Polymyxin-Coated Au and Carbon Nanotube Electrodes for Stable [NiFe]-Hydrogenase Film Voltammetry

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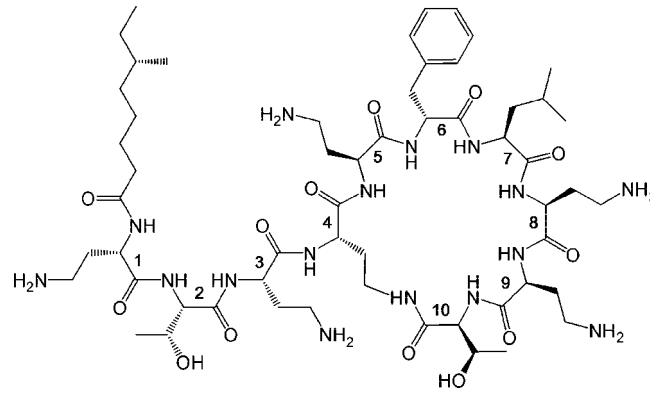
We report on the use of polymyxin (PM), a cyclic cationic lipodecapeptide, as an electrode modifier for studying protein film voltammetry (PFV) on Au and single-walled carbon nanotube (SWNT) electrodes. Pretreating the electrodes with PM allows for the subsequent immobilization of an active submonolayer of [NiFe]-hydrogenase from *Allochromatium vinosum* (*Av* H_2ase). Probed by cyclic voltammetry (CV), the adsorbed enzyme exhibits characteristic electrocatalytic behavior that is stable for several hours under continuous potential cycling. An unexpected feature of the immobilization procedure is that the presence of chloride ions is a prerequisite for obtaining electrocatalytic activity. Atomic force microscopy (AFM) relates the observed catalytic activity to enzymatic adsorption at the PM/Au(111) surface, and a combination of concentration-dependent CV and AFM is used to investigate the interaction between the enzyme and the PM layer.

Introduction

Owing to their high catalytic activity for the reversible interconversion of H_2 and protons,[1] [NiFe]-hydrogenases have become premier candidates for application in enzymatic fuel cells.[2] Apart from crystallographic[3] and spectroscopic techniques, protein film voltammetry (PFV)[4] has been indispensable in unravelling many details regarding their active site conformation and catalytic mechanism.[5] PFV requires hydrogenase immobilization on a suitable electrode material resulting in efficient electronic contact between the active site (via a prosthetic group in its electron-transfer chain) and the electrode. In this way, modulating the electrode potential yields direct electrochemical control of the adsorbed redox-active enzyme, which can moreover be subjected to various conditions (e.g., temperature, pH, or substrate concentration) in the surrounding buffer medium. The specific type of immobilization technique influences the activity and stability of the adsorbed hydrogenase and constitutes a considerable challenge.[6] For *Av* H_2ase used in the present study, coadsorption to edge-plane graphite (EPG) with the cyclic cationic lipodecapeptide polymyxin[7] (PM, Chart 1) has been particularly successful in obtaining an active enzyme coverage.[2a] The Armstrong group analyzed the electrokinetics of *Av* H_2ase on EPG in detail and measured a maximum proton reduction activity below pH 5.5 and a maximum H_2 oxidation activity above pH 6.[8] At high pH, *Av* H_2ase displays a characteristic reversible shut-off of the H_2 oxidation activity as OH^- binds to the active Ni(III) state at high potential (yielding the so-called "ready" state).[9]

Chart 1. Polymyxin B1 from *Bacillus polymyxa*, a Cyclic Decapeptide That is Positively Charged (Below pH ∼10) as a Result of Its Five Primary Amino Groups[a]



[a] (*S*)-6-Methyloctanoyl−L-DAB (1)−L-Thr (2)−L-DAB (3)−L-DAB (4)−L-DAB (5)−D-Phe (6)−L-Leu (7)−L-DAB (8)−L-DAB (9)−L-Thr (10) → (4) with L-DAB = L-2,4-diaminobutanoate.

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Although relatively straightforward, the electrostatic immobilization of enzymes by adsorption from solution often suffers from a lack of protein layer stability, decreasing the desirable voltammetric response.[8,10] Here we report on the wider applicability of PM as an electrode modifier, facilitating *Av* H_2ase film voltammetry on Au and SWNT electrodes, yielding an active and very stable *Av* H_2ase submonolayer. Apart from having a well-defined atomic and electronic structure, the high chemical stability and good conductive properties of SWNTs make them ideal candidates for use in fundamental charge transport studies.[11] Regarding use as nanoelectrodes for electrochemistry,[12] successful *Av* H_2ase immobilization on SWNTs or Au may prove interesting on the road to single-enzyme electrochemical studies. Au is widely implemented for nanofabrication purposes; moreover, the use of atomically flat Au(111) allows us to relate the observed enzymatic catalysis to molecular adsorption at the PM/Au surface using AFM.

Experimental Section

Materials. Polymyxin B sulfate (PM) was purchased from Sigma (product P1004, predominantly decapeptide B1, $C_{55}H_{96}N_{16}O_{13}\cdot 2H_2SO_4$). PM solutions were stored at −20 °C and generally used for 1 month. *Av* H_2ase was isolated and purified from *Allochromatium vinosum* and optimally activated by 30 min of incubation under 100% H_2 at 50 °C.[13] MES (2-(*N*-morpholino)ethanesulfonic acid) and CHES (2-(cyclohexylamino)ethanesulfonic acid) buffers (Sigma) were titrated with NaOH to pH 5.7 and 9.0, respectively. At pH 5.7, both proton reduction and H_2 oxidation activities are close to optimum[8] whereas oxidative inhibition is slow.[9] Ethanol suspensions of carbon nanotubes were prepared from HiPCO SWNTs (provided by R. E. Smalley, Rice University).[14] Deionized water (18 MΩ cm Milli-Q, Millipore) was used to prepare all solutions and to rinse samples and electrodes.

Electrochemistry. The working electrodes were Au disk electrodes (Bioanalytical Systems, BAS) with diameters of 1.6 mm (2.0 mm^2) and 5 μm (20 μm^2). The electrodes were polished with a water-based diamond suspension (Buehler, 6 μm particles for the macroscopic electrode and 50 nm ones for the microelectrode) and rinsed. Carbon nanotube multilayer electrodes were prepared from a suspension (∼1 g L^{-1}) of HiPCO SWNTs in ethanol. A droplet of the suspension was applied to a 2 mm^2 electrode, and the solvent was evaporated at 150 °C. This was repeated several times to obtain a uniform, dense layer of SWNTs.[15] These working electrodes were incubated with PM (specific concentrations and incubation times are mentioned in the main text) by immersing them upside down in a PM solution. After rinsing, a 20 μL droplet of buffer solution was placed between the working electrode, the reference electrode, and a Pt wire counter electrode as described by Hagen.[16] The electrodes were connected either to a BAS CV-50W or an EcoChemie Autolab potentiostat. The reference electrode was either a saturated calomel electrode (SCE, Radiometer K-401), +244 mV versus the normal hydrogen electrode (NHE), or a Ag/AgCl/3 M NaCl electrode (BAS RE-5B), +215 mV versus NHE. All potentials are given versus NHE. To measure sub-nanoampere currents with the microelectrode, the CV-50W potentiostat was used in combination with a BAS PA-1 preamplifier and a BAS C-3 cell stand with a Faraday cage. After recording background voltammograms, *Av* H_2ase was added to the buffer solution from a freshly activated stock solution of 10 μM in 5 mM TRIS (tris(hydroxymethyl)aminomethane) buffer (pH 8). The small volume required did not result in any pH change, which is also apparent from the observed correct midpoint potentials. The measurements were performed under a headspace of wetted Ar, N_2, or 3.2% H_2 in Ar (the latter only in the microelectrode experiment). CO and H_2 were added from 1 atm saturated stock solutions (0.99 and 0.78 mM, respectively).

Analysis of Titration Experiments. In the electrochemical experiments, the magnitude of the H_2 production current increases with increasing *Av* H_2ase concentration. The sigmoidal parts of the H_2 production curves, selected by subtracting a linear baseline from the derivative peak height, were fitted to a Langmuir isotherm of the general form $\theta = \theta_{max}/(1 + K_d/[H_2ase])$ with $K_d = 1/K_a$. From the AFM images, the *Av* H_2ase molecules were manually counted, and the coverage was fitted to the same equation.

Atomic Force Microscopy. Au samples for AFM were prepared by evaporating approximately 200 nm of Au on freshly cleaved mica (grade V-4, SPI supplies) that was degassed overnight at 400 °C and 10^{-7} bar prior to Au deposition. To obtain atomically flat Au(111) terraces, the Au was annealed in a butane flame. SWNT samples for AFM imaging were grown through chemical vapor deposition (CVD) from catalyst islands on a silicon wafer with a thermally grown oxide layer.[17] The samples were incubated with PM or *Av* H_2ase and then thoroughly rinsed with water, dried in a stream of N_2, and mounted in the AFM (Nanoscope IV, Digital Instruments). Images were recorded in tapping mode using silica cantilevers with a typical resonance frequency of 300 kHz, a spring constant of 42 N/m, and a nominal tip radius of 7 nm (Olympus).

Results and Discussion

Figure 1 shows characteristic voltammograms of *Av* H_2ase on polished Au (2 mm^2) that has been pretreated with PM. Experiments on *Av* H_2ase have to be performed under strictly anaerobic conditions because O_2 quickly inactivates the enzyme.[5a]

Under Ar, in the absence of H_2, the sigmoidal proton reduction wave and peak-shaped reoxidation of the accumulated H_2 are very similar to those observed on a stationary EPG electrode.[18] The catalytic response persists after immersing the electrode in a fresh buffer solution without *Av* H_2ase. Without the enzyme, no such response is obtained. Instead, the expected exponential proton reduction at the Au surface is observed at low potentials. The addition of H_2 yields a sigmoidal oxidation current and suppresses proton reduction.[8] The addition of CO, a known strong competitive inhibitor of *Av* H_2ase,[19] completely suppresses both proton reduction and H_2 oxidation. After the CO has been flushed out, the characteristic catalytic responses are restored, demonstrating that the inhibition is fully reversible. The magnitude of the H_2 production current increases with increasing *Av* H_2ase concentration, and the sigmoidal part of these curves can be fitted to a Langmuir isotherm with $K_d = 35$ nM.[20] This implies that the protein is firmly and stably adsorbed onto the PM-coated Au surface.

When *Av* H_2ase is added to bare Au, a small electrochemical response is sometimes obtained (typically less than 10% of the response on PM-pretreated Au). Furthermore, the coadsorption of *Av* H_2ase with PM from solution to bare Au yields a catalytic

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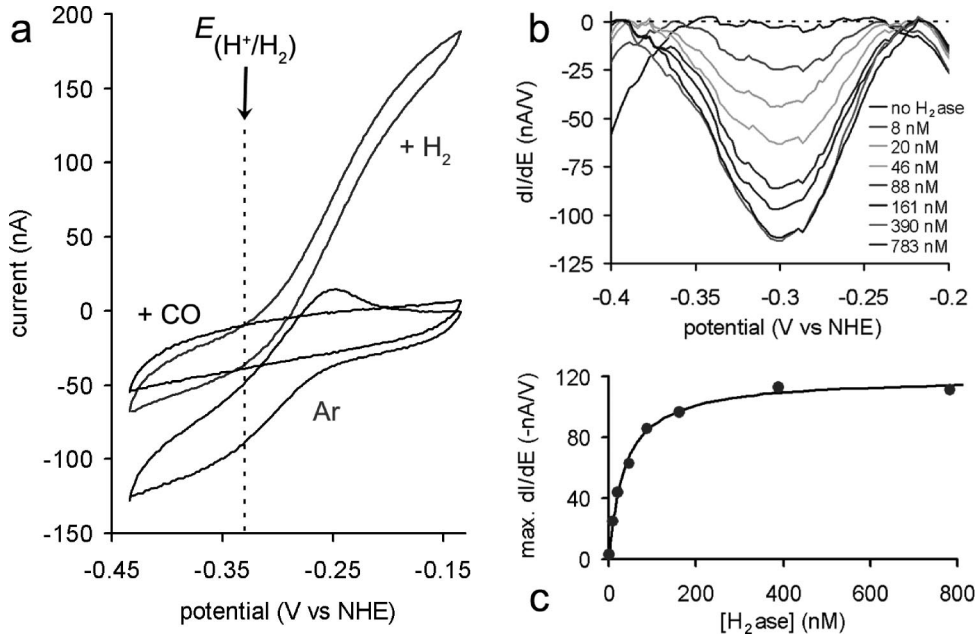

Figure 1. (a) CV of a Au disk electrode (2 mm^2) pretreated for 30 min with 50 g L^{-1} PM, rinsed with water, and then measured with 0.5 μM *Av* H_2ase in buffer under Ar, after adding 0.26 mM H_2, and after adding 0.26 mM CO. Scan rate: 5 mV s^{-1}. (b) Increasing derivative peak height (linear baseline subtracted) for a titration of *Av* H_2ase onto Au (2 mm^2) under Ar, pretreated for 30 min with 50 g L^{-1} PM, and (c) the corresponding Langmuir isotherm fit (K_d = 35 nM). The buffer contained 15 mM MES and 100 mM NaCl (pH 5.7).

response that is stable for only ~10 min, after which it diminishes invariably. The pretreatment method, however, yields enzymatic activity of superior stability. The constant (even slowly increasing) catalytic response persists for at least 2 h of continuous potential cycling.

To further verify that the observed catalytic response is indeed due to *Av* H_2ase and not due to the Au surface, the response at high pH was measured. In this way, the characteristic reversible shut-off behavior of the H_2 oxidation activity at high potential can be observed.[9] Because the typical slow oxidative inhibition and reductive reactivation of the enzyme become apparent only at low scan rate and when mass transport of H_2 is not rate-limiting, we used a Au microdisk electrode with a relatively low surface *Av* H_2ase concentration. The efficient hemispherical diffusion profile at such a microelectrode ensures that mass transport of H_2 is not rate-limiting.[21] Figure 2 shows the distinctive reversible switch-off behavior of an immobilized enzyme sample in the zeptomole range, which unambiguously proves that the measured activity is enzymatic.

An unexpected feature of the immobilization procedure is that the presence of Cl^- is critical for obtaining *Av* H_2ase electrocatalytic activity. When MES buffer is used without NaCl (more MES was added to obtain the same ionic strength) or in combination with either $NaClO_4$ or Na_2SO_4 as the supporting electrolyte, no catalytic activity is observed until NaCl is added (rapidly yielding an enzymatic response, Figure 3). This effect is not completely understood at the moment, but since Cl^- is not required for binding PM to Au (see the AFM data below), we suggest that the presence of Cl^- induces a morphological change in the 3D structure of PM. This change apparently induces the correct conformation for successful *Av* H_2ase attachment. In solution, PM is known to adopt a flexible tertiary structure that differentiates between monovalent and multivalent anions.[22]

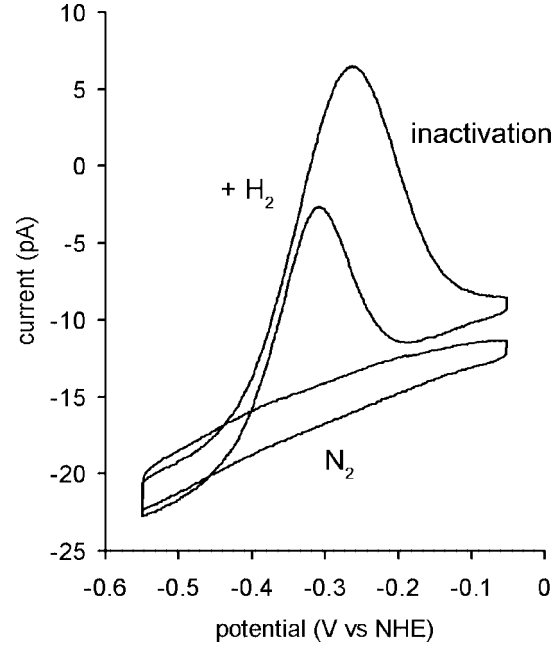

Figure 2. CV of a Au disk microelectrode (20 μm^2) pretreated for 30 min with 50 g L^{-1} PM, rinsed with water, and then measured with 20 nM *Av* H_2ase in buffer under N_2 and under 3.2% H_2 in Ar (26 μM H_2 in solution). Scan rate: 1 mV s^{-1}. The buffer contained 50 mM CHES and 100 mM NaCl (pH 9.0).

Direct binding of Cl^- to Au might also promote favorable PM or *Av* H_2ase binding. Although the very low activity due to the direct binding of *Av* H_2ase to Au does not increase significantly by adding Cl^- (Figure 3a), *Av* H_2ase may penetrate the PM layer, and the thus-stabilized enzyme may bind to Cl^--modified Au. However, no large changes in the morphology of the adsorbed PM were observed by AFM after incubating with H_2ase in MES/NaCl buffer (see below). The inset in Figure 3c shows that the Cl^--induced electrochemical response persists after rinsing the electrode, showing that Cl^- is necessary to attach *Av* H_2ase to

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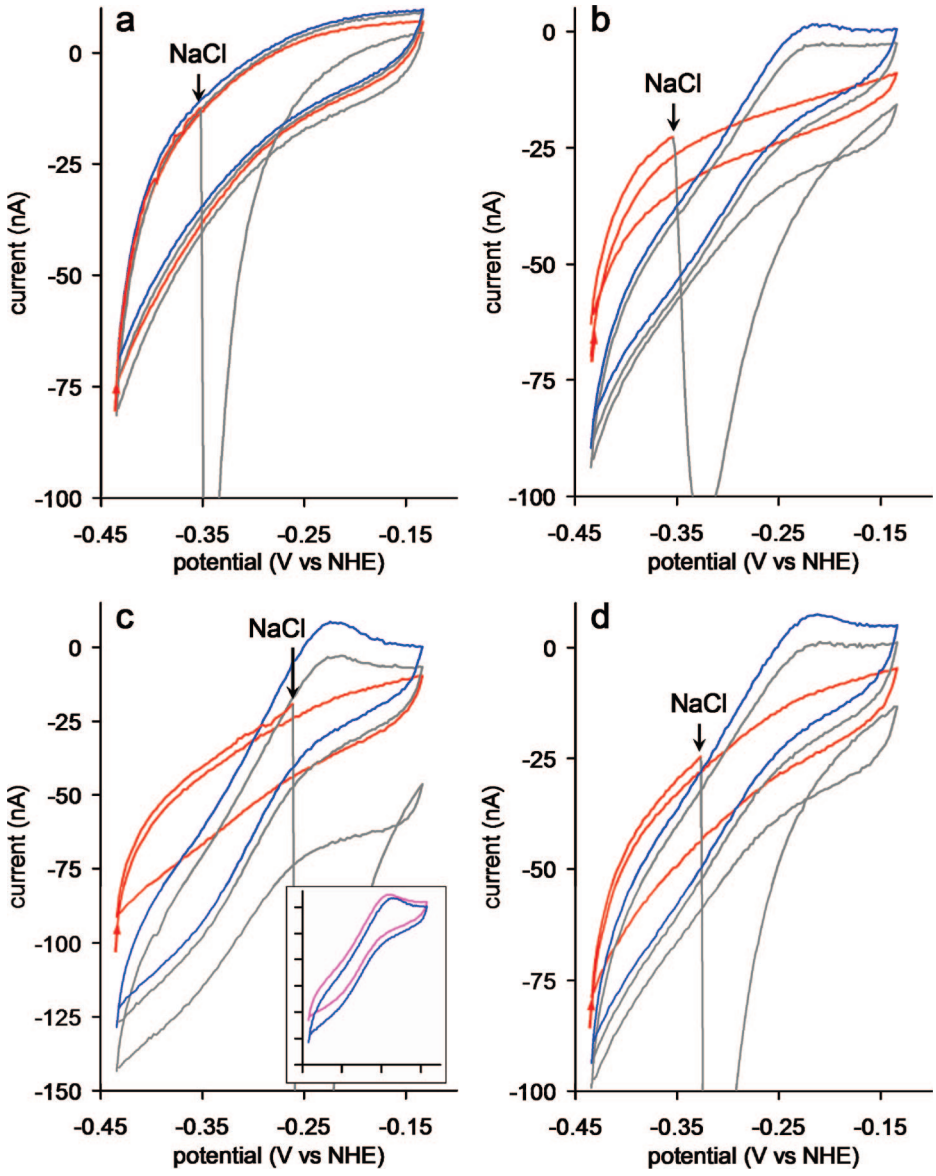

Figure 3. Effect of Cl^- on the response of *Av* H_2ase. CV of Au disk electrodes (2 mm^2) pretreated for 30 min with 50 g L^{-1} PM (b to d), rinsed with water, and then measured in the presence of 0.5 μM *Av* H_2ase in 200 mM MES/NaOH (pH 5.7) under Ar. For all experiments, scans 5 to 8 are plotted (5 mV s^{-1}), and a red arrow indicates the start of scan 5. During scan 6, 70 mM NaCl is added (black arrow). The current before adding NaCl is plotted in red, and scan 8 is plotted in blue (a) without PM pretreatment and no additional salt added initially, (b) no additional salt added initially, (c) with 90 mM $NaClO_4$ present from scan 1, and (d) with 90 mM Na_2SO_4 present from scan 1. The inset of c compares the electrochemical response in the eighth scan (blue), and after rinsing the electrode (purple, measured in 200 mM MES/NaOH, pH 5.7), the axes are equal to those in c.

the PM layer but that its presence is not required to retain *Av* H_2ase activity after attachment.

On an electrode consisting of a multilayer of HipCo carbon nanotubes[14] pretreated with PM, an active film of *Av* H_2ase is adsorbed, which persists after immersing the electrode in a fresh buffer solution without the enzyme. As plotted in Figure 4, the catalytic responses under Ar and after the addition of H_2, and the inhibition by CO, closely resemble those observed on PM-coated Au. However, the current density is an order of magnitude higher, presumably as a result of the large specific area of the nanotube mesh (even though only the top layers of the densely packed nanotube matrix are accessible to protein).[15] The catalytic *Av* H_2ase response persists and even increases slowly during 2 h of continuous potential cycling. Interestingly, the *Av* H_2ase film is even resistant to repeated cycles of rinsing and drying and storage under ambient conditions (in air, at room temperature) for several days. Subsequent reactivation of the adsorbed enzyme under 100% H_2 at 50 °C for 30 min restores the catalytic activity. This resistance to repeated rinsing, storage, and reactivation demonstrates that the adsorbed PM layer is very robust and that *Av* H_2ase is tightly adsorbed and extremely stable on PM-coated SWNTs (Figure 4b).

When the sigmoids for H_2 production at pH 5.7 on both Au and SWNTs are compared to the background in the CO-inhibited voltammograms (Figures 1 and 4), a residual catalytic slope is apparent at high overpotentials.[23] These residual slopes have also been observed for *Av* H_2ase adsorbed on EPG.[24] Léger et al. modeled this effect by introducing a dispersion in the interfacial electron-transfer rates due to a distribution of tunneling distances

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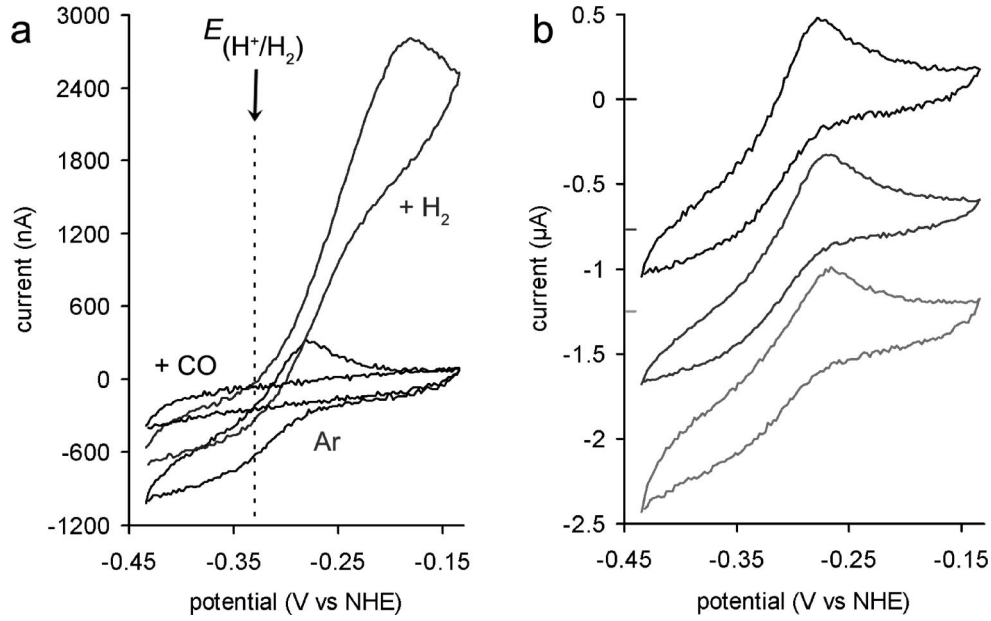

Figure 4. (a) CV of a SWNT multilayer electrode pretreated for 30 min with 50 g L^{-1} PM, rinsed with water, and then measured with 0.5 μM *Av* H_2ase in buffer under Ar, after adding 0.4 mM H_2, and after adding 0.2 mM CO. Macroscopic electrode area, ~10 mm^2; scan rate, 5 mV s^{-1}; and the buffer contained 15 mM MES and 100 mM NaCl (pH 5.7). (b) *Av* H_2ase response after scanning for 138 min (top); then the electrode was rinsed, stored overnight dry in air and the adsorbed enzyme reactivated (middle), rinsed again, stored dry in air for 11 days, and the adsorbed enzyme reactivated (bottom). The traces are offset for clarity with the actual zero levels indicated on the axis.

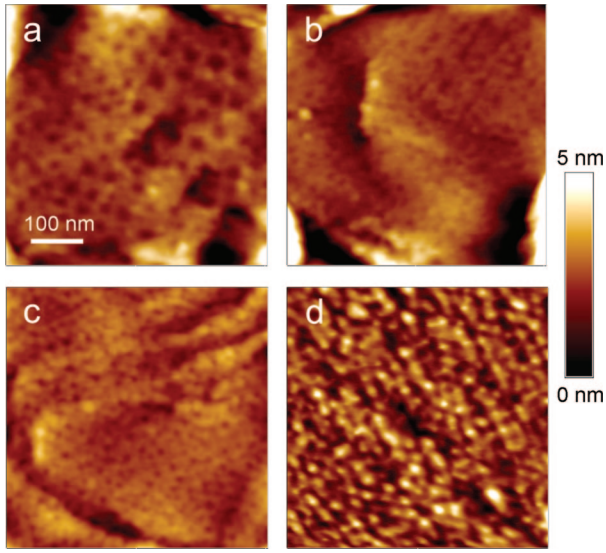

Figure 5. Tapping mode AFM (in air) of Au(111) incubated 15 min with (a) 0.1, (b) 1, (c) 10, (d) 100 g L^{-1} PM.

caused by different enzyme orientations.[25] The voltammograms suggest that the mode of *Av* H_2ase adsorption on PM-coated Au and SWNTs is similar to that on EPG.

Au(111), which has atomically flat terraces allowing the probing of its topology with AFM,[26] was prepared by flame annealing Au (on a mica support), and was subsequently incubated with PM solutions of varying concentration. After rinsing and drying under N_2 flow, a characteristic weblike structure was observed for all concentrations used (0.1−100 g L^{-1}, Figure 5), with ~1000 meshes μm^{-2}. The images for the lowest and highest PM concentrations show a PM network that seems to be incomplete and congested, respectively. The diameter of the openings varied roughly between 10 and 20 nm. The widest pits permitted the AFM tip to reach the bottom, yielding a depth between 1.5 and 2 nm.

A PM concentration of 10 g L^{-1} was chosen (Figure 6b) for visualizing *Av* H_2ase attachment. After incubation with *Av* H_2ase and subsequent rinsing and drying, the enzyme molecules could be clearly distinguished on the PM, resulting in features with heights of ~4 nm (Figure 6c,d). The number of adsorbed features increased when increasing the applied enzyme concentration and was fitted to a Langmuir isotherm with $K_d = 9$ nM and a maximum coverage of 1408 features μm^{-2} (corresponding to 0.23 pmol cm^{-2}). The K_d is lower than the value determined from the electrochemical experiments, which is to be expected because the rinsing procedure (required to prevent salt crystal artifacts) selects for the most tightly bound enzymes. Also, an apparent difference between the affinities determined from electrochemistry and from counting molecules could be related to differences in electrocatalytic activity due to the heterogeneity of the surface.

At low enzyme coverage (Figure 6c), part of the PM meshwork can still be observed, which implies that incubation with the enzyme does not alter the overall structure of the PM layer. Because of the lack of a clear spatial correlation in the meshwork, it is not immediately evident whether the *Av* H_2ase molecules bind directly on the PM layer or are selectively embedded inside the meshes. The heights of the enzyme features relative to the PM layer are mostly ~2 nm lower than the expected height of ~6 nm on the basis of the crystal structures of related enzymes,[3,27] which suggests that the molecules are adsorbed inside the meshes. However, the extrapolated maximum enzyme coverage of 1400 μm^{-2} (0.23 pmol cm^{-2}) is 40% higher than the density of pits (1000 μm^{-2}), suggesting that a fraction of the *Av* H_2ase molecules will adsorb to the top of the PM layer. We cannot fully exclude that the lowest features in the AFM images are due to (partially) denatured enzyme or monomerized subunits rather than embed-

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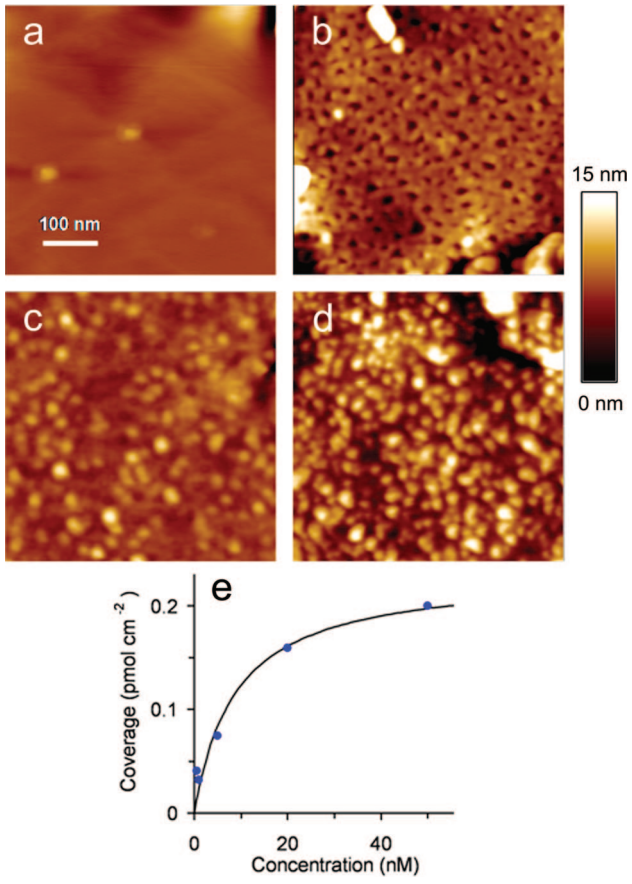

Figure 6. Tapping mode AFM (in air) of Au(111) with PM and *Av* H_2ase: (a) Au(111) on mica, (b) incubated 30 min with 10 g L^{-1} PM, (c) subsequently incubated with 5 nM *Av* H_2ase in buffer, and (d) subsequently incubated with 20 nM *Av* H_2ase in buffer. The buffer contained 50 mM MES and 100 mM NaCl (pH 5.7). (e) Corresponding Langmuir isotherm fit (K_d = 9 nM).

ded, structurally native protein. A crude estimate from Figure 2 (using a turnover rate of 10^3 to 10^4 e s^{-1})[18] yields an oxidation current density of 25 pA/20 μm^2 = 125 μA cm^{-2} corresponding to an active coverage of 1.3 to 0.13 pmol cm^{-2}.[28] Compared to a maximum coverage of 0.23 pmol cm^{-2} deduced from AFM, this suggests that the majority of adsorbed *Av* H_2ases is catalytically active and therefore adsorbed inside the meshes with their distal [4Fe-4S] cluster in close proximity to the Au surface. For *Av* H_2ase adsorbed on top of the 2-nm-thick PM layer, the long electron tunneling distance will render the interfacial electron transfer severely rate-limiting. The shallow slopes on the sigmoidal catalytic waves for H_2 production in Figures 1 and 4 might be attributed to this subset of the enzyme population.[25]

As an illustration of *Av* H_2ase binding to SWNTs, Figure 7 visualizes the binding of *Av* H_2ase to single nanotubes on SiO_2, as prepared by CVD. It can be seen that *Av* H_2ase preferentially binds to the sidewalls of PM-pretreated SWNTs compared to the SiO_2 surface. This selectivity was obtained, however, for much lower PM concentrations compared to those used to modify Au. In fact, the applied concentration (0.2 g L^{-1}) is comparable to that used in obtaining stable *Av* H_2ase voltammetry on EPG using PM as a coadsorbate.[9] At higher PM concentrations, the SiO_2 surface is also fully coated in the typical weblike structure,

(28) Because we cannot exclude the fact that the H_2 oxidation trace in Figure 1 suffers from H_2 mass transport limitation, we use Figure 2 for this estimation. We assume similar enzyme attachment behavior at pH 5.7 and 9.0.

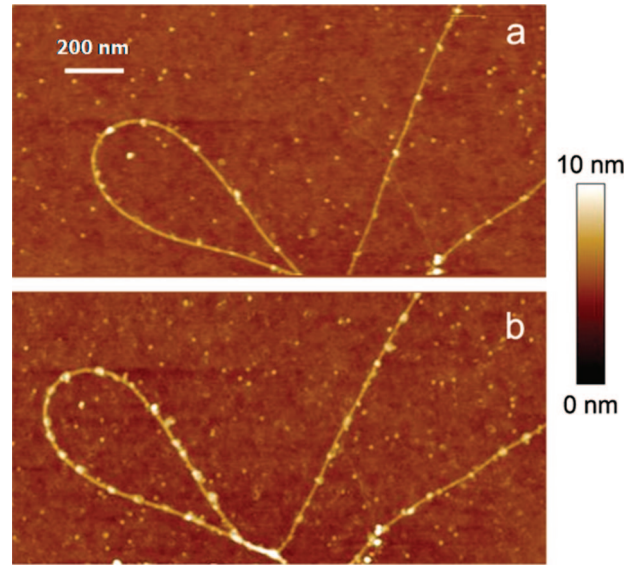

Figure 7. Tapping mode AFM (in air) of SWNTs on SiO_2 incubated for 10 min with 0.2 g L^{-1} PM and subsequently incubated for 10 min with (a) 10 nM *Av* H_2ase in buffer and (b) 20 nM *Av* H_2ase in buffer. The buffer contained 50 mM MES and 100 mM NaCl (pH 5.7).

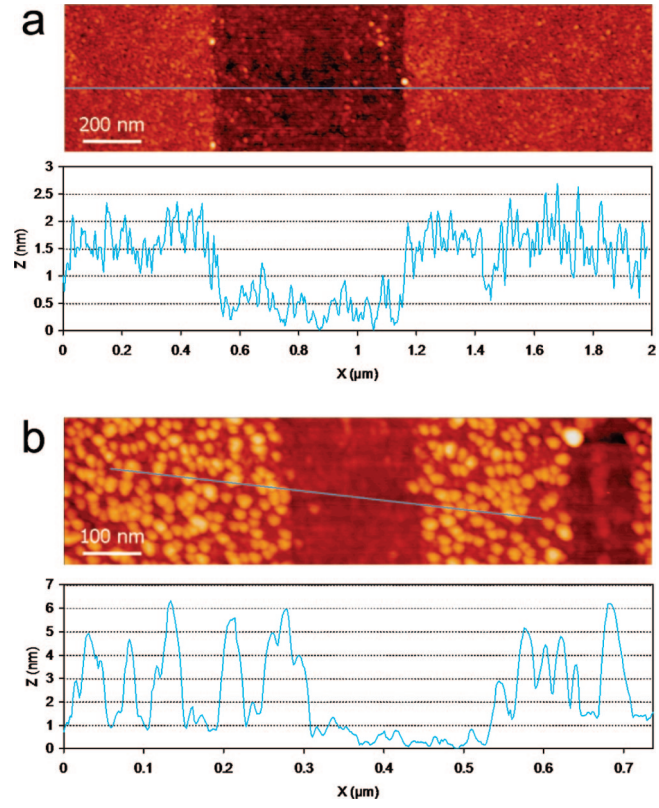

Figure 8. Tapping mode AFM (in air) of SiO_2: (a) incubated 10 min with 50 g L^{-1} PM and (b) another 10 min with 100 nM *Av* H_2ase in 50 mM MES with 0.1 M NaCl (pH 5.7). The trenches were created by contact mode AFM. The blue lines in the AFM images indicate the positions of the plotted height profiles.

with its average height being 1.1 nm (Figure 8a). This height was measured by locally removing the adsorbed PM layer using the AFM tip in contact mode, permitted by the hardness of SiO_2 (as opposed to Au). The subsequent addition of *Av* H_2ase results in a fully covered surface with features of 5 to 6 nm in height (Figure 8b). The height of the PM layer does not change significantly, indicating that its overall structure is preserved.

Taking into account enzyme adsorption on top of the PM layer as well as inside the meshes, the measured enzyme height suggests that the adsorbed *Av* H_2ase is structurally intact under the applied conditions.[3] Although electrochemically less relevant, these experiments on SiO_2 do corroborate that the AFM data on Au show structurally intact *Av* H_2ase, adsorbed inside the meshes of the PM layer.

Conclusions

We have presented a protocol for performing *Av* H_2ase film voltammetry on PM-modified Au and SWNTs. Pretreating the electrodes with PM yields a very stable immobilized enzyme layer with electrocatalytic behavior similar to that reported on EPG. Although the exact mode of action and the internal structure of the PM layer are unknown, our data shows that it prevents denaturing of the enzyme and permits the [4Fe−4S] cluster in its electron-transfer chain, near the enzyme's outer surface, to come within electron tunneling range of the electrode surface. Because Au and SWNTs are routinely used in nanofabrication techniques, PM modification of these materials facilitates the downscaling of *Av* H_2ase electrochemistry to the nanometer regime, thereby opening the route toward single-enzyme electrochemistry.

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