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# Retention of nativelike conformation by proteins embedded in high external electric fields

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In this Communication, we show that proteins embedded in high external electric fields are capable of retaining a nativelike fold pattern. We have tested the metalloprotein azurin, immobilized onto $SiO_2$ substrates in air with proper electrode configuration, by applying static fields up to $10^6$–$10^7$ V/m. The effects on the conformational properties of protein molecules have been determined by means of intrinsic fluorescence measurements. Experimental results indicate that no significant field-induced conformational alteration occurs. Such results are also discussed and supported by theoretical predictions of the inner protein fields. © *2005 American Institute of Physics*. [DOI: 10.1063/1.1902903]

Resistance of biomolecules to high electric fields is a main concern for nanobioelectronics/nanobiosensing applications, and it is also a relevant issue from a fundamental perspective, to understand the dielectric properties and structural dynamics of proteins. In nanoscale devices, biomolecules may experience electric fields as high as $10^7$ V/m in order to elicit charge transport/transfer. Understanding the effects of such fields on their structural integrity is thus crucial to assess the reliability of biomolecular devices. In this Communication, we show experimental evidence for the retention of nativelike fold pattern by proteins embedded in high electric fields. We have tested the metalloprotein azurin, deposited onto $SiO_2$ substrates in air with proper electrode configuration, by applying high static electric fields (up to $10^6$–$10^7$ V/m). The effects on the conformational properties of protein molecules have been determined by means of intrinsic fluorescence measurements. Experimental results indicate that no significant field-induced conformational alteration occurs. This phenomenon is discussed in terms of the intrinsic intraprotein electric fields. As the general features of such inner fields are not peculiar of azurin, the conclusions presented here should have general validity.

Azurin from *Pseudomonas aeruginosa* is an electron-transfer metalloprotein.[1] Its redox-active center contains a copper ion having five amino acid ligands arranged in a peculiar trigonal bipyramidal geometry. Thanks to its intrinsic stability, azurin has emerged as a good candidate for biomolecular nanoelectronics.[2] In this work, we have investigated the influence of strong electric fields on the protein conformational state by means of interdigitated electrodes, fabricated using standard photolithographic techniques. The structure consists of 500 interdigitated lines of 1 μm width and 1 μm spacing, resulting in an active area of $(1 \times 1)$ $mm^2$.[3] The electrodes geometry was properly designed both to allow the application of high electric fields to protein molecules and to detect the very weak fluorescence signal over a large area. Finite element electrostatic simulation of the electrodes was performed by a standard commercial code to assess the entity and spatial distribution of the electric field in the devices. A three-dimensional (3D) geometry was defined to evaluate the field bending close to the upper border of the electrodes. Fixed potential values were assigned to the electrodes, while a weak Neumann boundary condition (i.e., null tangential field) was imposed on the other outer surfaces. The interelectrode material was chosen to be vacuum. Figure 1 shows the color map of the electric field intensity, on a cross-section of the electrodes (growing values from blue to red): the field decays rapidly immediately outside the interelectrode region (where the field is predictably nearly uniform, except for the hot-spots on the corners, due to accumulated charge).

The protein was immobilized onto the interdigited electrodes structures, by cast deposition of a 20 μl drop of the protein solution (1.0 mg/ml in 50 mM $NH_4Ac$ buffer, *p*H 4.6); the incubation time was 15 min (at room temperature). After incubation, the buffer solution was removed, and the samples were accurately dried by high purity nitrogen flow. Such procedure results in protein immobilization both between electrodes, onto the $SiO_2$ substrate, and on top of the electrodes themselves. The presence of azurin molecules be-

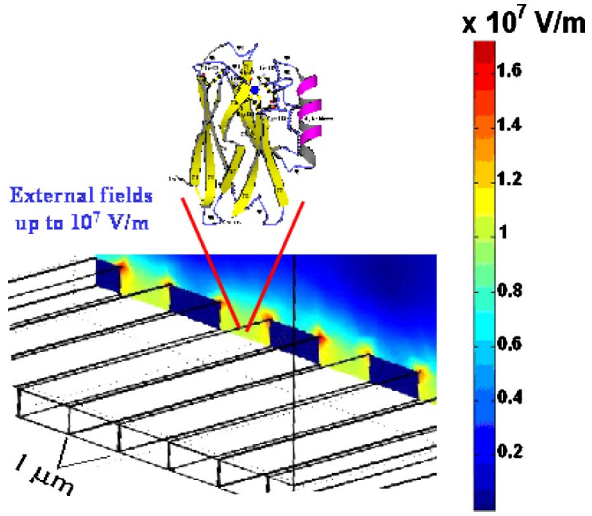



FIG. 1. Cross-section color map of the electric field intensity, obtained by the 3D finite element simulation. Between the electrodes, a nearly uniform field region may be observed, except for the hot-spots on the corners (red).

tween the micrometer-sized electrodes was carefully assessed by molecular resolution Tapping Mode Atomic Force Microscopy (TM-AFM) (Fig. 2). Therefore, the photoluminescence signals collected in our experiments includes contributions both from the protein molecules between the electrodes (which experience the full intensity of the applied electric field) and from molecules atop the electrode surfaces

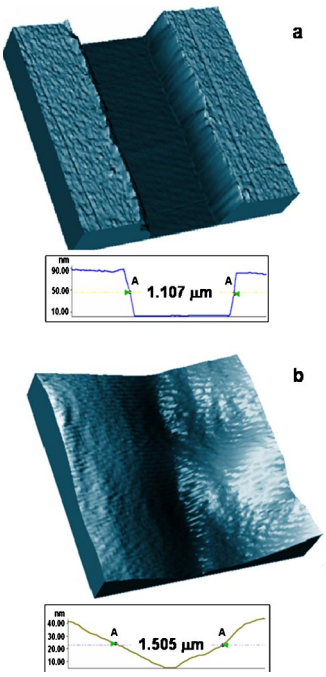


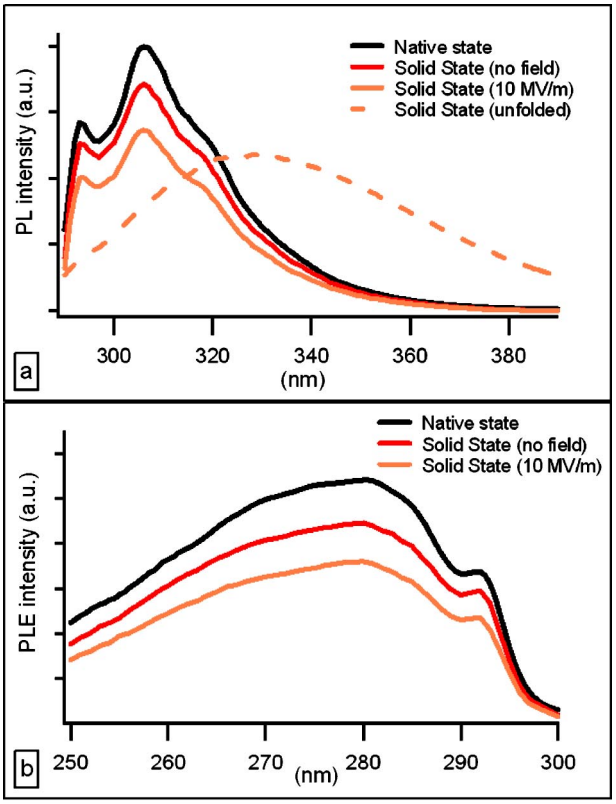

FIG. 2. AFM topography of the electrodes, before (a) and after (b) the deposition of azurin, in 3D and cross-profile representations. The apparently reduced electrode height and separation is due to azurin deposition, most of which occurs in the interelectrode region (see profiles). An important point is that the external field atop the electrode surface (which is 50%–60% of the peak value, as described in the text) extends, with a very good uniformity, for more than 100 nm from the metal surface (FEM analyses): this means that we have the *same* electric field for more than 25 protein layers (1protein=4 nm) onto the electrodes, i.e., remarkably more than the thickness reached in our experiments.

(which are exposed to about 50%–60% of the peak value, on the average, according to the FEM analysis).

Intrinsic fluorescence of *P. aeruginosa* azurin is due to a single tryptophan residue (Trp48).[4] Upon ultraviolet excitation, Trp48 exhibits an unusual "blue" emission ($\lambda_{max} \approx 306$–$308$ nm), owing to the highly hydrophobic surrounding microenvironment.[4,5] In the native state, azurin, apoazurin (i.e., without $Cu^{2+}$), and other metal derivatives, such as $Zn^{2+}$, exhibit identical fluorescence spectra, accounting for the lack of any structural change besides the metal site, as also predicted by crystallographic determinations.[5,6] However, since the different derivatives exhibit large variations in the fluorescence quantum yield, due to a strong quenching mechanism by the metal ion,[7] in this study we have used the apo-protein. As shown in Fig. 3(a) the emission spectra of apoazurin in the solid state and under high external fields exhibit the same line shape of the free native protein in buffer, and no emission shifts are detectable.[8] Since the intrinsic fluorescence in azurin is very sensitive to small perturbations of the protein folding,[9] this result reveals that the presence of such electric fields does not affect the overall fold pattern, indicating that the Trp residue remains embedded in the same hydrophobic environment.

The retention of the nativelike conformation in the protein films was also supported by the observed independence of their emission spectra of the excitation wavelength (not shown). This is an important point, since Az photoluminescence is not affected by $\lambda_{exc}$ if the protein is in the folded state, whereas its spectral line shape may be strongly influenced by the excitation wavelength if the protein undergoes conformational transitions. Importantly, also the excitation spectrum (PLE) was found to be unchanged upon electric field application [Fig. 3(b)] consistent with the absence of relevant perturbations in the physico-chemical conditions of the chromophore microenvironment. This confirms that such field intensities do not interfere with the conformational properties of the native protein. Moreover, it is interesting to note the clear retention of structured luminescence spectra (both PL and PLE) by solid state films, which is a peculiar feature of native Az conformation (in the native state, Trp48 is shielded in the rigid and highly hydrophobic core of the protein). This specifically contrasts with typical broad, red-shifted emissions observed in unfolded azurins, which are completely devoid of any spectral structure. In the case of azurin, the investigation of the microenvironment surrounding Trp48 is of particular relevance (see also Fig. 3, bottom), since such residue is thought to play an important role in the long-range ET processes through the molecule.[4,10] In addition, this result is important because it was obtained with the apo derivative, which is characterized by a lower structural stability with respect to the wild type copper protein.[9] Hence, the preservation of the nativelike conformation seems to indicate the lack of gross molecular rearrangements also under extreme experimental conditions, such as those normally achieved in biomolecular electronic devices.

It is worth noting that such remarkable results are supported by theoretical predictions. We have calculated the molecular electrostatic potential (MEP) for azurin in solution by solving the Poisson equation for a given charge



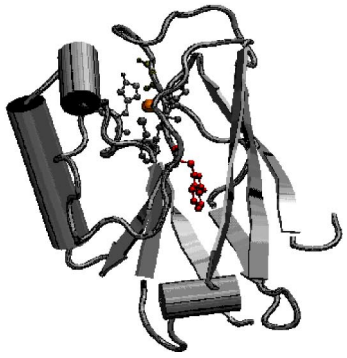



FIG. 3. Top, intrinsic fluorescence spectra of apoazurin in the native state and in the film environment with and without an applied external electric field. (a) Emission spectra (PL); (b) Excitation spectra (PLE). The fluorescence intensities are not in scale. The spectra of free native apoazurin were collected in 50 mM $NH_4Ac$ buffer, *p*H 4.6. All these experiments were performed at room temperature (20 °C), atmospheric pressure, 54% of humidity. The excitation wavelength was 280 nm (2 nm bandwidth). Az films were also excited at different wavelengths (250–300 nm) and no variations in the shapes of the PL spectra were observed. A fluorescence spectrum of a heat denatured sample (1 min at 100 °C) of the solid state protein is also reported for comparison. Fluorescence measurements were also performed in the presence of some solvent (i.e., without extensive drying of protein films by nitrogen flow) and identical results were obtained. Bottom, structure of azurin with focus on the relative arrangement of Trp48 and the electron-transfer active site. The residue Trp48, which is responsible for luminescence, is shown in red in ball&stick rendering. The active site is shown in gray in ball&stick rendering (the large orange ball represents the Cu atom). The rest of the protein is shown with the conventional features of the secondary structure, $\alpha$-helices and $\beta$-sheets, backbone.

distribution.[11,12] The subsequently obtained inner electric field present in the protein was found to have a mean value as high as $2.45\times10^9$ V/m and peak intensities up to $17\times10^9$ V/m, as shown by the field intensity profile plotted in Fig. 4. According to this analysis, the Az inner fields are

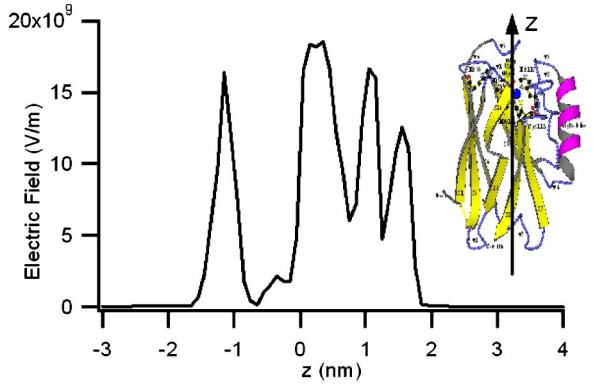



FIG. 4. Profile (along *z* direction) of the inner electric field in azurin as computed by solving the Poisson equation (see Ref. 12 for details). As shown in the inset, the *z* direction can be represented by the axis of the $\beta$-barrel that defines the protein shape, which is approximately along the line that can be traced between the Cys3-Cys26 disulfide bridge and the copper site (the field profiles along *x* and *y* axes are indeed very similar).

orders of magnitude higher than those applied in our experiments; thus, it seems reasonable that the fold pattern of the biased proteins is unperturbed by external field intensities in the range of $10^6$–$10^7$ V/m.

Although such inner field intensities may sound unrealistic, our calculated values are in very close agreement with recent studies,[13] in which the intriguing result on the higher density (with respect to bulk water) of the first hydration shell[14] was theoretically elucidated in terms of water molecules "electrostriction" induced by the huge electric field values ($\sim10^9$ V/m) at the surfaces of the biomolecules. In addition, molecular dynamics simulations by Xu *et al.* (on trypsin inhibitor) have demonstrated that field strengths in the range of $10^8$ V/m do not alter the overall structure (or temperature) of the protein (such fields are shown to be within thermal fluctuations), and only fields higher than $10^9$ V/m can induce significant structural changes.[15] Finally, hybrid quantum chemical molecular dynamics computations have predicted average fields within the range $5\times10^8$–$8.5\times10^9$ V/m.[16]

All these considerations agree with our experiments, indicating that the conformational state of the protein is not significantly perturbed with respect to the native state, upon external field application, resulting in the retention of the proper folding also under these conditions. It is noteworthy that the reported characterization on azurin is, to our knowledge, the first experimental demonstration of such effects.

In conclusion, the available evidence suggests that: (i) proteins in the solid state are capable of maintaining the tightly bound hydration shells and a nativelike conformation, even after solvent removal and application of high external fields; (ii) such surprising effects are consistent with the occurrence of huge intensity inner fields in the proteins. These results may also have importance for protein crystallization experiments, since recent studies have demonstrated that the application of high external electric fields (in the range of $10^5$ V/m) during the crystallization of protein samples can positively influence the nucleation rate and kinetics, increasing the size of the crystals and improving their crystallographic quality.[17]

## ACKNOWLEDGMENTS

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[1]E. T. Adman, Adv. Protein Chem. **42**, 145 (1991).

[2]R. Rinaldi, A. Biasco, G. Maruccio *et al.*, Adv. Mater. (Weinheim, Ger.) **14**, 1453 (2002); Appl. Phys. Lett. **82**, 472 (2003); Q. Chi, Z. Zhang, J. U. Nielsen *et al.*, J. Am. Chem. Soc. **122**, 4047 (2000).

[3]Interdigited electrodes were fabricated on thermally oxidized silicon wafers, using the standard photolithographic techniques. The structure, defined in AZ5214 resist, consisted of 500 interdigited gold electrodes with a line-space period of 2 μm, covering an active area of $1\times1$ mm$^2$. After exposure, samples were developed in AZ726MIF. The metal electrodes were obtained by *e*-beam metal evaporation (Cr/Au) followed by lift-off in acetone bath for more than 10 min at moderate temperature (35 °C-40 °C).

[4]S. J. Kroes, G. W. Canters, G. Gilardi *et al.*, Biophys. J. **75**, 2441 (1998).

[5]H. Nar, A. Messerschmidt, R. Huber *et al.*, J. Mol. Biol. **221**, 765 (1991).

[6]E. N. Baker, J. Mol. Biol. **203**, 1071 (1988); H. Nar, A. Messerschmidt, R. Huber *et al.*, FEBS Lett. **306**, 119 (1992); H. Nar, R. Huber, A. Messerschmidt *et al.*, Eur. J. Biochem. **205**, 1123 (1992).

[7]J. E. Hansen, J. W. Longworth, and G. R. Fleming, Biochemistry **29**, 7329 (1990); J. A. Sweeney, P. A. Harmon, S. A. Asher *et al.*, J. Am. Chem. Soc. **113**, 7531 (1991).

[8]Fluorescence spectra were recorded in photon counting mode, by using a 450 W Xenon lamp as the source of excitation and double monochromators both in excitation and emission. For measurements in solution, the emitted light was observed at right angles to the excitation radiation. Photoluminescence spectra of control samples, without protein, were recorded and subtracted from the experimental samples to correct for background interference. To monitor azurin luminescence from protein in the solid state, the exciting radiation was focused on the protein film with 30° angle of incidence, while the front surface emission was collected at 90° from the excitation. The intensity losses were negligible or less than 5% depending on the experimental conditions, thus excluding appreciable Trp photodegradation during acquisition.

[9]J. Leckner, P. Wittung-Stafshede, N. Bonander *et al.*, Biochim. Biophys. Acta **1342**, 19 (1997).

[10]S. Larsson, A. Broo, and L. Sjoelin, J. Phys. Chem. **99**, 4860 (1995).

[11]J. D. Madura, J. M. Briggs, R. C. Wade *et al.*, Comput. Phys. Commun. **91**, 57 (1995).

[12]The MEP of azurin from *Pseudomonas aeruginosa* was computed on the x-ray oxidized protein structure at *p*H 5.5 (molecule A in the PDB file 4azu. http://pdb.gmd.de). The University of Houston Brownian Dynamics (UHBD) software [Ref. 11], which was employed for the calculation, solves the Poisson equation for the charge distribution that characterizes the protein residues in the protonation state that depends on the *p*H. The OPLS [W. L. Jorgensen and J. Tirado-Rives, J. Am. Chem. Soc. **110**, 1657 (1988)] nonbonded parameter sets for atomic charges and radii were assigned to the protein residues [Ref. 2(a)]. In particular, the histidines (His35, His83, His46, His117) were considered protonated, with a proton on each N atom of the pentagonal ring, as pertinent for the acidic *p*H. The electric field has then been obtained by performing the numerical spatial gradient on the computational grid. Obviously, such a model is not meant to calculate accurate values for the intraprotein electric field, but only to estimate its order of magnitude. This is fully satisfactory for the kind of comparisons that we would like to perform in the present work.

[13]F. Merzel and J. C. Smith, Proc. Natl. Acad. Sci. U.S.A. **99**, 5378 (2002); I. Danielewicz-Ferchmin, E. Banachowicz, and A. R. Ferchmin, Biophys. Chem. **106**, 147 (2003).

[14]D. I. Svergun, S. Richards, M. H. J. Koch *et al.*, Proc. Natl. Acad. Sci. U.S.A. **95**, 2267 (1998).

[15]D. Xu, J. C. Phillips, and K. Schulten, J. Phys. Chem. **100**, 12108 (1996).

[16]P. R. Callis and B. K. Burgess, J. Phys. Chem. B **101**, 9429 (1997).

[17]C. Charron, C. Didierjan, J. P. Mangeot, and A. Aubry, J. Appl. Crystallogr. **36**, 1482 (2003).