Bioenergetics at the gold surface: SEIRAS probes photosynthetic and respiratory reactions at the monolayer level

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Abstract

The present study surveys a novel approach to studies of membrane proteins whose catalytic action is driven by the redox potential or by the membrane potential. We introduce SEIRAS (surface-enhanced IR absorption spectroscopy) to probe a monolayer of membrane protein adhered to the surface of a gold electrode. SEIRAS renders high surface sensitivity by enhancing the signal of the adsorbed molecule by approximately two orders of magnitude. It is demonstrated that reaction-induced spectroscopy is applicable by recording IR differences of cytochrome c after stimulation by the electrical potential. The impact of the membrane potential on the function of a membrane protein is demonstrated by performing light-induced difference spectroscopy on a microbial rhodopsin (sensory rhodopsin II) under voltage-clamp conditions. The methodology presented opens new avenues to study the mechanism of electron-triggered and voltage-gated proteins at the level of single bonds. As many of these catalytic reactions are of vectorial nature, control on the orientation of the membrane protein is mandatory. Approaches are presented on how to specifically adhere photosynthetic and respiratory proteins to the electrode surface and reconstitute these membrane proteins in the lipid bilayer. Functionality of such biomimetic systems is assessed in situ by spectro-electrochemical methods.

Introduction

The rapidly growing number of atomic-level structures obtained by X-ray crystallography provide the platform to describe protein function in terms of physical chemistry. Inherent to conventional crystallography, these structures are static, but the function of proteins requires structural changes to take place. Vibrational spectroscopy is an exquisite technique that combines high structural sensitivity with appropriate temporal resolution. Among the various vibrational spectroscopies, IR spectroscopy is a powerful method for the functional study of proteins and other biomaterials [1]. Most proteins, membrane proteins in particular, contain sufficient atoms to give rise to approximately $10^4$ vibrational modes. In addition to the modes arising from the peptide backbone and the amino acid side chains, there can be contributions from cofactors as well as water, buffer, lipids and detergent molecules present in the sample. Ultimately, there is an intractable number of overlapping vibrational bands that complicates the process of extracting details about the role of individual groups involved in the molecular mechanism. This task is simplified by the use of difference spectroscopy [2–4], which examines the differences in absorbance before and after inducing a perturbation that alters the state of the protein, usually simulating part of the physiological reaction that produces an intermediate state. This reaction alters a subset of the total vibrations; only those vibrations altered in the transition appear in the difference spectrum. The resulting changes to the absorbance spectrum can be less than 0.1% of the total absorbance. With this approach, the contributions from functional groups that are involved in the enzymatic reaction are extracted, including alterations as minute as protonation reactions, modulations in H-bonding of single-amino-acid side chains or even of internally trapped water molecules [5] with a time resolution as high as a few nanoseconds [6].

During the last few decades, FTIR (Fourier-transform infrared) difference spectroscopy has contributed significantly to the molecular understanding of the reaction mechanism of membrane proteins. In general, the transport and receptor function of membrane proteins is triggered by external stimuli, e.g. light (as in the photosystems and the rhodopsins), electrons (as in the respiratory chain complexes), ligands (as in G-protein-coupled receptors, the cysteine-loop superfamily and ATP-activated cationic channels) or a change in membrane potential (as in voltage-gated ion channels) (Figure 1). Owing to their central role in cellular function, membrane proteins are the target of more than 50% of all pharmaceutical drugs. Despite the biomedical relevance, molecular-level knowledge of the structure and function of these therapeutical targets is still scant.

In conventional IR spectroscopy, stacks of membrane protein layers are usually used to achieve an adequate difference.
signal from the sample. Typically, more than 200 layers are probed by either transmission spectroscopy or ATR (attenuated total reflection) spectroscopy [7]. Yet, such stacks of layers do not represent appropriate models for membrane proteins, which usually exist as monolayers in the cellular membrane. This is particularly evident when the impact of the membrane potential is investigated, a potential which is impossible to apply homogeneously across the whole stack of protein layers. Similarly, electron injection for triggering electron transfer in respiratory or photosynthetic proteins is possible only for the first layer adjacent to the electrode surface. Thus working at the level of a monolayer is a mandatory prerequisite for those experiments that use electrodes to stimulate protein activity. As a consequence, a novel IR spectroscopic approach must be developed to achieve acute sensitivity for the detection of reaction-induced differences from the monolayer of membrane protein.

**SEIRAS (surface-enhanced IR absorption spectroscopy)**

Monolayers of proteins and their functional changes are difficult to detect by conventional IR spectrometers as the amount of protein is of the order of a few pmol per cm² (10⁻¹² mol/cm²). This obstacle was overcome by the development of SEIRAS. SEIRA represents the phenomenon whereby the vibrational absorption bands of molecules that are adsorbed on to a nanostructured metal film are enhanced by a factor of 10⁻¹² to 10⁻¹⁰ [8]. The acute sensitivity of SEIRAS permits the detection of minute spectral changes of the adsorbed protein at the level of (sub-) monolayer coverage. The enhancement is restricted to within a short distance from the surface (<10 nm). Such an optical near-field effect essentially eliminates vibrational contributions from the bulk in the IR spectrum and selectivity detects signals from the adsorbed monolayer alone [9].

SEIRA active metal films of Au, Ag, Cu or Pt are usually prepared by high-vacuum evaporation or chemical deposition on IR-transparent materials, such as Si or CaF₂. These procedures typically create an island-like structure on the surface of the metal film, which is crucial for surface enhancement. The internal reflection (ATR) geometry is preferred in our set-up, with a gold thin metal film overlayer atop the reflection surface of the ATR prism (Kretschmann configuration). The advantage of this optical geometry is that the sample surface is freely accessible during the IR measurement. Details of the experimental set up can be found elsewhere [10].

**Protein binding to metal surfaces**

Vectorial catalysis (pumping and transport of ions and solutes) across the cellular membrane requires unidirectional orientation of integral membrane proteins. Biosynthetic pathways in the cell make use of protein integration machinery to insert proteins into membranes in an oriented manner. *In vitro* studies suffer from a random orientation of the membrane protein when the vectorial function is addressed. Most of the available methods for immobilizing proteins on to solid supports have traditionally relied on non-specific adsorption or on the reaction of naturally occurring chemical groups within proteins (mainly amines and carboxylic acids) with complementary reactive groups chemically introduced on to the solid support. In both cases, the corresponding proteins are attached to the surface in random orientation, which may cause the loss of the protein's biological activity. The use of recombinant affinity tags binds and orients membrane proteins when the solid surface is properly modified [11].

The chemical modification of the solid gold surface is performed by using heterobifunctional cross-linkers that typically comprise a thiol group at one end with a functional headgroup at the other end. The sulfur of the thiol group spontaneously binds in a quasi-covalent manner to the metal surface and an SAM (self-assembled monolayer) is formed [12]. The functional headgroup at the other terminus, which is accessible to the bulk solution, interacts specifically with the target protein to immobilize the latter at the metal surface. A variety of different immobilization strategies have been used which exploit covalent or non-covalent interactions of the molecule with the functional headgroup. Typical chemoselective tethering approaches are depicted in Figures 2(a)–2(c).

In the electrostatic approach, the attractive interaction is exploited between the positively charged surface patch (amino acid side chains of lysine, histidine and arginine residues) and the negatively charged carboxy-terminated SAM. This concept is effective in binding a small, polar protein such as cytochrome c, a protein that mediates electron transfer between the integral membrane protein complex of the respiratory chain. Cytochrome c adsorbed on carboxy-terminated SAM-modified electrode surface reveals direct electron transfer to the metal electrode, such that the redox state of surface-bound cytochrome c is controlled by the externally applied electrochemical potential [13–15]. This system is regarded as a model to study electron transfer to its native binding partnerCcO (cytochrome c oxidase).

Although the immobilization through electrostatic interaction works efficiently on small, water-soluble, polar proteins, this strategy is not effective for larger (bulky), non-polar...
Figure 2 | Surface reconstitution of membrane proteins

Scheme for surface tethering of a protein through (a) electrostatic interaction, (b) His tag/Ni-NTA chelating interaction, (c) strep-tag/streptavidin/biotin interaction, and (d) reconstitution of a surface-tethered protein into the lipid bilayer by detergent removal: (i) detergent-solubilized membrane protein adsorbed on to the chemically modified solid surface in the presence of detergent in solution, (ii) addition of Bio-Beads to adsorb detergent, and (iii) lipid molecules replace the detergent and form a bilayer around the hydrophobic moiety of the membrane protein.

proteins. When the protein is large, or its polarity is low, the electrostatic force is not sufficiently strong to orient the protein on the surface. This situation is particularly relevant to membrane proteins, because the detergent used for solubilization or the high salt concentration may screen the electrostatic interaction among the polar headgroups. Under these circumstances, a more specific and stronger interaction is required for surface tethering.

The use of affinity tags is a promising approach to fulfill this condition. A typical example is the tethering via a genetically inserted oligohistidine stretch (His$_6$ or His$_{10}$ tag) to the Ni-NTA (Ni$_{2+}$-nitrilotriacetate)-modified gold surface (Figure 2b) [16,17]. The interaction of the imidazole side chain of two histidine residues of the tag with the two available ligation sites from the Ni-NTA moiety provides a specific binding of the membrane protein on the metal surface. This approach is also superior in controlling the surface orientation of the protein, because the affinity tag can be introduced at virtually any position along the surface of the membrane protein by modern genetic techniques. Thus putting the His tag on opposite sides of the membrane surface essentially reverts the orientation of the protein with respect to the solid surface [18]. Such a control of the orientation of membrane protein is crucial when the vectorial function of the protein is addressed [17].

Surface modification comprises various steps. The bare gold surface is first exposed in a solution containing DTSP [dithiobis(succinimidyl propionate); a cross-linking reagent]. DTSP spontaneously forms a monolayer through cleavage of the S–S bond and covalent linkage of the sulfur group with the metal. Figure 3(a) depicts the SEIRA spectrum of the TSP [thiobis(succinimidylpropionate)] monolayer formed on the gold surface measured during the adsorption (in situ). The appearance of IR bands of the three C=O stretching modes (at 1809, 1783 and 1793 cm$^{-1}$) indicates the formation of the TSP monolayer [16]. In the next step, the TSP SAM layer is immersed in an aqueous solution of ANTA (aminonitrilotriacetate). The primary amine group of ANTA reacts with TSP to form a carboxamide linkage (Figure 3b). Again,
Figure 3 | Tethering recombinant PS2 to the gold surface

(a) SEIRA spectrum of TSP monolayer adsorbed on the Au surface. The reference spectrum is taken from the solvent DMSO in contact with the bare Au surface. (b) SEIRA spectrum of the NTA monolayer formed by coupling of TSP with ANTA. The reference spectrum is taken from the TSP-modified Au surface. (c) Time series of SEIRA spectra of PS2 during adsorption to the Ni-NTA-modified Au surface. The adsorption time is given in minutes.

After ligation of the central Ni$^{2+}$ ion by the NTA layer, detergent-solubilized membrane protein with a His tag is introduced (Figure 2d, i). Figure 3(c) depicts a time series of SEIRA spectra recorded during immobilization of recombinant PS2 (Photosystem II) on the Ni-NTA SAM [19]. Two bands evolve at 1661 and 1550 cm$^{-1}$, which are assigned to the amide I and II vibrations respectively of the protein backbone. The peak positions of these bands are characteristic of an $\alpha$-helical protein, in accordance with the predominant secondary-structure element of PS2 [20,21]. The position of the amide frequency represents a qualitative means to gauge the structure of the surface-bound protein, e.g. for competitive adsorption of two different proteins (with different secondary structures) (X.U. Jiang, A. Zuber, J. Heberle and K. Ataka, unpublished work) or for the identification of structural changes. The molecular sensitivity of IR spectroscopy represents a significant advantage of SEIRAS in comparison with other surface-probing techniques, such as surface plasmon resonance [12,23] or the quartz crystal microbalance [24], which mainly provide quantitative information.

After oriented binding of PS2 to the surface, full functional integrity is regained by embedding the membrane protein into a lipid layer [16]. This process is schematically shown in Figure 2(d). The surface-tethered membrane protein is incubated with detergentdestabilized lipid vesicles (liposomes) and microporous Bio-Beads are added. The amount of detergent is reduced by its adsorption in the Bio-Beads, while the concentration of liposome stays constant because of its larger size than the pore size of the Bio-Beads. As the detergent concentration gradually decreases, a lipid layer spontaneously assembles around the hydrophobic moiety of the membrane protein. Lipid reconstitution increases the stability of the membrane protein.

In electrochemical experiments, the redox properties of the Ni-NTA may interfere with the process being studied at potentials $>0.5$ V [compared with an NHE (normal hydrogen electrode)]. For those experiments, an alternative is represented by the use of the strep-tag instead of the His tag (Figure 2c) [23–25]. This method employs the specific interaction between surface-tethered streptavidin with a genetically introduced strep-tag of the protein. A streptavidin monolayer is formed through binding to a self-assembled biotin monolayer on the Au surface. Then, the target
protein binds via the strep-tag, which is structurally related to biotin, to the unoccupied binding site of streptavidin. We have recently applied SEIRAS to the investigation of the surface structure and membrane protein binding properties of the protein/streptavidin/biotin-SAM layer [22]. The biotin/streptavidin interlayer is superior to the Ni-NTA-modified Au surface with respect to selectivity and regeneration properties. A disadvantage of the biotin/streptavidin system is its size. As the decay length of the surface plasmon is approx. 10 nm, the connecting layer of the target protein should be as short as possible. The Ni-NTA layer is much thinner (≈1.5 nm) than the biotin/streptavidin layer (≈5 nm). With the former linker, the surface-tethered protein is closer to the gold surface where it experiences a stronger enhancement of the vibrational bands. In conclusion, the interlayer for selective binding of the protein to the solid surface must be carefully chosen to suit the purpose of the experiment.

Functional studies on surface-tethered proteins

The advantage of SEIRAS becomes most beneficial when it is applied to functional studies of the tethered membrane protein monolayer. Functional studies of proteins are performed by the application of external stimuli, such as light or chemicals, to initiate the enzymatic reaction. In addition to these stimuli, the use of a metal electrode enables either a charge imbalance to be created across the membrane (membrane potential) or electrons to be injected directly into the membrane protein (change in redox potential).

After setting up the biomimetic system, the functionality of surface-bound protein must be carefully checked. For redox-active proteins, electrochemical methods are very powerful since electrical current (or voltage) can be directly applied to the adsorbed protein monolayer. It is possible to combine SEIRAS with electrochemistry by using the Au film as a working electrode. The short distance between the electrodes allows a fast response of the cell after a sudden potential step (∼100 μs for a potential step of 800 mV). This response time represents the upper limit in our recently developed time-resolved FTIR experiments. Reversible oxidation and reduction currents are observed in cyclic voltammetric experiments on cytochrome c electrostatically adhered to the surface of a SAM of MPA (mercapto-propionic acid) [26]. The formal potential of +228 mV (compared with an NHE) is in the range of what was observed for cytochrome c in solution [27]. Thus the surface modification preserves the full functionality of cytochrome c. Redox-induced SEIDA spectra were recorded from the monolayer of cytochrome c when the electrode potential was cycled. The frequencies of the vibrational bands are identical with cytochrome c in solution, but the band intensities differ in amplitude when the chemical composition of the SAM headgroup is altered [28]. The former observation suggests that the functionality of cytochrome c is fully preserved, independently of the surface modification, while the latter observation is attributed to the differences in surface structure, i.e. the orientation and the relative distance of cytochrome c from the surface. The redox-induced SEIDA spectrum of cytochrome c when adsorbed to the carboxy-terminated headgroup bears great resemblance to the SEIDA spectrum of cytochrome c when adsorbed to a CeO-modified monolayer [18]. This result implies that the CeO-binding site is mainly composed of negatively charged amino acid side chains, such as glutamate or aspartate. This observation is in good agreement with the result of the X-ray structural analysis, which identified residues Glu148, Glu157, Asp195 and Asp214 (for CcO from Rhodobacter sphaeroides) as the major interaction partners with cytochrome c [29].

The proper function of surface-attached and lipid-reconstituted CeO was demonstrated by cyclic voltammetry. When cytochrome c, the native electron donor, is added to the reconstituted CcO surface, a catalytic current is observed that is due to oxygen reduction [16]. The activity of surface-immobilized PS2 was demonstrated by detecting a photocurrent during illumination of the monolayer measurements. The variation in excitation wavelength produced an action spectrum that tallies with the absorption spectrum of chlorophyll [19]. All these results demonstrate the feasibility of the experimental approach that controls the orientation, stability and functionality of the membrane protein monolayer.

Although each surface modification step was followed in situ by SEIRAS, the recording of a surface-enhanced IR difference spectrum of a membrane protein was not possible at that stage. Therefore we started with a model system whose reaction is conveniently triggered by light [30]. In Halobacteria, the two sensory rhodopsins [SRI (sensory rhodopsin I) and SRII] in complex with their respective transducers (HtrI and HtrII) trigger the movement of the flagellated cells towards favourable or away from harmful conditions in a wavelength-dependent manner (phototaxis [31]). We used recombinant SRII and bound the protein via a C-terminal His tag to the gold surface. Reaction-induced SEIDAS was performed under photostationary conditions. We succeeded in recording an IR difference spectrum on a monolayer of a membrane protein for the first time [32]. The signal-to-noise ratio was sufficient to even study the influence of a transmembrane electrical potential on the activity of the photosensory protein. The observed SEIDA spectra reveal that the application of negative membrane potentials leads to the selective halting of the light-induced proton transfer at the stage of Asp75, the counter ion of the retinal Schiff base, but leaves the other structural rearrangements unchanged. These results demonstrate that SEIDAS can be used to study the influence of the membrane potential on the reaction mechanism of a membrane protein at the molecular level.

References


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