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We have studied the morphological, conformational, and electron-transfer (ET) function of the metalloprotein azurin in the solid state, by a combination of physical investigation methods, namely atomic force microscopy, intrinsic fluorescence spectroscopy, and scanning tunneling microscopy. We demonstrate that a ''solid state protein film'' maintains its nativelike conformation and ET function, even after removal of the aqueous solvent. © *2004 American Institute of Physics.* [DOI: 10.1063/1.1828038]

In recent years, much effort has been devoted to the development and investigation of organic molecules and biomolecules as electron-conductive materials for applications to nanoelectronics and sensors. Biomolecules such as proteins hold great promise in this connection: They constitute nano-sized building blocks, carry out highly specific reactions, and can often self-assemble, which opens up the prospect of nanoscale surface patterning as an alternative to optical lithographic approaches. Progress in these areas must rely on the development of techniques for the controled fabrication of high-quality molecular monolayers, such as self-chemisorption.[1] The assessment of the immobilization process and its consequences on biomolecule function are therefore important issues, which are relevant to all planar molecular devices (e.g., field effect transistors and biosensors) operating under either quasiphysiological or ambient conditions. While the interaction of proteins with a solid substrate may lead to denaturation and/or to aggregation, with consequent loss of functionality, this may be prevented or reduced in the presence of a suitable buffer. So far, chemisorption procedures on biomolecules that avoid conformational transitions have been demonstrated in solution by several groups by means of STM/STS experiments.[2,3] On the other hand, the behavior of a biomolecule in air upon immobilization in the solid state, and particularly its morphological and structural stability, are difficult to predict and, to our knowledge, have hardly been investigated. Yet this information is crucial for the development of protein-based solid state devices, which depend on specific biomolecular functions.

Azurin (Az) is an electron-transfer (ET) metalloprotein, probably involved in the oxidative stress response of the bacterium *Pseudomonas aeruginosa*.[4] Its redox-active center contains a copper ion coordinated by five amino acid ligands arranged in a trigonal bipyramidal geometry. Thanks to its intrinsic stability, azurin has emerged as a good candidate for biomolecular nanoelectronics. In particular, in the last few years, the possibility of eliciting a current flow through the redox level of a single Az molecule has been demonstrated.[3,5,6] Moreover, it has been recently shown that the Az ET activity can be exploited for the realization of solid state biomolecular transistors working in air and at room temperature.[7] On the biochemical and biophysical side, an important question is whether the protein structure and functions are conserved under nonphysiological conditions. This is a key technological point for the realization of such biodevices, but it is also a striking issue from a fundamental viewpoint.

In the present paper, we carried out atomic force microscopy (AFM), intrinsic fluorescence spectroscopy, and scanning tunneling microscopy (STM) experiments on Az monolayers in air, in order to assess, respectively, the morphological, conformational, and functional state of Az molecules. We conclude that the immobilized proteins do not undergo denaturation even after removal of the aqueous solvent.

Our Az monolayer was realized with azurin immobilized onto a silicon dioxide surface that had been functionalized with thiosilane ($(CH_3)_3Si(CH_2)_3SH$). This procedure results in protein anchoring through the sulphur atoms of Cys3 and/or Cys26, since, apart from Cys112, which is involved in Cu coordination, these are the only residues available for S–S bonding.[8] The morphological characterization of such monolayer was performed by a height distribution analysis of AFM images. In the case of smaller globular proteins, like azurin, AFM is limited by the intrinsic low resolution (10–20 nm) in lateral measurements, owing to the finite size of the probe tip. This limitation, however, can be overcome by a direct measurement of the height of individually adsorbed proteins, i.e., by taking the maximum height of each molecule and subtracting the height of the local background (i.e., the uncovered substrate).[9] This approach, which utilizes the angstrom resolution of the AFM along the vertical (z) direction, is marginally affected by probe tip geometry. An analysis of the height distribution of Az monolayers reveals three main components [Fig. 1(a)]. The major molecular species,

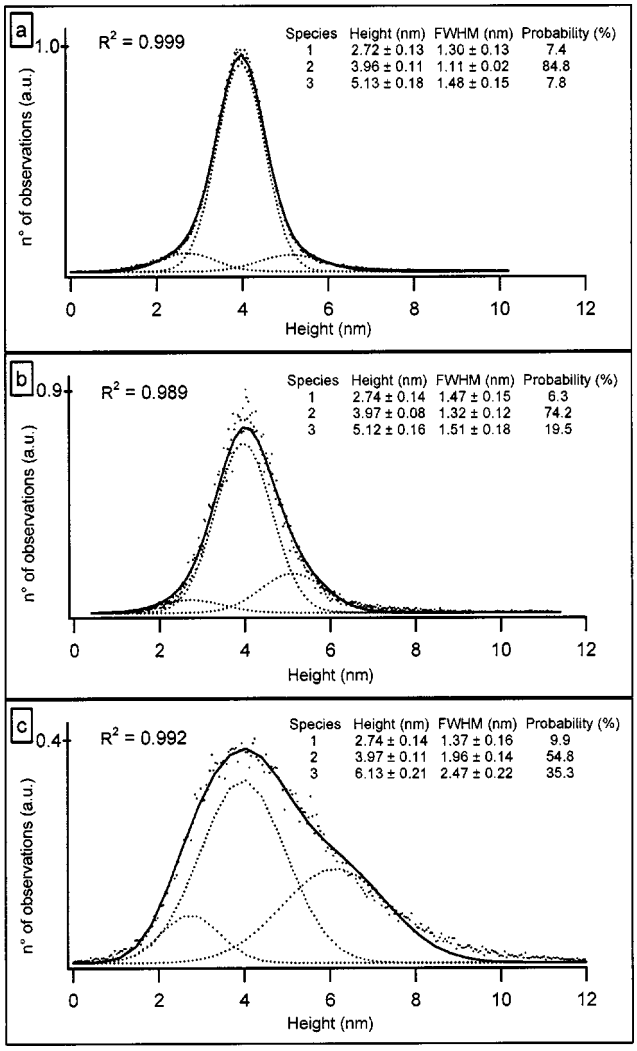

FIG. 1. AFM height distribution analysis of (a) an Az monolayer, (b) an Az monolayer heat-denatured in air after chemisorption (1 min at 80 °C), (c) a monolayer prepared with azurin heat-denatured in solution (1 min at 80 °C). A height distribution was plotted based on 10 independent AFM images (scan size: 2×2 μm). Curve fitting of the experimental data was performed iteratively by means of a combination of Gaussian functions (Ref. 9). Protein monolayers were prepared from a 4×10^{-2} μM Az solution (50 mM NH_4Ac buffer, pH 4.6). After incubation, the substrates were rinsed copiously with deionized water, and accurately dried by high purity nitrogen flow. Importantly, these structures were not present on bare silanized substrates, or if the same protein immobilization was carried out by using a mutant Az lacking the Cys3-Cys26 disulphide. Insets: calculated parameters of protein films according to the AFM height distribution analysis.

centered around 4 nm, corresponds to native azurin, since its size is in good agreement with that determined from x-ray crystallography[10] (see also insets of Fig. 1). Two additional peaks occur at 5.1 nm, which may correspond to unfolded or aggregated azurin, and at 2.7 nm, which may correspond to proteins of smaller conformation (i.e., proteolytic fragments). At any rate, these minor peaks altogether account for only a small fraction (~15%) of total immobilized protein, suggesting that the majority of Az molecules is not denatured. In particular, azurin aggregation, if any, must only occur to a limited extent in the protein films. This result is very important, since an efficient ET process in azurin requires molecules retaining their proper conformation.[11] In order to verify this conclusion, we performed two additional experiments in which azurin was intentionally denatured. Az thermally unfolds above 84 °C, with localized unfolding around Trp48 already appreciable at ~74 °C.[12] In the first experiment, dry protein films were heated at 80 °C for 1 min on a hotplate before AFM measurements. In the second experiment, azurin in solution was heated at 80 °C for 1 min before chemisorption on SiO_2, and subsequent AFM analysis. The distribution of the thermally treated Az films [Fig. 1(b)] shows that the prevalent species is still centered around 4 nm, though with a broader distribution, accounting for ~74% of the adsorbed protein. The other two species reveal structural features which are similar to the reference film, even if the species with a larger size (presumably denatured proteins) seems to be more abundant in the heated samples. Overall, the thermal treatment of the immobilized Az in air does not seem to affect strongly the protein conformation. A very different situation is found when the thermal treatment is performed in solution before immobilization [Fig. 1(c)]. In this case a remarkable conformational disorder is observed. The monomer band (4 nm) is still present in the height distribution, but it is now broader and accounts for only one half of the adsorbed proteins. In contrast, the size of the larger species has shifted to 6.1 nm with a pronounced broadening, and its abundance has increased to ~35% of the total, suggesting more extensive denaturation. Interestingly, this confirms peak assignation, indicating that dry protein films are more resistant to heat denaturation than soluble protein, and shows that AMF is a sensitive technique to detect protein denaturation. These findings suggest that Az chemisorption does not result in significant denaturation, and in particular in aggregation, and that ''compact'' globular proteins are capable of maintaining their native structure upon adsorption even after solvent removal.

The second step was to analyze the conformational properties of the immobilized azurin. This is important since the ET rate is strongly dependent on distance[13] and chemisorption may result in dislocation of the redox centers. The conformational properties of the Az monolayers were investigated by intrinsic fluorescence spectroscopy. Azurin from *P. aeruginosa* has only one tryptophan residue (Trp48), which is responsible for the protein fluorescence.[14,15] Due to the hydrophobic microenvironment surrounding Trp48,[10,15] ultraviolet excitation ($250\div300$ nm) of Az results in a structured photoluminescence with a maximum around 308 nm.[14] While holo- and apo-azurin exhibit identical fluorescence spectra, the emission of the holo-protein is strongly quenched by the presence of copper in the active site, though the exact mechanism underlying the quenching process is still matter of debate.[16] Therefore, we utilized the apo-form of azurin for the optical investigation of the protein monolayers on SiO_2. Such an approach is correct because the structural stability of the wild type copper protein exceeds that of the apo derivative.[17] Compared to the fluorescence of free apo-azurin in buffer, the emission spectrum of the immobilized protein is characterized by a similar lineshape (Fig. 2), although slightly red-shifted (~2–3 nm). This contrasts with the broadband, red-shifted emission of a denatured sample of Az (see also the inset of Fig. 2). We thus

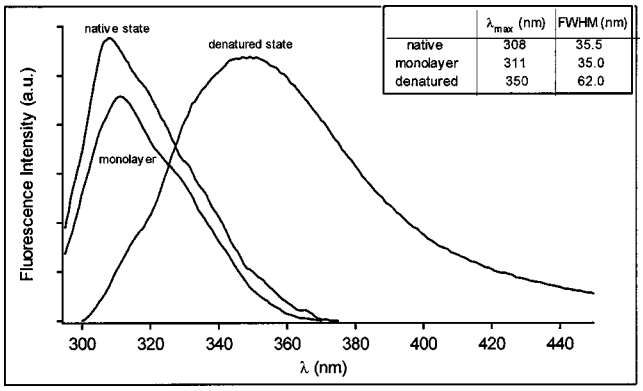

FIG. 2. Fluorescence spectrum of a monolayer of apo-azurin chemisorbed on SiO_2 compared to the spectra of apo-azurin in solution, in both the native and the fully denatured states (6 M guanidine hydrochloride). Fluorescence intensities are not to the same scale. Inset: optical parameters of the 3 spectra: wavelength of emission maximum (λ_{max}) and full width at half maximum (FWHM). All the emission spectra were recorded at room temperature (20 °C), atmospheric pressure, 54% humidity. The excitation wavelength was 280 nm (2 nm bandwidth).

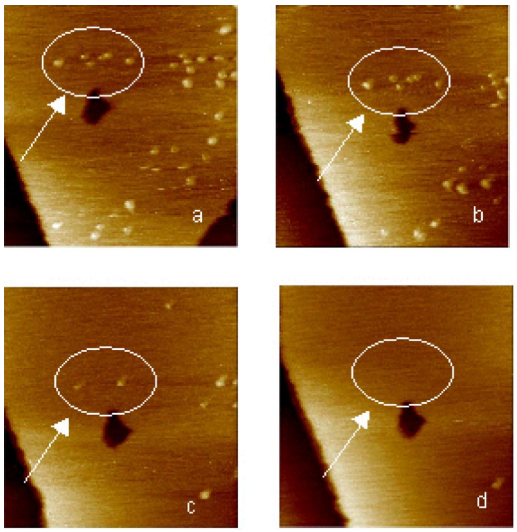

FIG. 3. STM images of Az (in 20 mM HEPES buffer, pH 4.6) on a Au(111) substrate as a function of bias voltage: (a) −400 mV, (b) −200 mV, (c) −20 mV, (d) +50 mV (bias applied to the substrate). Tunneling current: 1 nA, Scan area: 170×170 nm^2, Vertical range: 2 nm, Scan rate: 5 Hz.

conclude that the tryptophan residues in native and immobilized proteins are embedded in similar locations and that chemisorption does not significantly affect the folding of the native protein. The small spectral shift suggests the possibility of a very weak internal rearrangement due to the binding to the substrate, which does not affect the overall folding pattern. Importantly, the investigation of the microenvironment surrounding Trp48 is of particular relevance in the case of azurin, not only because Trp fluorescence is a very sensitive probe of protein conformational state,[17] but also because Trp48 is thought to play an important role in the long-range ET processes through the molecule.[15,18] The preservation of the nativelike conformation demonstrated by the immobilized species seems to indicate the absence of any significant surface effects on the biomolecules, which may be interpreted in terms of a strong capability of azurin to retain the protein hydration shells also under ambient conditions. It is noteworthy that the reported optical characterization represents, to our knowledge, the first evidence of such effect.

An additional proof that chemisorption on solid surfaces under nonphysiological conditions does not affect the proper folding of Az molecules is given by STM experiments. This technique allows direct investigation of the ET mechanism through the tunneling current, providing crucial information on the integrity of the copper active site, and, hence, on the functional state of the protein.[19] STM experiments were carried out, both in buffer solution and in air, on Cu azurin molecules directly immobilized onto gold substrates via the surface disulphide bridge Cys3-Cys26. Such immobilization procedure is thus analogous to that utilized for chemisorption on SiO_2. Two intramolecular ET routes have been characterized in azurin, the ''His46 pathway'' and the ''Trp48 pathway,'' *both* proceeding from Cys3 (the residue directly involved in the chemisorption) to different copper-ligating residues.[18,20] Figure 3 shows a sequence of STM images acquired in buffer at different bias voltages (without potentiostatic control). The proteins were clearly detectable on the gold surface as bright spots with a globular shape, in the size range of 4–6 nm, in good agreement with the crystallographic data[10] and with our AFM data (see above). This finding indicates that a favorable level alignment is elicited between the Au substrate, the molecular levels of Az and the tip, which permits ET to occur through the active site. Interestingly, STM detection of azurin at the surface was strongly dependent on the applied bias between the tip and the gold substrate, with protein images fading rapidly above and below an optimal gap voltage, owing to the occurrence of unstable tunneling conditions (results underlined by arrows in Fig. 3). These results are in good agreement with published work in which the visualization of gold-adsorbed Az by *in situ* STM, under electrochemical control, was found to be strictly potential-dependent.[6] Importantly, similar conditions of resonant tunneling are also found for immobilized azurin under ambient condition (Fig. 4), although the value of the

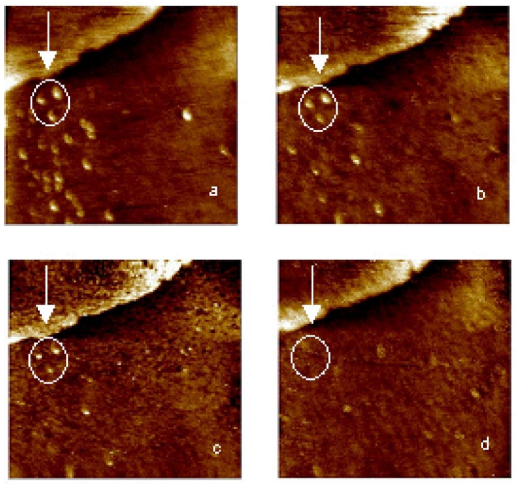

FIG. 4. STM images of Az in air on a Au(111) substrate as a function of bias voltage: (a) +100 mV, (b) +300 mV, (c) +600 mV, (d) +800 mV. Tunneling current: 500 pA, Scan area: 150×150 nm^2, Vertical range: 1 nm, Scan rate: 2 Hz.

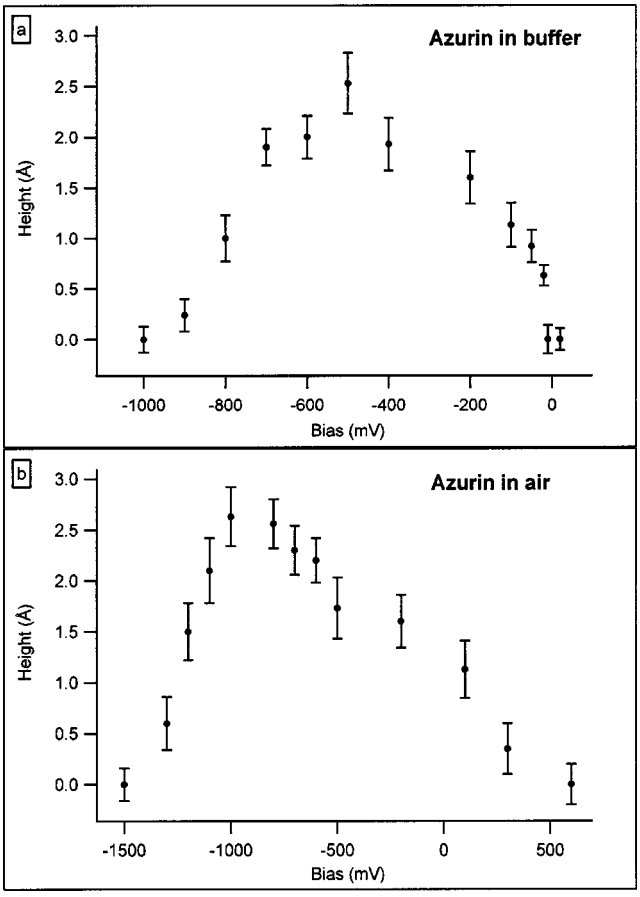

FIG. 5. Analysis of the apparent height of individual Az molecules as a function of the bias voltage: (a) in HEPES buffer, (b) in air. The height was measured by taking a line scan profile over groups of proteins and then averaging the results within the same image, i.e., for each bias. The tunneling current was set to fixed values while changing the bias.

optimal gap voltage depends on the particular experimental conditions. This dependence of tunneling involving the Az redox center on the gap voltage indicates the lack of gross molecular rearrangements upon immobilization in the solid state under nonphysiological conditions. A direct comparison between the Az behavior in air and in buffer solution under STM injection is shown in Fig. 5, where we plot the apparent height of individual Az molecules as a function of the applied gap voltage. In both cases, a clear variation of the measured height values of the protein is evident, ranging from 0 (no protein visible, bad tunneling conditions) to 2.5 Å (bright spots corresponding to good and stable tunneling conditions across the protein). These trends can be ascribed to the on/off resonance conditions in the tunneling processes entailing the molecular levels. A resonance behavior is found, with a peak around -1 V for tunneling in air, and -0.5 V for the liquid environment. The different voltages for the air and buffer measurements are only partially due to the relative dielectric constants (ϵ_r) of the two media (air and buffer), and can be mainly ascribed to the different tunneling current values in the two experiments (0.5 and 1 nA, for ambient and liquid conditions, respectively), which fix a different distance between the tip apex and the sample surface. By increasing the current, the tip is closer to the sample and the electrostatic field felt by the protein is larger. The experimental evidence that a suitable external bias induces intramolecular ET in the immobilized protein in air is thus very important, because it reveals that the Az functionality is not lost. This suggests that the charge distribution on the protein surface remains the same both in air and in liquid environment (likely due to the retention of the hydration shells) resulting in the optimal structural robustness of the protein in both cases.

In conclusion, we have demonstrated that Az chemisorption in the solid state does not significantly alter its conformation and the redox site structure even after the removal of the water solvent, and is therefore likely to preserve the azurin ET function(s). This discloses very interesting perspectives for the development of hybrid nanodevices operating in nonliquid environments.

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