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Observation of Giant Conductance Fluctuations in a Protein

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Abstract

Proteins are insulating molecular solids, yet even those containing easily reduced or oxidized centers can have single-molecule electronic conductances that are too large to account for with conventional transport theories. Here, we report the observation of remarkably high electronic conductance states in an electrochemically-inactive protein, the ~200 kD $\alpha_V\beta_3$ extracellular domain of human integrin. Large current pulses (up to nA) were observed for long durations (many ms, corresponding to many pC of charge transfer) at large gap (>5nm) distances in an STM when the protein was bound specifically by a small peptide ligand attached to the electrodes. The effect is greatly reduced when a homologous, weakly-binding protein ($\alpha_4\beta_1$) is used as a control. In order to overcome the limitations of the STM, the time- and voltage-dependence of the conductance were further explored using a fixed-gap (5 nm) tunneling junction device that was small enough to trap a single protein molecule at any one time. Transitions to a high conductance (~ nS) state were observed, the protein being “on” for times from ms to tenths of a second. The high-conductance states only occur above ~ 100mV applied bias, and thus are not an equilibrium property of the protein. Nanoamp two-level signals indicate the specific capture of a single molecule in an electrode gap functionalized with the ligand. This offers a new approach to label-free electronic detection of single protein molecules. Electronic structure calculations yield a distribution of energy level spacings that is consistent with a recently proposed quantum-critical state for proteins, in which small fluctuations can drive transitions between localized and band-like electronic states.

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Conflict of Interest

SL is a cofounder of Recognition AnalytiX, licensee of this technology. SL, PZ, PP and YZ are inventors on patent applications resulting from this work.

Introduction

Proteins are aperiodic systems with high barriers to electron or hole injection and strong vibronic coupling, all features that argue against conduction-band mediated transport. [1] Analysis of electron-transfer (ET) processes based on localized molecular-orbital analysis has had considerable success [2] even in explaining very long range transport in bacterial nanowires, for example. [3] Yet many questions remain. In a tunneling model, ET rates fall with distance as $e^{-\beta x}$. Typical electronic binding energies should result in $\beta \sim 2.2 \text{ \AA}^{-1}$, whereas direct measurements yield $\beta \sim 1$ to $2 \text{ \AA}^{-1}$. [4] In particular, STM break junctions measurements on peptide chains yield $\beta \sim 0.87 \text{ \AA}^{-1}$. [5] Even this smallest value for β rules out tunneling as a transfer mechanism in STM studies of redox proteins. For example, several independent measurements have established that the conductance of a single azurin molecule is $10^{-5} G_0$ (G_0 is the quantum of conductance, $77.5 \mu\text{S}$). [6] Based on the diameter of azurin ($\sim 3 \text{ nm}$) ET should be limited by tunneling alone to a maximum conductance of $\sim G_0 \cdot e^{-\beta x}$ or about $10^{-12} G_0$, a discrepancy of seven orders of magnitude. Alternative mechanisms like hopping or fluctuating resonance [4] are thermally activated, yet measurements of the temperature dependence of ET in Ferritin [7] and bacterial nanowires [8] and bacteriorhodopsin [9] show a weak temperature dependence, more characteristic of metallic conduction, so that the transport mechanism remains open to question. A recent review describes the observation of anomalously high conductances in many protein transport measurements. [10] It is even more difficult to understand long-range transport in proteins that do not contain any readily accessible redox centers, such as the Dronpa family [10] and bacteriorhodopsin. [9] Other examples include STM imaging of electrochemically-inactive proteins with set-point currents on the order of nA [11–13] and electron-spin polarization in chiral peptides. [14, 15]

Here, we report direct measurements of the conductance of a large, electrochemically-inert protein (ca. 10 nm diameter) using both STM and a fixed gap device to explore long-range transport. We chose to use a $\sim 200 \text{ kD}$ protein, the $\alpha_V\beta_3$ extracellular domain of human integrin (Figure S1 [16]) because it lacks obvious redox centers and binds a small cyclic (RGD) peptide selectively. [17] By modifying electrodes with a cyclic RGD containing a cysteine, we can control the attachment point of the protein as well as retarding the denaturation often observed when proteins bind bare electrodes. [18] This scheme also allows us to use a similar protein ($\alpha_4\beta_1$ that lacks the divalent metal-ion binding site required for strong RGD attachment [19]) as a control. [20] STM break-junction measurements show current peaks in excess of a nA at gap distances in excess of 5 nm. However, the STM gap is difficult to characterize. In order to overcome the limitations of the STM, the time- and voltage dependence of these high-conductance states were explored using a multilayer edge molecular electronic device (MEMED). This consists of a sandwich of two Pd layers separated by a layer of Al_2O_3 dielectric deposited by atomic layer deposition (ALD) to a thickness of 4 to 5 nm. [21] The electrode areas were small enough that capture of just a single molecule was possible, simplifying the interpretation of signals. Motivated by evidence for quantum coherent effects in proteins [22, 23] we carried out electronic structure calculations to compare the distribution of energy levels to the predictions of an analysis based on quantum-critical states in proteins. [24]

STM Break Junction Measurements

Figure 1A shows a schematic layout of the STM break-junction measurement. A Pd wire probe, etched to a sharp point and insulated to within a few tens of nm of its apex, was used in an Agilent PicoSPM together with a Pd-coated substrate as described elsewhere. [25] Pd, rather than Au was used because tunneling devices made with Pd are more stable. [26] Probes and substrates were functionalized with cyclic RGD peptide (inset Figure 1B) as described in Methods. Measurements were carried out in 1mM phosphate buffer (pH=7.4) with the potential of the substrate controlled at a voltage V_r with respect to an Ag/AgCl reference. The reference electrode was connected to the measurement cell via a salt-bridge (SB) because we found that adsorption of protein onto the surface of a simple silver-wire quasi reference resulted in instabilities in the reference potential. Integrin itself is electrochemically-inert, but the Pd surface has a rich electrochemistry (Figure S2). Cyclic voltammetry of a Pd surface functionalized with cyclic RGD peptide is shown in Figure 1B, where a prominent cathodic peak (“HA”) is associated with hydrogen adsorption. [27] Addition of $\alpha_v\beta_3$ protein blocks access to the surface, resulting in ~30% reduction in the cathodic peak (from 1.3 to 0.9 C/cm²), showing that the integrin is electrochemically inactive in this potential range. In order to avoid both this cathodic peak and the onset of Pd oxidation at positive potentials, both electrodes were kept at potentials between 0 and 0.5V vs Ag/AgCl (see s). The STM gap was set to an initial current of 4 pA (13 pS conductance at $V_t=0.2V$, corresponding to a distance, Z_0 , of about 2nm [28]) and the probe was then slowly (0.17 nm/s) retracted. The tunnel current decayed rapidly (~0.2 nm decay length – inset in Figure 1E) in buffer alone. However, when $\alpha_v\beta_3$ protein was added to a final concentration of 10 nM, remarkably large current spikes were observed at retraction distances up to $Z=3$ nm (overall gap, $Z_0+Z \sim 5$ nm) as shown in Figure 1D. When the experiments were repeated with $\alpha_4\beta_1$ protein substituted for $\alpha_v\beta_3$, the event frequency dropped from 26% of all retractions to 12%, and peak currents and the values of Z at which current spikes occurred were significantly reduced (Figure 1E). Summaries of combined measurements from four separate experiments with each protein are shown in Figures 2A and B. Many current points for $\alpha_v\beta_3$, exceed 0.1 nA (i.e., conductances > 0.5 nS) at distances $Z_0+Z > 3$ nm (Figure 2A). Clearly these currents cannot arise from a tunneling process with $\beta \sim 0.87 \text{ \AA}^{-1}$ [5] for which $G_0 \cdot e^{-\beta x}$ yields values smaller than 10^{-16} S.

The charge transferred in these events (obtained by integrating the current peaks) spans a large range of values (Figure 2C), but even the smallest ($Q \sim 1$ pC) correspond to transfer of about 10^7 electrons, a charge too big to be explained by polarization events on this length scale.

Table 1 summarizes the frequency with which these large current pulses are observed in STM measurements, showing also the effect of increasing bias. Large current events are barely seen below 0.1V, their frequency rising with increasing bias above this threshold. We also repeated the experiments without a reference electrode, finding essentially identical results over the range of 0.1 to 0.5V (Table 1) presumably because the large-area substrate used in the STM experiments serves to stabilize surface potential somewhat. The existence of a threshold bias, below which these high-conductance states do not occur, implies that they are not an equilibrium property of the protein.

We investigated the molecular specificity of the $\alpha_V\beta_3$ -RGD binding by comparing the selectivity of RGD functionalized surfaces with the selectivity of surfaces functionalized with cyclic RGE, a cyclic peptide of the same charge (RGD, pI=6.09; RGE pI=6.18) and differing by only one carbon atom (Figure S3). We also investigated the effects of functionalizing only the probe or substrate. The results of these experiments are summarized in Table 2 (no events were observed without functionalization). Essentially the same frequency of events was observed with only one electrode functionalized as with both, consistent with the known single binding site for RGD peptide. [17] Significantly more selectivity was obtained with RGD peptide than with RGE ("Ratio" column in Table 2). We can conclude that one chemical contact is required for the high conductance state to be observed, and that the specificity and frequency of events increases with the chemical specificity of the contact. In addition, specific binding results in larger current peaks at larger retraction distances than obtained with less-specific binding. The peptide coating probably retards the denaturation often observed when proteins bind bare electrodes, as suggested by the specificity of the binding. [18] These results are intriguing, but STM measurements are difficult to interpret. We changed the gap with time in order to explore a wide range of gaps, so that contact conditions were changed during the course of a measurement. Fixed gap measurements of molecular binding are possible with small, well defined molecular systems[29] but difficult to carry out with a large protein. The absolute value of the gap at any one time is not known with precision. In addition, the bias cannot be changed in a constant current mode without changing the gap when the servo control is active. The nanoscale geometry of the electrode surfaces is not known, so many molecules could interact with the probe at any one time. Finally, STMs are subject to electrical and mechanical noise that can sometimes generate current spikes spontaneously.

Fixed Gap Device Measurements

A fixed gap tunneling device has significant advantages over STM measurements. These are: (1) The gap does not change with electrical operating conditions, so that the bias may be changed without changing the gap; (2) The gap may be determined with some precision by TEM measurements; (3) The device is much smaller, so the associated electrode capacitances are smaller and the corresponding frequency response is higher. (5) With small enough electrode dimensions, the binding of a large number of molecules at once becomes improbable, and the constant electrical properties of the junction permit the detection of a single-molecule binding event via the generation of distinct two-level signals. The layout of our MEMED is shown in Figure 3A. It is fabricated much as described in our earlier paper [21]. The device is fabricated on top of a 25 nm thick SiN membrane with a first 10 nm thick Pd electrode, onto which 4 to 5 nm of Al_2O_3 is deposited by ALD, followed by a second 10 nm Pd electrode. The choice of gap size was dictated by the largest distances $Z_0 + Z$ at which events were observed in the STM. The device is passivated on top with SiO_2 . Devices were fabricated in the Nanofabrication Facility at ASU on top of substrates provided by Norcada Inc. (Edmonton, Alberta). A TEM image of a cross section of the junction (cut out using a focused ion beam mill) is shown in Figure 3B. Our original devices had large (micron-scale) openings, but we wanted to restrict the region over which proteins could be captured to dimensions small enough so that the capture of multiple molecules in the tunnel

junction was unlikely (illustrated schematically by the capture of a single integrin in Figure 3A). We used RIE to thin the underlying SiN substrate to ~ 15 nm, followed by e-beam lithography using a hard mask and further RIE etching (Figure 3C and Methods). In this way, we produced slits that were 50 to 100 nm across and up to 400 nm long, though the active area was defined by the top electrode widths of about 100 nm (Figure 3C). After cleaning and functionalization (Methods) the device was immersed in 1mM phosphate buffer (pH=7.4) with the electrode potentials controlled with respect to an Ag/AgCl electrode coupled via a salt bridge (SB in Figure 3A). Potential control is more important for small-area junction devices [30] because the area of the electrodes exposed to the electric double layer is minute ($\sim 10^{-15}\text{m}^2$) so that small changes in interfacial charge can change the surface potential significantly (In contrast to the STM where a macroscopic substrate stabilizes the potential).

Our devices often suffer from failure of the passivation layer (SiO_2 in Figure 3B) so that, even in the dilute electrolyte, leakage currents can be substantial (e.g., a few nA, Figure 3D). Control experiments show that these currents are essentially the same when measured between any one of the tunneling electrodes and a reference electrode in solution, and that they increase as damage to the top passivation layer becomes more obvious (Figure S4). Thus, these are currents that pass from exposed regions of the top electrodes via the electrolyte, and they are not a consequence of tunneling or electrochemical leakage across the tunnel gap itself. These leakage currents (Figure 3D) show no distinctive features beyond the shot- and system electronic noise (distributed as a Gaussian, Figure 3E). When 100 nM $\alpha_4\beta_1$ protein was added (Figure 3F) the background current falls a little, presumably because of passivation of the exposed electrodes by non-specific adsorption of this protein (see the cyclic voltammograms in Figure S2). Once again, the signal is featureless, displaying the same intrinsic noise as observed in buffer alone (Figures 3F,G). (In contrast to the STM measurements, no features have been observed in the fixed-gap devices in any measurement taken in the presence of $\alpha_4\beta_1$.) However, once $\alpha_v\beta_3$ protein is added to the same device, dramatic jumps occur in the current (Figure 3H). The background leakage current is reduced by about 20% on addition of the protein, consistent with the passivation effect seen in the macroscopic electrochemical measurements (Figs 1B, C) and presumably reflecting passivation of exposed regions of the top electrodes. The interesting new feature observed is that the signal jumps up from the baseline to two well defined levels (Figure 3I) in this case. The levels are separated by nearly 0.5 nA, corresponding to ~ 1.5 nS conductance ($V_t = 0.3\text{V}$). This is consistent with the surprising result observed in the STM measurements but observed here in a device of a known and fixed 5 nm gap (Figure 3B). Even if multiple molecules bind in the gap, their number cannot exceed about ten, so these conductances cannot be accounted for by a large number of parallel conduction paths. Events like this have been recorded in two devices held under potential control, and in three other devices in which potential was not controlled but very similar behavior was observed (Figure S5). In order to eliminate the possibility that the signal comes from a damaged region of the junction that might be small enough to sustain conventional tunnel currents, we tested sample chips from each wafer with the chemistry we have used previously for tunneling detection of DNA nucleotides, [30] obtaining no signals at all.

A series of current-time traces taken at biases from $V_t=100$ mV to 300 mV ($V_t=0$) are shown in Figure 4. Below $V_t=100$ mV no features are observed and the traces have an identical noise distribution to the controls (Figures 3D–G). At 100 mV, jumps in current start to occur. These are quite similar over periods of many seconds and give rise to a distinctive “bump” in the amplitude distribution (see the histogram to the right of the trace). Such two-level signals (2LS) are indicative of a single switching event, therefore most likely of the binding of a single molecule (other examples of 2LS switching are given in Figure S5). As the bias is increased, both the amplitude and frequency of switching events increases, with a transition (in this case) to three-level signals (observed at $V_t=250$ and 300 mV). The slits are big enough to capture more than one integrin, so we cannot rule out the possibility that signals with more than two levels reflect the simultaneous binding of more than one molecule, though the upper limit, dictated by the device size, is about ten molecules.

The traces shown in Figure 4 span 10s, over which time the signal amplitudes appear quite uniform. The signal amplitudes can change significantly over longer times. Accordingly, we have analyzed each stable run of signal separately for two devices at two reference potentials with the results summarized in Figure 4B. The vertical spread of data represented by the same symbol at the same bias gives a measure of the change of signal amplitude from one burst of 2LS signal to another, and could represent changes in the binding of a given molecule, or the capture of an entirely different molecule. In cases where three (or in one case four) levels appeared, they are analyzed separately and the current jumps referred to as peak 1 (2LS), peak 2 (3 LS) and peak 3 (4LS) in the caption of Figure 4. The first noticeable feature is that, in contrast to the STM data (where the gap was changed continuously) the distribution of switching currents is remarkably narrow (a factor of four at most in a given set of conditions). The switching currents all increase with bias, but with a ~ 0.1 V threshold for the onset of signals (as was observed with the STM measurements). The “on” state conductances are all on the order of 0.1 nS, quite a surprising result given that one of the two electrodes must contact the protein by means of a non-specific interaction that could be quite variable. The second feature of these data is the significant differences between different chips (e.g, traces f and g vs. traces a-d). Repeat runs with a given chip overlap somewhat (e.g., a and d, and f and g) though a given chip can change significantly in slightly different experimental conditions (c.f., a and e). Linear fits to runs a,b, f and g intercept the zero current axis between 120 to 200 mV, consistent with the observed threshold for the onset of signals. Linear fits to data sets d and e have a much smaller slope, but once again, no signals were observed below 0.1 V for these runs too.

In contrast to the limited range of measured current jumps, there is an enormous variation in charge transferred (Figure 4C) during each on-state. Many of the events overlap the distribution observed in STM measurements (Figure 2C, data overlaid on Figure 4C). In addition to the long-lived “on” states observed in the STM, the improved frequency response in these small devices reveals a population of fast events. A fast event is labeled “S” in Figure 4A, with the corresponding charge transfers labeled “S” in Figure 4C. The distribution for the longer-lived events is labeled “L” in Figure 4C with a typical corresponding pulse labeled “L” in Figure 4A. In these time resolved measurements it is clear that the wide distribution for the values of charge transferred is a consequence of the large spread of “on” times (Figures 5C,D). The short-time distribution of “on” times is

exponential and does not change significantly with bias, with a mean time of 31 ± 2 ms (Figure 5A). The ratio of the total “on” time to the total “off time” can be calculated from the number of points under the upper level peaks divided by the number of points in the baseline peak in the amplitude histograms for each run of 2LS signal (examples of these histograms are given to the right of Figure 4A). A plot of the means of these “on” to “off” ratios is shown in Figure 5B (where the error bars are ± 1 sd of the distribution of values at any one bias). The bias threshold of about 100 mV is clear, and the fraction of on-time increase roughly linearly with increasing bias.

A linear plot of decay times fails to capture the full range of events, so scatter plots of current vs. logarithm of the on-time for each event are presented in Figures 5 C and D. On this logarithmic display, they show the clustering of times that gives rise to the two distinct peaks in the histogram of $\log(Q)$ (Figure 4C). These plots also show that the on-currents are linearly related to the logarithm of the on-times for each event.

Discussion and Conclusions

Let us first recapitulate the main experimental findings. A large, electrochemically inert, protein can carry currents of nearly a nanoamp under a bias of a few tenths of a volt for times that can approach a tenth of a second, and over distances between electrodes of 5 nm or so. This effect appears only above a threshold bias of about a tenth of a volt, and the observed currents fluctuate on and off on ms to 0.1s time scales. The log of the “on-time” is linearly related to the observed peak current and the overall time in the conducting state increases as bias is increased. Much of the fluctuating current is a two-level signal. Taken together with repeated observations in gaps small enough to accommodate only a few (1 to 10 molecules) we conclude that the 2LS signals probably originate with a single molecule, likely explaining the rather reproducible current-switching levels observed at a given bias. Signals with multiple levels are larger, suggesting that they may reflect contributions from more than one molecule trapped in the gap. Finally, the signals are sensitive to the nature of the chemical tether used to link the protein to one of the electrodes. Events are more frequent with a specific (RGD) tether and its specific target ($\alpha_V\beta_3$) than with an off target protein ($\alpha_4\beta_1$) or a less selective tether (RGE). This observation is useful, in as much as it demonstrates that the proteins on the electrode are close enough to their native state for this recognition to occur. It also suggests that artifacts owing to contamination are unlikely to be responsible for these signals. The specific tethering has another important consequence, in that electronic features are enhanced by specific binding, manifested in both greatly increased peak currents, and much larger peak-current distances (Figure 2, Table 2). We note that other integrin proteins (e.g., $\alpha_V\beta_5$) bind RGD peptides with an affinity intermediate between $\alpha_V\beta_3$ and $\alpha_4\beta_1$ and could be used to further test the specific role of the binding ligand.

It is clear that these large currents cannot be accounted for by a pure-tunneling mechanism. It is known that high currents (nA) can be measured across large area junctions containing thousands of ferrocenes [31] but we are unaware of any widely-accepted mechanism for long-distance transfer of large amounts of charge at high (nA) rates over many nm in single molecules that lack redox centers. We examine some possibilities below.

Mechanisms involving localized intermediates

Long-range transfer of significant charge can occur in the presence of a high density of redox centers, spaced closely enough for coherent overlap to occur. [3] Contacting redox cofactors that are degenerate within the energy range of vibronic broadening can support coherent transport over significant distances via a flickering resonance mechanism. For example, in G-rich DNA where readily oxidized moieties are stacked in close contact, band-like transport can occur over distances of ~1.5 nm. [32] Systems much larger than this would require either significant tunnel transport ($\beta \ll 1$) to couple the delocalized regions, or intermediate redox active centers that store electrons between transport steps (but with a small enough reorganization energy to facilitate rapid redox turnover). The $\alpha_V\beta_3$ protein contains binding sites for Ca (or Mg in our case) ions, [16] but these are not electrochemically active in the accessible potential range here. Thus, transport involving intermediate redox centers seems highly unlikely.

Small gap electrochemistry

The discussion above is predicated on the macroscopic electrochemical measurements that show significant reduction of voltammometric features when protein is adsorbed onto the electrode surface (Figures 1B,C, Figure S2). How might the situation change when two electrodes are in close proximity? A redox-active species with fast turn-over kinetics in the gap can generate significant currents by cycling between the electrodes (reduced at one, oxidized at the other) when the electrode potential difference exceeds the formal potential. [33] The resulting current is given approximately by De/d^2 where D is the diffusion constant ($5 \times 10^{-6} \text{ cm}^2/\text{s}$ for a small ion) and d is the gap. For $d=5 \text{ nm}$ this is about 3pA, much smaller than the features observed here. To account for the observed currents one would have to postulate a mechanism that continued to release hundreds or thousands of highly reactive ions, and do so only when the target protein bound specifically.

Yet another possibility is that the presence of the protein could somehow generate rapid proton transport between the two electrodes. The maximum bulk currents for hydrogen adsorption are $\sim 60 \mu\text{A}/\text{cm}^2$ (Figure S2). For an electrode area of $400 \times 10 \text{ nm}$ (a maximum area for one of the MEMED electrodes) this would be 10^{-3} pA . The protein would have to enhance hydrogen transfer by a factor 10^5 or more, do so in way that is sensitive to the attachment chemistry, and generate a proton current from the hydrogen. This seems unlikely.

Transient Charging

Could the observed current pulses correspond to transient charging/discharging of ionized groups on the surface of the protein as a consequence of local pH changes near the electrode surface? At pH 7.4, the net charge for $\alpha_V\beta_3$ is $-45.1e$, and $-35.5e$ for $\alpha_4\beta_1$ (chargeable groups mainly include R, H, K residues (positive) and D, E residues (negative)). Millions of adsorbed proteins would be required to account for even the smallest charge transfers observed here.

Heating

Are we correct in assuming that the protein retains a native structure? Since the mechanism of charge transfer is unknown, we cannot assume that is dissipationless (as tunneling is). In the worst case where all the electrical power is dissipated in the protein, $P \sim 0.5$ nW (1 nA, 0.5V). The temperature rise at the surface of a hollow sphere of radius a immersed in an infinite heat bath of thermal conductivity K (0.6W/m.K for water) is given by $\Delta T = P/4\pi K a$ [34] giving, for $a=5$ nm, $\Delta T < 1$ K. In practice, the behavior of the junctions is reasonably reproducible (see the discussion above) when the bias is below 0.5V. Thus, thermal denaturation seems unlikely.

Is coherent transport possible?

It has recently been proposed that, rather than simply being aperiodic, many functional proteins may have rather special structures that poise them at a critical point (quantum criticality) that lies between the insulating states generated by disordered systems and the conducting states found in metallic systems. [24] In the space of all possible random arrangements, the quantum-critical state is extremely improbable, unless some (unknown) evolutionary pressure led to it. Systems at this point have the property that small fluctuations can push them into either a pure insulating or a pure metallic state. One indication of quantum-criticality is found in the distribution of energy-level spacings for a system. In the case of a metal, levels follow a Gaussian distribution

$$P_w(s) = \frac{\pi s}{2} \exp\left(-\frac{\pi s^2}{z}\right). \quad (1)$$

A random-Poisson distribution applies to insulators:

$$P_p(s) = \exp(-s). \quad (2)$$

At the quantum critical point, the distribution becomes a modified Poisson function:

$$P_T(s) = 4s \exp(-2s). \quad (3)$$

Analysis of a number of conducting polymers and insulators finds that they fit distributions (1) and (2), as expected, whereas many functional proteins fit distribution (3). This is not proof of quantum criticality (nor does it address the equally important question of coherence [23]) but we can at least ask if the electronic structure of $\alpha_v\beta_3$ protein is consistent with one of these distributions. Accordingly, we carried out electronic structure calculations using the known structure of the hydrated protein (Methods). At this stage, we have modeled only the conformation of the protein prior to RGD-binding. Conformational changes are expected on binding but their effect has not yet been modeled theoretically, so their impact on the electrical properties of the protein are currently unexplored. We have used cumulative distributions of energy level spacings because these avoid binning artifacts. The results are

summarized in Figure 6 (orange circles) together with the cumulative distributions corresponding to the three distribution functions listed. The calculated points lie on the quantum critical distribution (Equation 3). Thus, we cannot eliminate the possibility that collective quantum effects play a role. The nature of the conducting state, charge injection into the state, and the mechanism whereby a local field might drive the transition remain obscure.

Origin of the fluctuations

Assuming that an external field could indeed drive a protein into a conducting state, can we shed light on the subsequent fluctuations? They are reminiscent of the “contact” fluctuations observed in many molecular junctions. [29, 35] If, indeed, the protein is driven into a long-lived conducting state, then, given that one contact is well-defined (and long lived) via the RGD-protein interaction, the weak link would be the non-specifically bonded contact at the second electrode. A ‘weak link’ that dominates the system conductance might qualitatively account for the correlation between peak current and “on” time (Figures 5C,D). The matrix element connecting two sites A, B can be written in terms of the tunneling currents between all the intervening atoms according to the tunneling flux theorem [36]:

$$V_{AB} = -\hbar \sum_{\text{all coupled states}} J_{ab} \quad (4)$$

where J_{ab} is the tunnel current operator evaluated between all intervening atomic sites. Tunnel current increases with the magnitude of the matrix element that connects the sites, or, in the simplest possible case, $V_{AB} = fi$ where f is a scale factor (that could presumably be calculated using eqn. 4, given a structure). If V_{AB} also describes the bond strength coupling sites A and B, then the bond lifetime would be given by

$$t \propto \exp(V_{AB}/kT) \propto \exp(fi/kT).$$

Thus, the logarithmic dependence of current on bond lifetimes (Figures 5C and D) may reflect a direct correspondence between the matrix element for tunneling and the bonding energy of the link that dominates conductance.

Potential as a single molecule detector

The appearance of 2LS noise is a distinctive feature of specific binding and might therefore signal the detection of a target molecule even in a noisy environment, such as that which may be found in human serum, for example. As a first step in exploring this, we tested a device in Serasub (CST Technologies, Great Neck, NY), a protein-free serum substitute. In the undiluted serum, leakage currents were very high (5 nA initially, falling continuously with time) but the noise distribution in the presence of 0.1 nM $\alpha\sqrt{\beta}_3$ was strikingly different from that in the serum alone, or serum plus $\alpha_4\beta_1$ (Figure S6) where the appearance of 2LS is quite clear. Thus, the conductance fluctuations described here might be exploited for the development of a label-free single-molecule electronic detection system that could function in a noisy environment.

In conclusion, we have shown that an electrochemically-inert protein can be driven into a highly conducting state via the application of a small bias in a nano-junction device. This effect requires specific chemical attachment to one electrode in a fixed-gap device, offering a path to single molecule detection. Whether the specificity arises simply from an increased capture probability, or an electronic change on binding, is unknown at this point. The magnitude of the conductance fluctuations is completely unexpected, and without a known explanation, though the argument that the protein may exist in a quantum-critical state cannot be dismissed. If, as has been suggested, [24] other functional proteins are quantum-critical, then they too might exhibit conductance fluctuations, possibly accounting for some of the anomalous transport in proteins surveyed in the introduction to this paper. If so, this would raise the perplexing question of what evolutionary advantage could flow from this otherwise highly unlikely state.

Methods

Functionalizing STM probes and substrates

The STM probes were etched from a 0.25 mm Pd wire (California Fine Wires) by an AC electrochemical etching method, and then insulated with high-density polyethylene, leaving an open apex of a few tens of nanometers in diameter. [25] Before use, the probes were tested in 1 mM phosphate buffer (pH=7.4) at 0.5 V bias to ensure that the leakage current was <1 pA. For functionalization, the probe was gently cleaned using ethanol and water, respectively, and then dried in a flow of nitrogen. The probe was immersed in a 0.5 mg/mL peptide solution for ~20 h. After that, the probe was taken out, rinsed with water, dried with nitrogen gas, and used immediately. Palladium substrates were prepared by depositing a 200 nm palladium film onto a 10 nm titanium adhesion layer coated onto a 750 μ m thick silicon wafer using an electron-beam evaporator (Lesker PVD 75). The substrate was annealed with a hydrogen flame immediately before being functionalized by the same method as that for probe. Fourier transform infrared (FTIR) spectroscopy was used to characterize the functionalization (Figure S3b). Pd substrates were also treated using a UV- Ozone cleaner and then by H₂ gas reduction (to remove PdO). The FTIR spectra in Figure S3b demonstrate a successful modification with peptide.

Preparation of RGD and RGE

RGD peptide, cyclo(Arg-Gly-Asp-D-Phe-Cys), was obtained from Peptides International (Louisville, Kentucky). RGE peptide, cyclo(Arg-Gly-Glu-D-Phe-Cys), was synthesized by CPC Scientific (Sunnyvale, California) with a purity>95%. For chip and STM tip functionalization, peptides were dissolved to a final concentration of 0.5 mg/mL in a freshly degassed 1 mM phosphate buffer (pH=7.4), which was prepared using water from a Milli-Q system with a resistance of ~18 M Ω and a total organic carbon contamination below 4 ppb.

Reference electrodes

Ag/AgCl wires were prepared by soaking Ag wires in chloride bleach for at least 4 hours. The electrode was then housed in a pipette tip, using 3 M KCl as the electrolyte and 5% agarose gel as the plug (Figure S2c). As there was some KCl leakage from the gel plug into the STM cell (which can increase the tip leakage current) the KCl concentration was

reduced to 10 mM for STM measurements. This resulted in a shift of about 100 mV in reference potential, and it has been accounted for in references to V_r .

Cyclic voltammetry

Cyclic voltammetry was performed on a potentiostat (Model AFCBP1, Pine Instruments), with a Pt wire counter electrode, a salt-bridged Ag/AgCl reference electrode (3 M KCl), and a sweep rate of 5 mV/s. The area of Pd electrode was $0.5 \text{ cm} \times 1 \text{ cm}$. Successive sweeps are increased by $\pm 50 \text{ mV}$.

STM Measurements

Break junction measurements were carried out with a PicoSPM scanning probe microscope (Agilent Technologies). The Teflon liquid cells were cleaned in Piranha solution and then sonicated in Milli-Q water three times (Note that: the Piranha is highly corrosive and must be handled with extreme care). The current set point was 4 pA. Different sample bias was applied to the substrate as required. For better surface charge control, an Ag/AgCl with a salt bridge (10 mM KCl) was connected to the substrate (Figure S2c). The tip was approached to the substrate with an integral and proportional gain of 1.0 and then left to stabilize for 0.5 h. For break junction measurements, the tip was first brought into the set point position. After 5 s of waiting, the tip was retracted 10 nm from the substrate with a speed of 0.17 nm/s, during which a current-distance trace was recorded. Subsequently, the tip was re-engaged and the retraction process repeated. About one hundred traces were collected for each experiment and at least three independent experiments were conducted for each sample (using a different substrate and tip each time).

Fabrication of devices

Devices were fabricated on 25 nm thick suspended SiN membranes supported on 0.2 mm thick silicon wafers above $10 \mu\text{m}$ square openings. 10 nm thick Pd electrodes were deposited using electron beam evaporation, using EBL and lift off to define an electrode width of $20 \mu\text{m}$ in the active region of the device, with connections made to gold contact pads at the side of the device. Eight devices (each containing three junctions if separated, see Figure 3C) were fabricated on a single one-inch wafer, and the assembly was then coated with a 4.8 nm thick layer of Al_2O_3 using 22 cycles of atomic layer deposition. Top electrodes are placed over the ALD using a further 10 nm deposition of Pd followed by e-beam lithography to form nanowires of between 80 and 100 nm width as top electrodes. The ALD has a high density of pinholes, but these are generally avoided when narrow wires are used. Finally PECVD or ALD was used to cover the entire device with 20 nm of SiO_2 .

RIE Etching of devices

The SiN membranes were first thinned from below using a PlasmaTherm 790 RIE with CF_4 (50 sccm) and Ar (1 sccm) at 200W and 30 mT pressure for about 20 seconds, reducing the support membrane to 10 to 15 nm in thickness. 10 nm of Cr hard mask were grown thermally on the top of the SiO_2 passivation and either 80 nm of PMMA resist or 350 nm of ZEP520A spun on top of the Cr layer. The resist was patterned by EBL to expose the Cr

hard mask. The Cr hard mask was then opened by wet etching with Cyantek CR7s for 22 to 26 s

The underlying SiO₂ was opened by Floey RIE (CHF₃, 25 sccm, Ar, 25 sccm) using 200W at a pressure of 30 mT for 90s. The top Pd electrode was opened using a Plasma-Therm 790 RIE (CHF₃ 20 sccm, Ar 30 sccm and O₂ 5sccm) at a power of 200W and a pressure of 20 mT for times between 160 and 180 s. The Al₂O₃ dielectric was opened using Cloey RIE (BCl₃ 40 sccm) at 200W and a pressure of 15 mT for 55 to 60 s. The bottom Pd electrode was then etched in the Plasma-Therm RIE as described above, followed finally by Plasma-Therm RIE of the SiN support membrane (CF₄ 50 sccm, Ar 1 sccm) using 200W at a pressure of 30 mT for 240 to 300 s). The remaining Cr hard mask was removed using an Apex ICP-RIE (O₂ 8 sccm, Cl₂ 32 sccm, pressure = 10 mT, Coil RF = 800W, Platen RF = 20W, exposure time 8 s).

Characterization of device openings

Devices were imaged (Figure 3B) using a JEOL 2010 TEM in the HREM facility at ASU. Cross sections were cut using an FEI Novascan 200 Ga ion FIB. These were generally 100 nm thick and embedded in Pt/C.

Sample Preparation and Electrical Measurements

Chips were tested at each step to make sure no current could be measured across the tunnel junction (the Simmons formula[37] showing that currents should be negligible). Three top electrodes for each device were originally connected in a T (see Figure 3C) so that they could be tested for continuity prior to etching the openings. After formation of the openings, chips were cleaned using a UV-Ozone cleaner (Novascan) for 20 minutes on each side to remove organic contamination, followed by reduction of oxidized Pd in a flow of H₂ gas at 80°C. Chips were then soaked in 0.5 mg/mL RGD solution in 1mM phosphate buffer (pH=7.4) for 20 h. After that, chips were rinsed with water, blown dry with nitrogen gas and mounted in a custom cell with an integrated contact set for the tunnel junctions. An Ag/AgCl reference electrode coupled by a salt bridge was mounted in the sample chamber. The chamber was filled with 1 mM phosphate buffer (pH=7.4) and tested for leakage and to ensure that no spurious signals were being generated. Substantial (constant, dc) leakage was often measured at this point and it has been traced to a high density of pinholes in both the glass passivation layer and the alumina dielectric layer, often increasing with prolonged use of the device. However, no 2LS signals were observed. Integrin $\alpha_4\beta_1$ was purchased from R&D Systems (Bio-Techne), and $\alpha_v\beta_3$ from YO Proteins AB. 100nM integrin solutions were prepared using 1mM phosphate buffer and diluted into lower concentrations. The tunnel junction was biased using the voltage clamped output of an Axopatch 200B and current recorded using the Axopatch interface (Digidata 1550). The reference electrode was biased using a small, adjustable floating supply. The data acquisition rate was 100 kHz and the data were filtered with a 10 kHz low pass filter.

Electronic Structure Calculations

The exact numerical solution of the Schrödinger equation for the electronic states of large molecules such as proteins is a prohibitive task. Various approximations have been

developed, which reduce the problem to a one-electron problem in the effective field of the remaining electrons. Wave functions of molecules are usually written in the form of Linear Combinations of Atomic Orbitals (LCAO) $\phi_i = \sum_r C_{ir} \chi_r$, where ϕ_i is a Molecular Orbital (MO) represented as the sum of atomic orbital (AO) contributions χ_r . The one-electron eigenenergies and eigenvectors then can be determined from the generalized eigenvalue equation $HC = ESC$, where $S_{rs} = \langle \chi_r | \chi_s \rangle$ and $H_{rs} = \langle \chi_r | H_{eff} | \chi_s \rangle$ are the overlap and effective Hamiltonian matrices respectively. The effective Hamiltonian depends on the coefficients, which makes the problem nonlinear in C . This is the case in Hartree-Fock and Density Functional Theory (DFT) calculations, which then cannot be routinely carried out for proteins involving thousands of atoms. If we restrict our interest to the localization-delocalization problem in valence electrons and treat the two-electron part of the Hamiltonian as in the case of the electrons in metals, in an average sense only, we can apply semi-empirical methods. Once the positions of the atoms are known the Extended Hückel (EH) Molecular Orbital Method [38] is quite successful in calculating the MOs of organic molecules. The diagonal part $H_{rr}^{(EH)}$ of the EH Hamiltonian is given by the ionization energies of the AOs, while the off-diagonal elements are calculated from the diagonal

elements and the overlap matrix $H_{rs}^{EH} = \frac{1}{2} K (H_{rr}^{EH} + H_{ss}^{EH}) S_{rs}$ where the common choice for the empirical constant is $K=1.75$. This is similar in spirit to other tight binding Hamiltonians in solid state physics. For the numerical studies we selected X-ray diffraction data [39] of $\alpha_v\beta_3$ Integrin from the Protein Data Bank of RCSB. This structure does not include the RGD ligand, so the calculation cannot address the role of specific binding in modifying electronic properties. It consists of 959 amino acids and weighs about 190 kD including some coupled water molecules. The Open Babel tool [40] (The Open Babel Package, version 2.3.2 <http://openbabel.org> accessed April 2016) has been used to add hydrogen atoms to the X-ray diffraction structure (PDB ID: 4G1M). The EH calculations have been carried out by the numerical package YAeHMOP (<http://yaehmop.sourceforge.net/>, accessed April 2016). There are $N=65322$ valence electron AOs and the EH Hamiltonian and overlap matrices are of dimension 65322×65322 . The source code of YAeHMOP has been modified from 32 bit operations to 64 bit operations to accommodate such large matrices. The energy levels computed with the EH method have been analyzed with a statistical method of Ref. [41]. First, a number of degenerate energy levels have been removed from the spectrum. These degenerate levels do not belong to the spectrum of the protein, instead they come from many isolated identical molecules (such as H_2O) which do not interact with the protein, and are just artifacts in the structure file. We retain those water molecule states that are coupled strongly enough to perturb the protein spectrum. After this procedure $N=55837$ energy levels remained.

The distance between consecutive energy levels fluctuates in the spectrum. The raw distance between levels $\sigma_n = E_{n+1} - E_n$ can be normalized using the mean energy level distance $\Delta(E)$ at a given energy window around E . The ratio $s_n = \sigma_n / \Delta(E_n)$ is called the level spacing. The mean energy level distance is calculated as the average of k level spacings to

the left and to the right $\Delta(E_n) = \frac{1}{2k+1} \sum_{j=-k}^{+k} \sigma_{n+j}$. The choice of k depends on the variability of the density of energy levels. Statistical averaging would require large values,

while fast variation (especially singularities) in the density of energy levels restricts our choice to low values. We have found that a choice of $k=2$ ensures the stability of the distribution in our examples. Next, following standard procedures [42], the cumulative spacing $I(s) = \int_0^s P(s) ds$ is calculated and compared to the theoretical predictions. For the Poissonian statistics (Eqn. 2) $I_P(s) = 1 - e^{-s}$, for the Wigner surmise (Eqn. 1)

$I_W(s) = 1 - e^{-\pi s^2/4}$ and for the semi-Poissonian transitional statistics (Eqn. 3)

$I_T(s) = 1 - (2s+1)e^{-2s}$. In Fig. 6, we show the cumulative level spacing and in the inset we show the difference of the calculated to the theoretical $I_T(s)$. We can see that without any parameter fitting the spacings for $\alpha\sqrt{\beta_3}$ Integrin follow the critical theoretical curve with astonishing precision. Note, that no parameter fitting is involved in the procedure, the calculated spacing distribution has a less than 4% error like in the case of systems of critical quantum chaos [43], which are purely theoretical models as opposed to our case, where the positions of atoms in the protein are obtained experimentally. We should emphasize again, that finding a large tight binding Hamiltonian tuned exactly or almost exactly to the critical point by random chance can happen only with an astronomically low probability. So, finding just a single protein with about 1000 amino acids having this property at random is impossible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

STM current-distance traces show large current peaks at large distances when integrin protein binds specifically to an electrode. (A) schematic layout of the experiment. The probe tip and the substrate were functionalized with a cysteine-containing cyclic RGD peptide that binds $\alpha_v\beta_3$ integrin but not $\alpha_4\beta_1$. Electrodes were biased with respect to an Ag/AgCl reference connected via a salt bridge. Cyclic voltammetry on 0.5 cm² Pd-coated substrate modified with cyclic RGD peptide (inset) in 1 mM phosphate buffer (B) and after the addition of 10 nM $\alpha_v\beta_3$ (C). Logarithm of current vs retraction distance (ΔZ) for solutions containing 10 nM $\alpha_v\beta_3$ (D) or $\alpha_4\beta_1$ (E). Inset in E shows the rapid decay of current in buffer alone as the tip is retracted from its initial set-point (4pA at 200mV, corresponding to about a 2nm gap before retraction).

Figure 2.

Summary of current peaks observed in STM retractions. Scatter plots of logarithm of peak current vs. retraction distance (i.e., from the initial set-point gap of about 2nm to the current peak) for $\alpha_v\beta_3$ (A) and $\alpha_4\beta_1$ (B). (C) The distribution of charge in each peak (obtained by integrating each current-time curve) for the two proteins. The scale is logarithmic in charge.

Figure 3.

Fabrication of the fixed-gap tunnel chip and some typical results at a bias of $V_t=300$ mV (and $V_r=0$ V vs. Ag/AgCl). (A) shows the layered structure of the MEMED with an opening that exposes the electrodes to the solution, sized (in this illustration) so as to trap just one integrin molecule. Electrodes are functionalized with cyclic RGD peptide and biased as shown. (B) TEM image of a cross section of the junction showing the 4.8 nm dielectric layer. (C) TEM image of a “T” shaped top electrode that was milled with three RIE-etched slots (white openings). (D) Typical current trace in phosphate buffer. The high baseline current comes from stray leakage paths, and the noise distribution (E) is well-described by a single Gaussian. When the control protein, $\alpha_4\beta_1$ was added (F) the noise distribution did not change. When $\alpha_V\beta_3$ protein was added (H) large current jumps to well defined levels (I) were observed. The background leakage was also reduced (as was observed in the cyclic voltammetry) indicating that the adsorbed protein passivates the surface.

Figure 4.

Bias dependence of chip-signals from $\alpha v \beta_3$ protein. (A) Some selected current traces, showing the onset of current jumps above the baseline leakage, starting at a bias of 100mV, with fluctuations increasing as bias is increased (amplitude histograms are plotted to the right of each trace to the same current scale as each trace). Note that the current scales are different for each trace to show detail. The background leakage increases linearly with bias. (B) Scatter plot of the separation of peaks in the amplitude histograms as a function of bias. Sets of identical symbols at a given bias show the values obtained from an analysis of each burst of signal in a given run, so that the spread of points represents the variations from burst to burst of signal in a given recording. Data are from 2 chips, chip 6 (a-e) and chip 7 (f,g). Points d are for a first run with chip 6, and a,b, and c are for a second run. Here the b points are for a second peak in the distribution, and c are for a third peak. Runs were taken at a bias with respect to the reference of 0V except for e, and f which were taken at + 50mV. (C) Distribution (gray bars) of charge under each signal peak for the chip data at 300mV (gray) and for the STM data at 200 mV (red data). Over these ranges of potential and bias, both electrodes were in a region that avoids Faradaic currents.

Figure 5.

Time structure of the current fluctuations. (A) The short-time distribution of "on" times is Poisson and identical for two values of bias (as labeled). (B) The fraction of time spent in the "on" state as a function of bias (red squares). Additional black points are for the sharp additional features that appear as the larger current steps in the histograms at 250 and 300mV, possibly representing binding of more than one molecule in the junction. (C,D) Showing scatter plots of the "on" time vs. amplitude of the current peak for individual signal events. The time scales are logarithmic so the linear fits (red lines) demonstrate an exponential relationship between peak current and on-time. The fitted slope is 0.006 pA^{-1} in each case.

Figure 6.

Calculated cumulative level spacing distribution (orange circles) for $\alpha\sqrt{\beta_3}$ Integrin protein (PDB ID: 4G1M). Only one in every 2000 values are shown for clarity. For comparison the theoretical curves $I_P(s)$ (black line), $I_W(s)$ (blue line) and $I_T(s)$ (yellow line) are also shown. Inset: Difference between the data curves and $I_T(s)$ are shown. Each value is plotted. The error is below 4 % in probability. The calculated distribution is compatible with quantum criticality.

Fraction of STM retractions that showed large current events for three values of the probe bias with the substrate either floating with respect to the solution (Reference=“None”) or at 0V vs Ag/AgCl (Reference = “Ag/AgCl”). The fraction in parenthesis corresponds to events that overflowed the current limit of the amplifier (11 nA). Results are shown for the two proteins $\alpha_4\beta_1$ and $\alpha_v\beta_3$.

Table 1

Bias	0.5V	0.5V	0.2V	0.2V	0.1V	0.1V
Reference	None	Ag/AgCl	None	Ag/AgCl	None	Ag/AgCl
$\alpha_4\beta_1$	0.24 (0) N=133	0.3 (0.01) N=99	0.12 (0) N=144	0.15 (0) N=74	0 N=87	0.01 N=91
$\alpha_v\beta_3$	0.56 (0.09) N=137	0.58 (0.2) N=100	0.26 (0.03) N=136	0.32 (0.01) N=75	0.06(0) N=84	0.07(0) N=96

Table 2

Effect of electrode modification on the frequency of large current events. RGE is a cyclic peptide differing from RGD by only one carbon atom.

Ligand	Modification	$\alpha_4\beta_1$	$\alpha_v\beta_3$	Ratio
RGD	Tip + Substrate	0.19	0.57	3.1
	Tip only	0.21	0.55	2.6
RGE	Tip + Substrate	0.25	0.31	1.2
	Tip only	0.28	0.36	1.3
None		0	0	NA