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Genetic modulation of metalloprotein electron transfer at bare gold

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Engineered metalloproteins and enzymes can be self assembled on pristine gold electrodes in robust, electrochemically-addressable, arrays.

The mechanisms associated with metalloprotein electron transfer, central to fundamental biological processes, have been of considerable interest and much research during the past two decades.[1] Amongst this work, several studies have demonstrated cases where interprotein electron transfer (where protein partners communicate through evolved surface interaction sites) is gated, that is, preceeded by a chemical or physical process.[2] Heterogeneous electron transfer of metalloproteins at man-made electrode surfaces has been achieved through careful surface chemistry considerations.[3] The force balance imposed on a metalloprotein confined to an electrode surface differs from that in solution and this commonly leads to a loss of native conformation unless specific steps are taken to create a 'biocompatible interface'.[4,5] Though metalloproteins have been observed to exchange electrons with bare metal surfaces, these responses are usually transient and poorly reproducible.[6,7]

In recent years, we, and others, have developed methods whereby metalloproteins can be assembled on electrode surfaces in well-defined, electrochemically-addressable, molecular arrays.[8] We have specifically made use of the introduction of thiol-terminating cysteine residues into the surface of metalloproteins as a means of tethering them controllably to gold electrode surfaces.

Azurin is a well-characterised blue copper protein involved in bacterial electron transfer chains, such as those in *Pseudomonas aeruginosa*.[9] Though the wild-type form of azurin can be immobilized on pristine gold electrode surfaces through its exposed disulfide moiety, previous studies have reported that such arrays are electrochemically-inactive.[8,10] We have sought to control protein-electrode mechanical and electronic coupling through the use of site-selective mutagenesis. The designed introduction of gold-anchoring cysteine residues into the surface of the protein (Fig. 1) is achieved without significant structural/functional perturbation of the wild-type structure. In this paper we report results obtained with two such mutants and the wild-type protein.

Previous work has demonstrated that well-defined molecular adlayers can be assembled on pristine gold electrode surfaces by incubation of the freshly-prepared electrode in dilute solutions of the protein.[11] The topological structure of these protein adlayers can be obtained, at high resolution, by fluid-phase atomic force microscopy (AFM) or scanning tunnelling microscopy (STM) (Fig. 2).[5]

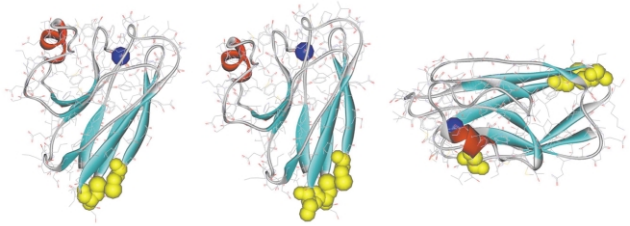

Fig. 1 Schematic representation of the wild-type protein (left), the K27C mutant (centre) and the S118C mutant (right) depicted in their respective surface-bound orientations. The copper centres are highlighted in blue and the cysteine/disulfide moieties yellow. The direct tunnelling distances (gold to copper) and molecular heights are 3.4–3.6 nm and 2.5–2.6 nm for both the wild-type and K27C proteins and 2.4–2.9 nm and 0.8–1.1 nm for the S118C protein.

The exquisite vertical resolution attainable by AFM provides a reliable means of determining molecular height provided suitable precautions are taken to minimize the influence of solution and probe contributions. Interaction forces between an AFM probe and a surface-confined biomolecule under fluid can be approximated by Deryagin–Landau and Verwey–Overbeek (DVLO) theory, in which electrostatic and van der Waals contributions are additive.[12] In this study, electrostatic forces were minimized by acquiring data under an ionic strength sufficient to screen surface charges. Compressional contributions (estimated to be 130–230 nN based on a damped harmonic oscillator model) were minimized by monitoring the effects of set point (oscillation damping) on measured molecular height and reducing this to the minimal value required for sharp surface tracking. By fitting height data to a guassian function, the peak maximum was found to vary by $< 6\%$ from experiment to experiment (quantitative height data were taken only from sharply resolved images obtained with good-quality probes). Height determinations were in good general agreement with the molecular orientations as depicted in Fig. 1. Specifically, there was no significant difference detectable between the wild-type and K27C protein forms. Height distributions obtained for the S118C mutant, however, were significantly (some 5–8 Å) lower. In view of the expected closer proximity of the S118C copper centre to the underlying gold electrode surface, one would expect the associated heterogeneous electron transfer kinetics to be significantly greater than observed with either the wild-type or the K27C forms.

With all three proteins, well-ordered molecular arrays can be formed, all of which exhibit facile electron transfer to the underlying bare gold electrode surface. The protein coverage, determined by integration of the voltammetric data, was 1.8–2.2 $\times$ 10^{13} molecules cm^{-2}. This is in good agreement with expectations based on a close packing of $\sim$3 nm diameter proteins (molecular footprint $\sim 7 \times 10^{-14}$ cm^2) and consistent with high retention of electroactivity on immobilization.

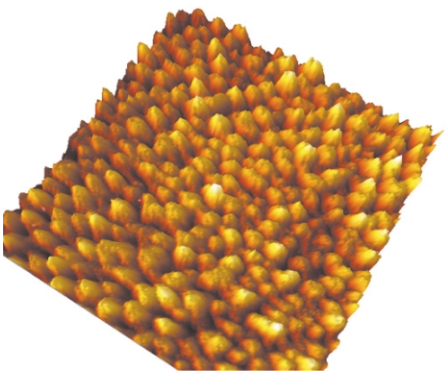

Fig. 2 Fluid Tapping Mode AFM (TMAFM) image (80 $\times$ 80 nm) of a typical electrochemically-addressable azurin K27C adlayer on Au[111]. Z-scale 0–32 Å. High-resolution fluid imaging is only possible if the biomolecules are robustly anchored to the solid support.

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Table 1 Electrochemical characteristics of the three azurin monolayers. Half wave potential is defined as $(E_{pa} - E_{pc})/2$ *vs.* SCE[16]

	Azurin WT	Azurin K27C	Azurin S118C
$E_{1/2}$*(mV *vs.* SCE)	165–175	165–175	120–145
E_{pa}/E_{pc}	1.2	1.2–1.4	1.1–1.2
Response stability[a]	Good	Good	Good
k_{et}/s^{-1}	300–400	210–320	300–570

[a] Defined as less than 5% decay in Faradaic response over 200 potential cycles.

Heterogeneous electron transfer rate constants were calculated by monitoring the variation in cyclic voltammetry peak separation with scan rate according to the method of Laviron and are summarized in Table 1.[13]† Though faster rates of electron transfer have been observed with azurin immobilized on pyrolytic graphite surfaces, these figures are indicative of facile communication between the copper centres and the underlying bare gold electrode surface.[14] Significantly, however, the rate of electron transfer observed with the S118C protein is not greatly different to that observed with the protein immobilized in a 'perpendicular' orientation, despite a greatly reduced copper-electrode separation. To further examine this apparent independence of redox kinetics on copper-gold tunnelling distance, the mutant proteins were immobilized on alkanedithiol spacer monolayers (one which one expects protein bound orientation to be the same as that on bare gold).[15] By varying the number of methylenes a distance decay factor of ~ 0.2 Å^{-1} was calculated, consistent, again, with minimal distance dependence. On increasing this spatial separation further by adsorbing the protein onto a C_{12} chain alkenthiol linker the rate of electron transfer was observed to significantly decrease to ~ 10 s^{-1}.

We believe that the cathodically-shifted half-wave potential observed with the S118C mutant protein is related to the relatively limited solvent exposure of this residue and the mild structural deformation that is known to accompany its covalent binding.[17] Significantly, this perturbation is not evident in data acquired from the other protein forms. Voltammetric peak widths (FWHM) were 60–65 mV, 75–80 mV and 85–110 mV for the S118C, K27C and wild-type metalloprotein forms respectively (Fig. 3). Since this parameter is diagnostic of electron transfer kinetics and/or homogeneity at the electrode surface,[18] and the kinetic differences are minimal (Table 1), these values are consistent with increasing degrees of heterogeneity in the protein monolayers. Predictably, this is greatest with the wild-type protein in which an appreciable amount of 'randomly' physisorbed molecular species may be expected.

The protocols outlined in this manuscript have been additionally applied to analyses with the blue copper protein plastocyanin in which robust electron transfer responses can be obtained in self-assembled protein adlayers (voltammetry with the wild-type protein is, in contrast, poorly resolved).[19] These results, coupled to those previously reported with enzyme systems, demonstrate that interactions at the bioelectrochemical interface can be controlled at the gene level.

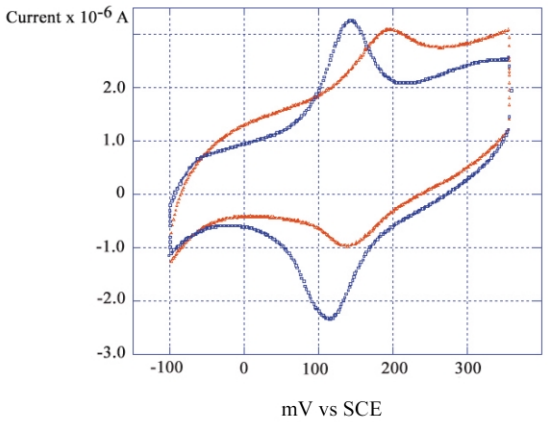

Fig. 3 Voltammetric responses characteristic of the S118C (blue) and K27C (red) molecular adlayer on polycrystalline gold (100 mVs^{-1}).

In summary, control of electronic coupling between metalloproteins and gold electrodes has been achieved through the introduction of anchoring residues into the biomolecule surface by site-directed mutagenesis. With the blue copper protein, azurin, well-ordered molecular arrays, exhibiting facile electron transfer on bare gold surfaces, can be prepared. At such proximity to the electrode surface, the electron transfer kinetics appear to be independent of copper–gold tunnelling distance, an observation consistent with the presence of a gating process.[2] By anchoring high-purity protein to carefully-prepared gold surfaces, rate constants comparable to those observed at modified electrodes can be obtained.[20]

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Notes and references

† Previously reported electrochemical analyses of wild-type azurin on gold have reported the absence of a Faradaic current detectable by CV and impedance-determined rate constants more than one order of magnitude lower than those we report here.[8]

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16 Electrochemical experiments were made with an Autolab system (Ecochemie, Netherlands) using a one or two-compartment, three-electrode setup. Electrodes were thoroughly cleaned by mechanical polishing with alumina followed by electropolishing in 1 M sulfuric acid All potentials are reported with respect to SCE.
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