The handle http://hdl.handle.net/1887/20986 holds various files of this Leiden University dissertation.

Author: Akkılıç, Namık
Title: Fluorescent electrochemistry : towards controlled redox-switching of a single metalloprotein
Issue Date: 2013-06-20
Chapter 4

Chemically-induced redox switching of single azurin molecules

Abstract

We use an optical method for the detection of the redox state of proteins based on excited state energy transfer (FRET) from an attached dye molecule to the protein redox center. This method is applied to azurin, a 14 kDa type I blue copper protein. We show that the fluorescence intensity of a single azurin molecule as a function of time, i.e. the fluorescence time-trace, shows an on-off switching behavior which depends on the redox conditions in solution. We observed a reversible change of the fluorescence intensity of Cy5 labeled azurin upon addition of oxidant or reductant: The fluorescence intensity in the oxidized state decreases by about a factor of 10 compared to that in the reduced state. Fluorescence lifetimes of surface-immobilized azurin were found to be $0.7 \pm 0.15$ and $1.8 \pm 0.2$ ns in the oxidized (fluorescence off) and the reduced states (on), respectively. Finally, using change point (CP) analyses of the fluorescence time traces from individual azurin molecules, we were able to measure their reaction kinetics and redox parameters as a function of the chemical redox potential in solution. As a result, to the best of our knowledge for the first time, we were able to determine the midpoint potential ($E_0$) of a single redox protein. We observed a significant heterogeneity in reaction kinetics and redox thermodynamics at the single molecule level.
4.1 Introduction

An overwhelming number of chemical reactions in nature, both in the living cell and in the inanimate world are redox reactions. When two compounds engage in such a reaction, one will be reduced, the other oxidized. Redox reactions play a major role in almost all metabolic pathways, as well as in the chemical and biochemical cycles that operate in the environment. The study of biological electron transfer (ET) reactions of proteins is not only crucial for our knowledge of many physiological functions such as cellular respiration, photosynthesis, and redox homeostasis (1, 2), but also for potential applications in biotechnology which drives the current proliferation of research on biofuel cells, protein biochips and biosensors (3, 4). The ability to monitor redox reactions of proteins and enzymes with high sensitivity is scientifically and commercially of great importance. Greatly enhanced sensitivity and specificity can be achieved with the FluRedox method (see chapter 1) by which redox turn-over can be monitored down to the single-molecule level, breaking new ground in redox enzymology.

As single-molecule techniques developed over the past two decades, their potential for the investigation of the reaction dynamics of proteins and enzymes was recognized early on (5): reaction mechanisms and associated kinetic models can be unraveled in unprecedented detail. Instead of monitoring the concentration change of the substrate or the product to measure the reaction rate, a single-molecule experiment follows individual catalytic turnovers in real time and records the waiting times ($\tau$) for completing individual reactions (5–7). Single-molecule kinetic theories have been developed to explore underlying reaction mechanisms and the associated kinetic models by analysing probability distributions and statistical properties of the waiting time parameters (8, 9). In one of the first experiments of Förster resonance energy transfer (FRET) at the single molecule level, Szabo et al. (10) demonstrated how to extract info from two-state single-molecule time trajectories which opened new doors in biomolecular research.
The observation of single electron transfer reactions will show the time-dependence of the redox properties of an individual protein, the distribution of redox properties over several proteins, and heterogeneity in the interaction with reaction partners. None of these important issues can be investigated equally well with existing ensemble methods. By combining the FluRedox method with potentiostatic or electrochemical control, we can relate the fluctuations of the fluorescence intensity to the exact conditions and properties of individual redox proteins and enzymes.

We have applied the FluRedox principle to study the chemically-induced redox switching of individual azurin molecules. Azurin (Figure 4.1A), from the pathogenic bacteria *Pseudomonas aeruginosa*, belongs to the family of T1 type redox proteins that contains a mononuclear Cu-site (11, 12) in which two histidines (H46 and H117), one cysteine (C312), and one methionine (M121) coordinate to the Cu-ion in a distorted tetrahedral manner (13–15). Azurin is a blue Cu protein with a mass of 14.6 kDa, which is common as an electron shuttle in ET chains of plants and bacteria, and is believed to play a role in oxidative
stress response (16). The hydrophobic patch around His117 at the protein surface, close to the redox-active site, is believed to mediate the formation of electron-transfer complexes (17). It has been shown that azurin can exchange electrons \textit{in vitro} with cytochrome c551, nitrite reductase, and several dehydrogenases, although its physiological partner has not yet been identified (16–21). Importantly, azurin has been reported to selectively induce and trigger apoptosis in several human cancer cells, most probably by stabilizing p53 (22, 23). Moreover, azurin can serve as a highly sensitive amperometric biosensor for the detection of superoxide radical that can be used to understand radical reactions at cellular level (24).

In its oxidized (Cu$^{2+}$) form, this protein has an intense absorption band in the visible region of 600 nm ($\varepsilon = 5700 \text{ M}^{-1}\text{cm}^{-1}$) that corresponds to a charge-transfer transition involving mainly the d$_{x^2-y^2}$ orbital of Cu$^{2+}$ and a 3p orbital of the sulfur atom of Cys112 (Figure 4.1B). This absorption disappears when the molecular orbital in the d$^{10}$ configuration of Cu$^+$ is filled, thus when the Cu site is reduced (25, 26). Previously, it has been demonstrated that this spectroscopic feature can be used to monitor the redox states of a protein with an attached fluorophore based on FRET, the so called “FluRedox principle” (25, 27), as illustrated in Figure 4.1A. Notably, fluorescence detection is highly selective with unmatched sensitivity, features that are very promising for biosensor applications (28–30). Most importantly, the enhanced sensitivity opens the doors for single-molecule detection of redox turn-over of proteins and enzymes (31–35). Recently, it was shown that the FluRedox principle can be combined with electrochemistry (36, 37), by which kinetic and thermodynamic heterogeneity of azurin on gold electrodes was investigated with a detection sensitivity as low as 100 protein molecules (38).

Herein, we report direct monitoring of electron transfer of single, fluorescently labeled azurin molecules covalently attached to a glass substrate, one electron at a time. We obtained continuous on-off switching of fluorescence, i.e. the
temporal fluorescence intensity profile, from the azurin construct under anoxyogenic conditions, which depends on the chemical redox potential in solution. Control experiments show that, this switching behavior is directly correlated with the redox state of the Cu-center in the azurin molecule. Using change point analyses (39) of the time traces we were able to measure the reaction kinetics and redox parameters of individual azurin molecules as a function of the chemical redox potential in solution. As a result, we were able to determine redox chemical behavior one molecule at a time, offering for the first time the distribution of key electron transfer parameters, such as the midpoint potential \( E_0 \) and reaction kinetics \( k_{\text{red}} \) and \( k_{\text{ox}} \) of the redox protein immobilized on a passive surface. We observed a significant heterogeneity in these parameters, in the lifetimes of oxidized and reduced azurin \( \tau_{\text{ox}} \) and \( \tau_{\text{red}} \), respectively, and in the switching ratio \( SR \) of individual azurin molecules.

### 4.2 Experimental Section

#### 4.2.1 Azurin Purification and Labeling

Wild type azurin from *Pseudomonas aeruginosa* was expressed in *E. coli* and purified as previously described (40). Cells from *E. coli* JM109 were transformed using a pUC-derived plasmid containing the azurin gene (41) followed by a signal peptide for periplasmic translocation. After culturing, cells were harvested and resuspended in a solution of 20% (w/v) sucrose in 30 mM Tris/HCl, pH 8.0, containing 1 mM EDTA for 20 min at room temperature. Subsequently, the solution was centrifuged at 8000 rpm for 15 min, and the supernatant was collected (sucrose fraction). The cells were resuspended in Milli-Q water at 4 °C, stirred for 20 min, and centrifuged at 8000 rpm for 15 min. The supernatant was collected and added to the above obtained sucrose fraction while the pellet was discarded. In *E. coli*, azurin is normally expressed in its apo-form. Therefore, after cells were lysed, copper sulfate was slowly added to the medium, to a final concentration of 600 μM in order to incorporate Cu in the polypeptide matrix.
Potassium ferricyanide was added to the solution to a final concentration of 100 μM to produce an oxidizing environment. A stepwise precipitation step was included by lowering the pH of the solution to pH 4 by adding concentrated acetic acid. The precipitated proteins were removed by centrifugation (8000 rpm, 20 min). The resulting clarified solution containing azurin was loaded on a home-packed CM Sepharose Fast Flow (Amersham Biosciences) column, and elution was performed using a pH gradient from pH 4 to pH 6.9 (50 mM ammonium acetate). Fractions containing azurin were collected and, after buffer exchange and reduction with sodium dithionite, loaded onto a home-packed DEAE Sepharose Fast Flow (Amersham Biosciences) column and eluted using a salt gradient from 0 to 50 mM of NaCl in 5mM Tris/HCl at pH 8.5. After a buffer exchange and oxidation using 1 mM potassium ferricyanide, azurin-containing fractions were loaded on a 5 ml HiTrap SP column (GE Healthcare) and eluted using a pH gradient of pH 4 to pH 6.9 (50 mM ammonium acetate). All the chromatographic steps were performed on an Äkta Purifier system (GE Healthcare). The purification process was monitored by checking the purity of the protein after each chromatographic step on SDS-PAGE and by means of UV/VIS spectroscopy (Cary 50 spectrophotometer, Varian Inc., Agilent Technologies, USA). The final product, after the last cation exchange column, appeared on an SDS-PAGE gel as a single band with apparent mass of ~14 kDa and showed an UV/Vis spectrum with a ratio Abs628nm/Abs280nm of ~0.57 which indicates full loading of the Cu-site (40).

Protein labeling was performed using a slightly modified version of a previously described protocol (27). Azurin was incubated in a molar ratio of 1:1 with the NHS-ester of the fluorescent label Cy5 (GE Healthcare, UK) in 20 mM HEPES buffer pH 8.3, for 2 hours. The unreacted label was then removed using a 5 ml HiTrap Desalting column (GE Healthcare). During the desalting step a buffer exchange to 5 mM Tris/HCl pH 8.5 was performed before the purification step described in the next section.
4.2.2 Purification of labeled species

Ion exchange chromatography (IEC) of the labeled protein species was performed on a 1 ml MonoQ column (GE Healthcare) using an Äkta Purifier (GE Healthcare) system (42). The labeled azurin fraction was loaded on the column (equilibrated with 5mM Tris pH 8.5) and subsequently protein species were eluted with a gradient from 0 to 100 mM NaCl in 5mM Tris pH 8.5 in 30 column volumes at a flow rate of 1 ml/min as recommended by the manufacturer. The elution process was followed by monitoring the absorbance at 280 nm (azurin) and 650 nm (characteristic absorption of Cy5) as shown in Figure 4.2A. The fractions corresponding to each peak were then collected and checked by means of UV/Vis spectroscopy to confirm the presence of protein (Figure 4.2B).

Figure 4.2. (A) Azurin was labeled with Cy5 and the resulting species were separated with anion exchange chromatography, recording both overall protein absorbance at 280 nm and the specific absorbance of the Cy5 label at 650 nm. (B) Display of the spectra corresponding to the peaks I-IV. The spectra of peaks III and IV strongly overlap, and are almost indistinguishable. The absorbance spectrum of Peak I has the same shape as the UV-Vis absorption spectrum of wt Cu-azurin from *Ps. aeruginosa* in the oxidized form, and is attributed to unlabeled azurin (confirmed by the ratio $\text{Abs}_{628\text{nm}}/\text{Abs}_{280\text{nm}}$ of $\sim0.57$, typical of wt azurin) The presence of protein is inferred from two spectral characteristics: the absorption at 280 nm and the typical sharp peak at 291 nm due to the only tryptophan in the sequence.
4.2.3 Absorption and Fluorescence Spectroscopy

Absorption spectra were measured using a Perkin Elmer Instruments Lambda 800 spectrophotometer with a slit width equivalent to a bandwidth of 2 nm. Fluorescence spectra and time courses in bulk were measured with an LS 55 commercial fluorimeter (Perkin Elmer, USA), with a red sensitive photomultiplier (R928, Hamamatsu, Japan), set to 5 nm band pass. Cy5 fluorescence was excited at 645 nm, and the fluorescence intensity at 665 nm was used for the analysis of the FRET efficiency.

To verify redox changes in bulk solution, fluorescence time courses were measured in a 5x5 mm quartz cuvette (Perkin Elmer) in 100 mM phosphate buffer at pH 7.0 buffer solution. The concentration of the Cy5 labeled protein was 100 nM. Protein reduction and oxidation during measurement was performed by adding reductants (dithiotreitol, DTT) and oxidant (potassium ferricyanide, K₃(FeCN)₆) from freshly prepared concentrated stock solutions (2-20 mM) directly into the cuvette to a final concentration of 5-20 µM, i.e. in 50 to 200-fold excess.

4.2.4 Azurin immobilization on glass

All glass slides were MENZEL GLÄSER Nr. 1 (Gerhard Menzel GmbH, Germany) which were cleaned by sonication in spectrometer grade acetone (45 min), then dipped in 10% NaOH/H₂O (45 min) and finally stored in methanol. Between each step, the slides were thoroughly rinsed and sonicated in deionized water (MilliQ). Before use the cover slips were blow dried under N₂ flow and ozone-cleaned (UVP PR-100 UV-ozone photoreactor) for 1 h immediately before silanization.

The surface of the cleaned glass slides was modified by depositing a layer of a 4:1 mixture of triethoxysilane (TES) and mercaptopropyl trimethoxysilane (MPTS) as described previously (32). All the silanes were purchased from Fluka and used
Figure 4.3. Protein immobilization in 3 steps: In step 1, glass slides were modified by depositing a layer of a 4:1 mixture of TES/MPTS with –SH groups exposed at the surface. Separately, in step 2, the NHS-PEO₄-maleimide linker is covalently bound to wt-azurin. Finally, the maleimide-end of the latter construct is attached to the exposed thiols at the silanized glass surface via the 24 Å long linker. The overall length of the linkers between the surface and the protein is about 31 Å.

Without purification. In the second step, wt-azurin was covalently bound through the succinimidyl-[(N-maleimidopropionamido)-hexaethyleneglycol] ester (NHS-PEO₄-maleimide, Pierce) to the modified glass slides (see Figure 4.3). Following the instructions from the manufacturer, the NHS-PEO₄-maleimide linker was added in a 100-fold excess of the protein, followed by a reaction time of 1 hour. The excess of the linker was then removed using a Centrispin-10 size exclusion column with a 5-kDa cut-off (Princeton Separations, Adelphia, NJ, USA) (32). Finally, 100 pM of NHS-PEO₄-maleimide-modified azurin was incubated on the silanized glass slide overnight at 4 °C and then rinsed with 10 mM potassium phosphate buffer at pH 7.0 to remove free azurin. This gave reproducible results of specifically immobilized individual, Cy5-labeled azurin molecules on the glass surface. The steps to functionalize the glass surface are summarized in Figure 4.3.
4.2.5 Single molecule imaging setup and single photon counting

The single molecule fluorescence measurements were conducted on a home-built sample scanning confocal microscope (Figure 4.4). The scanning confocal microscope was equipped with Time-Correlated Single-Photon Counting (TCSPC) capabilities. For fluorescence excitation a pulsed picosecond diode laser with 40 MHz repetition rate (PDL 800-B, PicoQuant GmbH) and an output wavelength of 639 nm was sent through a narrow-band clean-up filter (LD01-640/8-25, Semrock, USA), then coupled into a single-mode optical fiber, the output of which was collimated using a telescope system made of two achromatic lenses (+60 mm and +40 mm). The collimated beam was directed into the back entrance of an Axiovert 100 microscope (Zeiss), reflected by a dichroic mirror (Z 532/633 M, Chroma technology, USA) to a high numerical aperture (NA) oil objective (100× oil, NA 1.4, Zeiss, Germany) and then focused to a diffraction-limited spot (~300 nm) on the sample surface. A power density of ~0.4 kW/cm² was used at the sample to avoid excessive bleaching while recording fluorescence time traces. Epi-fluorescence from the labeled azurin was filtered with an emission filter (D 675/50 M, Chroma technology, USA) and focused with a +80 mm focal length achromatic lens on to the active area of an avalanche photodiode (Perkin-Elmer SPCM-AQR-14). The data acquisition was performed by the TimeHarp 200 TCSPC PC-board (PicoQuant, GmbH) operating in the special Time-Tagged Time-Resolved (T3R) mode, which stores the arrival time of each individual photon event. Samples were mounted onto a P-517 nanopositioner which was connected to a E-71 control unit, both from Physik Instrumente GmbH. Scanning, accurate positioning, data collection and lifetime analysis were performed by the SymPhoTime software package (PicoQuant GmbH).

Fluorescence images were acquired by scanning a 10 × 10 μm² area of the sample on the glass surface with a step size of 100 nm and a dwell time of 2 ms per point. A characteristic fluorescence lifetime image (FLIM) is shown in Figure 4.5. After imaging, the molecules in the scanned area were manually selected and
Chemical-switching of SM azurin

Figure 4.4. Single molecule fluorescence detection of azurin/Cy5 molecules. The redox state of the surface-imobilized protein was monitored using a mixture of DTT and \( K_3(FeCN)_6 \) as reductant and oxidant in the buffer solution. The redox potential of the solution was determined with a reference electrode (SCE) and counter electrode (platinum wire) connected to a voltmeter. The Az/Cy5-linker (orange curve, NHS-PEO4-maleimide) was covalently attached to the MPTS (yellow line)/TES (black line) covered glass surface (see section 4.2.4 for details).

Figure 4.5. The fluorescence lifetime image (10x10 μm^2) of immobilized azurin-Cy5 on a glass surface with a dwell time of 2 ms per pixel. The image contains, both, oxidized and reduced azurin in the presence of 200 μM \( K_3(FeCN)_6 \) and 100 μM DTT in the buffer solution.
an automatic recording procedure was started. During this procedure the scanner was moved successively to each selected spot and the fluorescence time trace was recorded over a time interval of up to 120 seconds before moving on to the next spot until all the selected spots had been processed. The fluorescence recorded from each single molecule was stored in a file to be further elaborated off-line.

4.2.6 Redox potential of buffer solution

Single molecule detection was performed in 100 mM phosphate buffer solution at pH 7.0 with freshly prepared K$_3$[Fe(CN)$_6$] and DTT as oxidant and reductant (Figure 4.4), respectively. The chemical redox potential of the buffer solution ($E$) in single molecule experiments was varied around the midpoint potential of azurin by adjusting the relative concentration of K$_3$[Fe(CN)$_6$] and DTT. The initial concentration of the K$_3$[Fe(CN)$_6$] was 200 µM in all cases and DTT was added to reach the final potentials of -20, 0, 20, 40, 60, 80 and 100 mV. The potential of the solution was measured with a voltmeter (Fluke 111) using a saturated calomel electrode (SCE) as a reference (RE) and 0.5 mm platinum wire as a counter electrode (CE). The experiments were performed in a sealed container under oxygen-free conditions by purging the solution with argon beforehand, and by maintaining an argon flow over the solution during fluorescence measurements.

4.2.7 Data elaboration and analysis

The files containing the time-tagged time-resolved data were converted to ASCII format, and processed using a changepoint-finding algorithm implemented in software which was kindly provided by Dr. Haw Yang, Princeton University (39). The changepoint-finding algorithm was run using a parameter value of $\alpha = 0.01$ for Type I errors (false positive) and a confidence parameter value of $\beta = 0.95$ to set the confidence interval around each change point. In essence, the changepoint-finding software performs a statistical analysis of the fluorescence time traces to determine the points where a change in the fluorescence intensity
occurs without making any prior assumptions. Furthermore, the software clusters the intensities in up to 5 different levels using an expectation-maximization algorithm and determines the most likely number of states for modeling the system, using a Bayesian information criterion (BIC) (39). The output of the changepoint-finding algorithm was further elaborated by using a home written algorithm to determine the time intervals associated with the oxidized and the reduced state, respectively. Only traces longer than 0.5 second and showing at least 2 transitions between different states (fluorescence on and off) were taken into account for further analysis. The in-bulk switching ratio was used as a threshold criterion to discriminate on-times (above the threshold) and off-times (below the threshold). In particular, the condition for a state change was imposed such that the intensity ratio before and after fell within ±2.5 standard deviations of the in-bulk switching ratio (90% confidence interval around the mean). Intensity drops to the background level due to blinking events were distinguished by assuming that the intensity of the oxidized state is at least a factor of 1.2 higher than the background level. Such blinking events were not counted as state changes. The on and off times from the selected single molecules were stored and subsequently analyzed. Data analysis was performed using custom-written algorithms in Matlab 7.9.

Histograms of the on- and off-times were built using a bin size of 1 ms. Fitting of the on- and off-times histogram was performed with a mono-exponential function defined as follows:

$$y = y_0 e^{-kx}$$  

where $x$ is the time bin number and $y$ is its occurrence. The fitted parameters are: $y_0$, the $y$ value at time zero and $k$, the rate constant of the decay. According to which distribution was fitted, an “on” or “off” subscript was added to $k$, corresponding to the reduced and oxidized state of azurin, respectively, to distinguish the two parameters.
The parameter \( \bar{P}_{\text{ox}} \) (\( \bar{P}_{\text{red}} \)) is used in the present work to describe the time-averaged probability that the molecule is in the oxidized (reduced) state (33). The value of \( \bar{P}_{\text{ox}} \) for each time trace was calculated as follows:

\[
\bar{P}_{\text{ox}} = \frac{\sum_{i=1}^{n} \tau_i \cdot \text{off}}{\sum_{i=1}^{n} \tau_i \cdot \text{off} + \sum_{j=1}^{m} \tau_j \cdot \text{on}}
\]  

(2)

where \( \tau_{i,\text{off}} \) and \( \tau_{j,\text{on}} \) are the \( i \)-th off- and \( j \)-th on-times, respectively; \( n \) and \( m \) are the total number of off- and on-time intervals in a single trace, respectively. A similar expression is used to calculate \( \bar{P}_{\text{red}} \). The denominator is essentially the total duration of the time trace (before bleaching).

### 4.3 Redox thermodynamics of single molecules

#### The Nernst equation

The electrode potential of a redox couple in solution, i.e. the free energy when referenced against a standard hydrogen electrode, is given by the Nernst equation in terms of the concentrations of reductant and oxidant ([\( \text{red} \)] and [\( \text{ox} \)], respectively),

\[
E = E_0 - \frac{RT}{nF} \ln \frac{[\text{red}]}{[\text{ox}]}
\]  

(3)

Here, \( R \) is the universal gas constant \((R = 8.314472 \ \text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1})\), \( T \) is the absolute temperature, and \( E_0 \) is the midpoint potential of the reaction. \( F \) is the Faraday constant \((F = 9.64853399 \times 10^4 \ \text{C} \cdot \text{mol}^{-1})\), and \( n \) corresponds to the number of electrons that are transferred in the reaction \((n = 1 \ \text{for azurin})\).

#### Redox system

In our experiments, we have a system consisting of three redox couples:

- Ferricyanide and ferrocyanide, \((E_{0,\text{FeCN}} = 116 \ \text{mV at pH}7, \ \text{vs. SCE})\):
  \[
  \text{[Fe (CN)$_6$]$^3^-$} + \varepsilon \Rightarrow \text{[Fe (CN)$_6$]$^4^-$}
  \]  

(4)
• DTT (dithiothreitol) and oxidized DTT, \( E_{0,\text{DTT}} = -574 \text{ mV at pH7, vs. SCE} \) (43)):

\[
C_4H_{10}O_2S_2 \rightleftharpoons C_4H_8O_2S_2 + 2H^+ + 2\bar{e}
\] (5)

• Oxidized azurin \( \text{Az}_{\text{ox}} \) and reduced azurin \( \text{Az}_{\text{red}} \), \( E_{0,\text{Az}} = 21 \pm 19 \text{ mV at pH7, vs SCE} \) (44)):

\[
\text{Az}_{\text{ox}} + \bar{e} \rightleftharpoons \text{Az}_{\text{red}}
\] (6)

At equilibrium, we have the following expression for the redox potential of the system, according to the Nernst equation:

\[
E = E_{0,\text{FeCN}} - \frac{RT}{F} \ln \left( \frac{[\text{Fe(CN)}_4^{3-}]}{[\text{Fe(CN)}_4^{4-}]} \right) = E_{0,\text{DTT}} - \frac{RT}{2F} \ln \left( \frac{[\text{DTT}_{\text{red}}]}{[\text{DTT}_{\text{ox}}]} \right)
\] (7)

Thus the redox potential can be calculated if we know the total concentrations of ferri- and ferrocyanide or of oxidized and reduced DTT.

The next treatment follows the theory as developed in Ref. (45). Depending on the redox conditions in the sample, an individual azurin molecule will continually switch between the oxidized and the reduced state at a rate which is determined by the concentrations of oxidizing and reducing species. We can evoke the ergodicity principle to formulate the Nernst equation in terms of the population distribution over time in such a two-state model (45).

The energy difference, \( \Delta E \), that is associated with the electron transfer reaction of azurin (Eq. 6), in the presence of the working electrode at a potential \( E \) is given by:

\[
\Delta E = ne\Delta V = ne(E_0 - E)
\] (8)

Here, \( e \) is the elementary charge, and \( n \) is the number of electrons involved in the reaction, \( i.e. \) the charge transferred in the reaction is \( ne \). Assuming that the time-averaged probabilities for the oxidized and reduced state of azurin, \( P_{\text{ox}} \) and \( P_{\text{red}} \), respectively, obey the Boltzmann distribution, we have
\[
\frac{P_{\text{red}}}{P_{\text{ox}}} = e^{ne(E_0 - E)/k_BT}
\]  

(9)

We have just retrieved the Nernst equation (Eq. 3), in terms of \( P_{\text{ox}} \) and \( P_{\text{red}} \), as can be seen when we rearrange this equation:

\[
E = E_0 - \frac{k_BT}{ze} \ln \left( \frac{P_{\text{red}}}{P_{\text{ox}}} \right)
\]  

(10)

This is clear when it is denoted that \( R = k_B \cdot N_A \) and \( e = F/N_A \). The only difference is that the concentrations of reduced and oxidized molecules are now replaced by the probabilities of being in either of the two states.

The time-averaged probabilities of being in these states must obey the condition \( P_{\text{red}} + P_{\text{ox}} = 1 \). Solving for \( P_{\text{red}} \) and \( P_{\text{ox}} \) gives

\[
P_{\text{red}} = \frac{1}{1 + e^{-\Delta E/k_BT}}
\]  

(11a)

\[
P_{\text{ox}} = \frac{1}{1 + e^{\Delta E/k_BT}}
\]  

(11b)

If we define the forward and backward rates in Eq. 6 as \( k_{\text{red}} \) and \( k_{\text{ox}} \), respectively, we can also write

\[
\frac{k_{\text{ox}}}{k_{\text{red}}} = e^{\Delta E/k_BT}
\]  

(12)

If a single molecule, which switches between the two states, is followed for a certain amount of time, we can determine the probability distribution of the dwell times, i.e. the times the molecule stays in either of the two states. For the present case, these distributions are given by

\[
P_{\text{ox}}(t) = k_{\text{red}}e^{-k_{\text{red}}t}
\]  

(13a)

\[
P_{\text{red}}(t) = k_{\text{ox}}e^{-k_{\text{ox}}t}
\]  

(13b)

We have thus established a firm relationship between redox properties of a single molecule and its dynamic behavior over time. Details for this description
Chemical-switching of SM azurin

of a two-state system can be found in the textbook on Biological Physics by Philip Nelson (45).

4.4 Results

4.4.1 Purification of labeled azurin

In vitro single-molecule experiments require a high level of protein purification on account of an inhomogeneous population which can otherwise significantly complicate the experimental results (46). Azurin contains a number of exposed lysines which compete with the N-terminus for binding of the Cy5-NHS ester, which may result not only in multiple labeled species, but also in multiple labels per protein molecule (42). To obtain a monodisperse and homogenous species for the single molecule experiments, azurin was further purified after labeling. The Az-Cy5 was fractioned using high resolution anion exchange chromatography, recording both overall protein absorbance at 280 nm as well as the specific absorbance of Cy5 at 650 nm. A typical chromatogram is shown in Figure 4.2A.

In the chromatographic separation we observed multiple peaks. The presence of protein in each fraction is inferred from two spectral characteristics: the absorption at 280 nm and the typical sharp peak at 291 nm due to the only tryptophan in the sequence. From the UV-vis spectral analysis of each eluting species (Figure 4.2B), fraction I shows the same spectrum as of the oxidized azurin, and is therefore ascribed to the unlabeled protein. The band around 628 nm in this case is due to the absorption of the Cu\(^{+}\) center. Moreover, in Figure 4.2B the ratio \(\text{Abs}_{628\text{nm}}/\text{Abs}_{280\text{nm}}\) = 0.57 for Peak I, the same as that of the unlabeled protein. The UV-vis spectra of fraction II-IV in Figure 4.2B display features of the protein as well as the label and are ascribed to labeled protein fractions. The spectra show an intense peak around 650 nm accompanied by a shoulder at 600 nm, characteristic of the Cy5 label.
We have used fraction II in all the single molecule experiments. The use of such a homogeneous sample proved to be essential for consistent recordings of the fluorescence dynamics of single molecules of labeled azurin.

### 4.4.2 Fluorescence switching in bulk

To assess the efficacy of fluorescence detection of redox-induced switching of Az-Cy5 we performed ensemble measurements using conventional spectrometry. The sample was excited at 645 nm and the fluorescence emission was monitored at 665 nm after addition of reducing or oxidizing agents.

It is well known for all type-1 Cu centers that the absorption band at 590-630 nm is only observed in the Cu\(^{2+}\) state, *i.e.* it is absent in the Cu\(^+\) state. Thus, it is inferred (the FluRedox principle) that resonance energy transfer from the fluorescent label to the Cu center occurs in the oxidized, but not in the reduced state of azurin. This is demonstrated in Figure 4.6 (black trace), where, upon addition of oxidant \( \text{K}_3(\text{FeCN})_6 \), the fluorescence intensity decreases, while subsequent addition of reductant (DTT) results in its increase. In principle, this reduction-oxidation cycle can be repeated indefinitely as previously reported \((25, 27)\). The slight decline in fluorescence amplitude is due to dilution of the sample upon repetitive addition of reactants. We define the switching ratio \((SR)\) of the Az-Cy5 construct as follows:

\[
SR = \frac{I_{\text{red}} - I_{\text{ox}}}{I_{\text{red}}} \times 100 \%
\]

(14)

where \(I_{\text{red}}\) and \(I_{\text{ox}}\) are the fluorescence intensity values in the reduced (on) and oxidized (off) state, respectively. For the Cy5 labeled azurin fraction associated with peak II in Figure 4.2, we found that in solution the \(SR \cong 90\%\).

The Cu ion in azurin can be reconstituted with Zn\(^{2+}\). This Zn-azurin moiety is not redox active, and we therefore used it as a control \((27, 31)\). Zn-azurin when labeled with Cy5 did not show any fluorescence switching upon addition of oxidant or reductant (grey line in Figure 4.6). Moreover, the control measure-
Figure 4.6. FRET-based on-off switching in bulk. 100 nM Cu-azurin labeled with Cy5 (black line) titrated with aliquots of reductant (DTT) and oxidant (K₃(FeCN)₆) in 100 mM phosphate buffer at pH 7.0. We observed a significant reversible change of the fluorescence intensity of Cy5 labeled azurin upon addition of oxidant or reductant. The fluorescence intensity in the oxidized state is reduced by about a factor of 10 compared to that in the reduced state. 50 nM Zn-azurin labeled with Cy5 (grey line) was used as a control and did not show any fluorescence switch.

ments also demonstrate that the Cy5 fluorescence intensity is not affected by the addition of reductant (DTT) or oxidant (K₃(FeCN)₆).

4.4.3 Resolving fluorescence time traces

In order to ascertain the redox-based fluorescence switching of the individual azurin molecules immobilized on a MPTS/TES coated glass substrate, we applied the fluorescence detection method that is based on the FluRedox principle. For this we tuned the chemical redox potential of the solution around the midpoint potential of azurin by means of varying the concentration of oxidant (K₃(FeCN)₆) and/or reductant (DTT). The final redox potentials of the solution were adjusted to −20, 0, 20, 40, 60, 80 and 100 mV, respectively, as measured with a reference electrode (standard calomel, SCE) and a counter electrode (a Pt wire) which were inserted in the droplet that covered the functionalized glass substrate. The two electrodes were wired to a voltmeter. We obtained only stable potentials
under oxygen-free conditions. A potentiometric titration of a solution of K$_3$(FeCN)$_6$ with DTT under anaerobic conditions is shown in Figure 4.7. The half-wave potential for the K$_3$(FeCN)$_6$/DTT couple obtained from the Boltzmann fit is about 31 mV vs SCE which is close to the midpoint potential of azurin (44).

As shown on Figure 4.3, Az-Cy5 was immobilized on a mixed monolayer of TES/MPTS on glass at low density such that fluorescence from single molecules can be resolved in the confocal microscope. Fluorescence time traces were obtained by successively parking the laser at each fluorescent spot in the image, and then recording the arrival times of the emitted photons. Time traces were obtained for up to 2 minutes duration. Photobleaching of Cy5 limited the time duration for which photon emission was observed to tens of seconds, and was marked by a drop of the count rate to background levels in a single step. The sample was de-aerated beforehand by bubbling with argon, and the measurements were performed under oxygen-free conditions in a sealed cell under an argon atmosphere. This not only improved the stability of the redox potential, but also enhanced the photostability of Az-Cy5, extending the time at which photobleaching occurred by at least an order of magnitude.

Typical time traces are shown in Figure 4.8 for two single Az-Cy5 molecules at
Figure 4.8. The real time fluorescence intensity traces of a single azurin molecule with a 10 ms bin size (black) and calculated intensity change-point states (purple) overlaid as a function of time. They exhibit an on-off switching behavior which depends on the redox potential in solution: A) at 20 mV vs. SCE, $P_{ox} = 0.52$, and B) at 60 mV, $P_{ox} = 0.76$.

Different redox potentials. They show a pronounced fluorescence switching behavior, and appear to be dominated by two discrete intensity levels, corresponding to the oxidized and the reduced state, respectively, of that particular Az-Cy5 molecule. This assignment is supported by the fact that the
control sample of Cy5-labeled Zn-Az did not show any fluorescence switching under the same condition (Fig. 4.9). The observed fluorescence time profile (Fig. 4.8, black line) of Cu-Az-Cy5 generally depends on the redox potential in solution. No redox switching was observed at potentials higher than 100 mV and lower than −20 mV: the molecule is either fully oxidized or reduced, respectively. At a potential of 60 mV (Fig. 4.8B) the Az-Cy5 molecules is mostly in the oxidized state ($\bar{P}_{\text{ox}} = 0.76$), while the reduced fraction increases at 20 mV ($\bar{P}_{\text{ox}} = 0.52$, Fig. 4.8A).

These numbers were obtained by a quantitative analysis of the time traces using the change point analysis program developed (and kindly provided) by Watkins and Yang (39). This program uses a generalized likelihood ratio test that determines the location of an intensity change point based on individual photon arrival times. Expectation maximization clustering and the Bayesian information criterion are then used for accurate determination of the true number of states accessible to the system. This procedure allows rigorous and quantitative determination of intensity change points without the artificial time resolution limitations that arise from binning and thresholding.

Figure 4.9. The real time fluorescence intensity trace of a Cy5 labeled single Zn-Az with a 10 ms bin size. The redox potential in solution is 20 mV vs SCE. Zn-Az does not show any fluorescence switching under the same conditions as Cu-Az (see Fig. 4.8).
Figure 4.10. FRET-based fluorescence switching ratio (SR) of individual azurin-Cy5 molecules immobilized on a glass surface. The histogram is based on data from 150 single molecules, and the average SR of 87 ± 5% correlates well with that of labeled azurin in bulk solution (see figure 4.6).

In Figure 4.8, the time traces that resulted from the change point (CP) analysis (purple lines) were overlaid with the experimental data which are accurately reproduced. The CP analyses were actually performed in terms of 5 intensity levels, because then we were able to reliably identify the occasional blinking event by the intensity drop to the background level. Blinking is attributed to the photophysical behavior of Cy5 (47–52). The events are relatively rare, and are not counted as a redox transition. The other 4 intensity levels were assigned as "on" (Az-Cy5 reduced) or "off" (Az-Cy5 oxidized) depending on the threshold level (dashed, horizontal line in Figure 4.8) that was based on the switching ratio measured in bulk solution (see Figure 4.6). Because of the high switching ratio the fluorescence intensity of an oxidized Az-Cy5 molecule is rather close to the background level.

We calculated the fluorescence SRs of individual Az-Cy5 molecules using Equation 14. Here, the values for $I_{\text{red}}$ and $I_{\text{ox}}$ were obtained from the CP fits similar to the ones shown in Figure 4.8 (purple lines). The histogram in Figure 4.10 shows the distribution of fluorescence SRs of individual azurin molecules immobilized on a glass surface. The maximum SR has a value of about 91%,
while the average is 87 ±5%, in good agreement with that of redox-induced fluorescence switching in bulk. However, we observe some heterogeneity in the distribution of single azurin SRs, possibly associated with (average) variations in distance of Cy5 to the redox center by virtue of the length of the linker.

4.4.4 Heterogeneity in fluorescence lifetimes

To provide further insight into the FRET-based redox changes, fluorescence lifetime studies were carried out on the immobilized Az-Cy5 molecules. Since we know (from the time-tag) whether each detected photon in a particular time trace is associated with the reduced or the oxidized state, we can reconstruct separate fluorescence decay curves for both states of the same molecule. The fluorescence decay curves of individual Az-Cy5 molecules were then fitted to mono-exponential decays convoluted with the instrument response function (IRF), to determine $\tau_{\text{ox}}$ and $\tau_{\text{red}}$, the fluorescence lifetimes in the oxidized and the reduced state, respectively.

The results are shown in Figure 4.11 for the reduced and oxidized form of Az-Cy5. In Figure 4.11A, we show an example of the fluorescence decays and their exponential fit of a single Az-Cy5. In this example, the lifetimes are $\tau_{\text{red}} = 1.8$ ns (red curve) and $\tau_{\text{ox}} = 0.8$ ns (blue curve). Here, the oxidized Az-Cy5 lifetime is reduced by the effect of FRET.

To investigate the heterogeneity in fluorescence lifetime of Az-Cy5 in its reduced/oxidized form we have analyzed more than 150 time traces. The distribution of the lifetimes of oxidized and reduced single azurin molecules are shown in Figure 4.11B which shows a significant heterogeneity for both the oxidized and the reduced forms of azurin. The experiments were repeated with the redox-inactive variant, Zn-azurin, in order to verify that the observed lifetimes were not affected by the redox components in solution. In contrast to Az-Cy5, the labeled Zn-azurin did not show any fluorescence switching, and the fluorescence lifetimes were similar to the lifetime of reduced Az-Cy5.
Figure 4.11. (A) Fluorescence lifetimes (dots) and fitted mono-exponential decays (lines) of surface-immobilized single azurin molecule in the oxidized (blue) and the reduced states (red): $\tau_{\text{red}} = 1.8$ and $\tau_{\text{ox}} = 0.8$ ns. (B) Fluorescence lifetime distribution of surface-immobilized single azurin molecules in the oxidized (blue) and the reduced state (red). Average lifetimes are $\bar{\tau}_{\text{ox}} = 0.7 \pm 0.15$ ns and are $\bar{\tau}_{\text{red}} = 1.8 \pm 0.2$ ns.

4.4.5 Redox parameters of a single Az-Cy5 molecule

The redox potential of the solution, $E$, was tuned by adding oxidant ($K_3(FeCN)_6$) and reductant (DTT) to the solution. The initial concentration of $K_3(FeCN)_6$ was 200 µM in all experiments, and DTT was added in small aliquots to adjust the potential. The redox potential ($E$) of the buffer solution covering the Az-Cy5 functionalized glass surface was thus adjusted to $−20, 0, 20, 40, 60, 80,$ and $100$ mV, respectively. At each potential we recorded a series of fluorescence time traces from individual Az-Cy5 molecules, and for each time trace we calculated $\bar{\tau}_{\text{ox}}$ and $\bar{\tau}_{\text{red}}$ (Eq. 2). From these results we calculated the midpoint potential of each single Az-Cy5 molecule using Equation 10.

The result of this analysis is summarized in Figure 4.12, which shows the distributions of the calculated midpoint potentials ($E_0$) at a number of solution potentials. The midpoint potentials range from $−100$ to $150$ mV which is consistent with electrochemical data (53). All the measured midpoint potentials are lumped together in the histogram shown in Figure 4.13.
Figure 4.12. The histogram of midpoint potential ($E_0$) of single azurin molecules at solution potentials ($E$) of $-20, 0, 20, 40, 60, 80$ and $100$ mV. The initial concentration of $K_3[Fe(CN)]_6$ was $200$ µM and DTT was added to adjust the redox potential in solution which was measured with a voltmeter using a saturated calomel electrode as a reference and $0.5$ mm platinum wire as a counter electrode. All the measurements were performed under anaerobic conditions in a sealed sample-holder under continuous argon flow. $E_0$ was calculated using the Nernst equation (see Equation 10 in the text).

We observe that the measured distribution of midpoint potentials shifts with the actual redox potential of the solution. This can be explained by the fact that the data contain a bias towards selection of Az-Cy5 molecules that are relatively
Figure 4.13. The histogram of midpoint potential ($E_0$) of about 200 single azurin molecules. The average midpoint potential was calculated from a Gaussian fit as $12 \pm 3$ mV with a fwhm = 92 mV vs SCE. $E_0$ of each single molecule was calculated using the Nernst equation (see Equation 10 in the text).

Because of the high switching ratio, molecules that are mostly in the oxidized state are under-represented because of low visibility. Furthermore, we have only analysed molecules that show fluorescence switching activity, which implies a bias by selecting molecules with a range of midpoint potential around the redox potential in solution. It is estimated that the fraction of molecules that are actively switching is about 50% at 20 and 40 mV, and is decreasing at higher and lower redox potentials, dropping to about 10% at −20 and 100 mV.

We note, however, that the overall distribution in Figure 4.13 is dominated by the measurements at 20 and 40 mV, both close to the average midpoint potential. For these measurements the bias is relatively small, and we believe that the distribution in Figure 4.13 is representative of the heterogeneity of the midpoint potentials of individual Az-Cy5 molecules. The histogram in Figure 4.13 for about 200 individual molecules was fitted with a Gaussian. The average midpoint potential is $12 \pm 3$ mV vs. SCE, and the distribution is characterized by a fwhm of 92 mV.
4.4.6 Electron transfer kinetics of a single azurin

The final aspect of fluorescence-detected redox switching of single Az-Cy5 molecules is the time scale, and thus the rate at which the switching occurs. The relevant parameters are the reaction rate constants of the electron transfer, $k_{\text{ox}}$ and $k_{\text{red}}$, for the oxidation and reduction reaction, respectively. They can be deduced for each molecule from the time traces through the distribution of dwell times, *i.e.* the times the molecule stays in a certain state before jumping to the

![Histograms of on and off times for a single azurin-Cy5 at solution potential of 20 mV.](image)

**Figure 4.14.** Histograms of on and off times for a single azurin-Cy5 at solution potential of 20 mV. The rate constant is given by the inverse of the characteristic time constants of the mono-exponential fits of the distributions. This result is obtained from the time trace in Figure 4.8A.

![The distribution of the reaction rates of individual Az-Cy5 molecules.](image)

**Figure 4.15.** The distribution of the reaction rates of individual Az-Cy5 molecules.
The reaction rate constants can be derived from the dwell time distributions using equation 13a and 13b. The dwell times of both states (oxidized and reduced) were measured for all the switching time traces.

The dwell time distributions obtained in this way were fitted to a mono-exponential decay for both states (oxidized and reduced), from which the characteristic time constant and its reciprocal, the reaction rate, were deduced. In Figure 4.14, we show the distribution of dwell times for both, the reduced and oxidized state of a single azurin molecule (see Figure 4.8A). From the characteristic time constants of the mono-exponential fits we find for this case that \( k_{\text{ox}} \) and \( k_{\text{red}} \) are equal to 10 s\(^{-1}\) and 6 s\(^{-1}\), respectively.

We have determined the reaction rate constants, \( k_{\text{ox}} \) and \( k_{\text{red}} \), for a limited number of molecules, because not all time traces were long enough to obtain a meaningful fit of the dwell times. Figure 4.15 shows that the distribution of reaction rates is rather broad.

### 4.5 Discussion

**Photophysics of Cy5:** In the present work, azurin was labeled with Cy5-NHS forming the donor in a FRET pair with the Cu redox center which acts as the acceptor. In FRET experiments Cy5 is notorious because of its complex excited state dynamics (for reviews, see (47, 54)). This involves the formation of triplet states and of long-lived conformational isomers, both of which are susceptible to photochemical transformations leading to permanent photobleaching. For the latter, not only the presence of oxygen, but also reactions with thiols play a key role (49). At the single-molecule level, the transient population of non-fluorescent states in Cy5 is the main cause of blinking, which may interfere with other signals that are being monitored via Cy5 excitation. In order to reduce the blinking and photobleaching rates, the effects of oxidizing and reducing agents (e.g., \( N,N \)-methylviologen, ascorbic acid) and triplet state quenchers (TSQs; e.g.,
Trolox, β-mercaptoethylamine) on the excited state properties of Cy5 in solution have been studied extensively (48, 55–58). It was shown that thiol-induced blinking of Cy5 can be overcome using Trolox in combination with a reducing/oxidizing system. Notably, the conjugation of Cy5 to biomolecules can also strongly reduce the efficiency of photoisomerization, resulting in a large increase of fluorescence quantum yield and lifetime (59–63).

In FRET experiments, photo-induced blinking of Cy5 seems to be most problematic when it is used as the acceptor, in which case the shorter-wavelength excitation of the donor may also affect the photophysics of Cy5 (64). Fluctuations in Cy5 fluorescence due to the population of transient, non-fluorescent states can be easily mistaken as fluctuations in FRET efficiency (65).

In the measurements on Cy5-labeled azurin described in this chapter, it proved, indeed, essential to remove oxygen from the solution. Adding oxygen scavengers to the solution was not an option because they may interact with the label or the chemicals in solution. Moreover, TSQs have key limitations, including poor aqueous solubility, problems with membrane permeability and biological toxicity (66,67). Rather, oxygen was removed by sparging with argon. It dramatically increased the number of emitted photons from the Cy5-label before bleaching. Under those conditions, and with 100-200 µM of reductant and oxidant in solution, we did not see any blinking in the control experiments with Cy5-labeled Zn-Az on the millisecond time scale. We can actually not exclude that blinking still occurs on the sub-millisecond time scale. It is quite possible that the redox components in solution contribute to triplet quenching and the suppression of subsequent photochemical reactions of Cy5 labeled azurin. The conjugation of Cy5 with azurin is also likely to reduce the propensity for photoisomerization. These possibilities were not explored in further detail.

**Fluorescence lifetimes:** The fluorescence lifetime of Cy5 is somewhat variable, depending on the solvent and the viscosity, for example. The fluorescence lifetime increases from 1.0 ns in water (68) to 2.0 ns when embedded in
polyvinylalcohol (PVA) (52). It is generally believed that this variability of the fluorescence lifetime is associated with the freedom of rotation of the polymethine chain (with heterocyclic moieties at each end) upon excitation of Cy5, which is associated with cis-trans photoisomerisation. This is corroborated by the relatively long fluorescence lifetime of Cy5 in a PVA matrix (52). For the average fluorescence lifetime of reduced Az-Cy5 we obtain a value of 1.8 ±0.2 ns, and similar values of Cy5-labeled Zn-Az. This is substantially longer than for Cy5 in water, and suggests that conjugation to azurin significantly affects the dynamics of the photoisomerisation process. Presumably the rate of photoisomerization is lower than that of the free dye in aqueous solution. The distribution of the fluorescence lifetimes in Figure 4.11B could be due, at least partially, to different ways at which the Cy5 label can arrange itself at the protein surface.

When Az-Cy5 is oxidized, the average fluorescence lifetime is reduced to 0.7 ±0.15 ns because of fluorescence quenching by FRET from the fluorophore to the Cu-center. From the fluorescence lifetimes we calculate a FRET efficiency of \( E = 1 - (\tau_{\text{ox}}/\tau_{\text{red}}) = 0.60 \pm 0.09 \). This number clearly deviates from the value of 0.87 that we calculate from the average switching ratios (Figure 4.10). It is an observation that we cannot explain at this moment, and that needs to be investigated in more detail.

**Midpoint potentials:** The average value of the midpoint potentials as measured by the single-molecule fluorescence time traces are consistent with other measurements, including those presented in Chapters 2 and 3 of this thesis. New in these experiments is the fact that we can obtain the distribution of midpoint potentials of individual Az-Cy5 molecules in solution, which is characterized by a fwhm of 92 mV. This value is significantly larger than the value of 14-15 mV found by FCV of Az-Cy5 adsorbed on an octanethiol SAM-coated gold electrode, both, at the single-molecule level (Figure 3.13, Chapter 3) and with high-density coverage (Figure 2.4a). The latter samples involve adsorption of Az-Cy5 on the thiolalkane SAM through hydrophobic interaction.
It is well established that the electrostatic environment of the entire protein and solvent system is a determining factor in fine-tuning the electronic properties of the metal-binding site of copper-containing proteins (69). In particular, hybrid quantum mechanics/molecular mechanics model calculations have shown that the electronic properties of the Cu-center in azurin are sensitive to long-range electrostatic interactions (70). These calculations also show a significant solvent rearrangement in a region close to the copper ion, specifically, around the copper-bound His117 residue upon reduction. It is claimed that the water rearrangement accounts for ~80% of the calculated value of the reorganization energy in this process. A similar conclusion was reached on the basis of experiments on electron tunneling in azurin crystals (71). Presumably the rearrangement is driven by modification of the electrostatic potential at the protein surface around His117, reflecting the change in the oxidation state of Cu. It may also affect the conformation of solvent-exposed side chains.

It thus seems reasonable to conclude that the local variations in the outer sphere around His117 can contribute to variations in the midpoint potential of individual azurin molecules. This residue, which has a key role in the electron transfer reaction of azurin, is normally in direct contact with the solvent. The experiments described in this chapter were designed to minimize the effect of the (modified) glass surface by using a rather long linker to covalently immobilize the protein. It may be assumed that under those conditions the Az-Cy5 behaves as a free molecule in solution with the His117 residue fully exposed to the solvent environment. This is different for the immobilization method that was used in Chapters 2 and 3. Here, the hydrophobic patch of the protein around His117 is largely shielded from the solution. In fact, at the hydrophobic interface between the protein and the SAM, most water molecules will be expelled. The result is a more homogeneous and a more static environment of the His117 residue than for azurin in solution. This is reflected in the relatively small heterogeneity of the midpoint potential, compared to that of Az-Cy5 in solution.
Dwell times and reaction rates: We observe a large heterogeneity in the forward and backward electron transfer reactions of Az-Cy5 with oxidants and reductants in solution (Figure 4.15). We attribute this heterogeneity to variations in the reorganisation energies from molecule to molecule. It would be interesting to extend these experiments to include different oxidants and reductants at a wider range of concentrations.

4.6 Conclusions

In summary, we have successfully immobilized Cy5-labeled azurin at the SM level by using a thiol-based, site-specific covalent linker. We have demonstrated that the fluorescence intensity of a single azurin molecule as a function of time shows on-off switching behavior which depends on the redox conditions in solution. Purified single Az-Cy5 molecules show a 87% switching ratio that is consistent with results of ensemble measurements.

To the best of our knowledge, we were able to determine for the first time the reaction kinetics and thermodynamic midpoint potential of individual azurin molecules. We have made a quantitative assessment of the heterogeneity of the midpoint potential, electron transfer rates and the lifetimes at the single-protein level. The local environment of the His117 residue was shown to be a determining factor for the distribution of midpoint potentials of single Az-Cy5 molecules. The FluRedox method could be easily applied to many other redox molecules, opening new doors for the application of fluorescence-based biosensor and molecular electronics.
References


17. Van de Kamp M, Silvestrini MC, Brunori M, Van Beeumen J, Hali FC, and Canters GW. 1990. Involvement of the hydrophobic patch of azurin in the electron-transfer reactions with


Chemical-switching of SM azurin


Chemical-switching of SM azurin