



Electrochemically changing potential

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Author :	Sebastiaan Van Mulken
Student ID :	0950815
Supervisor :	Biswajit Pradhan Michel Orrit
2 nd corrector :	To be determined

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Sebastiaan Van Mulken

Huygens-Kamerlingh Onnes Laboratory, Leiden University
P.O. Box 9500, 2300 RA Leiden, The Netherlands

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Abstract

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preamble.

Results and discussion

The experiments were performed with the blue copper protein azurin from *Pseudomonas aeruginosa*, labeled with ATTO655 (referred to as CuAz in the rest of the analysis). This small protein, with a molecular mass of 14 kDa, is involved in electron transfer (ET) reactions in a variety of both plants and bacteria. Its label, ATTO655, has been chosen since its properties have been well documented [?]. The labeling site used in this experiment is K122 (lysine at position 122), one of the closest labeling sites to the copper center of CuAz. In the future one could label ATTO655 to a labeling site further from the copper so that the inner-dye distance changes from less to greater than the Förster radius R_0 value for maximal sensitivity [?]. Beside the blue copper protein azurin, fluorescently labeled zinc azurin (ZnAz) was also used to perform experiments. This wild-type protein is used as a control since it does not show fluorescence switching when different potentials are applied.

Data collection

The sample, mounted on the scanning stages, was brought into the focal plane of the objective. Once an area was chosen, images of (80×80) (μm^2) were recorded as x-y scans. A positive and negative potential is applied and captured to detect the blinking CuAz. Images of typically (20×20) (μm^2) were recorded in which at least 10 blinking molecules were present. To collect data from a single molecule, the laser was parked on the blinking molecule and measurements were made for 30 seconds. Once all the blinking molecules of interest were measured, a new potential was applied and this process repeated. Many fluorophores bleach within sec-

onds, thus only the time traces of those molecules who survived all the different potentials were included for further analysis.

To make sure that the surroundings of the single molecules were indeed the intended potential, the I-t curves were recorded at the same time.

Time trace analysis

The software used for the fluorescence lifetime imaging and correlation software is SymPhoTime 64. With the help of this program, timetraces and its specific parameters are saved in .pt3 files. Older versions of SymPhoTime saved this data into .t3r files. Previous PhD student .. had written a program in MATLAB R2012b and C++ to read out the .t3r files and transfer the timetraces into multiple .dat files. This program has been adjusted in such a way it can read out the .pt3 files from the newer version of SymPhoTime 64. It allows you to manually select parts of the timetrace, as is shown in Figure 1.1.

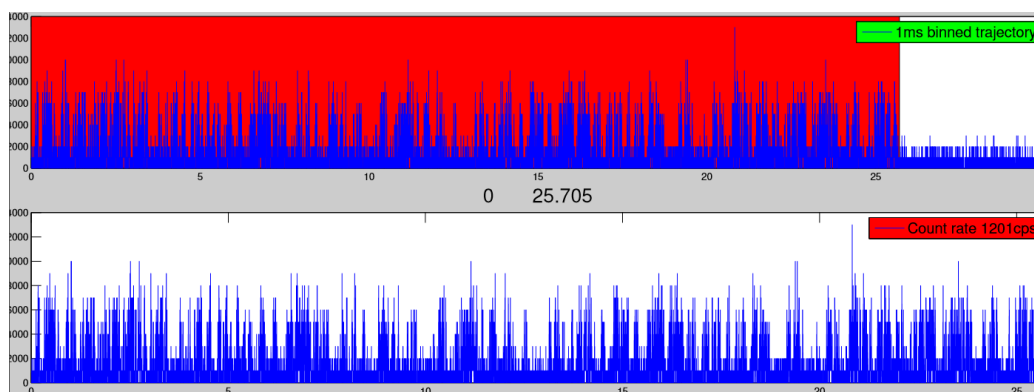


Figure 1.1: Selection of a timetrace with a program written in MATLAB R2012b and C to select the parts of the timetrace (in red) that will be put into .dat files. As in this specific case the Cu-Azurin bleached around 25.7 second mark and the timetrace once it is bleached is not of interest and therefore not selected.

Once the timetrace is selected and put into several .dat files, another C program is run to precisely calculate the on- (τ_{on}) and off times (τ_{off}). This program uses several mathematical methods to calculate the timestamps and intensity of intensity jumps in optical single molecule emission data that exhibit discrete intensity jumps, in detail described by Lucas P. Watkins and Haw Yang in their paper [?]. As an example, several on- and off times are visualized in Figure 1.2. These times are defined as the

duration of the CuAz being oxidized (τ_{on}) and reduced (τ_{off}). The intensity jumps that are controlled is what is referred to as 'switching' while the (often) unwanted and uncontrolled switching between the bright 'on' and dark 'off' states is called 'blinking'.

Results and discussion timetraces CuAz

Looking at the timetraces between 100mV and 0mV (Figure 1.3) a trend is noticeable on sight. For the higher potentials, the τ_{off} are long and the τ_{on} are relatively short which goes hand in hand with the low amount of events. When the potential decreases the amount of events start to increase and the τ_{on} becomes longer while τ_{off} becomes shorter. The explanation for this is simply the FluRedox principle. CuAz in oxidized form (copper is in Cu^{2+}) shows an absorbance maximum around 628nm which overlaps with the fluorescence emission of the ATTO655 dye as is shown earlier in Figure ???. Since FRET is high in this form, the fluorescence of the dye is quenched resulting in longer τ_{off} . When the potential is decreasing, the solution is reducing. When CuAz is reduced - since the absorption at 628nm disappears upon reduction - the FRET is low and thus the dye shows high fluorescence and longer τ_{on} times are expected. This principle seems to be the only role in play until the potential comes near 25mV. Here a new phenomenon is noticeable. Beside the expected increasing τ_{on} and decreasing τ_{off} due to switching of CuAz, a secondary timescale seems to be shown in the form of very short τ_{on} in very short succession. To explain this event, a closer look has to be taken at the chemicals involved in the chemical processes concerning the redox reactions. Apart from the intramolecular electron transfer between the label and the copper center, intermolecular reactions between the redox active components in the solution and the dye are also present. For higher potentials, the time scale of the events between dye and solution are relatively high. When the potential gets lower, the solution is more and more reduced. The interaction between the dye and the reduced ascorbate and ferricyanide happen on a smaller timescale and is now prominent in the timetraces. This is more apparent when looked at the autocorrelation. The probability $I(t)$ of emission of a photon by an excited state at time t is determined by

$$dI(t) = -\frac{1}{\tau(t)}I(t)dt. \quad (1.1)$$

The solution of equation 1.1 in the case of $\tau(t) = \tau_0 = \text{constant}$ is the classical single exponential decay function. At higher potentials the auto-

correlation can be fitted with this single exponential in the form of

$$g(\tau) = A_1 e^{-\tau/t_1} \quad (1.2)$$

where t_1 and A_1 are constants. These constants are related to the on- and off-times via the equations

$$\tau_{on} = t_1 + \frac{t_1}{A_1} \quad (1.3)$$

and

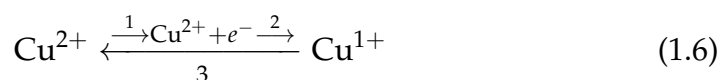
$$\tau_{off} = t_1 + t_1 A_1. \quad (1.4)$$

When the potential is lowered towards the 25 mV and below, a single potential does not fit the autocorrelation but rather a sum of two exponentials fits the autocorrelation:

$$g(\tau) = A_1 e^{-\tau/t_1} + A_2 e^{-\tau/t_2}. \quad (1.5)$$

The constant-pair t_1 and A_1 and t_2 and A_2 are related with the on- and off-times according equation 1.3 and equation 1.4. The longer τ_{on} are the ones from the switching of the CuAz, while the shorter τ_{on} is the one due to the blinking of the dye.

Another way of looking at the τ_{on} and τ_{off} is by plotting the distribution of the on and off times in the time traces. This is done in Figure 1.4. The fit through these histograms will give the average on- and off-times. A very interesting phenomenon is observed in the off-times however. Where the on-times are fitted with a single exponent, the off-times have a different form. Very short off-times seem to be relatively rare. To get a deeper understanding of this a closer look has to be taken at the electron transfer process between the solution and the copper azurin.



The reduction of Cu^{2+} to Cu^{1+} is a multi-step process. Bringing the electron close to the copper center (step 1) and reducing the copper (step 2) is happening in two short time scales. Contrary to the reduction of copper azurin, the oxidation is a single step process. This should go faster than reducing and thus shorter off times are expected to be much more present. This is in contradiction to the histograms which show an absence of these short times. Something in step 3 is delaying the oxidation. With the model presented above, this cannot be explained and further research to this subject is needed.

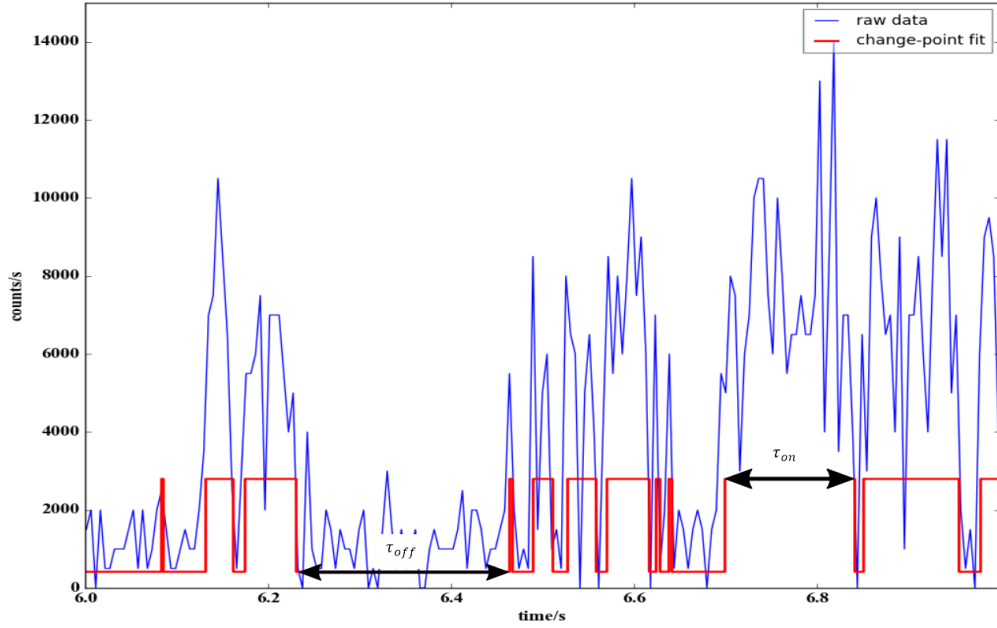


Figure 1.2: Same timetrace as Figure 1.1 but a smaller range. In red is plotted the calculated intensity jumps according to the program written by Lucas P. Watkins and Haw Yang. The time when the intensity is low is referred to as the τ_{off} and the time the molecules intensity is high is the τ_{on} .

To give the switching of the CuAz a more quantitatively meaning, the average τ_{on} ($\bar{\tau}_{on}$) and average τ_{off} ($\bar{\tau}_{off}$) can be related to the Nernst equation since the switching is due to the redox reaction of CuAz. Rewriting equation ?? we get

$$E = E_0 + \frac{k_B T}{ne} \ln \frac{\bar{\tau}_{off}}{\bar{\tau}_{on}}. \quad (1.7)$$

At midpoint potential $\bar{\tau}_{on} = \bar{\tau}_{off}$ or differently written $\frac{\bar{\tau}_{off}}{\bar{\tau}_{on}} = 1$. Using $\frac{k_B T}{ne} \approx 13\text{mV}$ and rewriting equation 1.7 to

$$\frac{\bar{\tau}_{off}}{\bar{\tau}_{on}} = \exp\left(\frac{E_0 - E}{13}\right) \quad (1.8)$$

the intersection in Figure ?? between $\frac{\bar{\tau}_{off}}{\bar{\tau}_{on}} = 1$ and the fitted line of $\frac{\bar{\tau}_{off}}{\bar{\tau}_{on}}$ will give the midpoint potential of each protein.

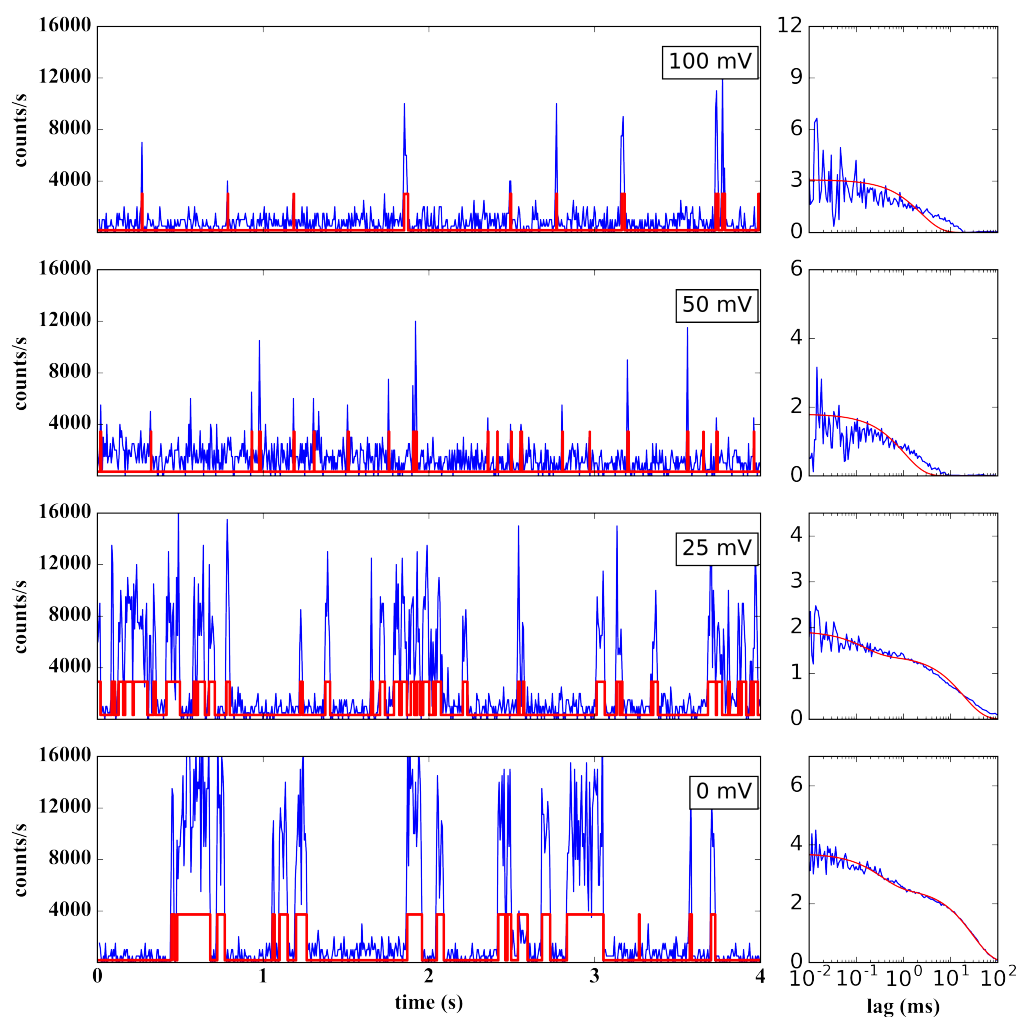


Figure 1.3: Timetraces together with its autocorrelation of the same CuAz molecule under different potentials. A clear difference between τ_{on} and τ_{on} under different potentials is visible: shorter τ_{on} for higher oxidizing potentials, longer τ_{on} for reducing lower potentials. For potentials below 25mV this pattern gets disturbed due to blinking of the ATTO665. This phenomenon is shown in the autocorrelation by changing from a single exponential fit to a multi-exponential fit.

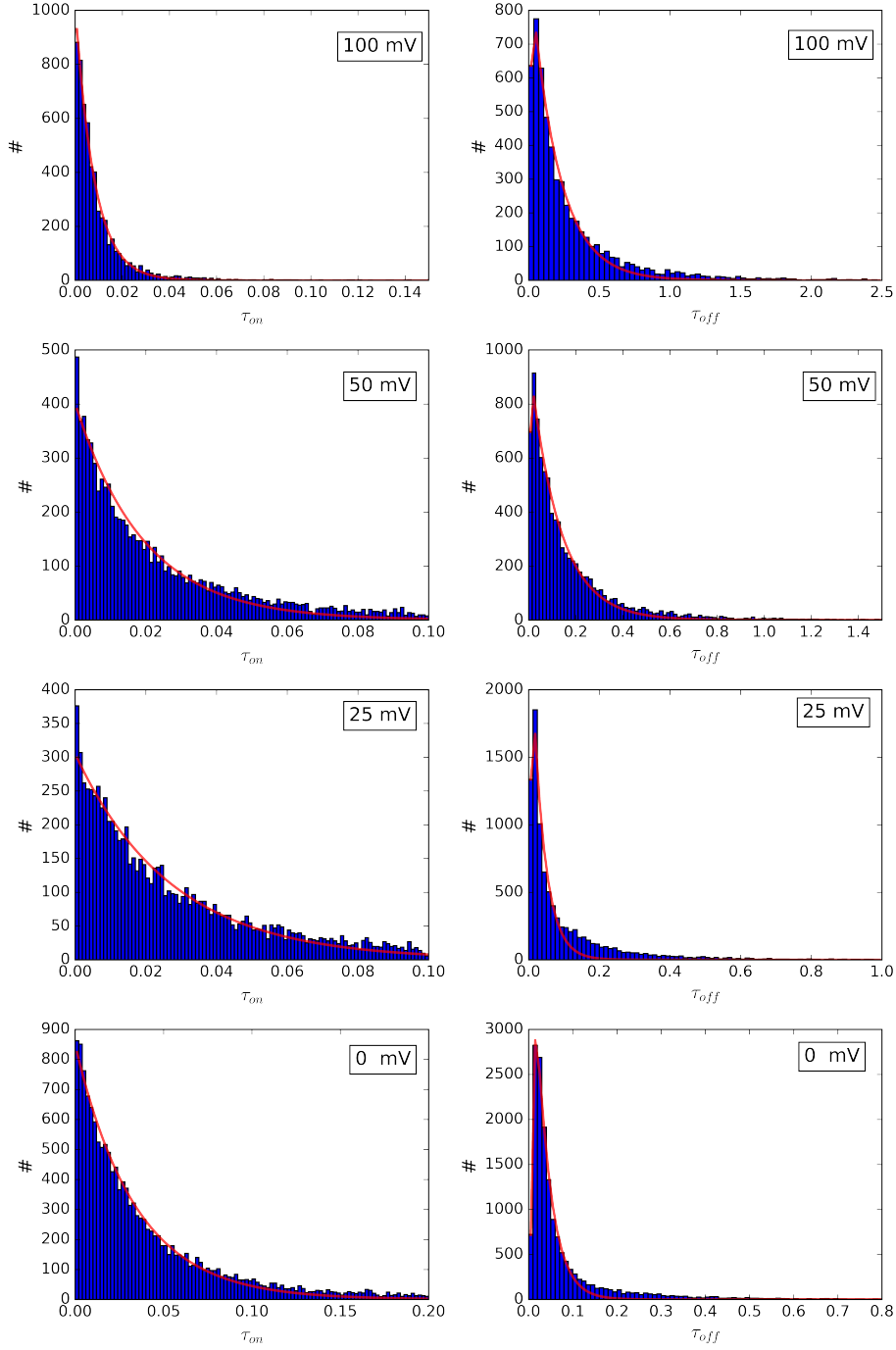


Figure 1.4: Histogram distribution τ_{on} and τ_{off} at different potentials.