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Active site structure and redox processes of cytochrome *c* oxidase immobilised in a novel biomimetic lipid membrane on an electrode

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Membrane-bound cytochrome *c* oxidase was attached to an electrode *via* a His-tag linker and studied by surface enhanced resonance Raman spectroscopy, demonstrating intact redox site structures and electron transfer between the electrode and the immobilized enzyme.

Nanostructured and mesoscopically scaled electronic devices including biomolecules as molecular building blocks represent one of the main topics in contemporary nano-biotechnology.[1] As a prerequisite for these devices, biomolecules such as enzymes must be immobilised under preservation of their native structures and functions. In this respect, immobilisation of membrane proteins still represents a challenge since it requires the embedment of the protein into a biomimetic system of lipid (analogues) on a solid support. To this end, various types of solid supported lipid membranes have been developed, including bilayers floating freely on quartz, indium tin oxide or gold surfaces, to polymer-, polyelectrolyte-supported and tethered bilayer lipid membranes.[2] Cytochrome *c* oxidase (CcO), a structurally, functionally, and spectroscopically well characterised membrane protein,[3–7] is one of the few complex enzymes that has been incorporated in supported lipid membranes.[8,9] Although redox activity toward cytochrome *c* (Cyt-c) as well as concomitant proton transport has been shown, these studies do not allow conclusions about the full structural integrity of the enzyme that acts as a redox-driven proton pump.

In this work, we have utilised a novel approach,[10] in which a lipid bilayer membrane is tethered to an Ag electrode *via* the integral protein, the aa_3-type CcO from *Rhodobacter sphaeroides*.[4] This system offers the possibility to analyse the immobilised enzyme by surface enhanced resonance Raman (SERR) spectroscopy which selectively probes the protein cofactors and thus can provide insight into the interfacial protein redox processes on a molecular level.[11,12]

Histidine(His)-tagged CcO, attached to a metal ion chelating coating of the metal surface, serves as a building block for a protein-tethered lipid bilayer membrane (Fig. 1). In this construct, the His-tag has been introduced to the C-terminus of subunit (SU) II at the periplasmic surface of CcO,[14] *i.e.* at the binding domain for the natural reaction partner Cyt-c.

For binding His-tagged CcO, the electrochemically roughened Ag surface was immersed in a solution of dithiobis-(*N*-succinimidyl propionate) in dry DMSO (2 mg ml^{-1}) for about 60 min. After rinsing with dry DMSO and ethanol, the dried modified surface was placed into an aqueous solution (pH 9.8, 0.5 M K_2CO_3) of 0.15 M N_α,N_α-bis(carboxymethyl)-L-lysine (ANTA) for 3 h. Finally, the coordination sites for the His ligands were created by treatment with 40 mM $NiCl_2$ (50 mM acetate buffer, pH 5.5) for 30–60 min. After rinsing with buffer solution (100 mM KCl, 50 mM potassium phosphate, pH 8), the protein was immobilized by immersing the modified Ag surface for about 2 h into the buffer solution containing 0.5 μM CcO and 0.1% dodecyl maltoside (DDM). For reconstitution of the lipid enviroment, the bound protein was incubated with buffer/DDM solution containing additionally 0.05 mg ml^{-1} 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPGPC). Then the detergent was removed by addition of biobeads (Bio-Rad) to the solution.

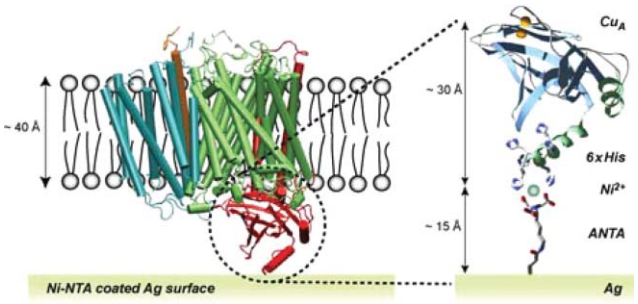

Fig. 1 Schematic representation of the CcO-tethered lipid bilayer. The left panel depicts the structure of CcO from *R. sphaeroides*[4] (sketch created by VMD[13]) embedded in the lipid bilayer on the Ag surface. The subunits SU I comprising heme a, heme a3 and CuB, SU II attached to the surface, SU III, and SU IV are displayed in green, red, cyan and ocher, respectively. The right panel shows the peripheral domain of SU II with the binuclear CuA center (yellow spheres) and the C-terminal his-tag that has been modelled into C-terminus with the program Swiss PDB viewer.

In this way, a protein-tethered lipid bilayer is created on the metal surface (Fig. 1). The exposed hydrophilic protein domain serves as a spacer between the membrane and the electrode and thus generates a submembrane space, which is electrically insulated against the bulk solution as demonstrated by impedance measurements.[10] Although the His-tag is attached to the rather flexible helix H of subunit II,[14] the surrounding phospholipid bilayer is likely to force the membrane-spanning helices of CcO in an approximately perpendicular orientation with respect to the electrode.

SERR and RR spectra display the so-called marker band region,[5,6] which allows distinguishing between the six-coordinated low-spin (6cLS) heme *a* and the high spin (HS) heme a_3 as well as determining the redox activity of the enzyme. In the RR spectrum of fully oxidised CcO in solution, characteristic heme *a* marker bands are at 1585 (ν_2), 1635 (ν_{10}), 1500 (ν_3), and 1647 cm^{-1} (formyl stretching) whereas the 1370 cm^{-1} band originates from the ν_4 modes of both heme *a* and heme a_3 (Fig. 2A). The doublet at 1477 and 1490 cm^{-1} results from the mode ν_3 of a ferric HS heme that is hexa- (6cHS) and pentacoordinated (5cHS), respectively. This finding indicates that for a fraction of the heme a_3–Cu_B catalytic site the bridging ligand is dissociated from heme a_3.[5] The 5cHS and 6cHS configuration can only be distinguished by the frequency of the ν_3 mode whereas for the ν_2 and the formyl stretching mode the bands of both coordination states coincide and give rise to the relatively strong bands at 1570 and 1671 cm^{-1}, respectively.

SERR spectra of the CcO-tethered lipid bilayers on the Ag electrode display surprisingly intense signals of the hemes *a* and a_3

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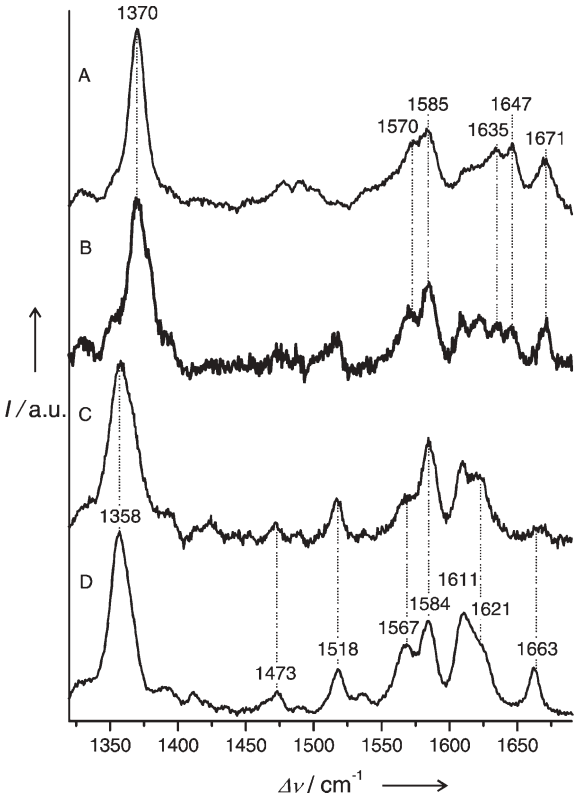

Fig. 2 SERR spectra of CcO tethered to a Ni-NTA functionalised Ag electrode and reconstituted with DPGPC, measured at (B) open circuit and (C) −0.65 V, compared with the RR spectra of CcO in solution, (A) in the fully oxidised and (D) dithionite-reduced form. Solutions contained 100 mM KCl and 50 mM potassium phosphate (pH 8) and, in RR experiments, 25 μM CcO and 0.1% DDM. All spectra were measured with 413 nm excitation. Further details of the spectroscopic experiments will be published elsewhere.

despite a separation of these groups from the metal surface of more than 50 Å (Fig. 1). At 0.0 V (*versus* Ag/AgCl) or at open circuit, the band frequencies reveal a good agreement with those of the RR spectrum of oxidised CcO (Fig. 2A,B). However, the shoulder at 1358 cm^{-1} on the low-frequency side of the prominent 1370 cm^{-1} band as well as the concomitant appearance of the characteristic 1517 and 1611 cm^{-1} bands indicates a small contribution from reduced heme *a* (*vide infra*), which may (partly) result from photoreduction by the exciting laser beam. Whereas under these conditions the immobilised enzyme is largely in the oxidised state, the SERR spectrum measured at −0.65 V (Fig. 2C) displays the vibrational band pattern of the reduced enzymes as revealed by comparison with the RR spectrum of fully reduced CcO in solution (Fig. 2D). Also for the reduced state, we note a very good agreement between the band frequencies in the RR and SERR spectra. A unique property of heme *a* is the large frequency downshift of the formyl stretching from 1647 to 1611 cm^{-1} which reflects a substantial redox-linked change of the hydrogen bond interactions of this substituent with Gln471 and Arg52.[4,6] Only a small downshift is observed for the stretching of the heme a_3 formyl group that remains in a largely hydrophobic environment.

Comparison of the RR and SERR spectra unambiguously shows that in both the ferric and the ferrous state the structures of the hemes *a* and a_3 as well as the specific interactions with the protein environment remain unchanged upon immobilisation. This is also true for the distribution between the 5cHS and 6cHS configuration in the ferric form of heme a_3, which has been shown to respond most sensitively to changes of the quaternary structure of the enzyme.[5] This finding can be taken as an indication that the entire enzyme structure is largely preserved in the immobilised state. The only noticeable differences between the RR and SERR spectra refer to relative intensities of the formyl stretchings that are weaker in the SERR spectra presumably due to the lower surface enhancement for the largely localised substituent modes as compared to the porphyrin modes.

The immobilised CcO can be reduced and re-oxidised by varying the electrode potential. No time-dependent changes are observed in the SERR spectra measured immediately after changing the potential. Thus, electron transfer reactions are fast compared to the time scale of the SERR experiments (< 1 min). Taking into account the considerable electron transfer distance to the primary acceptor Cu_A (> 40 Å; Fig. 1), we conclude that immobilisation of CcO *via* His-tags allows an efficient electron exchange with the electrode.

The redox potentials for Cu_A, heme *a*, and heme a_3 are estimated to be +0.09, +0.14, and +0.09 V, respectively.[15] However, complete reduction of the immobilised enzyme is not possible at electrode potentials positive to −0.65 V. Only to a minor extent, this large overpotential may result from the interfacial potential drops.[12] Instead, it is very likely that traces of oxygen already in solution, which were unavoidable in the present experimental set-up, cause the consumption of electrons at the catalytic heme a_3–Cu_B site. Under these steady state conditions, the oxidation states of the hemes are essentially controlled by the oxygen concentration in solution. This interpretation implies that the immobilised CcO is (largely) catalytically active. In fact, CcO that was immobilised to the electrode *via* a His-tag on the cytoplasmic side reveals enzymatic activity towards ferrous Cyt-c and is capable of transporting protons across the bilayer.[10]

In summary, we conclude that protein-tethered immobilisation of lipid bilayers allows the binding of CcO in a biomimetic environment and defined orientations while it is electronically coupled to the electrode. The structure of the heme sites is preserved and the catalytic activity appears to be retained. The present concept is not restricted to CcO but may be applicable to membrane proteins in general and thus opens new possibilities for studying and utilising immobilised proteins in fundamental and applied science.

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