Protein adsorption at a gold electrode studied by *in situ* scanning tunnelling microscopy

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The *in situ* adsorption, under physiological conditions, of azurin molecules at a gold electrode surface has been monitored at the molecular level by scanning tunnelling microscopy. The introduction of free cysteine residues into the surface of the redox protein by structurally conservative mutagenesis allows the immobilisation to be controlled in a manner in which the protein electrochemical activity is retained.

Scanning probe methods combine the ease of use and versatility of optical microscopy with a resolution, in three dimensions, comparable to that of electron microscopy; they thus constitute a powerful means of structural determination of biological molecules,[1–4] a wide range of which have now been imaged by both AFM and STM. AFM, in particular, is now a significant contributor in the field of structural biology. Since water is an integral part of protein tertiary structure, the advantages associated with the ability to carry out molecular-level imaging in a physiological environment are considerable.

It is likely that STM, on account of its lower contact area with a sample, will produce a higher spatial resolution than AFM. It also introduces the possibility of determining not only topography but also electronic properties. As with any form of microscopy, there are, however, significant problems associated with the use of biological samples which need to be considered. In addition to the possible collective mobility of proteins on a substrate surface, particularly under fluid[5] (where proteins are likely to be labile enough to escape imaging), tip-induced movement is perhaps the single biggest problem associated with the imaging of protein molecules by SPM methods.[6,7]

Though these problems can be at least partially alleviated through the use of appropriate scanning conditions, it is generally necessary to anchor the biomolecule to the substrate surface. Physical (hydrophobic and/or electrostatic) adsorption, while experimentally simple, is generally non-specific and may lead to a loss of native protein structure. Chemical modification methods, in which protein is covalently bound to the solid phase, have accordingly been developed to produce high levels of controlled protein binding. This process of adsorption must be carefully balanced so as to be sufficiently strong to inhibit movement of the molecules during scanning[8] whilst not directly leading to structural change. There are several ways in which this can be achieved. The macromolecule can be cross-linked to a functionalised substrate or a single molecular layer assembled on the substrate surface and this then used to bind the protein/enzyme. Silane chemistry has been commonly used in the production of protein-binding functionalised adlayers.[9–11] Proteins have also been immobilised on gold through the attachment to thiol-based self-assembled monolayers;[12,13] these may bind to proteins in a specific or non-specific manner.

An alternative, though less-used, strategy, is modification of the protein itself; for example, thiolation of protein amine functions with Traut's reagent (2-iminothiolane) has been used in binding to a gold substrate.[7] Such modifications are, however, non-specific and the orientation of protein binding remains largely random. In addition, such gross chemical modification may induce significant deviation from native structure. More recently, methods have been developed in which the orientation of the immobilised protein molecule can be controlled, to some degree,[14,15] through the use of histidine tagging[16,17] or the coupling of genetically engineered protein molecules to functionalised glass or silicon substrate surfaces.[9,10,18,19]

The inherent flatness, conductivity and ease of a preparation of a gold substrate makes it a particularly suitable surface on which to study protein adsorption. The aim of this study was to investigate the possibility of utilising the strong interaction between thiol functions, introduced into the surface of a copper-containing redox protein azurin by mutagenesis, in achieving stable, non-destructive protein immobilisation on a gold substrate and its consequent molecular-level monitoring by STM.

Azurins are, well-characterised, type I or blue copper proteins which function as electron transfer mediators at high redox potential.[20,21] They are relatively small (10–15 kDa) polypeptides arranged into two β-sheets, with little or no α-helical structure. They contain a single copper atom located strongly asymmetrically in a hydrophobic core and are known to be relatively stable over a wide range of temperature and pH conditions. Many mutants of azurin have now been produced and characterised.[22] The aim of this work was to mutate a serine into a cysteine; this is a conservative mutation in that (aside from the possibility of dimerisation), no significant protein structural change was anticipated.

Experimental

STM experiments were carried out with a Molecular Imaging Pico SPM (Model MS300) and a 'D' scanner in conjunction with a Nanoscope IIIa controller (Digital Instruments). Gold substrates were prepared by evaporation, at 0.2 nm s^{-1}, of gold (Goodfellows, Au 005220) on to freshly cleaved mica (Agar Scientific) at 250 °C and 1×10^{-5} bar (Edwards). Substrates were annealed in a butane flame, quenched in air for a few seconds, then immersed in pure water (Aldrich 27-073-3) within the molecular imaging fluid cell. The fluid cell was soaked overnight in a mixture of 30% H_2O_2 and 70% concentrated sulfuric acid, then rinsed in boiling Millipore water for at least 1 h prior to use. Tips were prepared by electrochemical etching of tungsten wire (0.25 mm diameter, Agar) in 2 M NaOH and coated with apiezon wax.

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The azurin (*Pseudomonas aeruginosa*) mutant S118 C, in which the serine at position 118 has been replaced by a cysteine, was expressed in *Escherichia Coli* strain JM109 and fermented in 301 LB media.[23] The introduced cysteine, which lies in the 'northern hemisphere' of the protein was selected on the basis of its vicinity (*ca.* 9 Å) to the copper centre. Mutagenesis was performed by polymerase chain reaction (PCR) methods.[24] The protein was purified by ion-exchange chromatography (CM-Sepharose FF and DEAE Sepharose) and stored at −20 °C in 20 mM HEPES buffer, 3 mM dithiothreitol (DTT), pH 7. Reconstitution was performed, after the removal of DTT by gel filtration, through the addition of 1.5–3 equivalents of copper(II) nitrate. The protein was characterised by SDS-Page and UV/VIS spectroscopy. The presence of the free thiol was confirmed through reaction with Ellman's reagent.[25]

Dc cyclic voltammetry experiments, at both edge-plane graphite and polycrystalline gold, were carried out in a standard two-compartment, three-electrode cell using an Eco Chemie PGSTAT 10 (Windsor Scientific, UK) in nitrogen-saturated, 20 mM HEPES, 100 mM KCl, pH 7 at 20 °C. Electrodes were polished with an alumina (0.015 μm particle size) slurry on polishing cloth (Beuhler UK). Gold electrodes were further cleaned by repeated cycling in 1 M sulfuric acid. All potentials are reported with respect to the standard calomel electrode (SCE).

For ambient imaging, freshly annealed gold substrate was immersed in a 150 μM protein solution (in deionised water) for 3–12 h, gently rinsed, blown dry with a jet of pure argon, and immediately imaged under conditions of high tunnelling resistance. Solution imaging was carried out in the same way, with the exception that the substrate was removed from the protein solution, gently rinsed, then immediately immersed under pure water or buffer (20 mM potassium phosphate, pH 7) and imaged. True '*in situ*' imaging was carried out by immersing freshly annealed gold substrate under pure water or buffer, then characterising both tip and substrate for at least 1 h. The observation of atomically flat, hexagonal facets confirmed the presence of a [111] surface.[26,27] The tip was then withdrawn a short distance from the surface (*ca.* 5 μm) and *ca.* 100 μl of 100–150 μM S118C in 20 mM phosphate buffer, pH 7, added. Feedback was then re-attained and imaging continued.

Presented images were subject to a first-order flattening and high-frequency noise was removed by low pass filtering.

Results

Electrochemical results

SDS-Page demonstrated that the protein remained monomeric during preparation and storage. The mutant displayed a good, quasi-reversible electrochemical response at a polished edge-plane graphite (EPG) electrode (Fig. 1), the potential of which was positively shifted with respect to the wild-type protein by *ca.* 30 mV ($E_{1/2}$ + 90 mV *vs.* SCE).[28] Whilst the wild-type protein displayed a poor, irreversible response at a bare gold electrode, the S118C mutant gave markedly different, stable electrochemistry (Fig. 2). A scan-rate study indicated that the electroactive protein was adsorbed on the gold electrode. Since these responses were obtained immediately on

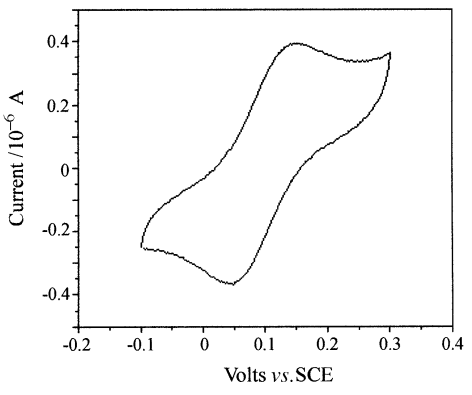

Fig. 1 Electrochemical response of 60 mM azurin S118C at a polished edge-plane graphite (EPG) electrode, in 20 mM HEPES pH 7, 100 mM KCl, at 20 mV s^{-1}

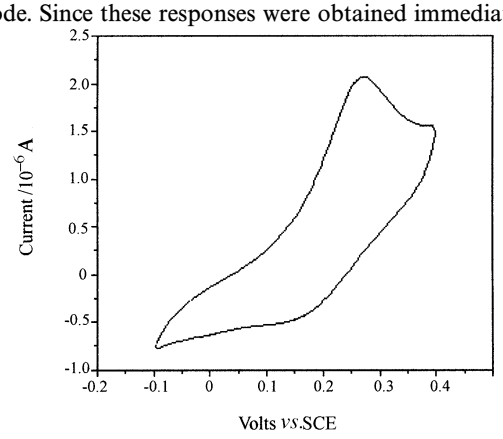

Fig. 2 Electrochemical response of *ca.* 70 μM azurin S118C at a polycrystalline gold electrode, in 20 mM HEPES pH 7, 100 mM KCl, at 20 mV s^{-1}

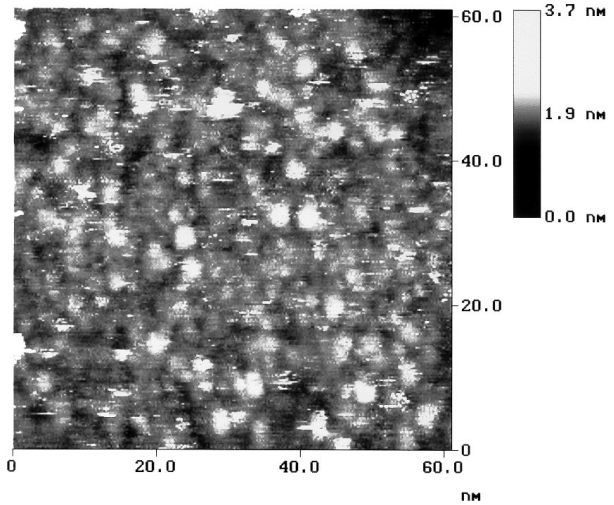

Fig. 3 Constant-current image of azurin molecules on Au[111] under ambient operation. Bias 1.7 V, set point 160 pA, scan rate 10 Hz

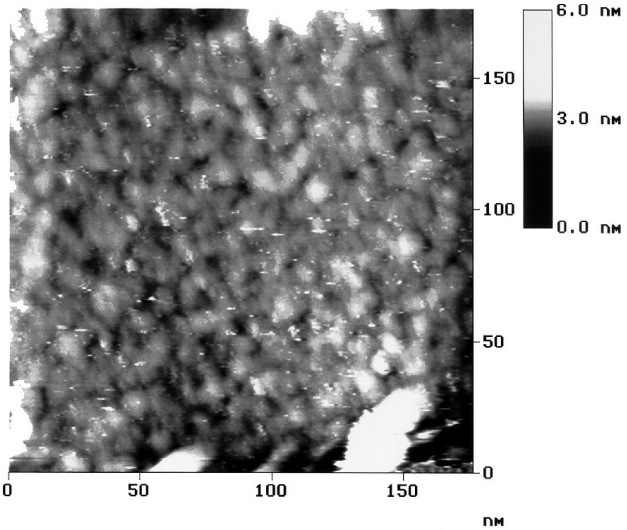

Fig. 4 Constant-current image of azurin molecules immobilised on Au[111] under 20 mM phosphate buffer, pH 7. Bias 920 mV, set point 650 pA, scan rate 8.7 Hz

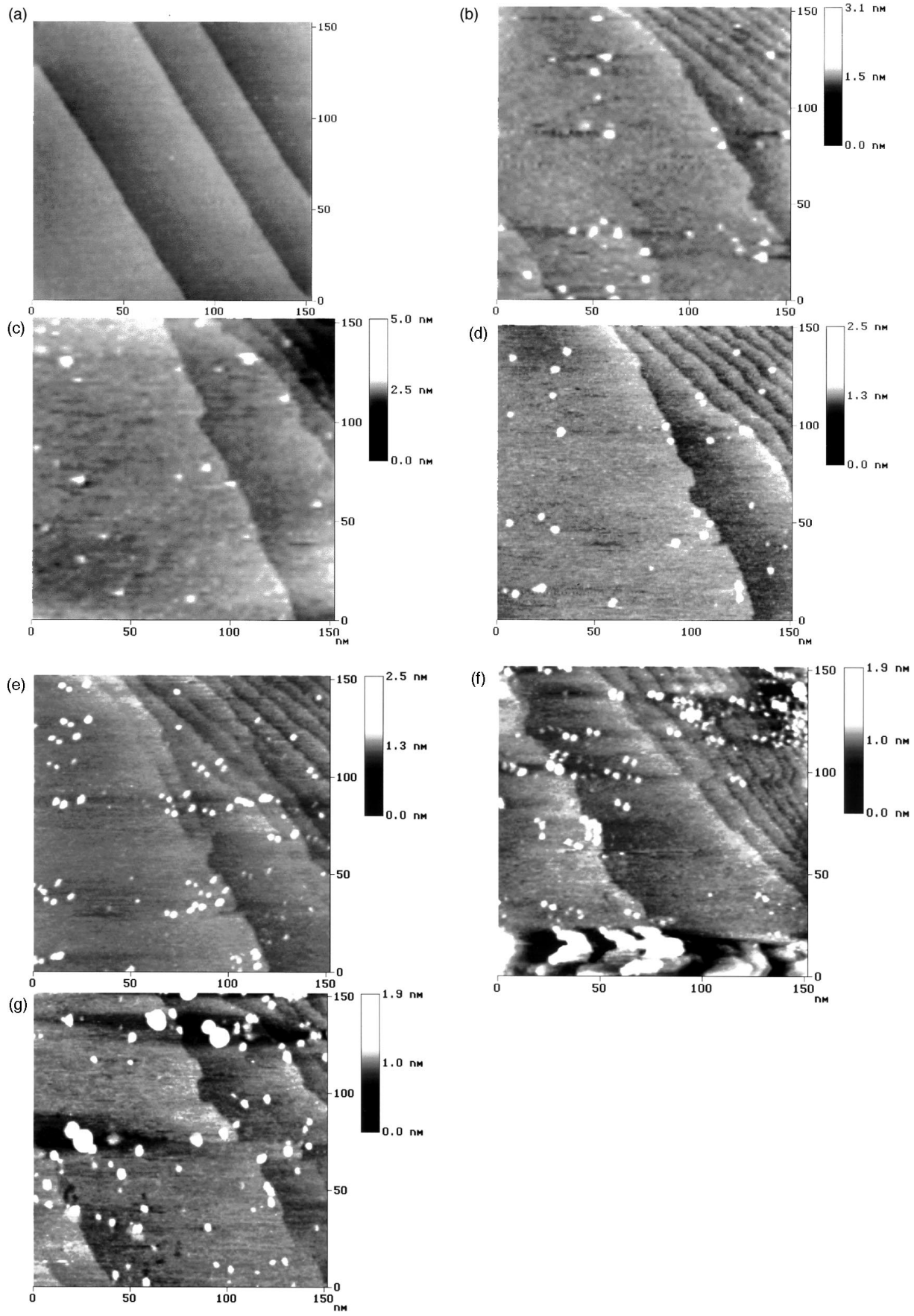

Fig. 5 Constant-current images of the *in situ* adsorption of S118C on an Au[111] surface under phosphate buffer. Bias 666 mV, set point 1.3 nA, scan rate 6 Hz. (a) The annealed gold substrate surface prior to the introduction of protein, *z*-scale 0–2.5 nm; (b) 3 min after the injection of 100 μl of 100 μM S118C solution; (c) 5 min after protein injection; (d) 10 min after protein injection; (e) 15 min after protein injection; (f) 18 min after protein injection; (g) 32 min after protein injection

immersion of a freshly polished gold electrode in protein solution (without pre-soaking), they are consistent with rapid adsorption. The role of the surface cysteine in this behaviour was demonstrated through the addition of β-alenoyl maleimide to the cell. Removal of the free surface thiol, through its reaction with the maleimide resulted in a dramatic change in electrochemical response—close to that observed with the wild type protein.

Scanning tunnelling microscopy†

Ambient imaging of protein-treated gold substrate demonstrated that the surface was covered with a large amount of soft material. At higher magnification (Fig. 3) this could be resolved into regularly sized, quasi-spherical molecules. Since such features were not observed if the substrate was immersed in protein-free deionised water and imaged under the same conditions, these must be azurin molecules. This is further supported by the fact that the smallest, clearly resolved features were *ca.* 4 nm in diameter (the long axis of the azurin molecule is *ca.* 4 nm in length). Images of similarly treated substrate taken under solution were noticeably sharper than those obtained in air. Individual molecules, and/or small aggregates, at high coverage, could, again, be resolved (Fig. 4).

Fig. 5 (a)–(g) shows a sequence of images taken following the injection of *ca.* 100 μl of 150 μM protein solution into the cell, to give a solution *ca.* 70 μM in protein. Though somewhat disrupted by the effects of thermal drift, the sequence of images significantly shows a clear evolution over time, with the amount of adsorbed protein steadily increasing. The size of these molecules was, again, consistent with the smallest of them being individual protein molecules. Occasionally, imaging would suddenly deteriorate (with tip leakage remaining low) and could not be re-established at an acceptable level. This could, perhaps, be attributed to protein adsorption at the tip. Importantly, it was generally observed that the material was stable to continual scanning. Unless immediately moved by the tip, individual molecules could be imaged in isolation and did not appear to possess inherent mobility on the surface. Images of individual isolated molecules are presented in Fig. 6, 7 and 8.

Discussion

Despite its importance in chromatography[28] and clinical diagnostics,[29] the adsorption of protein at a solid/liquid interface remains a poorly understood process[30,31] in which van der Waals, electrostatic and hydrophobic forces, amongst others, play a role.[9,32,33] At the present time, few analytical methods exist that enable this process to be studied at a molecular level.[29] Scanning probe microscopy offers the ability to carry out such studies *in situ* and at a truly molecular level.

These results demonstrate that the S118C mutant has a high affinity for a gold electrode surface. It should be noted that observations made with other proteins, such as cytochrome *c*[5,34] suggest that this affinity for the gold substrate is *not* inherent to all protein molecules. The observed lack of mobility of the azurin molecules on the surface is consistent with their covalent attachment through the surface cysteine residue. The importance of the introduced surface cysteine in the interaction of the protein with the gold surface was demonstrated by the dramatic change in electrochemical response observed on the blocking of this free thiol through reaction with a maleimide and is consistent with a specific chemisorbed, rather than physisorbed, state. The adsorption of L-cysteine itself on gold is known and believed to involve non-bonding electron pairs on the sulfur atom.[35,36] Since the electrochemical response of the mutant protein was stable to continual scanning, the adsorption must be in such a way that

† See http://www.rsc.org/suppdata/njc/1998/1119 for full colour versions of Fig. 3–8.

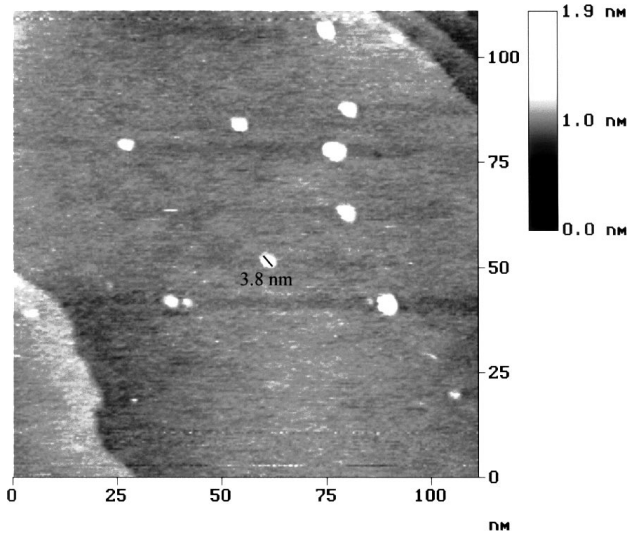

Fig. 6 *In situ* image of immobilised S118C molecules. Bias 666 mV, set point 1.3 nA, scan rate 6 Hz

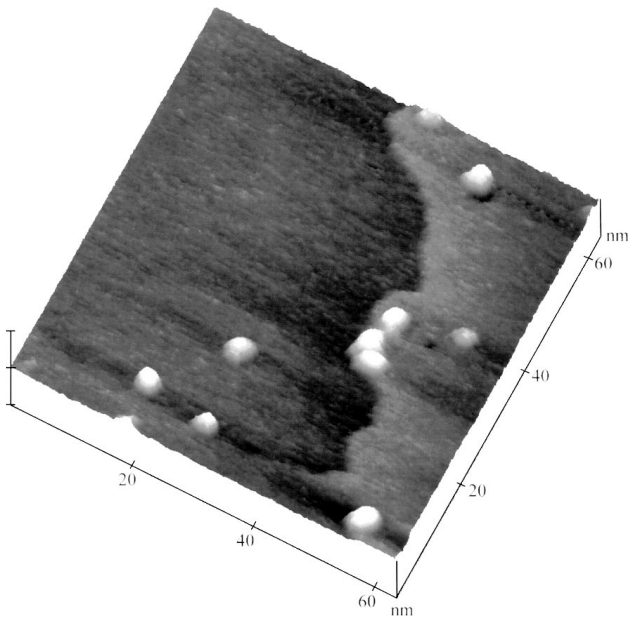

Fig. 7 A three-dimensional, constant-current image of immobilised azurin molecules on Au[111]. Bias 666 mV, set point 1.3 nA, scan rate 6 Hz

electron transfer between the copper centre and the surface is possible, and indeed, that the protein structure is essentially retained under such conditions. This behaviour may be related to the location of the introduced cysteine residue; the adjacent residue (117) is thought to lie on the electron-transfer pathway from the copper centre to the co-proteins of azurin.[37]

As this work was in progress, Friis *et al.*[38] published a combined AFM/STM study of wild-type azurin on gold. The authors concluded that the protein chemisorbs on the substrate surface *via* its disulfide group (Cys3-Cys26). Though it is possible that this functionality plays a role in bonding, the disulfide bond is an integral part of the protein structure and presumably must dissociate on adsorption (this is indeed supported by voltammetric studies[39]). In addition to allowing immobilisation to occur in a controlled orientation, the marked differences in electrochemical behaviour between the S118C mutant and the wild-type protein observed during this study (and a preliminary comparative STM investigation) support a significant role of this introduced surface cysteine residue. The electrochemical results do demonstrate that immobilisation through the surface cysteine residue does

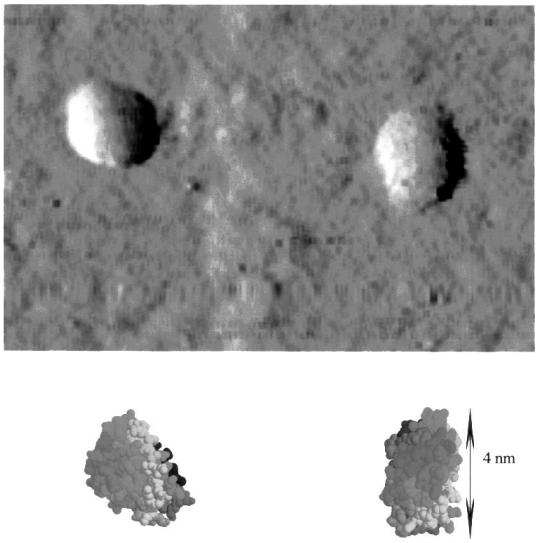

Fig. 8 A comparison of constant height images of two azurin S118C molecules (bias 770 mV, set point 940 pA, scan rate 9 Hz) and orientated space filling models of the protein. Scan size 12 nm × 16 nm

occur with retention of activity. Since the molecules imaged were stable to continual scanning and were, presumably, also anchored to the gold through this cysteine, it seems reasonable to propose that they are electroactive.

A comparison between high-resolution STM images and a space-filling computer graphical model (Brookhaven Protein Database) is presented in Fig. 8. If the models are orientated so as to direct the exposed surface cysteine residue towards an underlying substrate, the correlation, in terms of both size and shape, with the STM images is good (indicating that the tip radius of curvature was small enough to largely eliminate the effects of physical convolution).

Interestingly, imaging under fluid was considerably less disruptive (and subsequent images sharper) than that carried out under ambient conditions. In addition to this, the tunnelling current could be increased to more than 2 nA with no obvious detrimental effect on either adsorbate or image quality. The role of water in the STM imaging of protein molecules is now accepted.[7,40,41] Though it is unlikely that the azurin molecules under ambient atmosphere are 'dry', it is probable that they are less conductive.[12,41–43] If this is so, 'physical' interactions between the scanning tip and the adsorbed protein molecule will be greater in the former case.

Conclusions

In summary, the application of mutagenesis to the non-denaturing *in situ* immobilisation of protein for STM imaging is demonstrated. In view of the problems associated with the imaging of biomolecules by scanning probe methods, and the low number of molecular level *in situ* studies,[5,44,45] this represents a valuable advance.

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