus cyanide concentration trace.

Figure 3.

CVs of cytochrome *bo*₃ immobilized on 15 (A), 38 (B) and 56 nm (C) gold nanoparticles in air-equilibrated phosphate pH 7 buffer; 0.02 V.s⁻¹, 20°C; inset: TEM pictures of the nanoparticles samples.