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Real-time scanning tunnelling microscopy imaging of protein motion at electrode surfaces

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Abstract

A mutant (K27C) of the blue copper protein azurin [Eur. J. Biochem. 194 (1990) 109; J. Mol. Biol. 221 (1991) 765] for orientated immobilisation on gold surfaces was analysed by scanning tunnelling microscopy (STM) both in a resting state and following the application of a short potential pulse between the tip and sample.

Keywords: Protein immobilisation; STM; *Pseudomonas aeruginosa* azurin

1. Introduction

Proteins are desirable targets for immobilisation in biosensing devices due to their wide ligand specificities, which range from small molecules through to multiple protein assemblies. One important feature in ensuring the activity of an immobilised protein is that any ligand-binding or catalytically important motion of protein subdomains necessary for solution-based activity is retained. A means of investigating the motion of individual protein molecules on surfaces is provided by scanning tunnelling microscopy (STM). To this end, a mutant of the blue copper protein azurin [1,2] for orientated immobilisation on gold was analysed by STM both in a resting state and following the application of a short potential pulse between the tip and sample.

In order to achieve repeatable imaging of azurin by STM, it is necessary to immobilise the protein on a conductive solid substrate. A number of scanning probe microscopy studies have utilised the facile adsorption of azurin on hydrophobic self-assembled monolayers (SAMs) [3–5]. The immobilisation of azurin on STM substrates by Au–S chemistries has also been demonstrated. This was achieved by exploiting the interaction between a gold surface and a surface disulfide bridge (Cys3–Cys26) [3,4,6–8], or by the site-directed mutagenic introduction of a single surface cysteine residue. The oriented immobilisation of azurin on gold has been demonstrated by STM and cyclic voltammetry, using a genetically introduced surface cysteine residue, S118C, which specifically interacted with the gold surface [9].

In this paper, we demonstrate the STM imaging of the azurin mutant K27C, which unlike the mutant S118C rapidly dimerised in solution [10,11]. This dimerisation process was advantageous to our study as it was possible to induce the relative movement of the two protein components of the azurin 'pair' when immobilised. This is important to the study of multidomain proteins on surfaces, as relative movement of subunits is essential to their function in biosensors. For example, in the periplasmic maltose binding protein (MBP), a large conformation change is induced, such that two domains are brought into close contact upon binding of maltose [12]. The results presented here are thus preliminary to the study of multi-domain-protein conformational changes by STM.

2. Experimental

2.1. Protein expression and purification

The apo-form of K27C was prepared according to published methods [9,11]. Immediately prior to use, a 1-ml aliquot of K27C azurin was desalted and transferred into

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deionised water using a g25 desalting column (pd-10 column, AP Biotech). A 10 mM solution of $Cu(NO_3)_2$ was then added to a 1:1 copper/azurin ratio. Unbound copper was removed by a second passage through a desalting column. The concentration of azurin was determined using an extinction coefficient of ε_{280}=9.8 mM^{-1} cm^{-1}.

2.2. STM substrate preparation

STM substrates were Au (111) evaporated onto freshly cleaved mica and had been hydrogen flame-annealed by the supplier (Molecular Imaging). The STM tip used was composed of 80% Pt and 20% Ir. Gold substrates were extensively scanned prior to immobilisation to ensure that they were clear of surface features that could be mistaken for azurin. Substrates were then submerged in approximately 200 μl of azurin (6–60 μM) for between 20 min and 24 h, or a drop (~50 μl) of azurin solution was deposited on the gold. In both cases, samples were air-dried prior to imaging.

All STM imaging was performed on an ambient STM (WA Technology), in constant current mode. A Perspex box was constructed around the imaging head, within which the temperature and humidity could be controlled and monitored. Images are presented as raw data with global plane fitting.

2.3. Static secondary ion mass spectrometry (SSIMS) substrate preparation

A freshly evaporated gold substrate was immediately submerged in the same azurin solution as used for the STM and left overnight to allow protein adsorption. Prior to SSIMS analysis, each sample was removed from solution and rinsed with ultra pure water to remove unbound protein, followed by drying using an ultra pure argon gas jet.

3. Results and discussion

K27C azurin was immobilised on Au(111) STM substrates and imaged under different scanning conditions. Our aim was not to exclusively generate monolayers of K27C azurin though these were often seen, particularly at higher concentrations [5], but to be able to image well-resolved and isolated dimers and monomers of K27C azurin. It would then prove easier to try and address the motion of individual, isolated molecules. Fig. 1 shows typical images obtained when a drop of approximately 50 μl of 6 μM K27C azurin was deposited on an Au substrate and air-dried. Occasional dimers were observed, though not as many as might be expected from comparison with published studies of the bulk behaviour of azurin mutants with introduced surfaced cysteines [10,11]. This might be due to low copper incorporation in the sample of K27C used here, or because the rate of immobilisation on gold is sufficiently rapid that dimerisation is minimised. Indeed, on incubation at room temperature solutions of K27C were approximately 50% dimerised as determined by SDS-PAGE analysis (data not shown).

In order to confirm that the material immobilised on the gold was azurin, a concomitant study of the protein binding was conducted using the surface analytical technique, SSIMS, with similar freshly prepared gold substrates and the same protein stock solution. The mass spectra show the expected protein fragments [13], typically as ammonium ions. Additionally, the Cu centre was clearly observable, although that could result from the protein sample pretreatment. Results indicate coverage of approximately one monolayer, using this extended immobilisation time. This study will be described in more detail at a later stage.

The diameter of individual azurin molecules, whether isolated or in pairs was found to be 40±10 Å. The apparent height of individual molecules was 7±2 Å and was found to

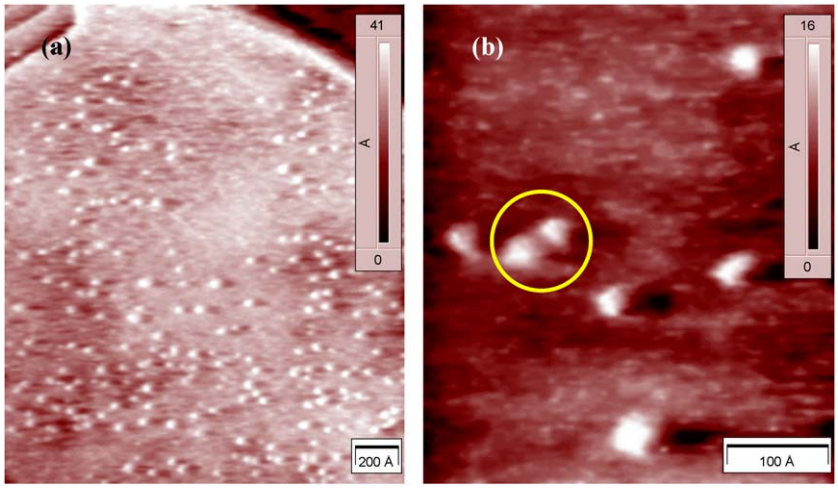

Fig. 1. STM images of K27C azurin showing individual isolated molecules and dimers (yellow circle) (colour available in the on-line version). Scanning conditions were bias voltage 0.1 V, current 0.1 nA, temperature 20±1 °C, relative humidity 35±5%.

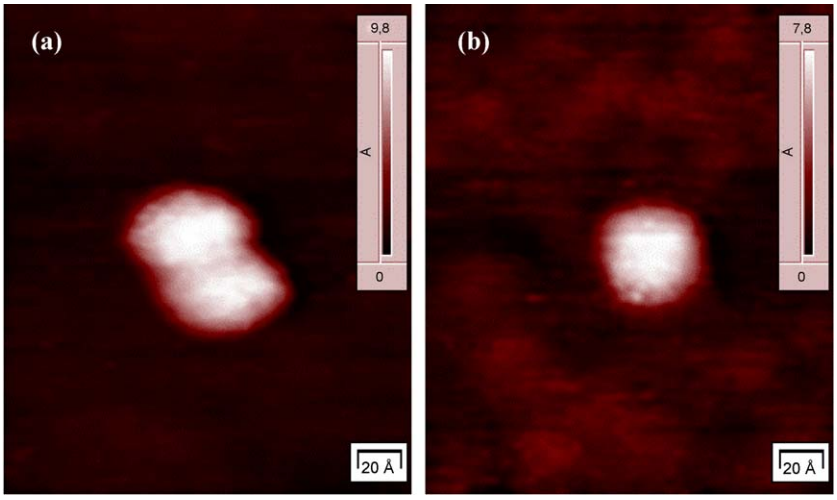

Fig. 2. STM images of tip-induced dissociation of K27C azurin dimer, (a) before and (b) after application of a voltage pulse. Scanning conditions were bias voltage 0.1 V, current 0.1 nA, temperature 20±1 °C, relative humidity 35±5%.

be dependent upon relative humidity. This dimension is significantly smaller than predicted from examination of the 3-D structure of azurin since the *z*-range measured by an STM is dependent upon the electronic density-of-states as well as topography [2,6]. As part of this study, wild-type and S118C azurin were also immobilised on gold substrates and imaged by STM. In both cases, monolayers of protein were readily observed similar to those already reported [5–9], particularly for gold substrates that were soaked in protein solutions. However, no dimers of protein were observed on gold when dilute solutions of wild-type and S118C were immobilised (data not shown). No movement of domains within the dimer pair was observed during STM imaging. Therefore, in order to cause physical (tip-induced) movement of the K27C dimer, it was necessary to apply a voltage pulse between the tip and sample.

The tip was positioned over the centre of the dimer, and an image acquired (Fig. 2a). A voltage pulse (3 V, 1 ms) was then applied to the tip, and an image of the azurin recorded immediately following this pulse. Image acquisition took approximately 60 s. As can be seen in Fig. 2b, the scan immediately following the voltage pulse revealed that the K27C pair had dissociated, and only one protein could be seen in the original location. From comparison with the background gold features, it was evident that thermal drift in the *xy*-plane was not significant during this time.

The nature of the interaction between sulphur-containing moieties and gold substrates has been intensively investigated by Chi et al. [3,6] and Friis et al. [7,8]. In wild-type azurin, an interaction between the Cys3–Cys26 disulfide bridge and the gold surface is strongly implicated [6], whereas in the mutant S118C the interaction between the protein and surface is dominated by the introduced free surface cysteine on the protein's surface [5,9]. The immobilisation interaction between the K27C dimer and the surface might therefore be from the Cys3–Cys26 disulfide (as in the wild-type), or through the disulfide between the surface-exposed introduced cysteines. The dissociation of the dimer pair with rapid movement of one azurin unit might imply a Au–S interaction between one azurin and the surface, and a voltage-induced oxidation of the Cys27–Cys27 disulfide, such that one partner of the dimer is then released and moves rapidly across the surface following application of the voltage pulse.

4. Conclusions

We have demonstrated that individual, isolated dimers of K27C azurin can be imaged by STM. Furthermore, dissociation of the dimer pair can be induced by the application of a voltage pulse between the tip and sample. This presents us with a technique for the engineering of protein distribution on biocompatible surfaces, with applications in nanobiotechnology devices, such as single molecule arrays, molecular transistors or biosensor elements. While a voltage-induced dissociation of domains within a naturally occurring multidomain protein would have severe effects on its activity, this might not be the case with the structurally simple and stable azurin monomers. Furthermore, dissociation of domains of a natural multidomain protein is much less probable than with the K27C dimer. In this study, azurin was not selected for its utility in a sensing device or array, rather for its known thermal and chemical stability. Therefore, the next stages will be the investigation of domain movement in immobilised multidomain proteins with applications in biosensing devices, such as periplasmic binding proteins, and the ligand, potential and current dependence [14] of such movements.

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