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Chapter 3

Fluorescence-detected electrochemistry of single redox proteins reveals the thermodynamic dispersion

Abstract

There are several parameters that can influence molecular electron transfer such as protein folding, electric field drop, and molecular orientation. Classical electrochemistry and spectroscopy are not sufficient to control and solve such influences at the molecular level. Redox-active metalloproteins tagged with a FRET-coupled organic fluorophore can be used to study electron transfer to a transparent conductive electrode in an optical configuration. Herein, we show that we can observe redox switching under potentiostatic control of individual, wild-type (wt) azurin molecules, tagged with Cy5 and immobilized on a semi-transparent gold electrode via an octanethiol self-assembled monolayer. The distribution of optically-determined midpoint potentials of individual azurin molecules gave a maximum value at 45.7 ± 0.5 mV with and fwhm of 15 mV (vs. SCE). The work presented here, shows direct measurement of controlled electron-switching, one electron at a time, of an individual redox protein, and its thermodynamic dispersion.
3.1 Introduction

The development of redox-gated (bio)molecular electronic devices and protein biochips is critically dependent on the electron transfer (ET) across the molecule-substrate interfaces (1–4). Such devices may incorporate metalloproteins which are at the central core of numerous chemical and biological processes and which have a remarkable ability to drive ET reactions in e.g., photosynthesis, cellular respiration and signaling (5, 6). Interfacial electrochemical ET has been extensively studied to investigate thermodynamic properties and quantitative electronic parameters of redox-active biomolecules, as well as their structure and distance dependence when adsorbed on conductive substrates (7–13).

It is considered a great challenge to increase the sensitivity of detection in the field of bioelectrochemistry, which drives the development of new methods and techniques. For example, electrochemical tunneling microscopy (ECSTM) has been used to map the tunneling resonance of single redox proteins (14–16), and to measure their electron transfer distance decay constants (16, 17). Furthermore, the miniaturization of the electrode construction (18) has been pursued, while another approach takes advantage of redox cycling in so-called nanogap transducers (19).

Being based on amperometric detection, these methods are inherently limited by the sensitivity of electric current measurements, requiring at least a 1000 electrons/s or so to obtain a measurable signal. In recent years, an enhanced sensitivity was achieved with spectroelectrochemistry which combines spectroscopy and electrochemistry. It takes advantage of the fact that the electronic energy transitions of many redox proteins exhibit an absorbance change in the UV-Vis region upon oxidation or reduction. It has been successfully applied to cytochrome c (20–22), blue copper proteins (21, 23) and redox enzymes (24, 25). Single-molecule sensitivity of spectroelectrochemistry at the
molecule-metal interface has been demonstrated by surface-enhanced Raman spectroscopy (26, 27).

In this chapter, we will show that individual electron transfer events under electrochemical control can be observed, and that the redox properties and thermodynamics of individual biomolecules can be accurately measured. The approach is based on the FluRedox method, described in Chapter 1, by which electron transfer events in redox proteins can be monitored by fluorescence detection which offers a dramatic increase in detection sensitivity. Proof of principle will be demonstrated by measurements on azurin.

Azurin is an electron-shuttling T1 type metalloprotein, with many interesting features which include applications in sensor technology and integration into nano-array devices (14, 29, 30). Azurin, from *Pseudomonas aeruginosa*, has a molecular weight of 14 kDa and contains one copper (Cu) ion which is the redox center that is located in the so-called northern region of the protein, buried in a hydrophobic patch at 7 Å distance from the surface (Figure 3.1A). The pentacoordinated Cu center, surrounded by its ligands His46, His117, Cys112, Met121 and Gly45, shows a distorted trigonal bipyramidal geometry (28) (Figure 3.1B).
Absorption spectra of Cu-Az in its oxidized (solid line) and reduced (dash line) form. Az absorption spectrum was measured at room temperature on 50 μM of Az in 20 mM Hepes buffer solution. The absorption spectrum of oxidized Cu-Az shows two main peaks at 280 nm and 628 nm (Cu$^{2+}$ absorption). Reduced Cu-Az shows only the 280 nm band.

The geometrical arrangement of ligands around the redox center affects the spectroscopic properties as well as the thermodynamic and kinetic features of azurin (31). Canters and coworkers, for example, rearranged the protein backbone by replacing His117 with glycine thus created a “hole” in the azurin coordination sphere. Addition of ligands such as imidazole rescues the T1 Cu center while anionic ligands induce a T2-character (32–34). ET reactions from azurin to its physiological partners occur through the aforementioned hydrophobic patch, along a pathway that involves His117 (33). Azurin in its oxidized state (Cu$^{2+}$) displays an intense ($\varepsilon = 5700$ M$^{-1}$cm$^{-1}$) ligand-to-metal-charge-transfer transition in the visible region of ~600 nm which gives rise to its fascinating blue color (35). Three strong ligands (His46, His117, Cys112) determine its main spectroscopic properties and are responsible for the very small Cu hyperfine coupling of T1 Cu contrary to T2 type proteins (36, 37). The optical absorption almost disappears when the protein is reduced to the Cu$^+$ state (see Figure 3.2).

In the so-called FluRedox principle, this spectroscopic feature of azurin has been combined with fluorescence resonance energy transfer (FRET) as a means to monitor redox changes of the Cu site (the energy acceptor) with an
Figure 3.3. An artistic representation of the fluorescence electrochemistry assembly combined with a fluorescence microscopy. A reference electrode (SCE) and counter electrode (Pt wire) were inserted in the sample solution which was contained in a sealed holder with a glass cover slip at the bottom. The cover slip was coated with a 10-12 nm thick gold layer which functioned as the optically transparent working electrode (OTE). Azurin labeled with Cy5 is adsorbed on the self-assembled monolayer of octanethiol which was deposited on the gold film. Azurin molecules are oriented (by hydrophobic interaction) with the copper center facing the electrode surface. According to the FluRedox principle, fluorescence of the dye label is quenched when the protein is oxidized because of FRET from the attached fluorophore to the metal-cofactor (dark blue sphere). In the reduced form of the protein (light blue sphere), quenching does not occur and emission of the fluorophore is uninhibited.

externally tagged dye label (the fluorescent donor) (38–40). For this, the absorption band of the protein has to overlap with the emission band of the dye in the FRET pair. The FluRedox principle has been successfully applied in biosensor technology (41–43) and single molecule enzymology (44–46).
Earlier we demonstrated the feasibility of fluorescence-detected cyclic voltammetry (FCV), i.e. a combination of the FluRedox method with potentiostatic control of the redox state of fluorescently labeled azurin (40). Azurin was adsorbed on a thiolalkane monolayer deposited on an optically transparent gold film (40). FCV allowed us to reveal kinetic and thermodynamic heterogeneity of azurin at a detection limit of a few hundred protein molecules. A mathematically rigorous, modified Butler-Volmer theory (47) was used for the analysis of fluorescent-detected voltammograms. The midpoint potential determined with FCV spans a range of tens of millivolts which was attributed to micro-environmental variance, protein-substrate and protein-protein interactions, or local electric field effects on the metal cofactor buried in the metalloprotein. In addition, Patil and Davis (48) reported a stepwise decrease of the spread of the midpoint potential (from 17 mV to 12 mV) with increasing alkanethiol chain length (hexanethiol to dodecanethiol). They suggest that the inhomogeneity of the interfacial alkanethiol monolayer on the gold electrode contributes to the thermodynamic dispersion.

In a follow-up on these experiments, we have improved the detection sensitivity by switching to a confocal fluorescence microscope equipped with an avalanche photodiode detector, where before we used a wide-field fluorescence microscope with a CCD imaging detector. Because of the improved signal-to-noise ratio, we could readily achieve single-molecule sensitivity. We report in this chapter direct monitoring of redox-gated and voltage-controlled fluorescence switching of individual wt-azurin molecules tagged with Cy5 on an octanethiol-modified optically transparent gold (Au) electrode (OTE) as illustrated in Figure 3.3. We uncover the dispersion in midpoint potential of azurin by analysis of fluorescence-detected voltammograms of individual azurin molecules which are immobilized on a gold-surface. The results shown in this work constitute the first direct observation of single-molecule fluorescence-electrochemistry and of thermodynamic dispersion in a protein film by measuring one molecule at a time.
3.2 Experimental Section

3.2.1 Sample preparation

Wild type azurin from *Pseudomonas aeruginosa* was expressed in *E. coli* and purified as previously described (49). Protein labeling was performed using a modified version of a previously described protocol (39). In short, wt (Cu) 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 at room temperature. Before labeling, the Cy5-NHS ester was dissolved in Dimethyl sulfoxide (DMSO) (Amersham Biosciences). Following the incubation step the unreacted label was removed using a 5 ml HiTrap desalting column (GE Healthcare). During the desalting step a buffer exchange to 5 mM TRIS/HCl at pH 8.5 was performed, required for the purification step (see below). To check for possible non-redox-dependent contributions to the fluorescence, zinc-reconstituted wt azurin was used as a control sample. This was purified and labeled in the same way as the native Cu azurin.

Purification step: labeling with the Cy5-NHS ester is not 100%, while the label can also attach to exposed lysines, although the N-terminus is assumed to be the preferred labeling site. To obtain a homogenous, 100% labeled sample, 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 (50). Before the labeled azurin fraction was loaded, the column was equilibrated with 5mM TRIS at pH 8.5. 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 absorbance at 280 nm (azurin) and 650 nm (characteristic absorption of Cy5) (Figure 3.4A).

The fractions corresponding to each elution peak were then collected and checked by means of UV/Vis spectroscopy to confirm the presence of protein
Absorption spectra were measured using a Lambda 800 spectrophotometer (Perkin Elmer Inc., USA) with a slit width equivalent to a bandwidth of 2 nm.

### 3.2.2 Fluorescence time courses in bulk

To verify redox switching in bulk solution, reduction and oxidation of Cy5-labeled azurin was performed by adding reductant (dithiotreitol, DTT) and oxidant (potassium ferricyanide, K$_3$(FeCN)$_6$) from freshly prepared, concentrated stock solutions (2-20 mM) directly into an optical cuvette to a final concentration of 5-20 µM, i.e. in 50 to 200-fold excess. In these experiments the fluorescence was monitored using a Cary Eclipse Spectrophotometer (Varian Inc., Agilent Technologies, USA). Fluorescence time courses of the labeled protein upon addition of oxidant or reductant were recorded by exciting the sample at 650 nm and monitoring the emission at 685 nm at room temperature in a 3-windows quartz ultra-micro cell with 100 µl total sample volume (Hellma Analytics, Müllheim, Germany). In order to minimize second order diffraction effects of the monochromator gratings suitable optical filters were placed, both, in the excitation and the emission path. The excitation/emission slits were set to 5 nm band-pass. The concentration of labeled protein was 50-100 nM in 100 mM phosphate buffer solution at pH 7.0.

### 3.2.3 Azurin immobilization on gold

The working electrode was prepared in-house and consisted of a semi-transparent gold layer of about 10 nm thickness which was deposited on a microscope slide (1” diameter glass coverslip, thickness 0.14-0.17 mm (#1), Menzel). Prior to gold deposition all glass slides were sonicated in spectrometer grade acetone (45 min) and stored in methanol. In between these steps, the slides were thoroughly rinsed and sonicated in deionized water (MilliQ). Before use the cover slips were blow-dried under a gentle nitrogen flow and then
ozone-cleaned (UVP PR-100 UV-ozone photoreactor) for 1 h and used for gold coating immediately.

The thin gold films were prepared by RF sputtering (ATC 1800F, AJA International) according to the protocol described by van Baarle et al (51). Good adhesion of the gold layer to the glass was ensured by first sputtering a layer of Molybdenium-Germanium (MoGe) of \(\sim 2\) nm thickness under 100% argon pressure. During subsequent gold sputtering, 5% partial oxygen pressure was added which helps to increase surface flatness (51).

The gold-coated slide was covered with a self-assembling monolayer (SAM) of 1-octanethiol (C8, for short) which serves as an intermediate layer. Freshly sputtered Au films were soaked overnight (15-18 h) in 1 mM solution of C8 in ethanol. A hydrophobic patch on the protein surface which is close to the azurin Cu center is believed to orient towards the hydrophobic alkanethiol head groups, leading to a favorable orientation for electron transfer (13, 16). The Cu lies only \(\sim 7\) Å beneath the protein surface at this hydrophobic patch (28).

Before incubation with azurin, the alkane-thiol/gold coated slide was rinsed with ethanol, blown dry, rinsed with milliQ water and blown dry again. The dry SAM-coated slide was then turned into a working electrode. For the confocal experiments on wt azurin, about 300 µL of a 100 pM azurin solution was put on the modified Au electrode and left to incubate overnight at 4 °C. This gave reproducible results of specifically immobilized wt-azurin without protein aggregation at the surface. For the immobilization step, a degassed 10 mM low-salt potassium phosphate buffer \((\text{K}_3(\text{PO}_4))\) at pH 7 was used to minimize aggregation of azurin on the surface. After incubation, unbound azurin was removed by rinsing 4 or 5 times with fresh buffer. The pH 7 buffer which was used to cover the surface layer during the measurements had a potassium phosphate concentration of 100 mM. All the measurements were performed in anaerobic conditions in a sealed sample-holder under continuous argon flow.
3.2.4. Surface characterization

The roughness of the bare 10 nm semi-transparent Au layer and azurin adsorbed substrates was determined by tapping mode atomic force microscopy (AFM) with a Digital Instrument multimode microscope (Veeco) equipped with a Nanoscope IIIa controller. Cantilevers with a resonance frequency of 70 kHz and a spring constant of 2 N/m (Olympus Corp.) were used for ex-situ imaging of the functionalized gold surfaces. Before imaging, the substrates with adsorbed proteins were rinsed with protein-free buffer to ensure the removal of loosely bound proteins and then imaged dry. For image analysis, WSxM 4.0 Develop 12.4 (Nanotec Electronica S.L.) scanning probe microscopy software was used (52).

3.2.5. Fluorescent electrochemistry setup

Fluorescently labeled, immobilized wt azurin was monitored with a confocal microscopy setup for simultaneous electrochemical and fluorescence intensity measurements.

**Single molecule imaging.** The sample-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) with an output wavelength of 639 nm was sent through a narrow-band clean-up filter (LD01-640/8-25, Semrock, USA), 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 focal lengths, respectively). The collimated beam was directed into the back entrance of an Axiovert 100 microscope (Zeiss), reflected by a dichroic mirror (Z532/633 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 on the sample surface. 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 a
single photon 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 in a file that we refer to as a fluorescence time trace. Samples were mounted onto a Physik Instrumente P-517 nanopositioner. Scanning, accurate positioning, data collection were performed by the Picoquant SymPho-Time software (PicoQuant GmbH).

A 10 by 10 μm area of the thiolalkane SAM-modified gold surface, covered with sparsely distributed, Cy5-labeled single azurin molecules, was scanned with a step size of 100 nm and a dwell time of 2 ms per point. A characteristic fluorescence intensity image corresponding to such a region is shown in Figure 3.9A. After imaging, the coordinates of the molecules in a scanned area were registered and an automatic recording procedure was started. During this procedure the scanner successively moves each selected molecule into the laser focus and at each position a fluorescence time trace was recorded for a duration of up to 300 seconds as a function of experimental conditions. The data were further elaborated off-line.

**Electrochemistry.** Azurin was immobilized on the semi-transparent gold working electrode as described in section 3.2.4. The working electrode was connected via a copper wire to a potentiostat (CH Instruments, model Chi832b). The counter electrode, consisting of a Pt wire, and the saturated calomel (SCE) reference electrode (Radiometer Analytical/BASI) were inserted into the 5 mL volume of buffer solution (100 mM K$_3$(PO$_4$) at pH 7.0) which covers the working electrode with the immobilized Cy5-azurin. Conventional staircase cyclic voltammetry (CV) was applied at scan rates of 10 mV/s to 5 V/s, scanning the applied potential from −0.10 to +0.20 V vs. SCE in potential steps of 1 mV. Alternatively, the chronoamperometric mode was used, in which a square potential waveform was applied, switching the potential between −0.10 to +0.20 V at a rate of 0.1 to 4 s$^{-1}$. The fluorescence rate of single, Cy5-labeled azurin
molecules was measured as a function of the applied potential, which results in a fluorescence-detected voltammogram.

The potential of the working electrode was recorded by one of the analog inputs of a data-acquisition (DAQ) card (National Instruments, PCI-MIO-16XE-10) which was connected to a microcomputer for control and for storing the data. A fluorescence time trace was recorded in synchrony with the potential scan. The two data files were processed by a home-written Labview application, which ties each photon emission event to a specific potential.

3.3 Results

3.3.1 Purification of Az-Cy5

We have purified azurin after labeling with Cy5 (NHS-ester) in order to get more homogenous species for the surface immobilization. The sample was fractionated using high resolution anion exchange chromatography (Figure 3.4A), recording both overall protein absorbance (280 nm) as well as the specific absorbance of Cy5 (650 nm). In the chromatographic separation we have observed multiple peaks because of the variety of labeling sites (exposed lysines) on the azurin surface. From the UV-vis spectral analysis of the eluting species (Figure 3.4B), one sees that fraction I shows the same spectrum as the oxidized azurin, which is, therefore, ascribed to unlabeled protein. In this case the band around 628 nm is solely due to the absorption of the Cu$^{2+}$ center, the ratio $\frac{\text{Abs}_{628\text{nm}}}{\text{Abs}_{280\text{nm}}} \approx 0.57$ being the same as for the native, oxidized protein. The UV-vis spectra of fraction II-IV in Figure 3.4B display features of the protein as well as the label and are ascribed to singly labeled protein fractions. The spectra show an intense peak around 650 nm accompanied by a shoulder at 600 nm, which indicates the presence of label in the sample. The presence of protein is inferred from two spectral characteristics: the absorption at 280 nm and the
FCV on single azurins

(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 peak 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 *P. aeruginosa* in the oxidized form. The band around 628 nm in this case is attributed to the absorption of the Cu$^{2+}$ center of unlabeled azurin in the sample, corroborated by the fact that the ratio Abs$\lambda_{628}$/Abs$\lambda_{280}$ ~0.57 is the same as for 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. Since we got the highest concentration of the label in fraction II, we used this fraction in all the single molecule experiments. Thus, we avoided immobilization of unlabeled or heterogeneously labeled species.

3.3.2 Fluorescence switching ratio (SR) in bulk

It is very well known for all the type-1 Cu centres that, while an absorption band is present at 590-630 nm in the Cu$^{2+}$ state, this band is absent in the Cu$^+$ state. Thus, one also expects to see a significant resonance energy transfer from the fluorophore to the Cu center in the oxidized but not in the reduced state of azurin. For instance in Figure 3.5 (black trace), upon initial addition of oxidant ($K_3$(FeCN)$_6$), the fluorescence intensity drops due to FRET between the attached
FRET-based on-off switching in bulk. A 100 nM Cy5-labeled Cu-azurin sample was titrated with aliquots of DTT and K$_3$(FeCN)$_6$ (2 mM) in 100 mM phosphate buffer at pH 7.0. We observed a large, 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. Zn-azurin labeled with Cy5 (grey line) was used as a control and did not show any fluorescence switch.

**Figure 3.5.** FRET-based on-off switching in bulk. A 100 nM Cy5-labeled Cu-azurin sample was titrated with aliquots of DTT and K$_3$(FeCN)$_6$ (2 mM) in 100 mM phosphate buffer at pH 7.0. We observed a large, 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. Zn-azurin labeled with Cy5 (grey line) was used as a control and did not show any fluorescence switch.

Fluorophore and the non-fluorescent Cu-center. Subsequent addition of reductant (DTT) produces an increase in the fluorescence intensity. In principle, this redox cycle can be repeated indefinitely as previously reported (38, 39). In fact, the fluorescence intensity in the oxidized state is reduced by about a factor of 10 compared to that in the reduced state in bulk. As a result, we obtained about 90% for the fluorescence switching ratio (SR) of Az-Cy5. The SR is defined as follows:

$$SR = \frac{I_{RED} - I_{OX}}{I_{RED}} \times 100$$  \hspace{1cm} (1)$$

where $I_{RED}$ and $I_{OX}$ are the fluorescence intensity values in the reduced bright and oxidized dark state, respectively. Furthermore, Zn-azurin (a redox inactive form of wt azurin, reconstituted with Zn instead of Cu) labeled with Cy5 was used as a control. It did not show any fluorescence switching upon addition of oxidant or reductant (grey trace in Figure 3.5).
3.3.3 Topography of Az-Cy5-functionalized thin Au film

For epifluorescent microscope imaging of labeled proteins immobilized on a gold surface it is necessary to use an ultrathin Au-film deposited on transparent substrates. We used optically transparent Au films at a thickness of about 10 nm which are homogeneous, very flat and conductive over the full area of the microscope slide (47, 53). These properties make them perfectly suitable for use as a working electrode in electrochemical studies in combination with simultaneous fluorescence detection in an epifluorescent microscope (see Figure 3.3).

To verify the topology of the surface, electrodes were imaged with AFM. A typical AFM image of the sputtered gold surface (10 nm thick) modified with an C8-SAM is shown in Figure 3.6A. The gold surface was polycrystalline, i.e. not atomically flat, but very homogeneous with a roughness of approximately 3 Å RMS over a range of tens of microns. This value is consistent with the previously reported roughness of the bare 10 nm gold film (47, 53).

![Figure 3.6.](image-url) Tapping mode AFM images. A) octanethiol modified 10 nm semi-transparent Au surface. B) wt-azurin molecules immobilized on octanethiol modified 10 nm semi-transparent Au surface. SAM of octanethiol were formed during overnight incubation. A 10 nm thick gold layer, with MoGe attachment layer, was sputtered on a standard 1" microscope slide, thickness #1 (0.14–0.17 mm). C) Height profiles of the green lines in image A (black line) and image B (red line). Sections showed clear ~4 nm high features in image B, which indicates the presence of single azurin molecules adsorbed on the Au electrode.
A typical *ex-situ* tapping mode AFM image of Az-Cy5 adsorbed on an C8-SAM-covered Au film is shown in Figure 3.6B. The observed globular features that are clearly discerned in the AFM image indicate individual Az-Cy5 molecules adsorbed on the 1-octanethiol SAM-covered Au films. Section analysis in Figure 3.6C shows that these features are clearly distinguished from the background of SAM/Au substrate and are about 4 nm high which is the typical size (14, 53) of a single wt-azurin as determined by X-ray crystallography (28). The small height variations of the individual proteins across the Au film can be attributed, at least partially, to the small height variations of the underlying gold substrate (on the order of 0.1-0.3 nm). At the same time, the height and width distributions are relatively narrow which suggests the absence of aggregation and a uniform orientation with respect to the surface.

### 3.3.4 Electrochemical Measurements

Protein film voltammetry (PFV), developed by F. Armstrong and co-workers (10), is a method to observe direct electron transfer (DET) between redox proteins and electrodes. PFV can be achieved by immobilizing Az-Cy5 on an optically-transparent gold (~10 nm) electrode through adsorption on an intermediary C8-SAM (see in Figure 3.3; C8 is short-hand for 1-octanethiol). Adsorption of Az-Cy5 on the C8-SAM layer presumably occurs by hydrophobic interaction between the terminal methyl group of the SAM and the hydrophobic patch at the protein surface around the Cu-containing redox center. Previously, it has been shown that the rate constants for electron transfer between the electrode and the Cu center, across the alkanethiols, are significantly higher than of the protein in the direct adsorption mode (54). Thus, the molecular orientation of the azurin on the Au/alkanethiol surface is towards the aforementioned hydrophobic patch which is favorable for direct electrons transfer with the Cu center facing the electrode as illustrated in Figure 3.3).

Figure 3.7 shows typical cyclic voltammograms (CV) of wt azurin adsorbed on
**Figure 3.7.** Typical background-subtracted cyclic voltammograms for wt azurin adsorbed on a semi-transparent gold electrode (working electrode, 10 nm) via an octanethiol SAM. Reference electrode SCE, counter electrode Pt gauze, electrolyte 100 mM phosphate buffer at pH 7.0. Incubation time for the 0.94 μM wt azurin labelled with Cy5 was 10 minutes. After rinsing the surface 5 times with the buffer, CV of immobilized protein was measured at a wide range of scan rates: A) 10-200 mV/s and B) 200-5000 mV/s (vs SCE). Arrows show the oxidation (forward) and reduction (reverse) cycles, respectively. Peak separation (ox-red) increases with scan rate.

**Figure 3.8.** Trumpet plot showing the scan rate dependence of peak separation for CV. Sweep rates range between 10 and 1000 mV/s. CV peak potentials at the maximum for oxidative (round dots) and reductive (diamond) cycles were fitted (solid line) based on the Butler-Volmer theory (explained in chapter 2). The midpoint potential (35 mV vs SCE) was calculated from the trumpet plot fitting.
gold modified with the C8-SAM, at scan rates that range from 10 mV/s to 5000 mV/s. The CVs have the shape expected for an immobilized redox protein, with the reductive and oxidative peaks almost coinciding along the horizontal axis at slow scan rates. For an immobilised redox protein, the ability to observe electrocatalytic activity is a crucial indicator that the functionality of the protein has not been significantly perturbed by the electrode attachment. The electroactive coverage \( \Gamma_{ea} \) of azurin was calculated as 3.4 pmol cm\(^{-2} \) as was determined from the anodic and cathodic charge of adsorbed protein:

\[
\Gamma_{ea} = \frac{A}{nFvA_{RE}}
\]

(2)

where \( A \) is the area of the observed CV peak, \( n \) is the number of electrons transferred (\( n = 1 \) for azurin), \( F \) is the Faraday constant, \( v \) is the potential scan rate and \( A_{RE} \) is the geometrical electrode area.

Figure 3.8 shows the resulting trumpet plot, in which the peak positions for oxidation and reduction potentials are plotted as a function of the logarithmic scan rate. This is a useful method of displaying and analyzing the characteristics of the PFV response over a wide time domain (13). Direct electron transfer of metalloproteins when immobilized on a conductive electrode can generate important information about their intrinsic thermodynamic, kinetic and mechanistic properties (10–12). At low scan rates, the potentials at the maximum of the oxidation and reduction peaks are close, and their average corresponds to the midpoint potential of the Cu site. As the scan rate increases, the oxidation and reduction peak potentials separate. Furthermore, the dashed lines in the trumpet plot show the Butler-Volmer based fit of the I-V curve (see chapter 2). As a result, the ET rate \( k_0 = 26 \text{ s}^{-1} \) and the midpoint potential \( E_0 = 35 \text{ mV vs SCE} \) as calculated from the trumpet plot fitting which corresponds well to previously reported data (47). Moreover, the surface coverage we obtained is consistent with a high retention of electroactivity on immobilization which is comparable with other results (47, 48, 53).
3.3.5 Fluorescence-electrochemistry on SM azurins

A typical fluorescence intensity image of individual azurin molecules is shown in Figure 3.9A. Wt azurin molecules labeled with Cy5 were immobilized on the gold electrode which is covered by a C8-SAM, as described above. The image shows a region of $10 \times 10 \, \mu m^2$ with a dwell time of 2 ms per point. An intensity profile taken along the center of a molecule in the image (red line in Fig. 3.9A) is shown in Figure 3.9B (red dots). The Gaussian fit of the intensity profile has a fwhm of 335 nm, very close to the diffraction-limited resolution (~280 nm) at the given excitation wavelength.

A reference electrode (standard calomel, SCE) and a counter electrode (a Pt wire) were inserted in the droplet of buffer solution that covered the functionalized gold layer which was configured as the working electrode. The three electrodes were wired to a potentiostat. The effect of the applied potential on the fluorescence of surface-immobilized, labeled azurin is shown in Figure 3.10A-D in which the working electrode is switched back and forth from a reducing potential (~0.2 V vs. SCE) to an oxidizing potential (+0.2 V). The bright-
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The fluorescence intensity images of the individual azurin molecules. SM azurins immobilized on gold surface via 1-octanethiol SAM, correspond to a region of 5 x 5 μm² with a dwell time of 2 ms per point. In images A and C, SM azurins were reduced by -0.2 V applied potential where the same molecules oxidized by +0.2 V (vs SCE) in images B and D.

![Figure 3.10](image)

**Figure 3.10.** The fluorescence intensity images of the individual azurin molecules. SM azurins immobilized on gold surface via 1-octanethiol SAM, correspond to a region of 5 x 5 μm² with a dwell time of 2 ms per point. In images A and C, SM azurins were reduced by -0.2 V applied potential where the same molecules oxidized by +0.2 V (vs SCE) in images B and D.

ness of the spots in the image, which correspond to individual azurin molecules, varies by about a factor of 5 to 7: a high fluorescence intensity (ON) is observed in the reduced state, whereas it is weak (OFF) in the oxidized state. This is similar to what is observed in a bulk solution when labeled azurin is chemically oxidized and reduced (53) (See Discussion part for more details).

This ON/OFF switching behavior is more clearly demonstrated in Figure 3.11 by monitoring the fluorescence intensity of a single Cy5-azurin molecule as a function of time. This is achieved by adjusting the scanning stage to move and then hold one of the molecules in the focus of the laser beam. Subsequently, the fluorescence intensity (black, solid line) is monitored while the potential (black, dashed line) is switched in a square-wave pattern (so-called chronoamperometry, Fig. 3.11A) at a period of 5 s (54) or linearly swept in the forward and reverse directions at a scan rate of 100 mV/s (cyclic voltammetry, Fig. 3.11B). The data in Figure 3.11 show the fluorescence count rate with a bin-size of 10 ms, together with the applied voltage pattern (dashed line). The fluorescence time trace follows an on-off switching behavior which depends on
The real time fluorescence intensity time traces (10 ms bin size) showing FRET-gated emission of Cy5-labeled SM-Cu-Az (black, solid line) on an octanethiol-modified gold layer (10 nm) in response to an applied potential (black, dashed line). A) Chronoamperometric potential was cycled from 0.2 to -0.1 V with a 5 s pulse width. B) Cyclic voltammetry was applied at 100 mV/s scan rate. Counter electrode Pt gauze, electrolyte 100 mM phosphate buffer at pH 7.0. Zinc inactive form of the wild-type protein (grey line) was used as a control, and did not show any fluorescence switching with the same applied potential.

Figure 3.11. The real time fluorescence intensity time traces (10 ms bin size) showing FRET-gated emission of Cy5-labeled SM-Cu-Az (black, solid line) on an octanethiol-modified gold layer (10 nm) in response to an applied potential (black, dashed line). A) Chronoamperometric potential was cycled from 0.2 to -0.1 V with a 5 s pulse width. B) Cyclic voltammetry was applied at 100 mV/s scan rate. Counter electrode Pt gauze, electrolyte 100 mM phosphate buffer at pH 7.0. Zinc inactive form of the wild-type protein (grey line) was used as a control, and did not show any fluorescence switching with the same applied potential.

the applied potential. Fluorescently labeled zinc azurin, the redox inactive form of the wild-type protein, was used as a control. With zinc azurin no fluorescence switching was observed at the same applied potentials (grey line).
The more conventional method of cyclic voltammetry consists of ramping the potential up or down linearly in time. An example is shown in Figure 3.7, where amperometric detection is used to measure the current response of the azurin sample. The current has a maximum near the midpoint potential where the rate of the electrode reaction is largest, at least at slow scan rates when the electron transfer rate is not rate limiting. A similar result is obtained (see Fig. 3.11B) by monitoring the fluorescence intensity of labeled azurin on the working electrode as a function of the applied potential (similar to the measurements reported in Figs. 2.1, 2.2 and A.2.4.) In this case the shape of the curve will be sigmoidal since the fluorescence intensity follows the relative populations of the oxidized and reduced states (47, 48). The result of such a measurement is shown in more detail in Figure 3.12, which can be called an ‘optical voltammogram’. It reflects the response of an individual azurin molecule, obtained by recording single electron transfer events. This is the first time that a fluorescent-detected voltammogram of a single redox protein has been obtained.

The relative population of the oxidized and the reduced state of the azurin is determined by a Boltzmann distribution. By fitting the fluorescence intensity ($I$) to a Boltzmann sigmoidal equation we can fit the fluorescence-detected voltammogram, and determine the midpoint potential ($E^0_f$) of an individual azurin. This will be equivalent to the potential at which the rate of change of the fluorescence intensity is maximum. Thus, by taking an average of optical peak potentials for a forward and backward sweep, the optical midpoint potential can be calculated as (48):

$$I = I_{\text{min}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + \exp \left( \frac{E - E^0_f}{d} \right)}$$  \hspace{1cm} (3)

where $I_{\text{min}}$ and $I_{\text{max}}$ represents the fluorescence intensities when the protein is at the fully oxidized and reduced states, respectively, and $d = RT/F$. In fact, Equation 3 is just another representation of the Nernst equation (See Chapter
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![Optical voltammogram of an individual wt-azurin molecule](image1)

**Figure 3.12.** An optical voltammogram (dots) of an individual wt-azurin molecule for 100 mV/s scan rate in 100 mM phosphate buffer, pH 7.0 at room temperature. The red line represents the Boltzmann sigmoidal fit to the data according to Eq. (3). The optically determined midpoint potential ($E^0_o$) (arrows) is the calculated value from the sigmoidal fit ($E^0_o = 33$ mV).

![Histogram of optically determined midpoint potentials](image2)

**Figure 3.13.** Histogram of optically determined midpoint potentials of 66 individual azurin molecules on an octanethiol-modified 10 nm Au at an applied potential (-0.1 to 0.15 V). The Gaussian fit is centered at 45.7 ± 0.5 mV with a fwhm of 15 mV.

1.4.1.2) with $n = 1$, where it is assumed that the increase in fluorescence intensity upon reduction is proportional to the concentration of reduced Cy5-azurin.
Figure 3.12 shows a typical optical voltammogram with the fit of the Boltzmann sigmoidal (red line) to the fluorescence intensity vs. applied potential. The optically determined midpoint potential (arrows) for this single azurin molecule is calculated as $E^0 = 33$ mV (vs SCE) from the sigmoidal fit, at which the rate of change of fluorescence is maximum.

The dispersion in the optically determined midpoint potential of 66 individual azurin molecules on C8-SAM-modified semi-transparent Au film is shown in Figure 3.13. The distribution of midpoint potentials was fitted to a Gaussian and gave a maximum value at 45.7 mV ($\pm 0.5$) with a fwhm of 15 mV (vs SCE).

### 3.4 Discussion

We have selectively immobilized individual, fluorescently labeled azurin molecules by stable adsorption on a self-assembled monolayer of thiolalkanes deposited on semi-transparent gold films (see Figure 3.3). This construct was incorporated as a functionalized working electrode in an electrochemical cell. It was shown that changes of the redox state of an individual azurin molecule by applying an external potential can be monitored in fluorescence using the FRET-based FluRedox principle (38–48, 53). This result implies a significant leap in sensitivity by which single-electron events at the working electrode can be monitored in real time with high time resolution.

It proved essential to include a purification step after labeling of azurin with the NHS-ester of the Cy5 dye molecule. It is generally assumed that the Cy5-NHS ester preferentially binds to the N-terminus at a pH of 8.3 (39). However, a recent, detailed mass-spectrometry analysis by Andreoni et al. (50) of Atto-655-labeled azurin has shown that this is not necessarily the case. A mixture of labeled species was obtained with the Atto-655-NHS ester, which included not only covalent binding of the label to the N-terminus, but also to lysines 24, 27, 122 and 128. Doubly labeled azurin molecules were also observed. This is problematic in single-molecule experiments, because the switching ratios of the
different azurin species differ significantly from one another. The analysis showed that Lys-122 was the preferred labeling position for the Atto-655-NHS ester at pH 8.3 (50) consistent with the nucleophilic character of this lysine residue.

Chromatographic purification of Cy5-labeled azurin also yielded a distribution of labeled species (see Fig. 3.4). The different species were not analysed further, and therefore it is not clear what the exact labeling positions are in this case. We have used peak II in Figure 3.4 for all the experiments described in this chapter. It turns out that this azurin-Cy5 species also has the largest switching ratio in solution of 90% (see Fig. 3.5). This is higher than values previously reported (40) but the latter were based on the average value of the mixture of azurin-Cy5 species immobilized on the gold electrode.

Cy5 is notorious not only for the role of the triplet state in the photophysical properties of this molecule, but also for conformational flexibility and its effect on their electronic structure (55, 56). It leads to pronounced blinking, and more importantly, an increased susceptibility for photobleaching. The presence of molecular oxygen, typically at a concentration of 0.5 mM (57) under ambient conditions in buffered solutions, has a large effect on the photostability of dye-molecules. Upon excitation, the dye molecule will every so often end up in the triplet state by intersystem crossing. Efficient energy transfer from the triplet state of the dye to molecular oxygen results in population of the O$_2$ singlet state which is highly reactive, and therefore -- directly or indirectly -- destructive of other molecular species in solution, including the fluorescent dye. Moreover, there is a possibility that the presence of oxygen will interfere with the electron transfer reactions at the electrode (25). For these reasons, 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 electrochemical measurements.
Under those conditions one would expect that the triplet life time of Cy5 and associated blinking events are extended into the ms-domain. This is, however, not observed. In control experiments with Cy5-labeled Zn-azurin, immobilized on a thiolalkane SAM-covered electrode, we do not observe any blinking at this time scale. Even more remarkable is the high photostability of the azurin-Cy5 construct under the conditions of the electrochemical measurements: photobleaching was virtually absent, and fluorescence time traces could readily be obtained for arbitrary duration. (For practical reasons, they were limited to several minutes.) This in stark contrast with azurin-Cy5 immobilized on a gold (53) or glass surface (see Chapter 4) without potentiostatic control of the electrode potential, where time traces are limited to at most tens of seconds because of photobleaching, even under an argon atmosphere.

The absence of blinking (at least at ms-time scale and longer), and the strongly enhanced photostability of the azurin-Cy5 construct under conditions of cyclic voltammetry are observations that we cannot fully explain. It appears that the triplet state of the Cy5-label is strongly quenched. This could be due to interaction with the metal electrode, although the thickness of thiolalkane layer is probably too large for such a coupling. However, a local reorganization of the thiolalkane layer under cyclic voltammetry conditions might bring the azurin-Cy5 molecule in closer contact with the gold surface. An indication for such an effect is the gradual decrease of the fluorescence rate in the voltammetric time traces, superimposed on the electrochemically induced changes. This may be due to increased quenching of Cy5 fluorescence by the gold layer. This would be consistent with the fact that we observed a somewhat lower fluorescence switching ratio (60%, see Figures 3.10 and 3.11) of azurin-Cy5 upon electrochemical oxidation and reduction at the working electrode than with chemically-induced redox switching on glass (see Chapter 4). In the latter case the switching ratio is about 90%. Previously, Elmalk et al. (53) showed that K27C azurin immobilized on Au surface through the mixed SAMs depicts chemically-induced fluorescence intensity switching of about 70% which is similar to the
electrochemically obtained SR of single wt azurin molecules. Thus, the lower switching ratio may also signify partial quenching of Cy5 fluorescence by the gold layer.

The distribution of optically determined midpoint potentials (Figure 3.13) of single azurin molecules is centered at 45.7 ±0.5 mV vs. SCE and the graphically determined width was found to be 15 mV. The average midpoint potential corresponds well with the value of 35 mV found using conventional CV (see Figures 3.7 and 3.8). The difference may be due to the higher packing density in the case of conventional CV, but could also be caused by small variations in the results of CV measurements, which are not uncommon.

The width of the distribution of midpoint potentials in Fig. 3.13 is in good agreement with the results obtained from the samples with high azurin surface coverage described in chapter 2 (see Figure 2.4). Moreover, the dispersion in the single-molecule data is also consistent with a recently published report by Patil and Davis (48) where a similar $E^0_f$ was calculated (~38 mV for about 1200 azurin molecules) with a fwhm of its distribution of 16 mV for wild-type azurin molecules tagged with Atto 655 and immobilized on a C8-SAM. The agreement among these different data sets suggest that the heterogeneity in ensembles of up to 1200 azurin molecules is the same as observed in the case of single molecules. This could mean that it is largely determined by the local environment at the scale of the region of interest (up to 600 x 600 nm) in chapter 2 and in ref. (48). It also follows that protein-protein interactions between azurin-molecules at the electrode surface have little effect on the observed midpoint potentials.

There are several parameters that can affect the reduction potential of copper proteins, such as electronic coupling, electric field drop or molecular orientation (58–61). The midpoint potential of azurin is particularly sensitive to outer sphere interactions which affect the redox site and its electronic structure through electrostatic interactions (59, 62, 63). It should be noted, however, that
the observed width of the midpoint potential distribution contains a thermal contribution as well, which is probably of the same order of magnitude (kT is about 25 meV at room temperature). The results in this chapter thus must be considered as an onset for a more detailed study of the heterogeneity in the azurin redox properties.

3.5 Conclusions

In this work, we have shown that it is indeed possible to control the redox state of individual wt azurin molecules immobilized on an electrode, by an applied voltage under potentiostatic control. The FRET-based electrochemical detection of individual azurin molecules reveals the heterogeneity of redox parameters in this system. It opens the door to more detailed studies of protein-electrode interactions. Apart from measurements of the midpoint potential, the dynamics of electron transfer processes at the electrode can be exposed by analysis of the switching dynamics in time-tagged fluorescence time traces.

References


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