Supporting information for “Protein binding and orientation matters: Biased-induced conductance switching in a mutated azurin junction”

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for
Protein binding and orientation matter:
Bias-induced conductance switching in a mutated azurin junction

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Introduction

This document contains additional supporting experimental protocols, data, figures, and discussions that are details of supplementary information to the main text, and support for the presented results. The information presented is:

1. The employed materials and methods
2. UV and vis absorption spectra of N42C Az
3. XPS results of N42C and WT Az on Au surface
4. PM-IRRAS data
5. Overlay of the IETS spectra of WT and N42C mutant Az
6. Reproducibility checks
7. AFM data
8. Arrhenius plot
9. UPS data
10. I-V curve fitting analysis
1. Materials and methods:

Proteins:

Wild type Azurin was isolated from P. aeruginosa using the published procedure of Ambler and Brown\textsuperscript{1, 2} except that the azurin elution buffer for the CM-cellulose column was pH of 5.0 instead of 4.6. The mutated azurin \(\text{N}42\text{C Az}\) was constructed and expressed following the published procedures\textsuperscript{3, 4}. It was isolated in its apo-form in the presence of dithiotreitol in order to prevent binding to other thiol containing molecules. To produce the holo-\(\text{N}42\text{C Az}\), a slight excess of Cu(II) together with a five-fold ferricyanide were added to the apo-form. That yielded the typically blue-colored, \(\text{C}42\) disulfide linked dimers which were employed for producing the monolayers.

\textbf{Figure S1:} Three-dimensional structure of holo-\(\text{N}42\text{C Azurin dimers}\)\textsuperscript{4} (based on PDB 4AZU)

\textbf{Gold nanowires:} The Au nanowires were fabricated using the process described by Martin et al\textsuperscript{5}. The Au metal is electrodeposited in on a nanoporous alumina membrane with 200 nm diameter nanopores. By electrodeposition from 35 mM AuCl\textsubscript{3} solution at a constant current of 1 mA for 4 hrs, Au nanowires of \(\sim 5-6 \mu\text{m}\) long were formed in the membrane. These metal replicas are then released into the solution by dissolving the alumina membrane in an aqueous base of KOH and suspending them in water, which served as the transport medium during the assembly process. They were 100-300 nm in diameter and 5-6 \(\mu\text{m}\) long.

\textbf{Protein monolayers formation on Au surface:} Au microelectrodes (1 \(\mu\) thick), were fabricated on top of a Si wafer by using photolithography, yielding a substrate that contains 260 devices. The Au electrode surfaces were initially cleaned by bath sonication in acetone/ethanol (3 min each)
and thoroughly rinsed in Milli-Q (18 MΩ) water. The Au surface is activated using ozone (UVO-cleaner Model No: 3422A-220) for 10 mins, followed by treatment with hot ethanol for 20 mins. The activated Au substrates were then rinsed with water and immediately used for incubating with the examined protein solution. Az monolayers were prepared by immersing substrates in a 1 mg/mL solution of WT Az or N42C Az mutant for 3 hrs followed by rinsing with clean water, before drying with a mild nitrogen stream. After monolayer formation, Au nanowires were dielectrophoretically trapped to complete the circuit/junction 6.

**Dielectrophoretic trapping:** The Au substrate samples modified with the protein monolayer were placed under an optical microscope to position probes on the junctions before applying an AC voltage. 25µL of the nanowire’s suspension was dropped gently on the protein monolayer and an AC Voltage ($V_{AC} = 1.5 \, V_{p-p}; \, f =1 \, MHz$) was applied to the substrate electrode for 20 min using a function generator (Model 8122, Tabor electronics Ltd.).

During the dielectrophoretic trapping process Au nanowires form bridges between the two micro-electrodes. Empirically it has been found that one of the two nanowire-electrode bridge contact is always shorted (See Fig. 2A). The likely cause for this shorting is the force by which the nanowire is pulled into the gap between the electrodes, which is exerted on the first electrode with which it makes contact. This force is apparently sufficient to destroy the monolayer on that electrode. The wire’s other end then performs a “soft landing”, preserving the monolayer on that electrode. Such nanowires can be observed using a standard optical microscope, allowing quick marking of useful junctions, i.e., those where the nanowire is clearly bridging the electrodes (see Fig. S2). Such selection left some 10% of all the contact pairs, i.e., between 20-30 out of the 260 contact pairs contain successfully trapped nanowires.

The final architecture of all measured junctions contains only a single Au nanowire between the two gold microelectrodes. Cases of two or more nanowires bridging between the two contact pads were rather rare and could be easily detected using an optical microscope, prior to the electronic transport measurements.
Figure S2: Bright-field optical microscopic image of microelectrode A) & C) Illustrate situations where the AuNW are not aligned/trapped. B) Shows where a AuNW bridges the microelectrodes.

**Current-voltage and conductance-voltage Inelastic Electron Tunneling Spectroscopy, IETS:**

The samples were loaded on an electrically floating sample stage and placed in a cryogenic probe station (Lakeshore TTPX). Current-voltage (I-V) measurements were performed to assess the transport efficiency across the junctions, using a Keithley 6430 sub-Femtoamp Source-Meter Unit, with a voltage scan rate of 20 mV/s. For all measurements, the substrate (bottom electrode of the junction) is grounded, while the AuNW electrode was biased, in a consistent manner (to ensure the bias polarity was in the same direction for all measurements). In each sets of experiments, scans were acquired that started and ended at 0 V (i.e., voltage sweep was from 0 to -1.2 V, -1.2 V to 1.2 V, 1.2 V to 0 V). This was done in order to check for a possible effect of the polarity of the initial voltage on the measured I-V characteristic. The measurements were done in 10^{-4} to 10^{-6} mbar vacuum, with the higher vacuum at lower temperatures.

Due to the mesoscopic nature of the junction, the variation in absolute currents was large (approximately 2 orders of magnitude). However, about a quarter of the junctions (from 30-40 successfully trapped nanowires per preparation), measured by I-V revealed high current magnitude. Such junctions that were identified as partially shorted were not analyzed in the current-voltage distribution.
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For conductance and IETS measurements, the system was cooled to ~10K (p=1×10^{-6} mbar). First and second derivatives of the current were recorded as the first and second harmonic signals, respectively, using lock-in techniques (two Stanford 830 lock-in amplifiers) with a DC bias added to an AC signal at a frequency of 443 Hz and applied to the samples. The measurements were done with a DC voltage step of 4 mV and an applied AC voltage of 8 mV. In control experiments on the electrode surface without proteins, only shorts (mA range currents) were observed, and no open circuit junctions were formed. The open circuit situation arose by the drift of the Au nanowires after being trapped, which eventually removes the top electrical contact yielding an open circuit.

**UPS and XPS Characterization:**

UPS and XPS measurements were carried out with Kratos AXIS ULTRA system using a concentric hemispherical analyzer for photo-excited electron detection. UPS was measured with helium discharge lamp, using He I (21.22 eV) and He II (40.8 eV) radiation lines. Total energy resolution was less than 100 meV, as determined from the Fermi edge of Au reference sample. All UPS spectra were measured with a -10 V bias applied to the sample to observe secondary electron photoemission cut-off at low kinetic energies.

XPS measurements were performed using a monochromatic Al Kα X-ray source (hν = 1486.6 eV) at 75W and detection pass energies ranging between 20 and 80 eV. Curve fitting analysis was based on linear or Shirley background subtraction and application of Gaussian-Lorentzian line shapes.
2. UV-Vis absorption spectra of N42C Az mutant:

![Graph of UV-Vis absorption spectra](image)

**Figure S3:** UV-Vis absorption spectrum of N42C Az.

The UV/Vis absorption spectrum of the N42C Az dimer is essentially identical to that of the wild type Az\(^8\), \(\lambda_{\text{max}}\) 630 nm which indicates that no structural changes around the copper site have occurred.

**Overlay of the absorption spectra of N42C Az and WT Az**

![Overlay of absorption spectra](image)

**Figure S4:** Overlay of the typical LMCT band of the type 1 Cu(II) site of the N42C Az mutant with that of the WT Az.

3. XPS results:

The presence and characteristics of the N42C Az mutant protein on the Au surface was verified and examined by XPS measurements. High resolution XPS spectra of C 1s, N 1s, Cu 2p
and S 2p regions of the Au surface, onto which the N42C Az mutant was adsorbed, are shown in Fig. S5. The shape of the C 1s peak showing three main carbon components (-C-C(H)-, -C-N and –C=O(-N-C=O)) is characteristic of protein molecules on the surface. The shape of the Cu 2p peak for the N42C Az mutant differs somewhat from that for WT Az (see Fig. S5C), as, in addition to the main Cu 2p3/2 component at ~ 932 eV (Cu 2p3/2), in the N42C Az mutant there is also a component at ~ 933.5-934.5 eV (Cu 2p3/2), which may be due to some Cu(II)-SOxH2y bonds. This may be due to traces of Cu(II) sulphate used in the process of holo protein formation. The S peaks (Fig. S5D) show those of S bound to metal (Au and Cu; at lower BE), as well as S bound to C (in cysteine and methionine). WT Az has 3 Cys sulfurs, one bound to the Au, one with free SH and one coordinating the Cu, and the N42C Az has 2 Cys sulfurs as it misses a one Cys residue. Both proteins have 6 methionines, one that coordinates the Cu in each of them. The much higher signal for the WT Az could be due to the stronger attenuation by the polypeptide matrix in case of the mutant.
Figure S5: High-resolution XPS spectra of A) C 1s of the N42C Az, B) N 1s of the N42C Az, C) Cu 2p of the N42C Az and WT Az and D) S 2p of the N42C Az and WT Az on the Au surface.

4. Polarization Modulation Infra-Red Reflection-Absorption Spectroscopy (PM-IRRAS):

In addition to XPS, PM-IRRAS was used to find evidence for protein presence on Au surface. Figure S6A and S6B shows the PM-IRRAS spectrum of N42C Az, at 2750-3050 cm\(^{-1}\) and 1200-1800 cm\(^{-1}\) wavelength ranges, respectively. The bands associated with the different functional groups of the monolayer are clearly visible in the spectrum. The bands located at 2850, 2930 and 2970 cm\(^{-1}\) are assigned to the symmetric (\(\nu_{as} \text{ CH}_2\)), asymmetric (\(\nu_{as} \text{ CH}_2\)) and asymmetric (\(\nu_{as} \text{ CH}_3\)) stretching vibrations of the CH\(_2\) and CH\(_3\) groups. The bands at 1677 and 1547 cm\(^{-1}\) are attributed to the amide I and amide II modes of the group, respectively.
Figure S6: The PM-IRRAS spectrum of Az grafted on a gold surface, expressed in absorption units ranging from A) 2750 - 3050 cm\(^{-1}\) B) and 1200 - 1800 cm\(^{-1}\).

5. Overlay of the IETS spectrum of \textit{WT Az} and \textit{N42C Az}:

Fig. S7 shows the IETS spectrum of A) \textit{N42C Az} and C) \textit{WT Az} at high bias, up to \(\pm 1.2\) V for the conductance data given in the main text Fig. 4. Figs S7B and S7D show the focused IETS spectrum of the same given in Figs. S7A and S7C in the low bias region. Fig. S7E, shows the overlay of the IETS spectrum in the low bias region, indicating that Au-S bonds of the \textit{N42C Az} and \textit{WT Az} have not changed or been destroyed at high bias, which might yield artifacts in the observed conductance spectrum.
Figure S7: IETS spectrum of A) WT Az and C) N42C Az at high bias up to ±1.2 V. B) and D) show the zoomed spectra in low bias region. E) show the overlay of the low bias IETS region (the same bias scale) of the N42C Az and WT Az.
6. **Reproducibility checks:**

**Figure S8: A-D)** Shows the I-V (black) and Conductance-Voltage(dI/dV) plots (brown) of N42C Az junction at high bias, at 10K carried out on different samples.

Fig S8 shows the overlay of current and dI/dV-Voltage plots of four different N42C Az junctions measured at different times. These four different junction plots of N42C Az clearly show the conductance switching behavior taking place upon increased bias, where the conductance values reach $\sim 200$ nA/V at 1.0 V. The majority of the measured junctions of the N42C Az mutant were very stable till $\sim 0.5$ V (for statistics see Fig. 3 main text). Once the bias is increased above 0.75 V the stability drops quickly. Therefore, after acquiring the results till 0.5 V, the bias was increased from 0.5 V to 0.75 V, and the conductance and I-V data till 0.75 V were collected. When the junctions were still stable, we moved to 1.0 V and finally to 1.2 V. WT Az junctions show high stability at higher bias compared to those of N42C mutant. This could be due to the high current/conductance value reached via the mutant Az compared to the WT Az at a given bias.
Fig. S9 shows the current-voltage and dI/dV-Voltage plots of two different WT Az junctions, where no conductance switching behavior is observed at high bias. The results presented here (Figs. S8 and Fig. S9) are similar to those presented in the main text Fig. 4, i.e., conductance switching behavior is observed only for N42C Az mutant.

**Figure S9: A-B)** Shows the I-V (black) and Conductance-Voltage(dI/dV) plots (brown) of WT Az junction at high bias, at 10K carried out on different samples.

7. **AFM data:**

Fig S10 presents the tapping mode topographic image of N42C Az mutant on Au surface.

**Figure S10:** A 1 × 1 μm tapping mode topography image of N42C Az on a Au surface.
8. Arrhenius plot:

Fig. S11 presents the ln(current) vs. 1000/T(temperature) plots measured at negative bias voltages from 50-500 mV, for the temperature dependence lnI-V plots that are given in the main text Fig. 2C.

![Arrhenius plot](image)

**Figure S11:** ln(current) at applied 50-500 mV bias voltages, as function of 1000/T, for the N42C Az junctions in the negative bias region.

9. UPS data:

![UPS data](image)

**Figure S12:** Semi-log plots of the UPS spectra near the Fermi level (UPS intensity on log scale) for the three studied surfaces, viz. clean Au, Au with an N42C Az and with a WT Az monolayer, showing E\textsubscript{HOMO} values deduced from the onset of photoemission with respect to E\textsubscript{F}.
As shown in Fig. S12, the UPS intensity logarithmic results yield much smaller HOMO onsets than those derived from the linear intensity vs. Binding Energy UPS plots shown in Fig. 5B of the main text. Most importantly is that of the N42C Az surface is \( \sim 1 \) eV closer to the electrode Fermi level than that of the WT Az, one which agrees with the result of the linear UPS plot presented in the main text. Due to the expected vibrational broadening of the HOMO, there will not be one correct value, but the log plot is probably the more representative one for tail states. In earlier work based on our Azurin transistor measurements \(^9\), we found that the LUMO of the WT Az is slightly above the electrode Fermi level, which could fit with the end of the tail, based on the estimate derived from Fig. S12. Naturally, the energetics can be further affected by the top contact, especially for the WT Az.

**Figure S13:** The UPS spectra of N42C Az and WT Az monolayers on Au using A) He I and B) He II excitation.

Fig. S13 shows the full spectra of WT Az and N42C Az mutant obtained using He I (\( h\nu = 21.22 \) eV) and He II (\( h\nu = 40.8 \) eV) photon excitation sources. He II spectra of the WT Az and N42C Az mutant shows \( E_{\text{HOMO}} \) values (2.1 eV for the N42C Az and 3.0 eV for the WT Az) close to those obtained from the HeI spectra (2.0 eV for the N42C Az and 3.0 eV for the WT Az) (see main text Fig. 5B).
10. I-V curve fitting analysis:

We fit the experimental I-V curves of both the WT Az and the N42C Az mutant from Fig. 3A and 3D (main ms.) to third order cubic polynomials and then extract the required parameters using a simplified one-energy level Landauer model\(^\text{\textsuperscript{10}}\) (see Fig. S14). Using this method, we extracted the electrode-molecule coupling strength values (Γ), the effective energy barrier (ε\(_0\), in eV), effective conductance at zero bias (G\(_{eq}\)= dI/dV) and a dimensionless symmetry parameter (α = 0 for a symmetrical I-V plot). All the experimental I-V curves can be fit to third-order polynomials with a high correlation (R\(_2\) ~ 1). See Table-1 for the extracted parameters and their values. For a detailed description regarding the single-level Landauer model and curve fitting analysis, we refer to the SI of our previous publication\(^\text{\textsuperscript{11}}\).

**Figure S14:** Third order polynomial fit for the I-V plot of A) N42C Az and B) WT Az Junction.

| Protein   | V range | ε (eV) | Γ meV | G\(_{eq}\)(nA/V) | α    |
|-----------|---------|--------|-------|-----------------|------|
| WT Az     | 0 – 0.5 V | 0.66   | 0.3   | 7               | 0.51 |
| N42C Az   | 0 – 0.5 V | 0.59   | 1.1   | 100             | 0.51 |

**Table-1:** The parameter values extracted from fitting the I-V curves of both the WT and the mutant N42C Az to a third order polynomial.

The electronic coupling (Γ) and the energy barrier values (ε\(_0\)) that we find, give us an idea of the effective energy scales involved in the ETP process. Comparing the results for the WT Az and N42C Az mutant shows that over the given bias range, the Az mutant ‘s coupling to the electrodes is higher than that of the WT. The calculated effective barrier off both the WT Az and the N42C Az mutant are > 0.5 V, consistent with that, below 0.5 V ETP via both the WT Az and the N42C Az is by off-resonant tunneling transport (further supported experimentally by the temperature
independence of the current, Fig. 1 main ms.). In general, though, in off-resonant tunneling regime, both the WT and the mutant behaves similarly, with a slightly higher current and the conductance values for the mutant than the WT Az, which can be traced due to better coupling to the electrodes.

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