Probing Electron Transport in Proteins at Room Temperature with Single-Molecule Precision

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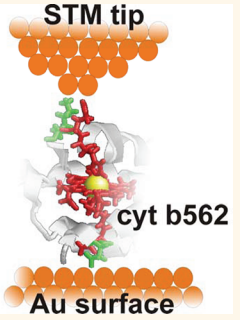
ABSTRACT Studying electron transport through immobilized proteins at the single-molecule level has been of interest for more than two decades, with a view on the fundamentals of charge transport in condensed media and applications in bioelectronics. Scanning tunneling microscopy (STM) is a powerful tool in this context, because, at least in principle, it should be possible to address individual proteins on an electrode surface reproducibly with single-protein precision. As reported in this issue of *ACS Nano*, MacDonald and colleagues have now achieved this for the first time at room temperature for covalently immobilized cytochrome b562, combining imaging and tunneling spectroscopy in a custom-built, ultralow drift STM, with single-protein precision. Using site-directed mutagenesis, cysteines introduced in specific locations in the amino acid sequence of the protein allowed the team to investigate conduction along different directions through the protein, namely along its short and long axes.



Imaging of single biomolecules such as DNA or proteins has attracted significant attention since the early days of scanning tunneling microscopy (STM), first *ex situ* (*i.e.*, in air or vacuum) and then *in situ*, namely, in solution environments and under electrochemical potential control.[1−3] Solution environments are more suitable for proteins and especially enzymes, since their stability and structure—and hence their function—are affected by the immediate electrostatic environment. While early studies on proteins mainly focused on exploring the capabilities of the then new technique of STM, and on comparing real-space STM images with X-ray crystallographic data, attention soon shifted to fundamental aspects of the charge-transport process itself,[4,5] the potential use of biomolecules in molecular electronics,[6] and the study of single-molecule catalysis.[7,8] Electrochemical environments offer particular features in this context: since the potential at the redox site of a protein or enzyme is determined by strong interfacial fields, bipotentiostatic control of the respective electrodes (*e.g.*, tip and substrate in a STM experiment) enables controlled and reversible switching between different redox states ("redox gating").[9−11] A protein or enzyme may thus be switched on or off, depending on the applied potential; analogies to electronic transistors were recognized early.[4,5] Ulstrup, Stimming, and their co-workers were the first to probe the catalytic activity of functional redox enzymes by STM in solution, even though effects were apparently not very strong. The dimensions of multiredox center enzymes, about 5 nm in height from the substrate surface, seem to be at the limit of what can be imaged by STM due to the strong distance dependence of the tunneling current.[12]

A Step Change in Probing Individual Biomolecules. While STM imaging of small proteins on electrode surfaces is now more or less routine, room-temperature operation *ex situ* or *in situ* does have its limitations. For example, thermal or mechanical drift is typically much larger in these systems than it is at low temperatures in vacuum environments, where manipulation of surface atoms or specific probing of particular chemical bonds was demonstrated years ago.[13,14] Thus, in order to probe the electronic conductance of individual (redox) proteins, experimentalists either had to resort to imaging (in constant-current mode), where STM contrast needs to be converted into conductance changes *a posteriori*, or by employing tunneling spectroscopy on random samples of protein on the surface. The spatial precision was typically insufficient to probe a preselected protein on the surface reproducibly. As described in this issue of *ACS Nano*,[15] MacDonald and colleagues have now overcome this significant shortcoming by developing a custom-built, ultralow-drift STM that can operate at room temperature, in air and in solution, at drift rates routinely in the low pm/s range.[11]

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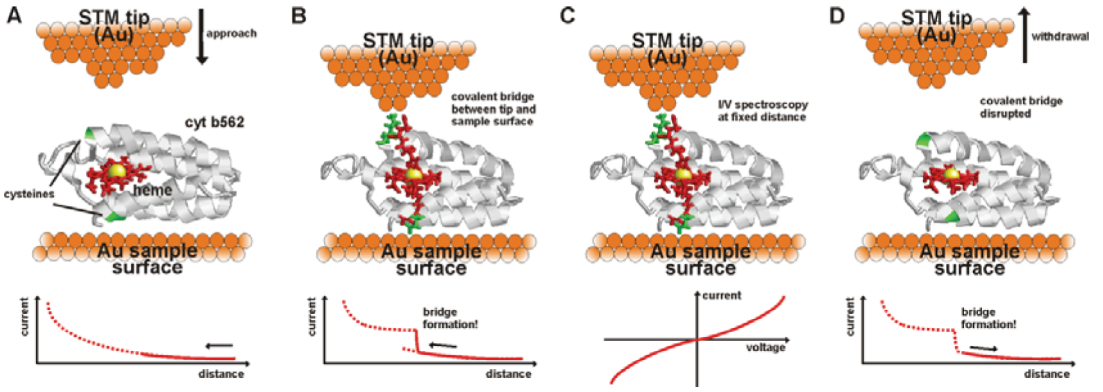

Figure 1. Illustration of the experiments performed by MacDonald and colleagues. A particular protein on the surface is identified by STM imaging. Protein engineering was used to introduce cysteines in specific locations (here Asp5 and Lys104). The thiol groups of the cysteine rapidly form strong bonds to the respective Au surfaces; this thiol group initially leads to protein immobilization on the surface. (A) The protein is approached with the STM tip. The tunneling current increases exponentially with decreasing distance to the surface. (B) Once the STM tip is close to the cysteine, a molecular bridge forms between the tip and surface; the tunneling current increases in a step-wise manner. The electron transport pathway could include amino acids cys5-asn6-met7-heme-his102-gln103-cys104. (C) At fixed tip position, *I*/*V* tunneling spectroscopy is performed to characterize the charge-transport properties of the protein. (D) Upon withdrawal of the tip, the molecular bridge is disrupted and the tunneling current drops again. The bonding between protein and sample surface could, in principle, also break. MacDonald and colleagues therefore confirmed the state of the protein after the spectroscopy experiments by subsequent STM imaging. [crystal structure of cyt b562 for illustration; pdb code, 1QPU].

This allows them not only to image cytochrome b562, a small electron-transfer protein found in *E. coli*, but also to identify and then to probe individual proteins on the surface reproducibly. The team used site-directed mutagenesis to replace two amino acids in the sequence by cysteines, whose thiol functionality allows covalent binding to the gold substrate surface and the STM tip (also Au; see Figure 1). Interestingly, the team compared two different protein variants: in one design, they replaced amino acids in positions 5 and 104 (Asp and Lys, respectively), placing the thiol linkers on opposite sides along the short axis of the protein ("SH-SA"). In the second design, they replaced aspartic acids (Asp) in positions 21 and 50, leading to a structure with thiols on opposing sides along the long axis of the protein ("SH-LA"). For steric reasons, only one of those linker groups can attach to the gold substrate *via* a strong gold–sulfur bond, leaving the second one dangling in the air (or solution). When the gold STM tip is brought close to the protein, a second gold–sulfur bond is formed, establishing a well-defined molecular bridge between the STM tip and the substrate (see Figure 1B).

The electronic coupling between the two surfaces *via* the protein bridge leads to a marked increase in the tunneling current. Its magnitude depends on the molecular details of the bridge, its coupling to the electrode surfaces, and the applied bias voltage. The tunneling conductance of covalently bound cyt b562 was found to be an order of magnitude larger than for the physisorbed, wild-type protein. To characterize charge transport in the junction further, MacDonald and colleagues also performed current–voltage spectroscopy on the same protein (see Figure 1C), providing insight into the nature of the charge carriers (electrons *vs* holes), the HOMO/LUMO gap, the potentially rectifying behavior of the molecular junction, and so forth. Interestingly, they found two conductance states for SH-LA, which they argue is related to the different distances between the heme and the tip (3.1 and 2.1 nm), depending on the orientation of the protein in the junction. In SH-SA, the heme group is approximately in the center of the electrode gap and only one conductance state was observed. Withdrawal of the tip eventually leads to bond rupture and the tunneling current returns to its initial value (see Figure 1D). However, it is not clear *a priori* which gold–sulfur bond is going to break first—the one to the substrate or the one to the tip. One could argue that noncovalent interactions between the protein and the (flat) substrate surface are stronger than those between the protein and the tip. This would imply that the protein remains on the surface and the tip remains contamination-free for tunneling experiments on the next protein. This is obviously desirable from an experimental point of view, but, in general, one would expect a statistical distribution between cleavage at the surface and cleavage at the tip. Hence, another very valuable achievement of the work by MacDonald and colleagues is that they could image the same part of the surface even after the tunneling experiment. In this way, they could check (a) whether the protein was still attached to the surface and (b) whether it had changed shape—an indication of structural alterations in the protein. The latter may not be totally unexpected, given the force exerted by the tip and the strong local electric field.[16]

The benefits of the work by the Cardiff team are thus multifarious: extremely low drift and high precision enable tunneling spectroscopy on particular proteins in different local environments, such as close to step edges or to other proteins on the surface; and, in combination with

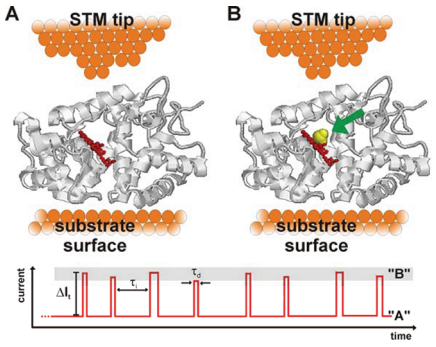

Figure 2. Illustration of catalytically modulated tunneling. An enzyme, here horseradish peroxidase (pdb code 1W4W), between the tip and substrate surface switches between an open and a bound state "A" and "B," respectively. After catalytic turnover (not shown), the enzyme returns to state "A." The two states have different tunneling conductances, hence the current displays telegraphic noise patterns. These may be analyzed to give statistical distributions of tunneling current modulation ΔI_t, the distribution of event duration τ_d, and interevent time τ_i.

protein engineering, charge transport may be probed in different directions through the protein and under conditions where its structure is well-defined. The exact nature of the electron transfer pathway is, however, less clear; the one suggested in Figure 1 is only one possibility. In reality, hydrogen-bonding networks can be quite complex and multiple pathways may have to be taken into account to gain a quantitative understanding of the charge–transport process. On the other hand, studies on entire proteins, rather than simple model systems, provide better insight into the role of the nuclear environment. Does a static, mean-field representation suffice? Or does one need to take into account nuclear fluctuations, which may occur on different time scales ranging from picoseconds to microseconds and slower?

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To this end, the work by MacDonald and colleagues may enable a new type of tunneling spectroscopy, namely tunneling current noise spectroscopy for biomolecular systems. Structural fluctuations in the protein and even catalytic activity in enzymes may induce modulations in the tunneling current, *e.g.,* as a result of alterations in the local potential distribution or changes in the electronic structure due to molecular binding events. Provided the time resolution of the current measurement is high enough, discrete events may be resolved and related to the functional properties of the biomolecule under study. Taking enzymes (or more generally catalysts) as an example, previous studies have exclusively focused on STM imaging in the hope of detecting catalytic activity in the image contrast.[7,8,17] Molecular binding events to the catalytic center may, however, be relatively rare if turnover is efficient and mass transport is slow. This means that, most of the time, the catalyst is in its open state and the time-averaged tunneling current modulation is much smaller than the individual modulation event. On the other hand, resolving the individual binding events at high temporal resolution offers significant advantages, as illustrated in Figure 2: not only can the full current modulation ΔI_t be detected, but so can its duration and interevent time distribution (τ_d and τ_i). These could provide information about the catalytic process itself, as well as the mass transport characteristics in the junction.

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However, such experiments can only be performed if mechanical and thermal drift of the STM relative to the surface is minimal. Otherwise the observed tunneling current modulations will be affected by changes in the tunneling distance—an effect that is difficult to deconvolute. Again, the work by MacDonald and colleagues is truly enabling, in that it demonstrates the precision needed for these new types of experiments.

In summary, the work by the Cardiff group clearly sets new standards in STM imaging and tunneling spectroscopy of biomolecules, and it will be interesting to see how this area evolves in the near future. Apart from the instrumental advances, the strategic replacement of amino acids by site-directed mutagenesis paves the way for charge-transport studies in realistic, well-defined protein model systems that are complementary to optical and electrochemical charge-transfer studies in solution and on surfaces.

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