Single azurin redox switching

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## Introduction

Introduction here.

Experimental Section

Protein synthesis

Azurin (wild type) from Pseudomona aeruginosa was expressed in E. Coli and purified as described before[1]. BL21 E.coli was transformed with PGK22 plasmid that has gene for azurin expression. The cells were cultured in luria broth (LB) medium. Then the cells were harvested and resuspended in 20 % (w/v) sucrose solution in Tris pH 8 buffer containing 1 mM EDTA. Then the solution was centrifuged at 8000 rpm and the supernatant was collected. Copper sulfate was added to the solution for insertion into the active site of azurin. The unwanted proteins were precipitated by addition of acetic acid until pH 4. Again the turbid solution was centrifuged to separate azurin that remained in the supernatant. The azurin solution was loaded on a CM Sepharose fast flow column and elution was performed in an Akta purifier (GE Healthcare) with a pH gradient from 4 to 6.9 in 50 mM ammonium acetate. Fractions containing azurin collected and reduced with sodium dithionite. At this moment

the solution both zinc and copper azurin. The azurins were putified in a DEAE sepharose column by a salt gradient of 0 to 50 mM NaCl in Tris pH8 buffer. Fractions containing copper azurin and zinc azurin collected and concentrated separately. The purity of the samples were checked by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and UV/Vis spectroscopy (Cary 50 spectrophotometer, Varian Inc., Agilent Technologies, USA). The azurins appeared on SDS gel page at $\tilde{1}4$ kDa. Both zinc and copper azurin showed a characteristic peak at $\tilde{2}90$ nm while Cu azurin showed an additional broad absorption peak at 620 nm as can be seen in figure S2 when oxidized. The ratio $O.D_{628nm}/O.D_{280}$ for Cu azurin was 0.56 which indcated that all the azurin molecules had a Cu atom. The concentrated protein was stored at -80^0C until further use.

Fluorescent labeling

The labeling protocol was based on the previous work.[2] ATTO 655 NHS-ester was bought from ATTO-TEC GmbH. The buffer containing azurin was replaced with HEPES pH 8.3 and all the amine containg impurities were removed. A mixture of 200 μM azurin and ATTO 655 NHS-ester (ration 1:1) was incubated for 45 min. The NHS-ester group reacts to one of the amine group on the protein. The unreacted dyes were removed by a HiTrap desalting column. The labeled protein was concentrated in Tris pH 8.5 buffer by centrifusing in a 3 kDa Amicon ultra filter. The labeled protein further purified by an ion exchange chromatography in a 1 mL MonoQ column (GE Health). The different peaks obtained (see figure S1) corresponds to the different number and position of the dye on azurin. The peak-III corresponds to the protein labeled at Lysine122 position.[2] This fraction was choosen for our single-molecule experiemnt for the high switching ratio explained later. The same protocol was used for Zn azurin labeling and similar peak separation observed.

Potentiostat

7

4

5

6

C$_6$H$_6$O$_6$

3

[Fe(CN)$_6$]$^{3-}$

2

Oxidized

1

8

9

Reduced
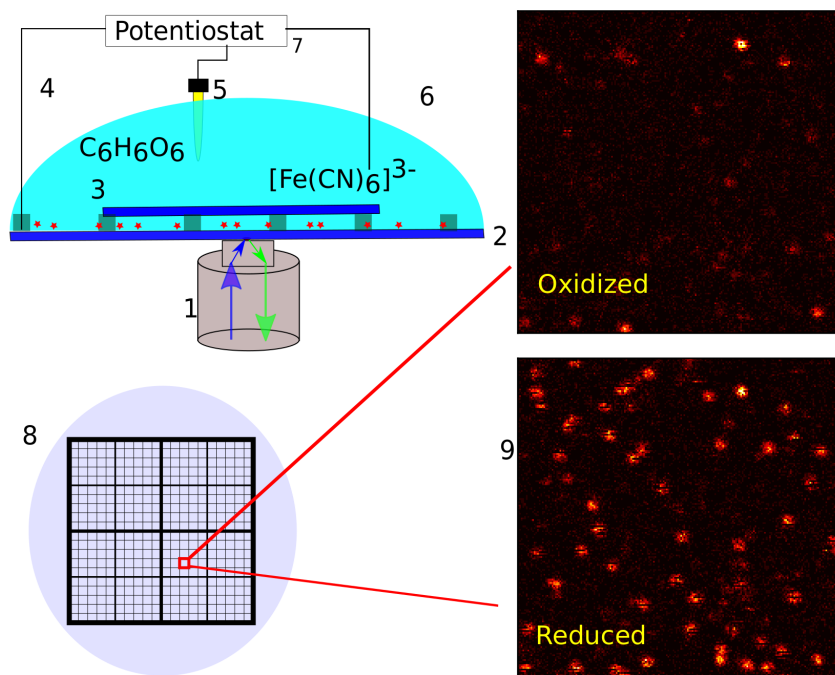
Scheme 1: The scheme picture of the final setup. **(1)** Objective through which light is irradiated on and collected from the sample. **(2)** The functionalized sample slide with on top the platinum grid and another small glass slide to press the grid on the sample slide, resulting in small confined volumes in the order of nanoliters. **(3)** The electron mediator solution consisting of 200 µM ferricyanide, 100 µM ascorbate in PBS (PH 7.4) buffer with a total volume of 4 mL. **(4)** The working electrode (Platinum wire) that is in contact with the platinum grid. **(5)** The saturated calomel reference electrode. **(6)** The Platinum wire (not touching the grid) as counter electrode. **(7)** The potentiostat (Model 800B Series Electrochemical Detector, CH Instruments) to which the electrodes are connected. **(8)**, **(9)** Top view of the sample slide and two images are showing the labeled Cu-Azurin reduced(brighter) and oxidized(dimmer).
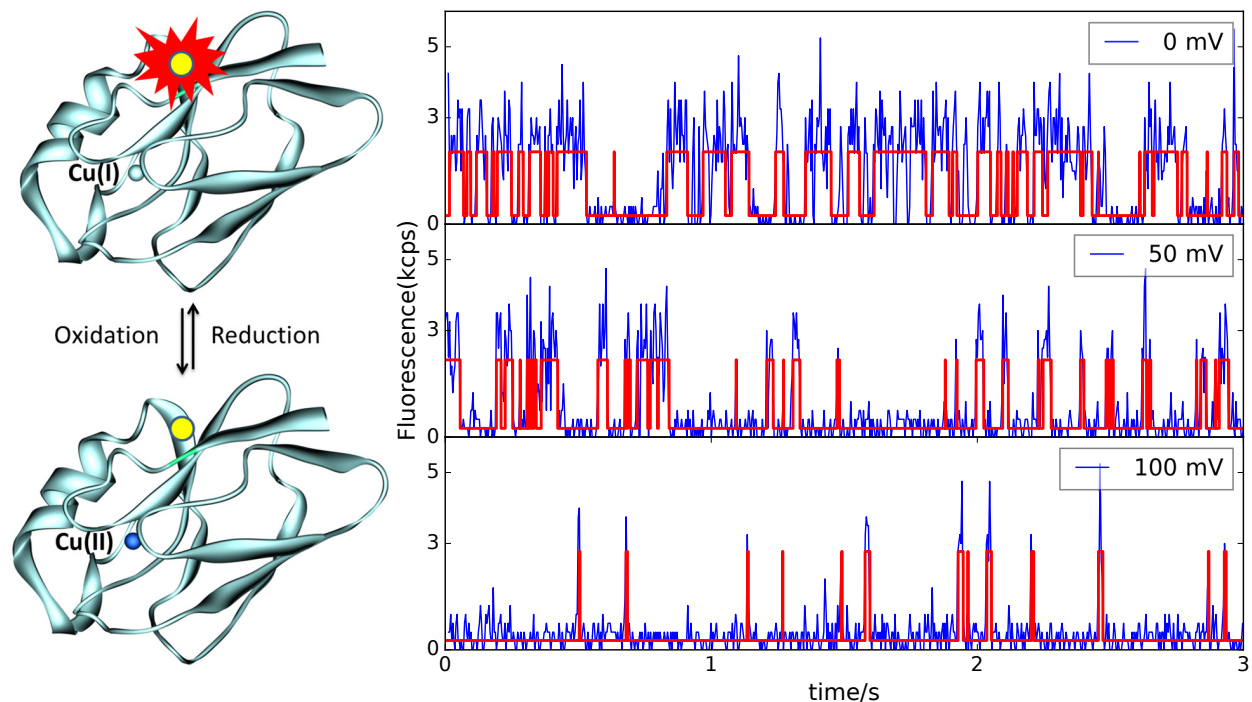
Figure 1: Time traces of Cu-Azurin labeled with ATTO655 at different potentials. The structure of the protein with properly positioned dye can be seen in the schematic picture in the left. In Cu(II) state (shown in bluish atom in the protein structure), the dye is non fluorescent because of FRET and in Cu(I) state (shown in gray atom), the dye is fluorescent. Notice the amount of time the protein spends on bright and dark state at different potentials. At lower potentials (e.g 0 mV) the protein is brighter most of the time because of the higher concentration of reductant.
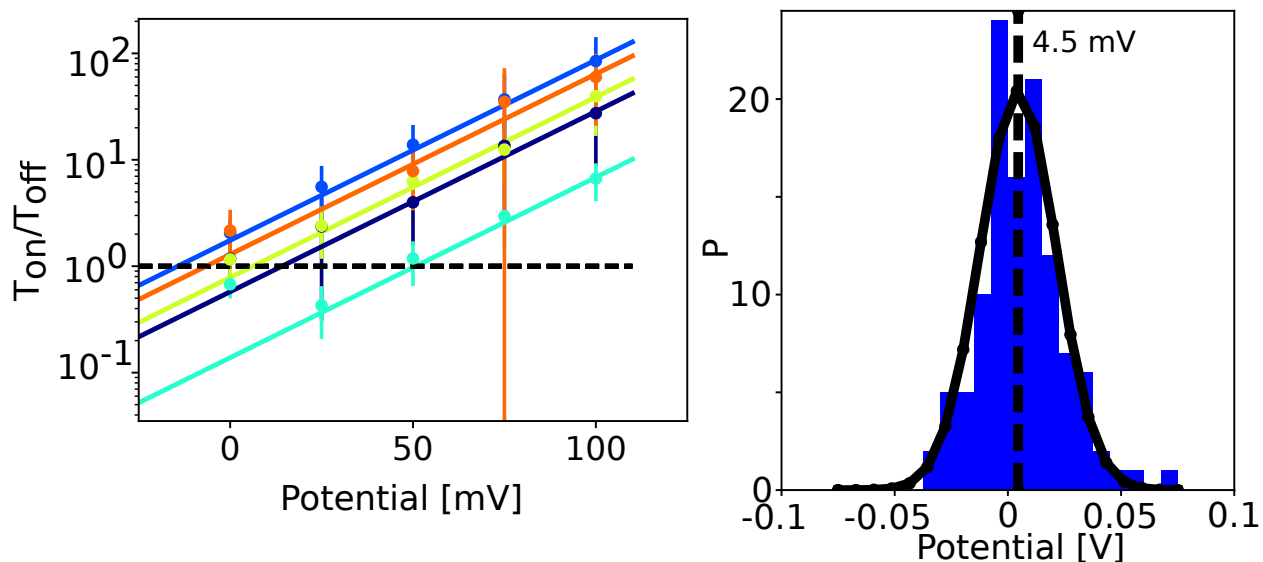
Figure 2: Ratio between on and off time as a function of applied potential for the same single-molecule. Different color represents different single-molecules. And the line connecting the data points is the Nernst fit for all the data points above 25 mV. The plot in the right is the histogram of midpoint potentials for 132 molecules with a gaussian fit with center 4.5 mV with respect to calomel electrode.
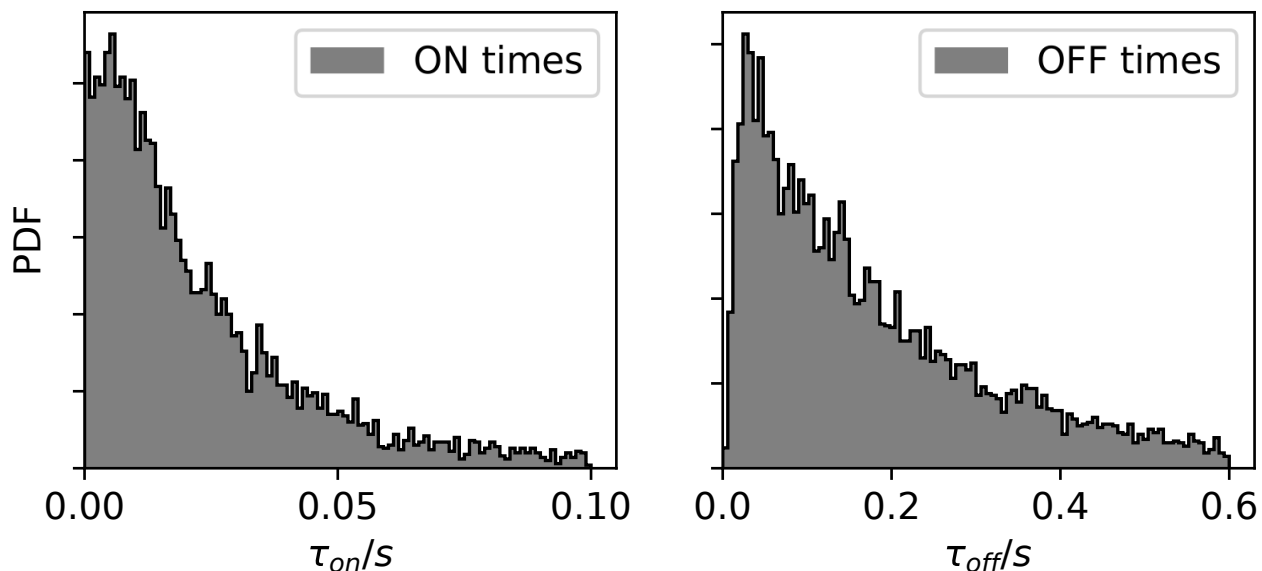

Figure 3: **On off histogram.** The histogram of on-times(a) and of off-times(b) of Cu-Azurin-ATTO655 showing rise time. This indicates that both oxidation and reduction of Cu-Azurin occurs through an intermediate step.
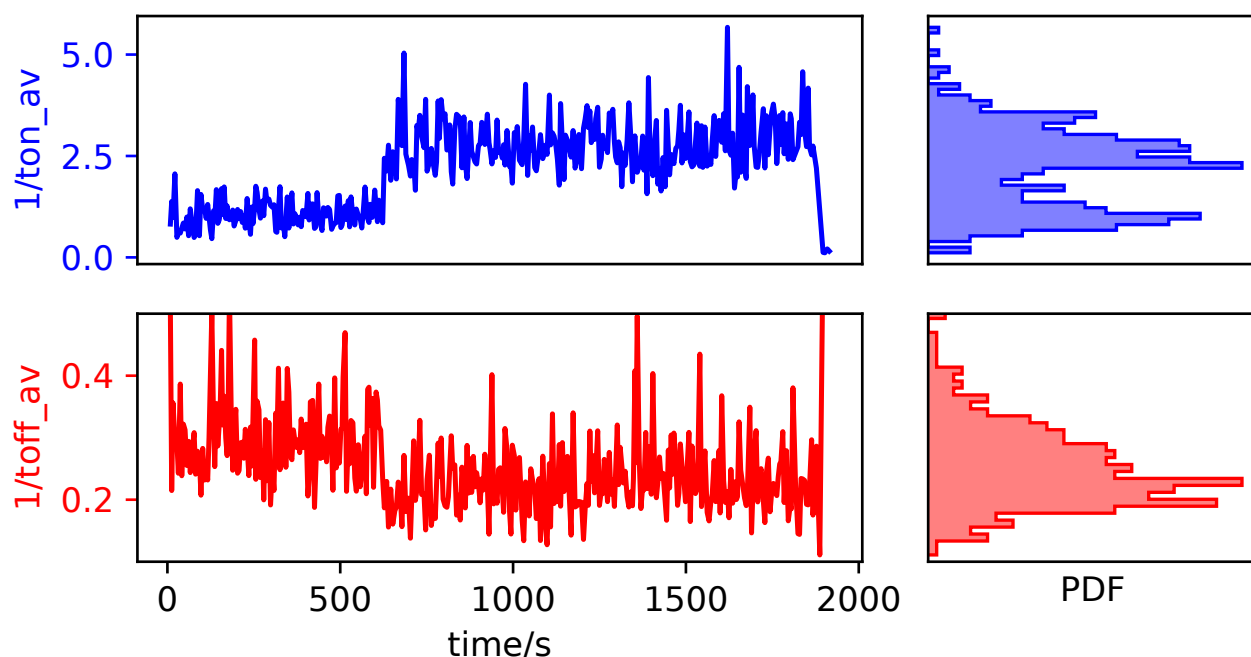
Figure 4: **Dynamics in the turnovers of the protein.**

Results and discussion

References

(1) Kamp, M. v. d.; Hali, F.; Rosato, N.; Agro, A.; Canters, G. *Biochimica et biophysica acta* 1990, *1019*, 283–92.

(2) Nicolardi, S.; Andreoni, A.; Tabares, L. C.; Burgt, Y. E. v. d.; Canters, G. W.; Deelder, A. M.; Hensbergen, P. J. *Analytical chemistry* 2012, *84*, 2512–20.
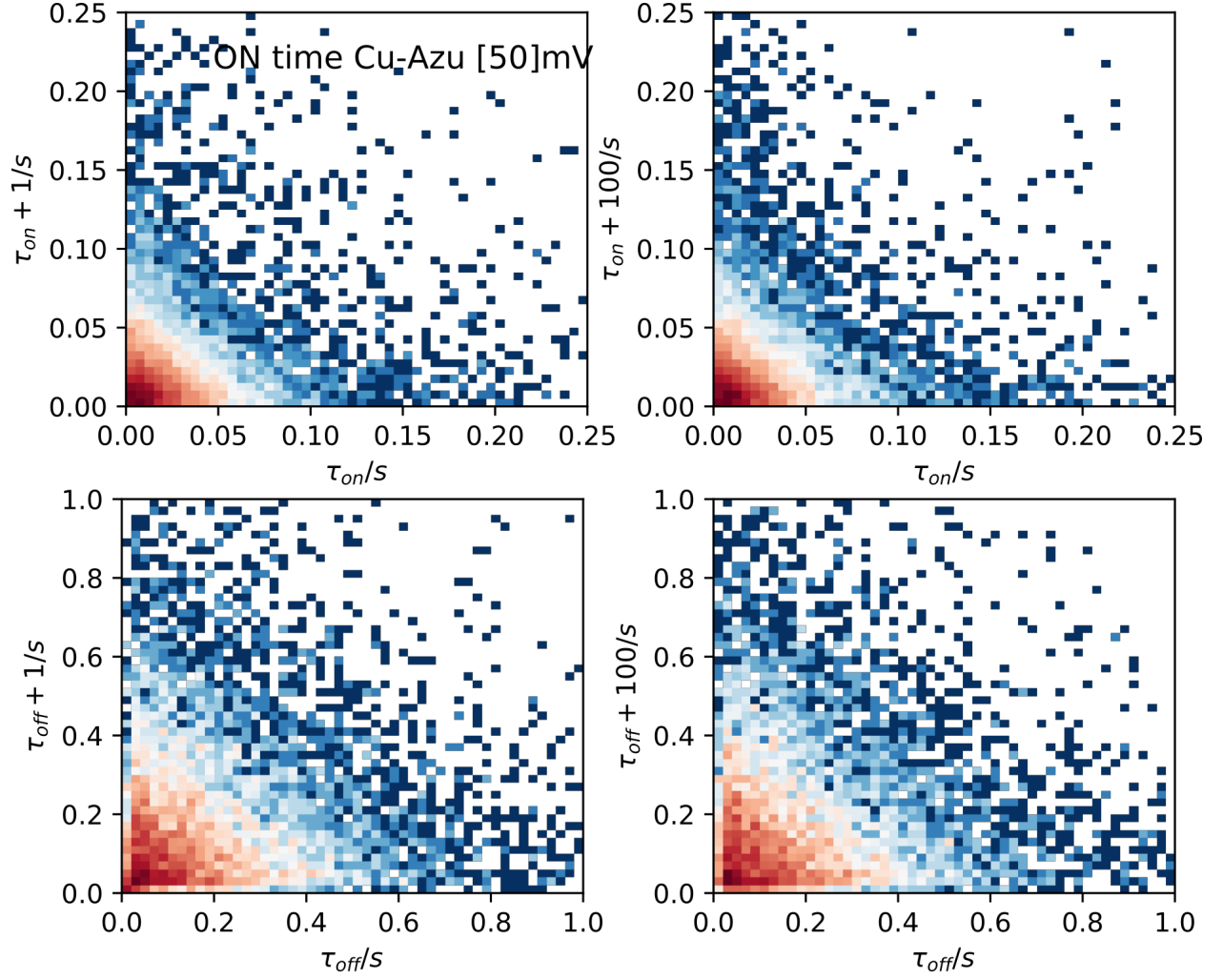
Figure 5: **2D histogram: Cu-Azurin.** 2D correlation plot of (a) on-times vs the next on-times (b) on-times vs the on-time after 100 turnovers (c) off-times vs next off-times (d) off-times vs the off-time after 100 turnovers.
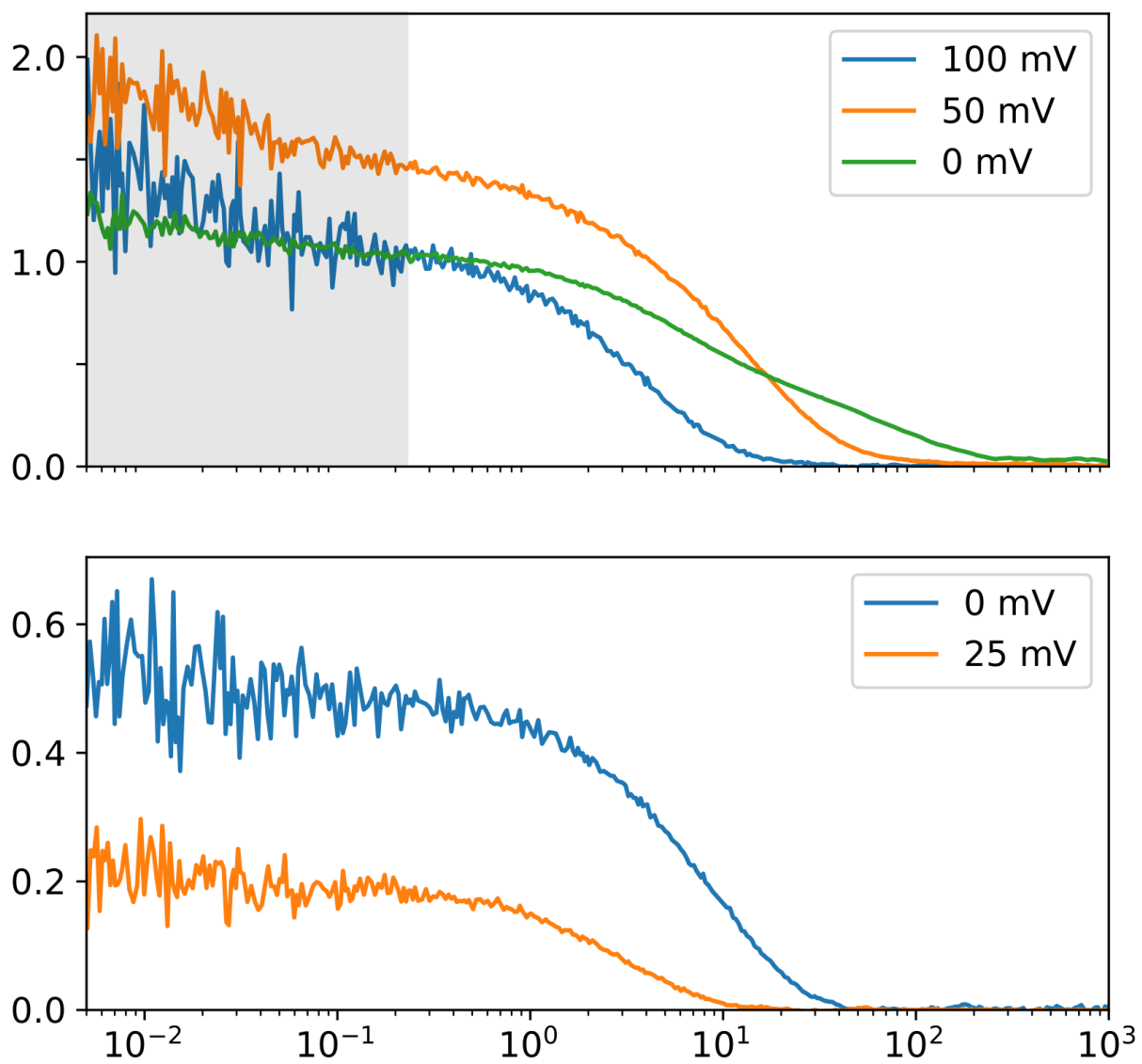
Figure 6: **Intramolecular electron transfer.** Shorter decay with time constant fo around 30 us

azurin