UC Irvine
UC Irvine Previously Published Works

Title
Single Molecule Enzymology Using Carbon Nanotube Circuits

Permalink
https://escholarship.org/uc/item/0sb6g164

Author
Collins, Philip G

Publication Date
2013

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

SINGLE MOLECULE ENZYMOLOGY USING CARBON NANOTUBE CIRCUITS

Y. Choi[1], P.C. Sims[1], T. Olsen[2], O.T. Gul[1], B.L. Corso[1], M. Iftikhar[2], G.A. Weiss[2,3], and P.G. Collins[1]
Departments of [1]Physics and Astronomy, [2]Chemistry, and [3]Molecular Biology and Biochemistry,
University of California at Irvine, Irvine, California 92697, USA

ABSTRACT

For over a decade, researchers have pursued sensitive, label-free biosensing and biodetection using nanoscale electronic devices like field-effect transistors. Prototype devices made with single-walled carbon nanotubes proved to have enough sensitivity to detect single molecule events, as well as sufficient bandwidth to monitor real time dynamics. Combining these characteristics has led to novel electronic measurements where the bond-by-bond chemical activity of single enzyme molecules is monitored in real time. The high bandwidth of the nanotube transistors allow every individual chemical event to be clearly resolved, providing excellent statistics from tens of thousands of turnovers by a single molecule.

KEYWORDS

carbon nanotube, sensors, lysozyme, enzymology, single molecule

INTRODUCTION

Most enzymes have high specificities for a particular substrate and therefore are highly specialized for detecting a particular microbe, a type of bacteria, or rare molecules that can constitute disease markers. This specialization makes enzymes a key reagent in virtually all analytical tests. However, the practical exploitation of an enzyme's specificity usually requires additional equipment, power, and reagents. In principle, nanotechnology provides opportunities to dramatically reduce these requirements. By working at the molecular scale, nanosensors could theoretically be no larger or energy intensive than the enzyme itself.

This premise has guided past research focused on building sensitive electrical nanocircuits that contain a single enzyme molecule. Specifically, tethering a single biomolecule to a carbon nanotube field effect transistor can produce a stable, high bandwidth transducer for monitoring protein motions, charge transfer events, and enzymatic activity. These tiny and low-power devices have recently been used for long duration monitoring of enzyme activity with single molecule resolution [1, 2]. The advance provides the necessary proof-of-concept to begin imagining practical and low-power electronic sensors that have the selectivity and specificity of any desired enzyme.

Here, we summarize recent measurements monitoring the dynamic, single-molecule processivity of three different enzymes: lysozyme [1-3], protein Kinase A [4], and the Klenow fragment of DNA polymerase I [5]. In all three cases, single molecule activity has been electronically monitored for 10 or more minutes, allowing us to directly observe rare transitions to chemically inactive and hyperactive conformations. The high bandwidth of the nanotube transistors further allowed every individual chemical event to be clearly resolved, providing excellent statistics from tens of thousands of turnovers by a single enzyme. Initial success with three different enzymes indicates the generality and attractiveness of the nanotube devices as a new tool to complement other single molecule techniques.

METHODS

Our devices are based upon field effect transistors (FETs) using individual, isolated, single-walled carbon nanotubes (SWNTs). SWNTs are high conductivity, one-dimensional conductors with relatively inert sidewalls. The diameter of a SWNT conductor is small compared to proteins, enzymes, and antibodies, which makes them unique components for building electrical circuits that incorporate biomolecules.

Next, one and only one biomolecule is noncovalently attached to each SWNT conductor (Figure 1). This precision control is achieved using a low density of anchor molecules such as pyrene-maleimides [1]. The pyrene groups provide good adhesion to the SWNT sidewall and the maleimide group provides for a covalent linkage to any cysteine in the desired biomolecule. Figure 1 shows example devices, in which one electrically-connected SWNT is labeled by a single lysoyzme attachment.

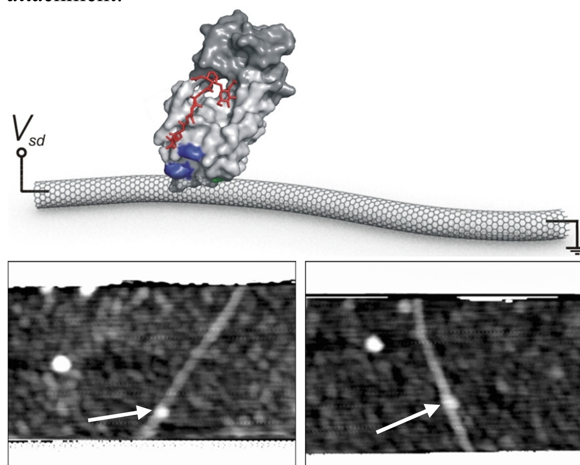

Figure 1: Single biomolecule nanocircuits. (top) Schematic representation of a device, showing the relative size of a nanotube to lysozyme. The drawing highlights lysozyme's two active domains (light and dark grey), which move with respect to each other when processing substrate (red). (middle) Example AFM images of single-walled carbon nanotube transistors, each labeled with a single T4 lysozyme molecule (arrows). Source and drain electrodes lay across the top and bottom of each image, protected under a polymer layer that passivates the device.

Devices like those in Figure 1 provide a sensitive electronic connection to monitor any enzyme's chemical activity. The mechanism of operation does not require electrical stimulation or self-heating of the enzyme, however. The electrical current $I(t)$ flows completely within the SWNT device, and the role of the enzyme is merely to act as a local, electrostatic gate able to modulate the FET channel.

As a specific example, Figure 2 shows a detailed image of T4 lysozyme attached to a SWNT. A cysteine amino acid (highlighted green), introduced by mutagenesis at residue 90 (S90C) provided a reproducible attachment site via the pyrene-maleimide anchor. $I(t)$ flowing through the SWNT was then affected by charged amino acids on lysoyzme's surface [3]. Figure 2 highlights two charged amino acids K83 and R119 that are in the vicinity of the attachment site. As the enzyme opens and closes upon its substrate, each amino acid moves slightly as depicted using light and dark grey for the open and closed conformations (Protein Data Base 1QTV and 148L, respectively). The 0.15-nm motions of charges at K83 and R119, highlighted in blue, are sufficient to partly gate the SWNT FET and change its conductance.

Devices were measured by submerging them in a buffer solution appropriate for the enzyme and then monitoring $I(t)$ under constant bias conditions. By measuring the same molecule with and without substrate or other co-factors, we could distinguish which components of $I(t)$ corresponded to enzyme activity. Typical measurements were recorded for at least 600 s using V_{sd} = 100 mV and with the liquid electrolyte held at 0 V using a Pt wire as a pseudo-reference. $I(t)$ was amplified (Keithley 428) and digitized at 100 kHz. The amplifier bandwidth of 40 kHz provided time resolution of approximately 25 μs. To protect the connective electrodes from the electrolyte, a polymer layer (poly(methyl methacrylate)) was patterned so that only a small portion of the SWNT would be exposed.

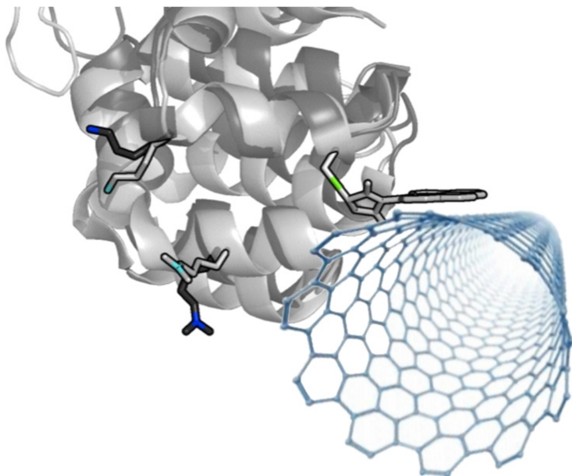

Figure 2: Detail of a typical biomolecular chemical linkage. The pyrene end of a pyrene-maleimide linker molecule noncovalently adheres to the SWNT sidewall. The desired protein is covalently linked to the maleimide group via a cysteine amino acid (highlighted green).

RESULTS AND DISCUSSION

Figure 3 shows the readout of activity from a molecule of c-AMP dependent protein kinase A (PKA). In buffer solution, the enzyme is inactive and it drives no particular dynamic signal in the SWNT FET (Fig. 3, top). In the presence of Kemptide substrate, however, a time series of spikes is observed corresponding to substrate binding and unbinding (Fig. 3, middle). By monitoring similar signals over long periods, the single-molecule rate constants k_{on} and k_{off} can be directly measured. To function properly, PKA must simultaneously bind a substrate molecule and a molecule of adenosine triphosphate (ATP). Probing the same device with ATP produces similar traces having slightly different timing and kinetic rates.

When both ATP and Kemptide are present in solution, the simultaneous binding of both leads to a fully-closed protein conformation that is catalytically active [6-9]. In this active state, the gamma phosphate of ATP is transferred to the Kemptide, and then the phosphorylated product is released.

PKA's fully-closed conformation produces a stronger gating effect on the SWNT than either ATP or Kemptide binding alone, leading to a distinguishable, third signal in $I(t)$ (Fig. 3, bottom). The combination of signals from the enzyme's open, partly bound, and fully closed conformations proved that phosphorylation could be monitored with single bond resolution. Analysis of enzyme activity over the course of many minutes uncovered surprising aspects of PKA's kinetics, including highly variable rates (Table 1) that corroborate its role as a promiscuous regulatory enzyme [4].

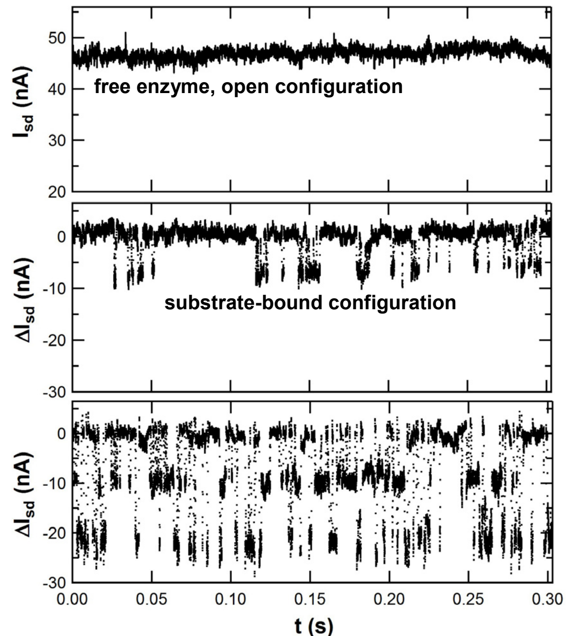

Figure 3: I(t) signals transduced by a single PKA molecule without substrate (top), with substrate (middle), and with both substrate and ATP (bottom). In the last case, a three-molecule complex forms for substrate phosphorylation, and the electronic signal transduces each step of the process.

Besides PKA, two additional enzymes have also been studied using SWNT devices. The Klenow fragment (KF) of DNA polymerase I has been attached to SWNTs to monitor DNA replication (Fig. 4) [5], and T4 lysozyme has been monitored while cleaving peptidoglycan [1-3].

In the case of KF, similar *I*(*t*) signals are observed during the processing of single-stranded DNA. KF functions by binding a template strand and a complementary nucleotide, and then closing upon the template to incorporate the nucleotide [10, 11]. The opening and closing actions moved KF surface charges with respect to the SWNT FET, generating two-level fluctuations in *I*(*t*). Example signals, highlighted in Figure 4, extended for hundreds of seconds in the presence of excess template molecules.

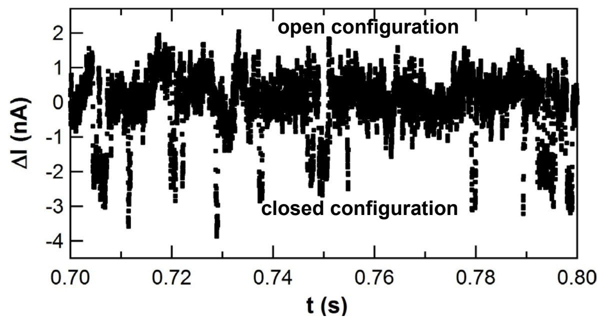

Figure 4: I(t) signals transduced by a single KF molecule in the presence of homopolymeric, single-stranded DNA (poly(dT)$_{42}$) and complementary dATP nucleotides.

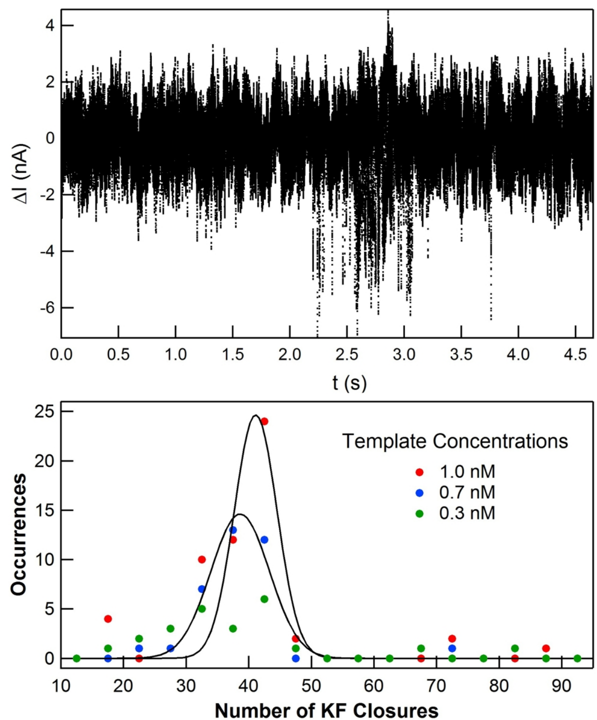

Figure 5: (top) A cluster of excursions as a single template molecule binds to KF and then is processed. (bottom) Histogram of the number of closures observed in different clusters, as measured using a 42-base template. The peak at 42 closures indicates that each closure corresponds to one nucleotide incorporation.

We have also monitored KF activity in the low-concentration limit of only 0.1 to 1.0 nM template. At these concentrations, the diffusive waiting time for arrival of a template molecule extends for many seconds, on average. In those experiments, the arrival of a single template molecule interrupted long inactive periods with one cluster of many KF closures, indicating the continuous processing of the template (Figure 5, top). Using a homopolymer templates with 42 bases, we accumulated and analyzed many similar clusters. As shown at the bottom of Fig. 5, analysis of these events showed that KF processed up to 42 bases, exactly matching the length of the templates used [5].

Finally, Figure 6 shows example *I*(*t*) signals observed from lysoyzme in the presence of its substrate, peptidoglycan. Unlike the other two enzymes, closure of lysoyzme upon its substrate produced transient increases in *I*(*t*), rather than decreases. This change in sign merely indicated the sign and relative motions of lysoyzme's surface charges. In fact, we have reversed the sign and maintained a constant magnitude by producing lysozyme variants with either positive or negative amino acid substitutions at K83 and R119 [3]. In a series of measurements using seven different variants, the electrostatic mechanism depicted in Fig. 2 was conclusively proven [3].

The data in Fig. 6 is an example of rapid closures that other single molecule techniques had previously been shown to be catalytically nonproductive. Lysoyzme motions at rates of 100 to 300 s^{-1} do not successfully cleave peptidoglycan. We showed that this type of fast motion nearly vanished when lysoyzme processed a synthetic, linear substrate [2]. Thus, the rapid, nonproductive closures are peculiar to the cross-links in natural peptidoglycan.

Much slower motions at rates of 10 to 60 s^{-1} correspond to successful catalytic hydrolysis of peptidoglycan's glycosidic bonds. The nonproductive and catalytic motions have different kinetic rates, but they correspond to nearly identical mechanical motions and they produced *I*(*t*) signals that were the same height. These findings further showed that the electronic transduction was caused by mechanical motions rather than by the enzyme's particular chemistry. A future goal for this electronic technique will be to try to distinguish chemical and mechanical causes, for example to address the longstanding question of whether chemical activity drives mechanical motion or *vice versa*.

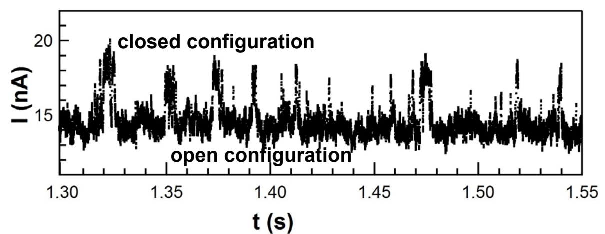

Figure 6: I(t) signal transduced by a single T4 lysozyme molecule. I(t) fluctuates between two levels in sync with the enzyme domains opening and closing on its substrate, producing a real-time electrical recording of the enzyme's activity.

Because the recordings extended for hundreds of seconds on any given molecule, excellent statistics could be generated for each type of motion. Table 1 summarizes the mean durations of open (unbound) and closed (bound) conformations of all 3 enzymes studied. For PKA, we note unusually large standard deviations, as the τ values varied widely from one second to another. For KF, we found that τ_{open} was nearly twice as long when forming AT and TA complementary base pairs than when forming CG and GC ones. And with lysozyme there were two characteristic sets of timing as described above, which depended on whether lysoyme was catalytically active or nonproductively stuck at cross-link.

Table 1: Durations of Open and Closed Conformations

Enzyme	$<\tau_{open}>^*$ (ms)	$<\tau_{closed}>^*$ (ms)
Protein Kinase A (PKA)		
ATP	5.0 ± 19	3.1 ± 4.3
Kemptide	1.7 ± 3.7	1.8 ± 2.9
Ternary compound		2.7
Klenow fragment (KF)		
CG/GC base pairs	38.5 ± 5.7	0.32 ± 0.06
AT/TA base pairs	67.5 ± 5.7	0.37 ± 0.09
Lysozyme		
nonproductive (fast)	2.90 ± 0.10	0.26 ± 0.01
catalytic (slow)	64.0 ± 2.0	0.95 ± 0.08

**All values stated as mean ± one standard deviation*

From the raw data, many additional statistics can be calculated. For example, average kinetic rates are given by $k = (<\tau_{open}> + <\tau_{closed}>)^{-1}$. Statistical variances helped reveal the presence of intermediate, rate-limiting chemical steps in each motion. Instantaneous rates were even more informative than average ones, since their distributions suggested sensitivity to conformational or environmental variability. Long-duration recordings revealed memory effects in which the instantaneous rates maintained unusual values through tens and even hundreds of catalytic cycles. These recordings allowed us to calculate the probabilities of rare transitions to chemically inactive and hyperactive conformations. Analysis of all such parameters has been described in detail previously [1-5]. The level of detailed analysis was uniquely enabled by the long-term stability of the electronic monitoring technique.

CONCLUSIONS

With all three enzymes, single molecules were electronically monitored for >10 minutes. The high bandwidth of the SWNT FETs allowed individual chemical events to be clearly resolved, providing excellent statistics from tens of thousands of catalytic turnovers. Besides establishing values for processivity and turnover rates, the measurements revealed variability, dynamic disorder, and the existence of intermediate states.

On the more practical side, devices that can directly indicate enzyme activity have potential applications in medical testing and diagnostics. For example, the lysozyme-SWNT devices are now well-enough understood that they could be deployed as bacterial sensors. Our immediate success generalizing the platform to three enzymes indicates that the technique is versatile and promising for both single molecule science and practical applications.

ACKNOWLEDGEMENTS

This work was supported by the National Cancer Institute of the NIH (R01 CA133592-01) and by the NSF (DMR-1104629 and ECCS-1231910).

REFERENCES

[1] Y. Choi *et al.*, "Single-Molecule Lysozyme Dynamics Monitored by an Electronic Circuit", *Science*, vol. 335, pp. 319-24 2012.

[2] Y. Choi *et al.*, "Single Molecule Dynamics of Lysozyme Processing Distinguishes Linear and Cross-Linked Peptidoglycan Substrates", *J. of the Am. Chem. Soc.*, vol. 134, pp. 2032-5, 2012.

[3] Y. Choi *et al.*, "Dissecting Single-Molecule Signal Transduction in Carbon Nanotube Circuits with Protein Engineering", *Nano Lett.*, vol. 13, pp. 625-31, 2013.

[4] P. C. Sims *et al.*, "Electronic Measurements of Single-Molecule Processing by Protein Kinase A", *J. Am. Chem. Soc.*, vol. pp. in press, 2013.

[5] T. J. Olsen *et al.*, "Electronic Measurements of Single-Molecule Processing by DNA Polymerase I (Klenow Fragment)", *J. Am. Chem. Soc.*, vol. pp. in press, 2013.

[6] J. A. Adams, "Kinetic and Catalytic Mechanisms of Protein Kinases", *Chemical Reviews*, vol. 101, pp. 2271-90, 2001.

[7] B. E. Kemp *et al.*, "Role of Multiple Basic Residues in Determining the Substrate Specificity of Cyclic Amp-Dependent Protein Kinase", *J Biol Chem*, vol. 252, pp. 4888-94, 1977.

[8] J. A. Adams, and S. S. Taylor, "Energetic Limits of Phosphotransfer in the Catalytic Subunit of Camp-Dependent Protein Kinase as Measured by Viscosity Experiments", *Biochemistry*, vol. 31, pp. 8516-22, 1992.

[9] D. A. Johnson *et al.*, "Dynamics of Camp-Dependent Protein Kinase", *Chemical Reviews*, vol. 101, pp. 2243-70, 2001.

[10] C. M. Joyce, "Techniques Used to Study the DNA Polymerase Reaction Pathway", *Biochimica Et Biophysica Acta-Proteins and Proteomics*, vol. 1804, pp. 1032-40, 2010.

[11] H. V. Aposhian, and A. Kornberg, "Enzymatic Synthesis of Deoxyribonucleic Acid .9. Polymerase Formed after T2 Bacteriophage Infection of Escherichia Coli - New Enzyme", *Journal of Biological Chemistry*, vol. 237, pp. 519, 1962.

CONTACT

*P.G. Collins, collinsp@uci.edu