Keysight Technologies
Scanning Probe Studies of a Metalloprotein

Application Note

KEYSIGHT TECHNOLOGIES

Introduction

Redox reactions are of central importance in sustaining all life and are mediated in nature by highly specific “interaction surfaces.” We are interested in the analysis and control of biological redox proteins/enzymes with electrode surfaces. Azurins are well-characterized blue copper proteins that function as “electron shuttles” in certain bacteria. Of the many enzymes known to catalyze redox reactions involving molecular oxygen, the cytochrome P450 monooxygenases are presently attracting much attention. These b-haem containing oxidases, which are widely distributed in nature, are able to catalyze the controlled activation of dioxygen and play a role in the biosynthesis of steroids, drug metabolism, the bioactivation of polycyclic aromatic hydrocarbons (PAHs) to carcinogens, and detoxification. Presently, much interest in P450 enzymes is associated with attempts to attain reproducible voltammetric responses (1). If these responses can be achieved with the enzyme immobilized in an active form, then one can postulate the generation of derived biosensing systems targeting specific compounds.

Since water constitutes an integral part of protein tertiary structure, the ability of scanning probe microscopy (SPM) methods to carry out molecular-level imaging of biological structure under quasi-physiological conditions is important. As with any form of microscopy, however, there are significant issues associated with attempts at high-resolution imaging of biological material under fluid using SPM. In addition to the mobility of material under solution, tip-induced movement of the molecules of interest remains an issue that must be solved. Though immobilization can be achieved through either chemical modification of the underlying substrate (2) or the protein itself (3), these methods are generally non-specific and the orientation of the immobilized biomolecule remains largely random. We have developed a strategy whereby proteins and enzymes are anchored to gold electrodes via use of site-specifically introduced surface cysteine residues (4). The application of genetic engineering methods allows the introduction of single immobilization sites onto the surface of a protein. The biomolecule can then be immobilized on electrode surfaces in a reproducible manner, a controlled orientation, and (in many cases) an active form.

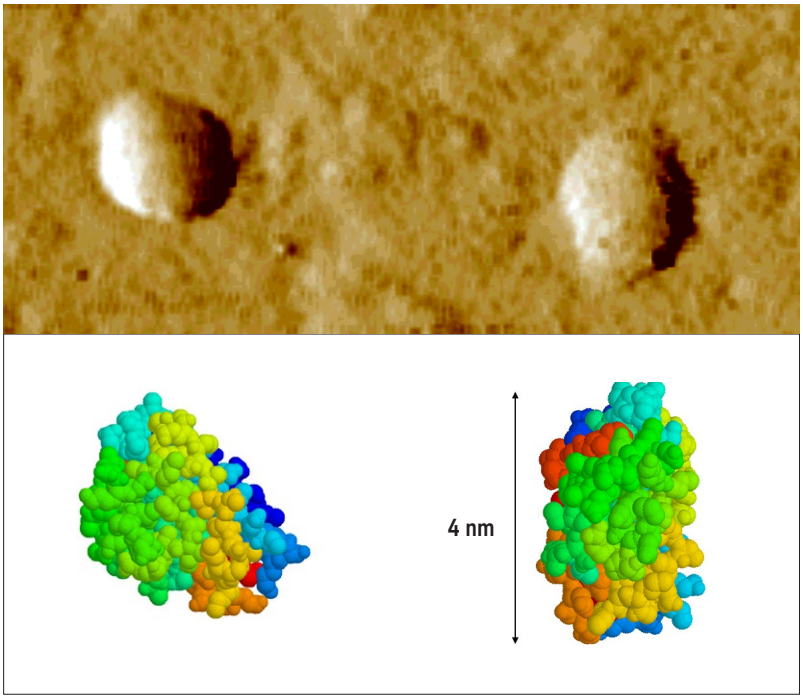

Figure 1. Comparison of a constant-height STM image of two azurin S118C molecules with orientated, space-filling models of the protein. Scan size: 12 x 16 nm.

This methodology has enabled high-resolution *in situ* scanning tunneling microscopy (STM) studies to be carried out on isolated azurin molecules (see Figure 1).

A surface cysteine residue, located in a region where the haem moiety is closest to the surface (see Figure 2), was site-specifically engineered and used to anchor cytochrome P450cam enzyme molecules covalently to a gold electrode

The structural and functional properties of the densely-packed protein/enzyme adlayers were subsequently probed electrochemically (cyclic and pulse voltammetry and by atomic force microscopy (AFM) and STM at nanometer resolution. More ordered adsorption occurs at high coverage and reproducibly with this K344C mutant than with the wild-type enzyme. In the absence of a “controlling” surface cysteine residue, one would

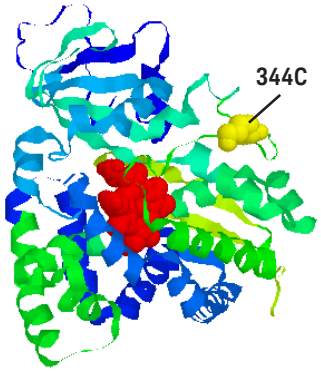

Figure 2. Computer graphical representation of the wild-type cytochrome P450cam with the K344C residue highlighted.

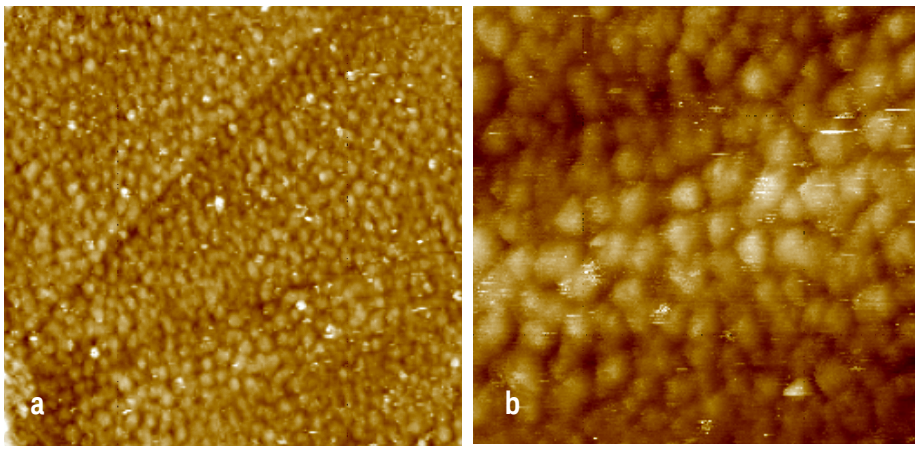

Figure 3. Ambient scanning tunneling images of an immobilized cytochrome P450cam adlayer on a single crystalline gold electrode surface. An almost complete coverage is evident. Bias voltage: 1.1 V. Tunnel current: 190 pA. Scan rate: 6.8 Hz. Scan size: (a) 210 x 210 nm, (b) 90 x 90 nm.

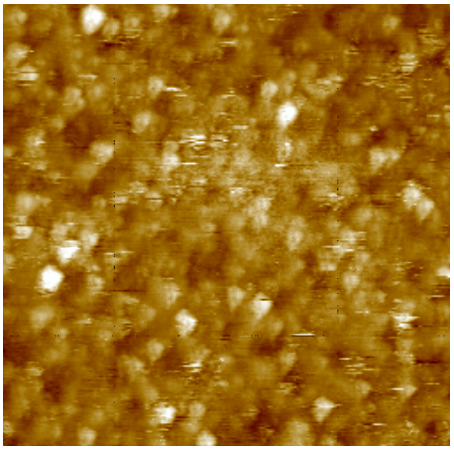

Figure 4. Fluid phase tunneling image of the immobilized cytochrome molecules. Scan size: 120 x 120 nm. Bias voltage: 1.5 V. Tunnel current: 200 pA. Scan rate: 5 Hz.

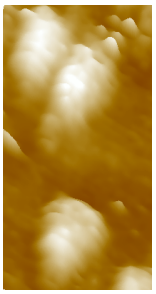

Figure 5. Individual molecules of P450cam K344C imaged under potassium phosphate buffer. Scan size: 10 x 20 nm. Bias voltage: 1.2 V. Tunnel current: 215 pA. Scan rate: 9.4 Hz

expect that the shapes and dimensions of the enzyme would be considerably more variable than those observed with the surface cysteine mutant, and this is indeed what is observed. The subsequently formed, close-packed monolayer arrays were probed by STM at high resolution under ambient conditions (see Figure 3) and under aqueous (buffered) solution (see Figures 4 and 5).

Though one does not necessarily expect the molecular dimensions of a surface-confined hydrated enzyme to mirror those derived from diffraction methods (especially under the pressures typically generated in the tunnel gap), fluid images were, in general, within 20% of crystallographic dimensions (4–5 nm x 5–6 nm). Under typical ambient conditions, much, if not all, of the tunnel current is carried by a thin film of water present at the surface of the biomolecule. Since current does not flow through the protein (only over its surface), only the gross (topOgraphic) structure is resolved. In view of the fact that recent fluid phase tunneling studies have been unable to resolve protein internal structure (5), it seems likely that a significant amount of current is carried by electrons tunneling through the molecule when it is immersed in solution.

Electrochemical studies indicate that electronic coupling of the mutant enzyme (the haem of which is somewhat buried within the protein and thus difficult to address) to the underlying gold electrode surface is enhanced in comparison to the native structure (for which there is no reproducible coupling). It is highly likely that this coupling is related to the position of the anchoring residue because the introduced cysteine lies on the proximal side of the enzyme where the haem is closest to the surface and the cysteine residues of the native enzyme do not lie in this region. Electron transfer to this adlayer is also sensitive to the addition of a suitable substrate, indicating that the enzyme is catalytically active as well as electrochemically addressable. Studies are underway to monitor *in situ* interactions between immobilized P450cam, with the distal surface anchored to a gold electrode, and its natural electron transport partner, putidaredoxin. The possible role of the haem moiety in mediating enhanced tunneling through the enzyme matrix is also being investigated.

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