Analyzing the catalytic processes of immobilized redox enzymes by vibrational spectroscopies

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Critical Review

Analyzing the Catalytic Processes of Immobilized Redox Enzymes by Vibrational Spectroscopies

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Summary

Analyzing the structure and function of redox enzymes attached to electrodes is a central challenge in many fields of fundamental and applied life science. Electrochemical techniques such as cyclic voltammetry which are routinely used do not provide insight into the molecular structure and reaction mechanisms of the immobilized proteins. Surface-enhanced infrared absorption (SEIRA) and surface-enhanced resonance Raman (SERR) spectroscopy may fill this gap, if nanostructured Au or Ag are used as conductive support materials. In this account, we will first outline the principles of the methodology including a description of the most important strategies for biocompatible protein immobilization. Subsequently, we will critically review SERR and SEIRA spectroscopic approaches to characterize the protein and active site structure of the immobilized enzymes. Special emphasis is laid on the combination of surface-enhanced vibrational spectroscopies with electrochemical methods to analyze equilibria and dynamics of the interfacial redox processes. Finally, we will assess the potential of SERR and SEIRA spectroscopy for *in situ* investigations on the basis of the first promising studies on human sulfite oxidase and hydrogenases under turnover conditions.
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Keywords surface-enhanced resonance Raman spectroscopy; surface-enhanced infrared absorption spectroscopy; cyclic voltammetry; enzyme immobilization, electron transfer; electrocatalysis.

INTRODUCTION

Immobilization of enzymes on electrodes has become a central topic in various fields of fundamental and applied life science. Redox enzymes wired to an electrically conducting support constitute an essential building block of bioelectrochemical devices in biotechnological applications that exploit the high specificity of enzymatic reactions for the development of biosensors, biocatalysts, or biofuel cells (*1*). Even though there is a major step from the proof-of-principle on the laboratory scale to viable industrial applications, the recent advances in materials chemistry, specifically the preparation of conducting nanoporous support materials (*2, 3*), generate a strong driving force in this research field. Typically, metals or semiconductors serve as the interface between the biomolecule and the electrical circuit such that the mode of enzyme immobilization is the crucial step for the proper functioning of such devices. In many cases, direct binding to metals or inorganic materials destabilizes the protein structure and frequently causes denaturation (*4*), which can be avoided by appropriate coating of the surfaces (*5–7*). Thus, various strategies for biocompatible enzyme immobilization have been proposed and tested including surface coating by polyelectrolytes, peptides, or amphiphiles (*5–7*). These latter compounds comprise lipids or lipid analogs and form self-assembled monolayers (SAMs) or bilayers on a solid support. Such coatings can also be considered as simple models for biological membranes, mimicking the natural environment of integral membrane proteins, or membrane-attached proteins (*8*). Here, the supporting electrode may exert two functions: it serves as an artificial reaction partner for donating electrons to or accepting electrons from immobilized redox proteins; in addition, it allows altering the potential drop across the membrane model for controlling the electric field acting on immobilized proteins. Thus, these membrane models are of particular interest for fundamental studies that aim at elucidating the processes of enzymes under conditions that are most closely related to their physiological reaction conditions (*8, 9*).

No matter if the studies of immobilized enzymes are motivated by potential applications or fundamental questions, there are several key issues that have to be addressed. Experimental conditions have to be optimized to achieve immobilization of the redox enzymes under preservation of the native structure,

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with good electrical contact to the electrode, and high catalytic efficiency.

Usually, electrochemical methods are used to probe the processes of immobilized redox enzymes (*10*). These techniques, among them particularly cyclic voltammetry (CV), are sensitive tools to monitor electron exchange reactions of the enzyme with the electrode under nonturnover and turnover conditions and thus allow for the *in situ* analysis of the biocatalytic processes. Electrochemical methods, however, exclusively probe the current as a response of the potential-induced interfacial reactions but do not provide information about the molecular structure and dynamics of the species involved. Thus, solely based on electrochemical data, the determination of enzymatic reaction mechanisms is hardly possible. To bridge this gap and to complement electrochemical techniques, spectroscopic methods have to be used, which are sufficiently sensitive and selective with respect to the immobilized proteins and allow identifying the nature of the various (intermediate) states formed during the catalytic cycle (*11*). These requirements are fulfilled by vibrational spectroscopies, specifically surface-enhanced resonance Raman (SERR) and surface-enhanced infrared absorption (SEIRA) spectroscopy (*7, 8, 12–14*).

In this contribution, we will review the current state of spectroelectrochemical analysis of immobilized enzymes using surface-enhanced vibrational spectroscopies. We will first outline the principles of the methodology, the potential of the techniques and their current limitations. The subsequent sections are dedicated to review the results obtained on (i) monitoring immobilization of enzymes and characterization of their structures, (ii) analyzing redox equilibria and electron transfer (ET) dynamics, and (iii) probing the enzymatic processes under turnover conditions. Finally, we will briefly describe promising developments of the approaches.

METHODOLOGY

Surface-enhanced vibrational spectroscopies exploit the enhancement of the oscillating electric field of radiation due to the dipole–dipole coupling with surface plasmons of a metal, that is, the oscillations of the electron gas (*14*). An efficient coupling and a strong field enhancement requires the frequency match of the incident radiation and the surface plasmons, the frequency of which depends on the surface morphology and the dielectric properties of the metal. According to these boundary conditions, Ag and Au surfaces with roughness dimensions smaller than the wavelengths of the incident light have turned out to exhibit the strongest field enhancement by a factor of up to 30, which affect the optical transitions of molecules in close proximity to the metal surface. As in Raman scattering, the fields of both the incident and scattered radiation are enhanced and the intensity is proportional to the square of the electric field, enhancement factors of up to 10^6 are noted for the surface-enhanced Raman (SER) effect. Particularly important for studying redox enzymes is the combination of the SER effect with the molecular resonance Raman (RR) effect that leads to the exclusive enhancement of Raman modes of a chromophore on excitation in resonance with its electronic transition. As a result, the SERR (= SER + RR) effect offers the possibility to selective probe the cofactor of immobilized enzymes.

Compared with the SER effect, the amplification of the infrared absorption (IR) signals in SEIRA is distinctly weaker (10^2–10^3), as only the electric field of the incident light is enhanced. Nevertheless, this enhancement is sufficient to selectively probe the IR signals of immobilized proteins.

The plasmonically enhanced field decays with the distance from the metal surface, leading to a drop of the enhancement factor to about 10–20% on separation of the molecules by about 3 and 6 nm in the case of SERR (SER) and SEIRA, respectively (*14*). Thus, it is possible to use SERR and SEIRA spectroscopy even if the enzymes are not directly adsorbed to the metal but immobilized on a biocompatible surface coating, a prerequisite for preserving the native protein structure in most cases (*vide supra*).

Because of its higher chemical and electrochemical stability, Au is preferentially used in SEIRA experiments but its applicability to SER/SERR spectroscopy is restricted to excitation wavelengths above 550 nm as strong d→d transitions at higher energies impair direct excitation of surface plasmons (*15*). However, as most of the cofactors of enzymes exhibit electronic transitions below 500 nm, the SERR spectroscopic analysis is restricted to Ag which provides plasmonic enhancement from 400 nm up to the infrared spectral region (*14, 15*).

SERR and SEIRA spectroscopy can be operated as spectroelectrochemical techniques, if the metallic support material serves as the working electrode (*11–14, 16*). Such devices allow determining the electronic communication of the immobilized enzyme with the electrode not only in static experiments (redox equilibria) but also in time-resolved measurements in conjunction with the potential jump technique (ET dynamics) (*17–19*). Time-resolved SERR and SEIRA spectroscopy can be applied to study reversible processes with a time-resolution of down to a few microseconds, depending on the response time of the electrochemical cell.

ENZYME IMMOBILIZATION AND STRUCTURAL ANALYSIS

Immobilization of integral membrane proteins and soluble proteins requires different concepts (Fig. 1) (*5–7, 16, 20–22*). For soluble proteins, the knowledge of the three-dimensional structures substantially facilitates the design of an appropriate immobilization approach which may be based on electrostatic, hydrophobic, or covalent binding. Proteins exhibiting well-defined positively or negatively charged surface regions may be attached via electrostatic interactions to SAMs (or bilayers) carrying oppositely charged head groups. Correspondingly, also proteins with hydrophobic binding domains may be specifically bound to layers of methyl-terminated amphiphiles. Frequently,

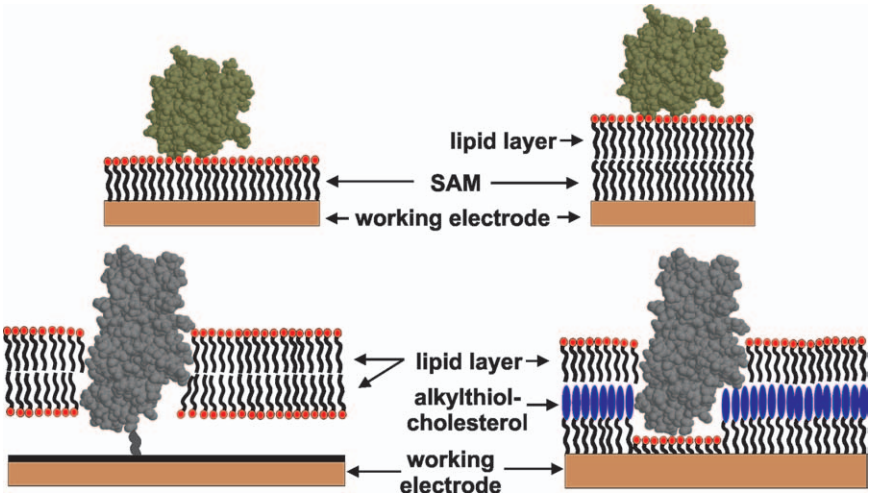

Figure 1. Illustration of various immobilization strategies for soluble (top) and integral membrane proteins (bottom). Further information is given in the text.

these regions constitute interaction domains for the redox processes with the natural partner proteins such that the orientation of the bound protein with respect to the SAM surface may be quite similar to the precursor complex of the natural template (*7, 8*). Many proteins, however, do not display clearly defined charged or hydrophobic binding domains such that mixed SAMs including amphiphiles with charged, polar (hydroxyl-terminated alkanethiols), or hydrophobic headgroups have to be tested (*23, 24*). An additional parameter to control protein binding is the ionic strength and the pH of the solution, specifically when SAMs including charged amphiphiles are used. These immobilization strategies guarantee a mobility of the adsorbed protein, which is essential for the interfacial ET process (*25*). The situation is different for covalent binding which can be achieved via cross-linking of preformed electrostatic complexes or via the thiol side chains of Cys residues, which may either be available in the native protein or introduced at different positions on the protein surface by genetic engineering. If covalent binding occurs via more than one attachment sides, the mobility of the bound protein is significantly reduced leading to a rather rigid configuration (*26*).

Integral membrane proteins may be immobilized in the solubilized form such that the interaction with the surface is largely mediated by the detergent molecules (*27*). This rather simple procedure does not ensure a uniform orientation of the protein, which in turn requires more sophisticated procedures to assemble membrane-like structures on the electrode surface, such as supported, tethered, or suspended membranes (*20–22*). Among them, only membranes tethered via a protein His-tag have been used so far for SERR- and SEIRA-spectroelectrochemical studies of enzymes (*28–32*). The assembly of these devices requires first the functionalization of the electrode with nitrolacetic acid (NTA) to form a Ni–NTA or Zn–NTA monolayer after incubation with Ni(II) or Zn(II) solution, followed by the attachment of the solubilized membrane protein via a coordinative complex using the imidazole residues of the protein's His-tag. Subsequently, the detergent molecules are removed by Biobeads to allow for the reconstitution of a lipid bilayer around the anchored proteins. In this affinity binding approach, denoted also as protein-tethered lipid bilayer, the attachment site of the protein is exclusively determined by the position of the His-tag such that a uniform orientation is guaranteed. A drawback of these assemblies is associated with the relative large distance of the protein's cofactor which attenuates the SERR intensities (*14*) and impairs the ET (*vide infra*) (*33*).

The efficiency of protein immobilization as well as the adsorption kinetics may be monitored by vibrational spectroscopies (*32, 34, 35*). In a first approximation, the intensity of the SEIRA and SERR signals can be taken as a measure for the amount of the bound protein, although surface selection rules have to be considered for relating intensities and surface concentrations. For symmetric cofactors, the surface-enhancement of the RR scattering critically depends on the relative orientation of the chromophore (and thus of the protein) with respect to the metal surface (*36*). In the case of heme proteins, optimum enhancement of the RR-active totally symmetric modes is achieved for a perpendicular orientation of the porphyrin such that variations of the absolute SERR intensities may reflect either changes of the surface coverage or a reorientation of the protein on the surface (*25*). Deviations from the perpendicular orientation, however, cause a relatively stronger enhancement of nontotally symmetric modes versus totally symmetric modes under off-resonance conditions, whereas a decrease of the surface concentration is associated with an intensity reduction for all porphyrin modes.

SEIRA intensities may exhibit an orientation-dependence, as the enhancement of the IR absorption bands is particularly strong for modes associated with a dipole moment perpendicular to the surface (*37*). For the amide I mode of α-helices, the dipole moment is aligned along the axis of the helix, affording

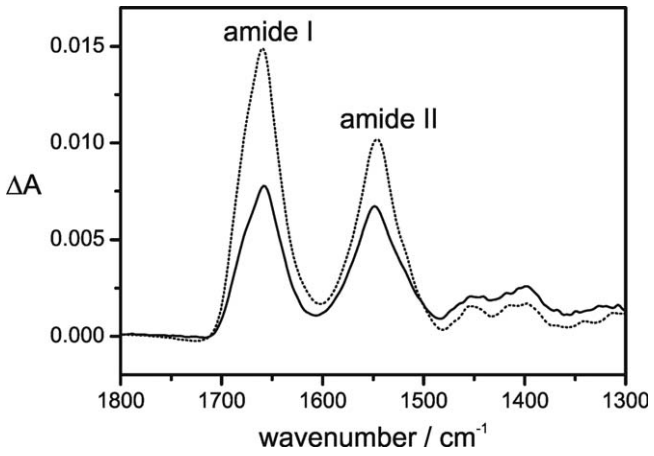

Figure 2. Absolute SEIRA spectra of the integral *B. japonicum* cbb_3 oxygen reductase immobilized on detergent coated electrode (solid line) and *via* the His tag tethered protein (dotted line). Further details are given in ref. *32*.

strong SEIRA intensities of the amide I for α-helices with perpendicular orientation with respect to the surface (*32, 34*). Thus, the SEIRA intensity ratio of the amide I and amide II modes is specifically useful for monitoring the immobilization of membrane-bound proteins including transmembrane helices. As an example, Fig. 2 illustrates the SEIRA spectra of the oxygen reductase cbb_3 (cytochrome cbb_3) from *Bradyrhizobium japonicum*, immobilized in two different modes: adsorption of the solubilized enzyme with a largely random orientation and immobilized via a His-tag in a tethered membrane with a uniform perpendicular orientation of the transmembrane helices, reflected by a low and a large amide I/amide II intensity ratio, respectively (*32*).

On comparison with the RR spectra of the enzyme in solution, SERR spectra offer the possibility to check the integrity of the active structure of the immobilized enzyme and to identify different (oxidation) states of the cofactor (*38*). This approach is particularly powerful for heme enzymes, as porphyrins display a rich vibrational band pattern which is strongly enhanced on excitation in resonance with the Soret transition at about 400 nm. Among them are the so-called marker bands which originate from modes, the frequencies of which are well-correlated with the oxidation, spin- and coordination state of the heme (*39, 40*). Furthermore, the RR/SERR spectra *in toto* represent a characteristic fingerprint of the specific heme–protein interactions.

A large body of SERR spectroscopic data has been accumulated on the heme protein cytochrome *c* (Cyt-c), an ideal model protein to explore the possible consequences of immobilization on the structure of the redox center (*7–9, 16, 26, 38, 40, 41*). Thus, it could be shown that electrostatic adsorption may cause a rearrangement of the heme pocket structure, if the interfacial electric field experienced by the immobilized protein exceeds a certain threshold (*38*). The primary target of the field-induced structural perturbation is the bond between heme iron and the axial Met ligand which, in the ferric state, is easily broken (*40*). The corresponding transition from the native six-coordinated low spin (6cLS) to a five-coordinated high spin (5cHS) state is usually followed by further structural changes in the heme pocket including the His binding to the vacant coordination site, leading to a new non-native 6cLS state. On the basis of the characteristic component spectra, SERR spectra can be disentangled to provide the relative spectral contributions of the various oxidation and coordination states. This is illustrated in Fig. 3 that displays SERR spectra of Cyt-c electrostatically immobilized at high electric fields.

The field-induced formation of a 5cHS species is not a unique property of Cyt-c but is also observed for a variety of heme proteins and enzymes such as cytochrome P-450 (P-450) (*42*), cytochrome b_{562} (*43*), cytochrome c_6 (*24*), cytochrome c_3 (*44*), cytochrome cbb_3 (*32*), the membrane-bound hydrogenase (MBH) from *Ralstonia eutropha* (*45*), and human sulfite oxidase (*46*). Correspondingly, immobilization conditions for ferric heme enzymes should be optimized with respect to maximum SERR intensity, corresponding to maximum surface coverage, but even more important, to minimum contributions from the 5cHS state. Note that this latter screening criterion only holds for heme enzymes which in the ferric state adopt a 6cLS, whereas it is not applicable for heme enzymes like cytochrome

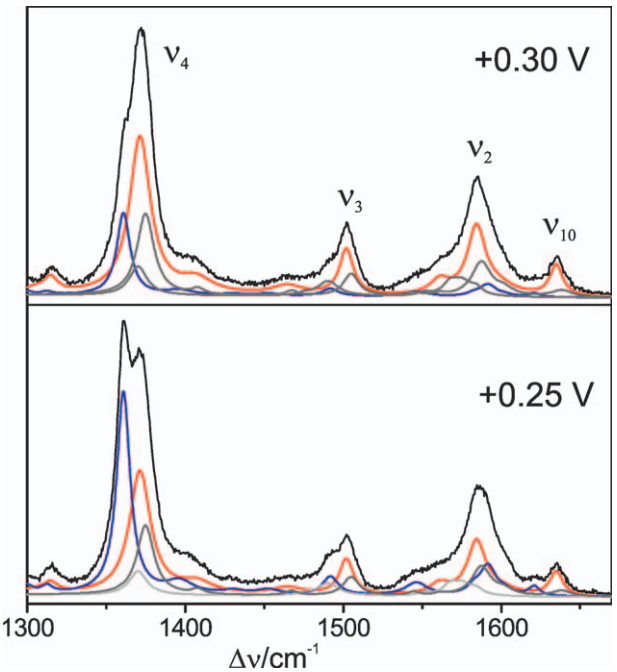

Figure 3. SERR spectra of Cyt-c immobilized on an Ag electrode coated with a SAM of mercaptoacetic acid, measured at different electrode potentials (vs. NHE). The blue, red, and gray lines represent the component spectra of the reduced B1, oxidized B1, and oxidized non-native states B2[6cLS] and B2[5cHS], respectively. Further details are given in ref. *9*.

c peroxidase, in which the 5cHS configuration is the prevailing species in the native state (*47*).

Immobilization of P-450 represents a particular challenge in as much as this enzyme easily undergoes a transition to its inactive form P-420 even in solution. So far, various attempts failed to bind the enzyme on SAM-coated electrodes under preservation of its native structure (*42*). It seems to be that hybrid bilayers on electrodes, consisting of a methyl-terminated SAM and a lipid layer on top, represent a more promising approach although the distance-dependent attenuation of the SERR signal was found to be significant (*48*). Thus, further amplification by addition of coated Ag nanoparticles was required to obtain a satisfactory SERR spectrum, which, however, displayed a vibrational signature similar to that of the RR spectrum of the native enzyme.

The local electric field strength experienced by the immobilized protein may be determined on the basis of the vibrational Stark effect using nitrile labels introduced at specific positions on the protein surface (*49*). The corresponding C≡N stretching mode displays a field-dependent frequency shift that can be probed by SEIRA spectroscopy. In this way, a field strength of about 10^9 V/m has been recently determined for electrostatically immobilized Cyt-c at a position close to the SAM/protein interface. The value is in good agreement with previous estimates derived from simple electrostatic models (*38*). To avoid field-induced denaturation, the local electric field may be weakened either by varying the thickness of the monolayer, its surface charge density via admixture of uncharged amphiphiles, or by increasing the ionic strength of the bulk solution (*24*).

The SERR spectroscopic analysis of redox enzymes containing cofactors other than hemes is significantly more difficult, mainly due to the much lower intrinsic SERR intensity. However, a convincing case study has been presented by Broderick et al. (*50*), who have investigated the nonheme iron enzyme chlorocatechol dioxygenase adsorbed on citrate-coated Ag nanoparticles. The authors demonstrated the preservation of the native active site structure of the enzyme on adsorption and, moreover, provided evidence for a significant catalytic activity, via a combination of enzymatic assays and SER spectroscopic product detection under turnover conditions.

DIRECT ELECTRON TRANSFER

Direct electrical communication (*i.e.*, without using soluble redox mediators) of immobilized redox enzymes with electrodes is usually tested by CV which, under nonturnover conditions, affords the redox potential. Furthermore, scan-rate dependent measurements allow distinguishing between diffusion-controlled and surface-confined ET and offer the possibility to determine the rate of the heterogeneous ET (*10, 33*). SEIRA and SERR spectroscopy are more time-consuming than CV but they provide deeper insight into the redox process at electrodes, as it has been documented particularly for the electron-transfer protein Cyt-c on SAM-coated electrodes (*8, 9, 16, 18, 19, 25, 26, 38, 41, 51*). Here, a large body of experimental data has been accumulated which have contributed to a comprehensive view about the molecular events associated with interfacial ET of redox proteins and enzymes in general. It has been shown that electron tunneling is the rate-limiting step only at weak interfacial electric fields (*9, 18, 51*). Increasing electric field strengths, which may be established by decreasing the thickness of SAMs carrying carboxylate headgroups, raises the activation barrier for the rotational diffusion of the bound protein that is usually required to adopt a protein orientation with optimum ET pathway (*25, 52*). Under these conditions, the experimentally determined ET rate constant k_{ET}^{app} displays an exponential distance-dependence in the low-field regime, characteristic of electron tunneling, but levels off when protein reorientation becomes rate-limiting (*25*), a behavior that has been observed for a variety of redox proteins and enzymes (*16*). A further increase of the electric field affects electron tunneling directly such that k_{ET}^{app} even decreases again at very short distances (*9, 16*). In this high field regime, also the dynamics of the redox-linked reorganization of the interfacial hydrogen-bond network is eventually slowed down, reflected by a kinetic H/D isotope effect of k_{ET}^{app} (*9*). Furthermore, such high fields promote the transition to non-native states (*vide supra*) (*38*).

As a consequence, optimization of the direct ET of redox enzymes on SAM-coated electrodes requires a SAM thickness that avoids electric-field perturbation of protein mobility but ensures efficient electron tunneling. Additional parameters for fine-tuning interfacial ET are the surface charge density of the SAM (*i.e.*, the SAM composition), as well as the pH and ionic strength of the bulk solution as demonstrated in recent studies on human sulfite oxidase (*vide infra*) (*46*).

Oxygen reductases constitute the most widely studied class of membrane-bound enzymes that has been studied in the immobilized state. In one of the first SERR spectroscopic investigations, the type B (quinol-dependent) oxidase from *Acidianus ambivalens* was immobilized in the solubilized form under retention of the native structure of the heme *a* and heme a_3 cofactor sites (*27*). Because of the characteristic vibrational signatures of both hemes, potential-dependent SERR measurements allowed the unambiguous determination of the redox potentials for heme *a* and a_3. Most surprisingly, the order of the redox potentials was found to be reversed compared to type A cytochrome *c*-dependent oxidases which points to a different mechanism of the electroprotonic energy transduction. In contrast to type A enzymes, the intraprotein ET of this aa_3 oxidase is already guaranteed by the order of the midpoint potentials at the onset of enzyme reduction and, therefore, does not require a complex network of cooperativities to ensure exergonicity.

In contrast to the direct adsorption of the solubilized enzyme, the concept of protein-tethered lipid bilayer has been used to immobilize type A cytochrome *c* oxidases (CcO). SERR spectra obtained from these devices were found to be basically identical to the RR spectra of the solubilized enzymes in solution, ruling out any immobilization-dependent structural change of the redox sites (*28, 29, 31*). Potential-dependent

SERR measurements by Hrabakova et al. (*29*) demonstrated that even at high overpotentials a complete reduction of the heme cofactors could not be achieved. Although heme *a* was found to be largely reduced, a considerable fraction of heme a_3 remained in the oxidized state. This was attributed to spurious contributions of oxygen in the electrochemical cell which could not be completely avoided even under continuous degassing of the solution with highly purified Ar. As a consequence, the reduction of oxygen at the catalytic site leads to a distinct steady state concentration of ferric heme a_3. This interpretation is consistent with time-dependent SERR experiments that reveal a very slow rate of heme *a* reduction of 0.002 sec^{-1}, independent of the orientation of the immobilized CcO. Taking into account the large separation of the heme *a* cofactor from the electrode in both orientations (*ca*. 5 nm), an electron hopping mechanism via nondedicated pathways has been proposed (*29*). The conclusions are in line with the electrochemical and SEIRA studies by Ataka et al. (*30*) on the same enzyme immobilized on Au instead of Ag electrodes. In that study, no CV response could be detected with an orientation of the electron entry site of CcO facing the electrode surface. Immobilization via the opposite orientation and the addition of Cyt-c - the natural electron donor of CcO - to the bulk solution affords a distinct CV signal indicative for the involvement of Cyt-c in the ET to CcO and the subsequent catalytic oxygen reduction. The function of Cyt-c as a mediator for the enzymatic process of CcO is further supported by SEIRA experiments demonstrating the complex formation of Cyt-c with CcO.

Consistent with these results are the data obtained for the cbb_3 oxygen reductase from *B. japonicum* immobilized according to the same concept (*vide supra*) (*32*). Here, both CV and SERR spectroelectrochemical measurements revealed the reduction of the heme cofactors and a electrocatalytic process only in the presence of the redox mediator N,N,N′,N′-tetramethyl-p-phenylendiamine.

Protein-tethered lipid bilayers ensure a uniform enzyme immobilization in a quasi-natural environment but, due to the multiple interfacial potential drops and the large separation of the enzyme's cofactors, these devices do not allow for a potential control at the reaction centers and provide only a poor direct electronic communication of the redox enzymes with the electrode such that an efficient electrocatalytic process requires the presence of redox mediators. In this respect, the more recent article by Friedrich et al. (*31*) presents rather puzzling conclusions. While ignoring the studies described above, the authors interpret the results in terms of an efficient electronic coupling between the electrode and the immobilized CcO, with the primary electron acceptor Cu_A oriented toward the electrode. In fact, a distinct CV signal is reported in the absence of a redox mediator albeit at an overpotential of about −450 mV compared with the redox potential of Cu_A. The CV was simulated in terms of a series of ET steps including the CcO cofactors and the His-tag coordinated Ni center. The simulation requires a transient ET step from the electrode to the Ni site with a rate constant $> 5{,}000$ sec^{-1} which, however, cannot be rationalized in view of the long distance (> 2 nm) and the endergonic character of the reaction ($\Delta E \approx 0.2$ V). As a support for the analysis of the CV, the authors refer to potential-dependent SERR experiments. However, a careful inspection of the poorly resolved spectra shown in that work indicates a very broad redox transition which is far from being complete at −0.35 V, which in turn is consistent with previous SERR spectroscopic results (*29*). Thus, the work by Friedrich et al. (*31*) does not question the critical assessment for this immobilization approach as outlined above.

CATALYTIC PROCESSES

So far, only a few systems have been investigated by SERR- or SEIRA-spectroelectrochemistry under turnover conditions. In the case of human sulfite oxidase (hSO), SERR spectroscopy has been used to analyze the catalytic cycle of hSO bound to electrode surfaces (*46*). This enzyme is composed of a large domain harboring a molybdopterin cofactor (Moco), an N-terminal cytochrome b_5 heme domain, and a large C-terminal dimerization domain (*53*). The Moco and heme domains are connected by a flexible polypeptide chain region. Catalytic sulfite oxidation takes place at the Moco followed by a fast intramolecular ET to the heme center and from there to an external redox partner (*54*). Catalytic efficiency in hSO is proposed to be strongly coupled to large domain motions as, prior to efficient intramolecular ET, re-orientation of the flexible heme domain toward the Moco binding site is required to form a complex with a short heme-Moco distance (*55, 56*).

Because of the low absorption coefficient of the Moco SERR, spectra of hSO immobilized on nanostructured Ag electrodes exclusively display the vibrational signature of heme b_5 (*46*). Optimum immobilization under nonturnover conditions with respect to preservation of the native heme pocket structure and efficiency of heterogeneous ET were found for mixed amino/hydroxyl-functionalized SAMs and high ionic strength. These studies were then extended to turnover conditions using a combined SERR spectroscopic-electrochemical approach. Catalytic currents derived from CV measurements were correlated with the rates of intramolecular ET on monitoring the heme oxidation state with SERR spectroscopy during the catalytic cycle. On increasing the ionic strength in the solution by raising buffer concentration from 5 to 750 mM, a reversible decrease in the SERR signal intensity (at constant potential) was observed, whereas the catalytic current and the intramolecular ET rate drastically increased under the same conditions (Fig. 4). In agreement with dipole moment calculations of the hSO homodimer, it was concluded that enzyme immobilization primarily occurs via the dimerization domain and, therefore, the activated Moco-heme conformation is associated with a larger distance of the heme to the electrode. At high ionic strength conditions, the heme domain is able to switch between a surface bound conformation reflected by a high SERR intensity and the conformation

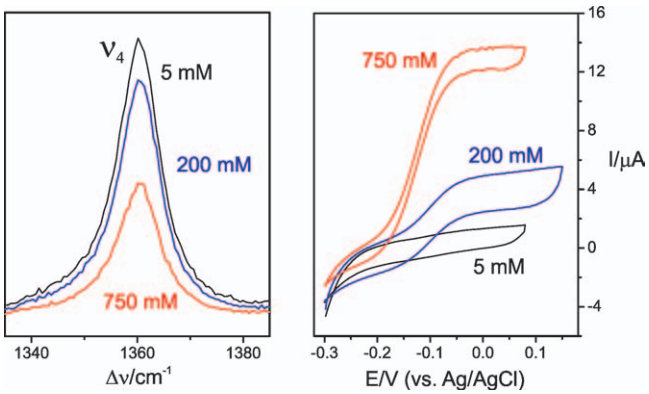

Figure 4. Left: SERR spectra of hSO (ν_4 region) bound to amino-terminated SAMs, measured at open circuit upon increasing buffer concentrations (from top to bottom). Right: CV of hSO under turnover conditions at increasing buffer concentrations (from bottom to top). Further details are given in ref. *46*.

with short Moco-heme distance exhibiting lower SERR intensity. At low ionic strength (corresponding to buffer concentrations $<$ 50 mM), the heme domain is predominantly bound to the electrode surface and thus domain motion, required for post-catalytic intramolecular ET, is inhibited.

[NiFe]-hydrogenases are enzymes capable to catalyze the reversible heterolytic cleavage of molecular H_2 into protons and electrons (*57*). They consist of at least two subunits: a large one, containing the active site (a bimetallic Ni-Fe center), whereas the small one harbors three [FeS] clusters that act as electron relay centers and connect the deeply buried active site to the protein surface. This small subunit can be used to anchor the enzyme on a Au or Ag electrode coated with SAMs in a way that the electrical contact between the terminal [FeS] cluster and the electrode is ensured, a prerequisite for spectroelectrochemical experiments (*45, 58, 59*).

The peculiar molecular structure of [NiFe]-hydrogenases with one CO and two CN-ligands coordinated to the Fe atom of the active site, makes this enzyme particularly appropriate for IR and SEIRA investigations, as it is possible to probe the ligand stretching modes without interference by spectral contributions from the protein matrix (*57*). The anaerobic [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF) has been electrostatically and covalently attached to Au electrodes using amino-terminated SAMs (*58, 59*). The integrity of the active site of the immobilized enzyme was probed by SEIRA spectroscopy upon gas exchange and by CV in the presence of hydrogen as a substrate (Fig. 5). Furthermore, spectroelectrochemical redox titrations of the immobilized enzyme could be achieved by applying constant electrode potentials in the presence and absence of substrate, while simultaneously monitoring variations of the redox state of the enzyme by SEIRA spectroscopy (Fig. 5) (*59*). Accordingly, this study allowed a direct comparison of the formal midpoint potential with the so-called switch potential, a quantity characteristic of the potential-induced anaerobic reactivation process determined by CV (Fig. 5C). Thus, the combined SEIRA and CV approach provided insight into the nature of species involved in the reductive reactivation.

In view of potential biotechnological applications, oxygen-tolerant hydrogenases that are capable of H_2-cycling in the presence of ambient oxygen concentrations are of particular interest for spectroelectrochemical characterizations (*34, 60*). So far, these investigations were restricted to the membrane-bound hydrogenase (MBH) of *R. eutropha* (*R.e.*) H16, which has been successfully immobilized via a His-tag such that the native active site structure remained unperturbed and a reversible reac-

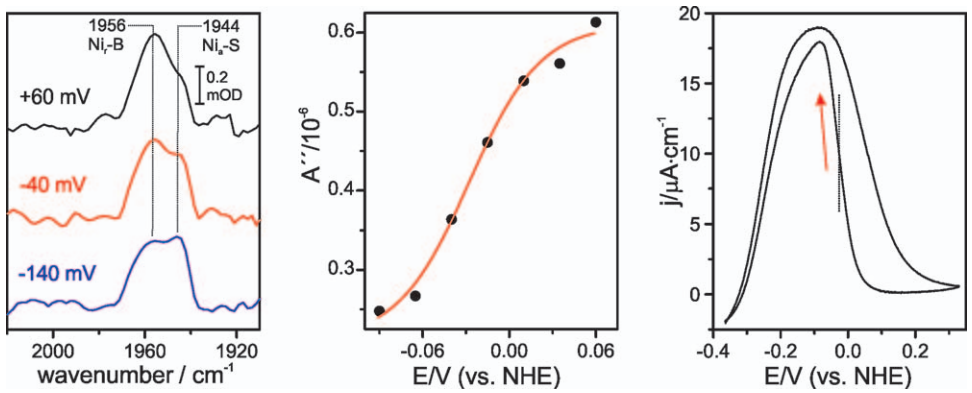

Figure 5. Left: SEIRA spectra in the CO stretching region of the immobilized *Dv*MF [NiFe] hydrogenase at different applied potentials under H_2 atmosphere. Center: Nernstian plot of the second derivative band intensities of the CO stretching mode of the Ni_r–B state determined from the respective SEIRA spectra ($E^{SEIRA}_{switch} = (-16 \pm 14)$ mV and $n = 1.07 \pm 0.2$). Right: CV traces of the same *Dv*MF protein film recorded with a scan rate of 1 mV sec^{-1} at room temperature. The arrow displays the reactivation sweep. The dashed vertical line indicates the switch potential. Further details are given in ref. *59*.

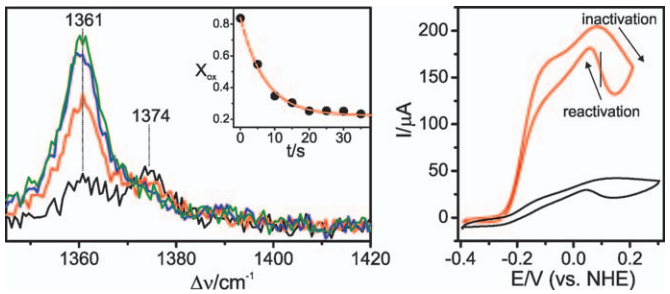

Figure 6. Left: SERR spectra of HoxZ obtained at open circuit in the absence of hydrogen (black) and after hydrogen incubation for 5 sec (red), 10 sec (blue), and 15 sec (green), reflected by the increasing SERR intensity. The inset displays the time-dependent decrease of the oxidized hemes *b* as derived from the SERR spectra. Right: anaerobic inactivation and reductive reactivation (marked by arrows) of the heterotrimer (red, top) and heterodimer (black, bottom) under hydrogen gas atmosphere (scan rate 5 mV sec^{-1}). The scan direction is indicated by the green arrow. The switch potential is indicated by a vertical line. Further details are given in ref. *45*.

tion with H_2 could be detected (*61*). However, in that study a proper electrical communication between the immobilized enzyme and the electrode could not be obtained. In this respect, an improvement could be achieved by electrostatic binding to a carboxyl-terminated SAM-coated (Ag) electrode, using both MBH-heterodimer and the heterotrimer which includes the hetero-dimer and the natural electron acceptor, diheme cytochrome *b* (HoxZ subunit) (*45*). In this case, a good electronic communication between the [FeS] cluster ET chain and the supporting SAM-coated (Ag) electrode was achieved as demonstrated by CV. Using SERR spectroscopy, which exclusively monitored the reduction and oxidation of the hemes *b*, it is shown that the HoxZ subunit is not primarily involved in the ET to the electrode. The bypass via the terminal FeS cluster to the Ag electrode was found to be faster than the heterogeneous ET via the heme cofactors, which, however, play a crucial role in stabilizing the hetero-trimeric enzyme on the electrode. This conclusion is derived from the hydrogen-induced reduction of HoxZ and the about 4–5 times higher catalytic current compared with the hydrogenase hetero-dimer (Fig. 6) (*45*).

[FeFe] hydrogenase, which are more effective hydrogen producers albeit more oxygen-sensitive than [NiFe] hydrogenases, have been successfully immobilized on Au electrodes as demonstrated by SEIRA spectroscopy monitoring the amide I and II modes (*vide supra*). Simultaneous CV studies exhibit catalytic currents, although the CN and CO stretching modes of the active [FeFe] site could not be detected by SEIRA spectroscopy (*62, 63*).

PERSPECTIVES

Including SERR and SEIRA spectroscopy into the toolbox for studying immobilized enzymes has substantially deepened the knowledge about the mechanism of electrocatalytic processes. This is particularly true when surface-enhanced vibrational spectroscopies are used in combination with electrochemical techniques on the same device. This requirement cannot readily be fulfilled in the case of SERR spectroscopy which in most cases requires the use of Ag instead of Au electrodes. As a consequence, SERR spectroelectrochemical studies are restricted to a narrower potential range, as the oxidation potential is distinctly more negative for Ag than for Au. To overcome these limitations, hybrid electrodes that combine the optical properties of Ag with the higher electrochemical stability of Au may represent promising alternatives (*15, 64, 65*). Furthermore, the use of coated colloidal Ag nanoparticles as local signal amplifiers, coadsorbed with the target enzymes on optically inactive electrodes, may substantially broaden the applicability of SERR spectroscopy for *in situ* spectroelectrochemical studies (*66*).

The present account has demonstrated that SERR and SEIRA spectroscopy may be successfully used to study highly complex enzymatic complexes including different cofactors. Moreover, even for *in situ* investigations of cellular systems, these spectroelectrochemical techniques may provide valuable information as shown by recent studies on metal-reducing bacteria grown on Ag electrodes (*67, 68*).

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