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Nanoscale Electron Transport Measurements of Immobilized Cytochrome P450 Proteins

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Abstract

Gold nanopillars, functionalized with an organic self-assembled monolayer, can be used to measure the electrical conductance properties of immobilized proteins without aggregation. Measurements of the conductance of nanopillars with cytochrome P450 2C9 (CYP2C9) proteins using conducting probe atomic force microscopy demonstrate that a correlation exists between the energy barrier height between hopping sites and CYP2C9 metabolic activity. Measurements performed as a function of tip force indicate that, when subjected to a large force, the protein is more stable in the presence of a substrate. This agrees with the hypothesis that substrate entry into the active site helps to stabilize the enzyme. The relative distance between hopping sites also increases with increasing force, possibly because protein functional groups responsible for electron transport depend on the structure of the protein. The inhibitor sulfaphenazole, in addition to the previously studied aniline, increased the barrier height for electron transfer and thereby makes CYP2C9 reduction more difficult and inhibits metabolism. This suggests that P450 Type II binders may decrease the ease of electron transport processes in the enzyme, in addition to occupying the active site.

Keywords

Cytochrome P450; Electron Transfer; Nanopillars; Biomolecular Electronics; Conductivity; Conducting Probe Atomic Force Microscopy

1. Introduction

The electron transfer (ET) properties of heme-thiolate monooxygenases have been extensively studied and, as a result, the ET process for a subset of these proteins has been

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well characterized.¹ One example of such a protein is cytochrome c, which has become a model for studying biological ET.² There have been comparatively fewer studies of electron transfer on monooxygenases involved in metabolic processes. The cytochrome P450s (P450s) are monooxygenases and part of a large family of heme containing proteins found primarily in the liver where they are bound to the smooth endoplasmic reticulum.³ The main function of P450s is to metabolize substrates into more water-soluble and readily excretable forms, primarily via oxidation (e.g., insertion of an oxygen atom, typically into a C-H bond, to form a hydroxylated product or demethylation). The transfer of an electron from a donor (cytochrome P450 reductase, CPR) to the P450 heme group is the controlling step for the initiation of the catalytic cycle.⁴ A better understanding of ET in P450s would allow exploitation of their unique properties for use in new biomedical applications, for example, in bioreactors for bio-transformations/metabolic engineering,^{5–7} biosensors for personal drug monitoring,^{8–10} or bioremediation.¹¹

Several factors have been suggested to affect ET, such as substrate binding and protein aggregation. Substrate binding has been observed to alter the spin state of the heme iron in P450s.¹² Spin state has been measured using absorption spectroscopy and some degree of correlation has been observed between changes in spin state and metabolic activity.^{13,14} Type II binders to P450 enzymes, are often inhibitors and not metabolized and have no effect on spin state, or stabilize the enzyme in the low spin state.¹² Moreover, there are many substrates, such as dapsone, that alter the spin state very little, but are nevertheless metabolized. The reduction of heme can also occur in the absence of substrate in some P450s, and equal reduction rates between low and high spin heme species have been measured.¹⁵ This suggests that other factors may be modulating ET other than spin-state changes caused by substrate binding.

P450s are known to aggregate, forming homo- and hetero-oligomers in solution, and a range of effects from these interactions can occur, such as alteration in catalytic rate or degree of binding to the coenzyme CPR.^{16–21} Attempts to prevent this aggregation using detergents, salts, and lipids have been shown to affect ET.^{22–24} Due to these difficulties, it has not been possible to determine, how oligomer formation alters metabolism, and it is very possible that aggregation affects ET. A related factor is whether CPR binding to P450s, an obligatory step in the P450 catalytic cycle, alters ET. For example, it is possible that the binding of CPR to a P450 may induce a conformational change that facilitates ET.^{25–28} It is also possible that substrate binding causes conformational changes that affect ET from CPR to P450,^{13,29} and by combined processes may also modulate ET.

The literature suggests that the binding of substrates may be key for facilitating ET in P450s,^{30–32} and a correlation between the rate of substrate metabolism and the ease of ET has been proposed.^{33–36} One approach used to explore this issue has been to immobilize P450 to an electrode in an attempt to achieve direct electron supply from the redox electrode to the redox active group of the P450.³⁷ A variety of techniques have been used to achieve immobilization, including the direct wiring of a flavin cofactor to the heme,³⁸ confining P450s inside thin films on electrodes,³⁹ and tethering of modified P450s.⁴⁰ These attachment procedures have permitted the use of electrochemical methods, including cyclic voltammetry, square wave voltammetry, and amperometry, thus providing a way to measure

critical parameters relevant to the redox processes in the proteins (e.g., the redox potential E°) under a variety of conditions.⁴¹ However, these electrochemical measurements tend to alter protein conformation and function,³² cause interactions of the heme with the electrode,⁴² and there is ambiguity of the aggregation state of the enzyme as a consequence of immobilization. Consequently, the results have not established a clear correlation between metabolism or metabolic rate and ET.³³

Recently, immobilization of unmodified P450s on electrodes using various self-assembled monolayers (SAM) has allowed the use of electrochemistry approaches to study P450s that retain their metabolic activity.^{7,9,10} Nanoscale approaches have also been used to probe single enzyme electron transport (ETp), including scanning probe microscopy,⁴³ mechanical break junctions,⁴⁴ and conductive probe atomic force microscopy (CPAFM).⁴⁵ We recently developed a methodology to measure the properties of non-aggregated P450s using CPAFM that allows the study of the same individual P450 enzyme in response to different bound substrates.⁴⁶ In this approach, electron beam lithography is used to pattern four 21×21 gold nanopillar arrays on doped silicon. The lateral dimensions of the nanopillars are in the range of 20 to 40 nm, allowing isolation of the P450 of interest through bonding to a SAM bonded to the gold nanopillar. Here, the SAM was comprised of ω -thioundecanoic acid which selectively bonds to gold and not silicon when dissolved in ethanol,⁴⁷ ensuring attachment of the P450 to gold pillars and not the surrounding silicon. Other laboratories have made similar arrays using analogous methods.⁴⁸ Using this indexed array of gold nanopillars unmodified cytochrome P450 2C9 (CYP2C9) was selectively immobilized, the electrical conductance measured with CPAFM on the same enzyme molecule under different conditions, and a correlation between ET, ETp, and enzyme metabolic activity was found.⁴⁶

Here we describe important advances in our CPAFM measurement technique. By measuring changes in pillar height prior to and after attachment, we confirm that the nanopillars have single layer P450 coverage, and not an aggregated enzyme. In addition, we demonstrate how the AFM tip force modulates the conductance of the proteins on the gold nanopillars. Finally, we demonstrate that the CYP2C9 inhibitor sulfaphenazole reduces ET, in agreement with our previous results with another inhibitor (aniline).⁴⁶

2. Materials and Methods

All chemicals were purchased from Sigma Aldrich (Milwaukee, WI) and were of analytical reagent grade and used without further purification unless otherwise noted. Acetone, isopropanol, methyl isobutyl ketone, mercaptoundecanoic acid (MUA), 8-octanethiol (OT), N-((3-dimethylamino)propyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), flurbiprofen, dapsone, and potassium phosphate-buffer (Kpi, 40 mM, pH 7.4, prepared from potassium mono- and dibasic phosphate). Ethanol (100%) was purchased from Pharmco-Aaper (Brookfield, CT). The 300 MIF developers, 495K poly(methyl methacrylate) (PMMA) and 950K PMMA, were obtained from Micro Chem (Newton, MA). Boron-doped [100] silicon wafers were obtained from University Wafer (South Boston, MA). AZ 5214 photoresist was obtained from AZ Electronic Materials (Capitol Scientific, Austin, TX). Cytochrome P450 2C9 enzyme (CYP2C9) was prepared by expression in an *E. coli* system, isolated, and purified as described previously.⁴⁹

Deionized water was obtained from a Nanopure Ultrapure (Thermo Scientific, Waltham, MA) water system and had a resistivity of 18.2 M Ω -cm.

Silicon wafers were cleaned by immersion in Buffered Oxide Etch (BOE) 1:10 hydrofluoric acid (49%):deionized water solution for 3 min and rinsed in a cascading water bath for 10 min. The wafers were then blown dry with nitrogen, and immediately spin coated with PMMA to prevent silicon oxidation. The wafers were placed in a Laurell WS-400B-6NPP/LITE spin-coater (North Wales, PA), and spincoated with 495K PMMA (4% in anisole) (5000 rpm, 30 s), placed on a hot plate (180 °C, 2 min) to evaporate solvent, and cooled to room temperature. Samples were then spin-coated with 950K PMMA (4% in anisole) (5000 rpm, 30 s), placed on a hot plate (180 °C, 2 min) to evaporate solvent, and cooled to room temperature.

Electron beam lithography was then performed to pattern the PMMA using a JEOL JSM-7600F field emission analytical scanning electron microscope (Tokyo, Japan) equipped with Nanometer Pattern Generating System software from JC Naby Lithography Systems (Bozeman, MT). The working conditions were chamber pressure 9.6×10^{-5} Torr, electron beam accelerating voltage 30.0 kV, working distance 8.0 mm, and probe current 40–45 pA. To index the nanopillars, a fiducial marker was made via a diagonal scratch, from one corner, on the PMMA. After focusing, the pattern was written under software control at the end of the fiducial scratch. Upon completion of electron beam lithography, samples were developed by rinsing in a solution of 1:3 methyl isobutyl ketone:isopropyl alcohol (70 s), then in 100% isopropyl alcohol (20 s), and then deionized water (10 s).

A Temescal BJD-2000 system (Edwards Vacuum, Phoenix, AZ) with an Inficon XTC/2 deposition controller (East Syracuse, NY) was used for metal evaporation. Chamber pressures were 1.0×10^{-5} Torr. Samples were rotated (3 rpm) and monitored for metal thickness using a crystal monitor with gold-coated 6 MHz quartz piezoelectric crystals (Kurt J. Lesker Co., Clairton, PA). Rates of 0.3–0.5 Å/s were maintained during the deposition of a titanium adhesion layer (2 nm) and a gold layer (15 nm). After deposition, samples were cooled to room temperature before being removed from the chamber. Lift-off of the photoresist was performed by placing the samples in warm acetone (60–70 °C) and swirling them. SEM images of the nanopillar arrays are shown in Figure 1.

SEM imaging was performed using a JEOL JSM-7600F (Peabody, MA) field emission analytical scanning electron microscope with a pressure inside the chamber of 9.6×10^{-5} Torr. AFM imaging was performed using an Asylum MFP 3D–BIO AFM (Santa Barbara, CA) in tapping mode using Asytec-01 silicon tips (Asylum Research, Santa Barbara, CA). Height images were taken in tapping mode to minimize tip effects. Gwyddion version 2.37 (Gwyddion.net) was used for analysis of tapping mode height images.⁵⁰ Height analysis was done by using a grain threshold to highlight the pillars and obtain a distribution of pillar height. This distribution was then fit to a Gaussian model to obtain a mean pillar height from the peak center.

Samples were washed in acetone, methanol, and deionized water, each, for 5 min, washed with ethanol, and immersed in an ethanolic solution of OT (10 mM) and MUA (2.5 mM) (18

h), and rinsed with ethanol and then 40 mM potassium phosphate buffer pH 7 (Kpi), three times each. Samples were immersed in Kpi containing EDC (2 mM) and NHS (5mM) for 2 h, and then immersed in a Kpi solution containing CYP2C9 (50 nM), flurbiprofen (40 μ M), and dapsone (40 μ M) for 24 h. After the CYP2C9 was attached, the samples were rinsed with 3.9 mM Cetyltrimethylammonium Bromide (CTAB) for 5 minutes to remove non-specific binding, then Kpi, and then blown dry with nitrogen.

Prior to CPAFM measurements, the tip cantilever spring constants were measured in accordance with the manufacturer's instructions on freshly stripped mica for all RMN 25Pt300B tips (Rocky Mountain Nanotechnology, Salt Lake City, UT) used. Spring constant calculations were used to apply the same force or modulation of force during all CPAFM measurements. Fiducial scratches were used to orient our samples to take measurements on the same nanopillars for each CPAFM measurement. CPAFM scans were taken in-air by starting the bias at 0 V, moving linearly to maximum bias (3 to 4 V), then linearly to minimum bias (−3 to −4 V), and finally returning to 0 V. Curves shown for each sample are the average of at least five scans taken from the maximum to the minimum voltage cycle. Substrates were applied by immersing chips with immobilized CYP2C9 in a Kpi solution containing 40 μ M of flurbiprofen and 40 μ M dapsone, or 40 μ M sulfaphenazole, and then blown dry with nitrogen. Between CPAFM experiments, samples were rinsed in deionized water for at least 1 h to wash small molecules (flurbiprofen, dapsone, or sulfaphenazole) from the enzyme. All processes were performed in an argon atmosphere at room temperature.

3. Results and Discussion

A unique aspect of the nanopillar array is that it allows for sequential measurements under different conditions to be conducted on the same bonded CYP2C9 molecule. This, in part, was made possible by using a scratch as a fiducial marker which allowed us to find the same nanopillar with the same CYP2C9 molecule in subsequent studies. Scanning electron microscopy (SEM) images of the nanopillar platform are shown in Figure 1. The images show that each nanopillar was between 20 and 40 nm in diameter. CYP2C9 was attached to the nanopillars as described in reference 46. Briefly, a self-assembled monolayer was bonded to the gold nanopillars containing ω -mercaptoundecanoic acid. The carboxyl groups of the SAM were activated toward amide formation such that the CYP2C9 was bonded to the gold nanopillars through the SAM. Bonding to the SAM can occur through the N-terminus or any surface accessible lysine of CYP2C9. While it has been suggested that the orientation of the heme (controlled by the orientation of the enzyme with respect to the pillar) plays an important role in ET,⁵¹ the reproducibility of our results indicate that our enzyme is either able to adopt a similar conformation across pillars or that heme orientation is inconsequential. Previous cyclic voltammetry studies done by our lab utilizing the same attachment scheme of CYP2C9 on a gold film revealed a peak width characteristic of a prevalent orientation of our bonded enzyme,⁷ and we hypothesize attachment is on a lysine near the N-terminus due to pKa and steric constraints.⁵² In addition, because of this experimental construct minimal changes are expected in conformation when measuring the same pillar under different conditions (except for changes caused by changes in force). One question about this immobilized system is whether the CYP2C9 that is bonded to the gold is

still active, and able to metabolize substrate. Previous work in our laboratory and the current work have demonstrated that this system maintains the ability to carry out CYP2C9-mediated metabolism of compounds known to be substrates, when endogenous co-factors and co-enzymes (i.e., NADPH and CPR) are present.⁵² Therefore, the ETp studies conducted here are on immobilized CYP2C9 proteins that retain their *in vivo* activity.

Since aggregation of the P450 proteins may alter the ET and ETp processes, to obtain unconfounded information about the P450 ET process, aggregation effects need to be identified or removed. Thus, studies like those herein which employ isolated P450 can be used to minimize aggregation and its effect on ET. The nanopillar lateral dimensions, on average, are approximately 30 nm and the diameter of a single CYP2C9 enzyme is approximately 6 nm.⁵³ Based on previous studies in our lab, we expect approximately 50% coverage depending on the CYP2C9 concentration used, on the top of each nanopillar. Thus, it is expected that a maximum of 13 CYP2C9 molecules could bind to the top of a nanopillar.⁵²

Monolayer attachment would show that we are not measuring through multiple enzymes, and leave only the possibility of lateral enzyme interaction(s). Changes in pillar height were used to evaluate attachment and aggregation of CYP2C9. While conductive studies were completed in contact mode, height images were obtained in tapping mode to avoid possibly moving bonded CYP2C9 off the pillar top during raster scanning.⁵⁴ Maximal z-axis resolution of AFM has been shown to be ~0.1 nm,⁵⁵ which is more than sufficient for distinguishing between a single CYP2C9 or two CYP2C9s stacked on top of another. Parameters to obtain monolayer coverage were evaluated on gold films, and height was assessed by scratching a small area clean in contact mode at a high force (160 nN), and re-imaging in tapping mode with a new tip.⁵⁶ This showed a height change of ~1.1 nm for SAM, and ~7 nm for SAM/CYP2C9, which is in good agreement with monolayer coverage (see supplementary data, Figure S1).

Figure 2a depicts an AFM height image of our platform with pillars highlighted through grain analysis in Gwyddion software. A height threshold was chosen to select only for the gold pillars and not the silicon surface, as our attachment scheme will only allow bonding of our enzyme to gold through amide bond formation with the SAM. A height distribution was extracted for an entire array (441 nanopillars) and fit to a Gaussian line shape to find the peak center. Figure 2b shows an overlay of the height distributions for bare gold pillars and pillars with SAM/CYP2C9 attached. A shift in pillar height of 3.5 nm from bare gold pillars to CYP2C9 immobilized pillars can be easily observed in the data. In addition, the peak width increased from 3.7 nm for bare gold pillars to 6.7 nm for SAM/CYP2C9 attached pillars. The increase in pillar height with attached SAM/CYP2C9 is in good agreement with attachment of CYP2C9 to approximately 50% of our pillars given the proteins spherical diameter of 6 nm.⁵³ This is further validated by the increase seen in the peak width, which implies that, after attachment, an increase in heterogeneity is observed on our arrays. This is consistent with some pillars bearing attached protein and some remaining without bonded protein. Furthermore, the change in height and peak width is too small to support the case where multiple proteins would be stacking on top of one another, providing strong evidence that we are measuring ETp through a single P450 layer.

CPAFM *I-V* curves provide conductance information which occurs through pathways known to dominate the ETp process.⁵⁷ Previously we observed that our gold nanopillars yield an ohmic response when voltage is applied, and that attachment of SAM leads to insulating behavior that is slightly reversed by bonding of CYP2C9.⁴⁶ To obtain data for CYP2C9, at least five curves were averaged, all of them going from a maximum positive to a minimum negative voltage. The *I-V* curves measured as a function of applied force for P450 attached to a gold nanopillar are shown in Figure 3 for the enzyme alone and in the presence of substrate (flurbiprofen). In both cases, the current increased with probe force, as expected for an organic medium.^{56,58} In contrast, no force dependent increase in current was observed for *I-V* curves taken on the silicon surrounding the pillars (see supplementary data, Figure S2). Force dependent studies demonstrate that a reliable electrical contact to our protein is made at 16 nN. At 32 nN the *I-V* curves indicate a significant loss in resistance, implying a large degree of compression.⁵⁹ Therefore, 16 nN was chosen as the applied force to obtain *I-V* data of CYP2C9 under various conditions.

Figure 4 shows substrates/inhibitor used in our CPAFM studies of three different nanopillars whose *I-V* data are graphed in Figure 5. All *I-V* measurements listed for nanopillars were made on the same pillar and thus the same CYP2C9 enzyme and with the same applied tip force (16 nN). To vary the substrate bound to the CYP2C9, the nanopillar was treated with substrate solutions under saturating conditions. Once a series of measurements were made, the substrate was washed out by immersion in phosphate buffer, and then immersed in a solution containing a different substrate at a saturating concentration in phosphate buffer. Substrates that enter the active site in solution cannot escape once the sample is removed from solution and blown dry, and thus they are stuck in the active site during the in-air CPAFM measurements. We note that curves were consistent over sequential measurements after removal and re-insertion of samples (data not shown), and that the platform provided reproducible curves in the case of no substrate/inhibitor presence within the active site (see supplementary data, Figure S3)

Here we compare CYP2C9, flurbiprofen/dapsone (metabolized by CYP2C9, see Figure 4) and sulfaphenazole (a CYP2C9 inhibitor, see Figure 4). First, *I-V* data were obtained on CYP2C9 by itself (Figure 5a). Next, the nanopillar array was immersed in a mixture of flurbiprofen and dapsone. Flurbiprofen and dapsone are substrates readily metabolized by CYP2C9. We have previously demonstrated that flurbiprofen and dapsone can occupy the active site simultaneously.⁶⁰ The *I-V* data demonstrate that with both flurbiprofen and dapsone present with CYP2C9, the enzyme conductance is increased (Figure 5b), in good agreement with our previous findings of simultaneous binding of both substrates in the active site.⁴⁶ We have previously demonstrated that flurbiprofen metabolism is activated by the presence of dapsone.⁶¹ Thus, the *I-V* data showing increased conductance agree with the hypothesis that the rate of P450-mediated metabolism accelerates as ET becomes easier.

Following the *I-V* measurements of the CYP2C9-flurbiprofen/dapsone system, the nanopillars were rinsed with water and treated with a solution of sulfaphenazole (Figure 4) under saturating conditions. Sulfaphenazole is a known inhibitor of CYP2C9-mediated metabolism, that is purported to stabilize the heme iron in the low spin state.⁶² The *I-V* data for CYP2C9 with sulfaphenazole bound indicates that ETp is more difficult than the

CYP2C9 alone or when readily metabolized substrates are bound (Figure 5c). This is in agreement with our previous data regarding aniline, another inhibitor of CYP2C9. Like sulfaphenazole, aniline is a Type II competitive inhibitor purported to stabilize the heme iron in the low spin state,⁶² and thus when bound to CYP2C9, it reduced the ease of ET. We have previously demonstrated that the ease of P450 reduction and spin state are not well correlated, as CYP2C9 complexes with either flurbiprofen or dapsone had identical conductance profiles,⁴⁶ despite flurbiprofen being known to stabilize the high spin state, and dapsone having little effect on spin state. In addition, ETp was much easier for dapsone than aniline, despite neither altering spin state. Our results with sulfaphenazole indicate that inhibitors may also block ETp. This result presented herein is now the second example of this type of behavior. It is also a novel result because P450 inhibitors are thought to act only by binding to the active site and blocking access to it, and/or stabilizing the heme iron in the low spin state. Finally, we note that sulfaphenazole was chosen due to its similar inhibitory properties in comparison to aniline. However, sulfaphenazole binds tightly to CYP2C9 while aniline binds only weakly.⁶² Therefore, the strength of binding does not seem to affect the inhibitor's ability to block ET.

A quantitative analysis was undertaken to better understand the information embedded in the *I-V* curves. While the Simmons model is generally used for CPAFM analysis at low bias,^{58,59} this was not a suitable model for this system due to the low conductivity near $V_B = 0$. Measurements of conductivity near bias voltage $V_B = 0$ were zero, and remained zero (to within the noise floor of the data, ± 3 pA) until the conductance turned on at a critical voltage (see Figure 5). This indicates that the tunneling barrier for the electrons transferred from the gold nanopillar to the CPAFM tip was large in energy. Instead, the data were fit with the Poole-Frenkel (PF) emission model⁶³ to compare with our previous findings and look for reproducible trends. In the PF emission model electrons are trapped in localized states and conduct by hopping from one localized state to another within an insulating layer. This model is appropriate for our system in which the protein-SAM, the insulator, and various functional groups within the protein serve as areas where localized states may occur and trap electrons between them.^{47,52,53,64}

In the PF model, the current to voltage ratio *I/V* is expected to behave as

$$\ln(I/V) = \ln C - \frac{q\Phi_B}{kT} + \frac{q}{kT} \left(\frac{q}{\pi d \epsilon_0 \epsilon_s} \right)^{1/2} V^{1/2}, \quad (1)$$

where *V* is the applied (bias) voltage, *q* is the charge of an electron, Φ_B is the effective voltage barrier that the electron must overcome to move from one localized state to another, *d* is the distance across which the voltage is applied, ϵ_0 is the permittivity of free space, and ϵ_s is the relative permeability of the material (in this case CYP2C9 and SAM) at high frequencies, assuming that there is no local polarization induced. Also, *k* is the Boltzmann's constant, *T* is the absolute temperature, *C* is a constant that depends on the intrinsic mobility of the charge carriers, the effective area of the electrical contact, and the effective distance *d* across which *V* is applied. A plot of $\ln(I/V)$ as a function of $V^{1/2}$ should therefore yield a straight line with the y-intercept proportional to Φ_B . The slope should be sensitive to changes in the effective size (*d*) of the protein and its effective dielectric constant. As shown

previously,⁴⁶ although it is not possible to solve for Φ_B because the value of C is unknown, it is possible to obtain relative changes in d and Φ_B assuming that C remains unchanged upon binding of different substrates.

Figure 6 contains a plot of $\ln(I/V)$ as a function of $V^{1/2}$ for the positive bias data for the nanopillars shown in Figure 5. The data were fit to Equation 1 for $V > 1$ V. Data from smaller voltages were poorly fit by the model as was observed in our previous work,⁴⁶ and it is possible another mechanism is at play in this region. The quantitative results are presented in Table 1, where Φ_B is the shift of barrier height from the run in which there was no substrate bound in the active site of CYP2C9. The fractional change d/d_0 , where d_0 is the distance between hopping sites, was also calculated. The data in Table 1 reveal that the slopes for all conditions were similar, implying that d and ϵ_s were similar in all runs as hypothesized. Thus observed differences in the y-intercept should be due to changes seen in Φ_B .

Table 1 shows that for all nanopillars, the addition of flurbiprofen and dapsone decreased the barrier height. In contrast, the addition of sulfaphenazole does not decrease the barrier height, and might slightly increase it. The general trend for the width of the barrier is that of an increase in the addition of substrate, except for sulfaphenazole in nanopillar 2. These trends are consistent with the general properties of CYP2C9 and how it interacts with these particular substrates. The reduction of CYP2C9 is easier in the presence of metabolized substrates (flurbiprofen and dapsone), but more difficult in the presence of inhibitor (sulfaphenazole).

Figure 7 depicts a plot of $\ln(I/V)$ as a function of $V^{1/2}$ for the positive bias data for the nanopillars presented in Figure 3. The data were fit to Equation 1 for $V > 1$ V as was done in Figure 6. For the enzyme alone (pillar A), measured with the largest force (32 nN), the plot reveals a possible phase transition as seen by the multiple linear regions. We fit all three possible linear regions to avoid biasing the data (see supplementary data, Figure S4). The lack of phase transition observed in the presence of flurbiprofen could be explained by the known stabilizing effect of substrate binding within P450s.⁶⁵ The quantitative results are plotted in Figure 8 with the same description of variables found in Table 1. The relatively large error bars for the Pillar B at 8 nN data are due to a limited linear regime to fit the PF model (see Figure 7). Using the low force (2–3 nN) data as the baseline, a decrease in the barrier height with increased applied force (both pillars) is observed. Fit 1 for Pillar A at 32 nN is in good agreement with the trend seen in Pillar B. Fit 2 and 3 both result in an increase in barrier height, which disagrees with the general trend that a decrease in barrier height is associated with an increased applied force.⁵⁸ We thus believe that Fit 1 is the correct region to analyze.

In both pillars there is no change in slope until the highest force is applied (32 nN). This difference in slope implies a change in d or ϵ_s , in contrast to our substrate/inhibitor analysis conducted at a single force, where only minimal changes in slope were observed. Using Fit 1 again for Pillar A at 32 nN, good agreement with Pillar B is obtained for d/d_0 values

Under increased force we expect the protein to become compressed.^{58,59} Compression of proteins has also been shown to correlate with a decrease in barrier height,⁶⁶ which agrees with our results in Figure 8a. Interestingly, Figure 8b shows an increase in the spacing of localized states with increased force. This would indicate that even though the P450 is being compressed, the spacing between localized states within the P450 increases on average.

These results can be explained by a zig-zag path of electron flow through the protein's different motifs between localized that are not aligned the vertical direction, as shown in Figure 9. This can be demonstrated by noting that the distance between two hopping sites is $d = \sqrt{a^2 + c^2}$, where a is the in-plane component and c is the vertical component of the vector $\mathbf{d}$ which extends from one state to the other. Small changes in a and c would result in a small change in d with respect to the change in c of

$$\frac{\delta d}{\delta c} = \frac{c}{d} + \frac{a}{d} \frac{\delta a}{\delta c}. \quad (3)$$

We now assume that the volume of the protein that contains the two hopping sites is proportional to $V \propto a^2 c$, and that this volume remains constant when the protein is compressed, that is, the protein deforms when it is pressed by the tip from above. Mathematically, this means that the change in volume

$$\delta V \propto 2ac\delta a + a^2\delta c = 0. \quad (4)$$

Solving equation 4 to obtain a relation between δa and δc , and combining this result with equation 3, yields a change of the total distance between hopping sites with respect to the change in vertical distance of

$$\frac{\delta d}{\delta c} = \frac{2c^2 - a^2}{2da}. \quad (5)$$

Equation 5 shows that decreasing the vertical distance ($\delta c < 0$) leads to either an increase or decrease in d depending on whether $2c^2 - a^2$ is negative or positive, respectively. Our data seem to indicate that, on average, the hopping sites are located such that $a > \sqrt{2}c$. Currently there is no way to determine which specific functional groups in the protein serve as these impurities in order to model their positions under compression by the AFM tip, and further work is necessary to elucidate this process. In any case, the force data further validate that our choice of 16 nN is appropriate and does not lead to large changes in our enzyme size due to CPAFM tip interaction.

4. Conclusions

We have developed an effective platform to measure the conductance of immobilized proteins that eliminates aggregation effects on ET studies. The data demonstrate that for immobilized CYP2C9 proteins, a correlation exists between the energy barrier height between hopping sites, Φ_B , and CYP2C9 metabolic activity. An inverse correlation between Φ_B and tip force was also observed which agrees with other prior work. Interestingly, the data indicate that the protein is more stable in the presence of a substrate when subjected to a

high tip force. This agrees with the substrate entry into the active site helping to stabilize enzyme conformation. For large forces, the relative distance between hopping sites d/d_0 also increases, possibly because protein secondary structures such as alpha helices and beta sheets often serve as pathways for ET,¹ and thus there are numerous ways in which electrons could travel through P450s to obtain this change in d/d_0 . Another inhibitor (sulfaphenazole), in addition to the previously studied aniline, was found to increase the barrier height for ETp and thereby makes CYP2C9 reduction more difficult and inhibits metabolism. Although P450 inhibitors are thought to compete for binding to the active site and not be metabolized,⁶⁷ our findings suggest that P450 Type II binders may also decrease the ease of ET processes in the enzyme. Overall we show that our nanopillar platform allows for repetitive and reproducible studies of the same unmodified immobilized molecule in sequential studies. Future studies can be undertaken with this platform to identify protein-protein effects using hetero or homo-dimers,⁶⁸ as well as substrate motifs important for metabolism using substrate analogs.⁶⁹

Supplementary Material

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Figure 1.
SEM image showing a section of one of the four nanopillar arrays created by electron beam lithography (left) and zoomed in to show the size of the nanopillars (right).

Figure 2. Height distributions for pillars before (blue squares) and after (red circles) CYP2C9 attachment. Fittings to a Gaussian model gives a peak center of 11.2 nm with a width of 3.7 nm for bare gold pillars (blue curve), and a peak center of 14.7 nm with a width of 5.9 nm after CYP2C9 attachment (red curve). Inset: AFM Height image of pillar array with SAM/CYP2C9 attached. Pillars (highlighted in red) were selected for height analysis through grain height thresholding.

Figure 3. Measured I - V curves as a function of force for two nanopillars with attached CYP2C9. Pillar A contains CYP2C9 alone, Pillar B contains CYP2C9 with substrate flurbiprofen attached. Data obtained from two different samples.

Flurbiprofen **Dapsone** **Sulfaphenazole**

Figure 4. Structures of the CYP2C9 substrates flurbiprofen and dapsone and the CYP2C9 inhibitor sulfaphenazole.

Figure 5.

I-V curves measured for three nanopillars of (a) CYP2C9 alone, (b) CYP2C9 with flurbiprofen and dapsone, and (c) CYP2C9 with inhibitor sulfaphenazole.

Figure 6.

Poole-Frenkel plot for the data obtained for positive bias voltages for nanopillars in Figure 5. CYP2C9 alone (black), CYP2C9 with flurbiprofen and dapsone (red), and CYP2C9 with inhibitor sulfaphenazole (blue). The symbols are the data and the lines are fits to a straight line.

Figure 7.

Poole-Frenkel plot for the data obtained for positive bias voltages for nanopillars in Figure 3 using same color code. Force was modulated with measurements (a) at 2–3 nN, (b) at 8 nN, (c) at 16 nN, and (d) at 32 nN. The symbols are the data and the lines are fits to a straight line. 32 nN on Pillar A was fit for all three linear regimes, with Fit 1 between 1.6 to 1.8 $V^{1/2}$, Fit 2 between 1.2 to 1.6 $V^{1/2}$, and Fit 3 between 1 to 1.2 $V^{1/2}$ (see supplementary data, Figure S4).

Figure 8.

a) Change in barrier height as a function of force. Lowest force used as the baseline to generate barrier height values. b) Change in d/d_0 as a function of force with lowest force used as a baseline to determine values. Data calculated using Poole-Frenkel as done in Table 1.

Figure 9.

Simplified depiction of electron transfer through hopping sites (red circles) within a protein immobilized to gold under non-compressed (left) and compressed (right) conditions. The vector $\mathbf{d}$ determines the relative position between the top two hopping sites and a and c indicate the horizontal and vertical distance components, respectively. The values of $\delta\mathbf{d}$, δa and δc are the changes in the vector and distance components when the protein is compressed by the tip.

Table 1Results of the Fits to the I - V data with the Poole-Frenkel model.^a

	CYP2C9	Flurbi+Dap	Sulfaphenazole
Pillar 1			
Intercept	−11.38	−8.59	−12.97
Slope	6.10	4.92	5.96
Φ_B	0	−0.07	0.04
d/d_0	0	0.39	0.04
Pillar 2			
Intercept	−9.76	−8.88	−12.01
Slope	5.32	5.25	5.65
Φ_B	0	−0.02	0.06
d/d_0	0	0.03	−0.12
Pillar 3			
Intercept	−10.90	−9.40	−10.77
Slope	5.64	5.09	4.77
Φ_B	0	−0.04	−0.003
d/d_0	0	0.19	0.31

^aThe units are $\ln(nS)/V^{1/2}$ for the slope, $\ln(nS)$ for the y-intercept, eV for Φ_B , and dimensionless for d/d_0 .

Abbreviations: Flurbi, flurbiprofen; Dap, dapsone.

^bA value of $T = 300\text{K}$ was used to calculate Φ_B . The error for intercept and slope values were $< 4\%$.