Fluorescence correlation spectroscopy on electron transfer reactions : probing inter- and intramolecular redox processes

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Summary

Biological processes such as photosynthesis, oxidative phosphorylation, respiration and much of the enzymatic catalysis occur via electron transfer (ET). In order to fulfil these tasks, a cell generally needs all its essential performers. Copper proteins are one of those major players, involved in a wide range of redox processes, predominantly in biological energy productions and metabolic processes. The last few decades have witnessed large efforts to understand ET mechanisms with cutting-edge experimental research combined with theories and molecular models of proteins. How ET flows through these proteins involving donor and acceptor and what determines the specificity is still a subject of debate. Azurin is a small blue copper protein that plays a role in ET processes in gram-negative bacteria. In this thesis, azurin has been chosen as the main redox protein because of its high stability and largely known structural, spectroscopic and mechanistic properties.

The availability of fluorescence based detection techniques has brought about a breakthrough in the optical studies of the properties of biomolecules e.g. enzyme mechanism, protein folding etc. To obtain detailed information on the ET reactions in proteins such as azurin, fluorescence correlation spectroscopy (FCS) has been used. This method can be easily applied to many other redox proteins and have potential applications in fluorescence based biosensors and molecular electronics. In the present thesis, a series of FCS experiments on ATTO655 labeled azurin was presented. The aim was to gain more insights in electron transfer processes.

In the introduction of **Chapter 1**, the general features of blue copper proteins and azurin are presented. Then an overview is given on the methods that are important to this thesis, e.g. fluorescence resonance energy transfer (FRET), fluredox principle, fluorescence correlation spectroscopy (FCS). This chapter also provides an outline on the theory of electron transfer.

Chapter 2 describes the calibration of the experimental setup used for the investigation on ET reaction in azurin. ATTO655, a red-absorbing dye has been chosen to calibrate the setup. The main goal of this work was to precisely determine the three-dimensional parameters (k and r_0) of the experimental setup. First an overview was given on the autocorrelation function (ACF), 'D' the diffusion coefficient of the molecule of interest, 'τ_D' the residence time of the molecule in the probe volume, the three dimensional parameters (k and r_0) and how they are related to each other by FCS equations. Then the calibration was performed using pure buffer and a 57.0% (w/w) sucrose solution containing different ATTO655 dye concentrations. Two approaches were undertaken to determine the effective volume of the setup. One way to determine the parameters was by plotting the average number of molecules in the probe volume as a function of the concentrations of the dye and the other way was by fitting of the obtained autocorrelation data with FCS equations. The first approach produced V_{eff} = 2.3±0.2 fL in buffer and in 57% (w/w) sucrose solution it was 2.9±0.1 fL. The second method resulted V_{eff} = 2.1±0.5 fL in buffer and V_{eff} =1.3±0.2 fL in 57% (w/w) sucrose solution. The experimentally obtained 'k' value in 57%

(w/w) sucrose solution was used for the fitting of the ACFs of labeled azurin samples (Chapter 3, 4 and 5). From the fits of the ACFs also τ_D of the dye was determined. The average diffusion times for the dye was found to be 0.10 ± 0.01 ms (in buffer) and 2.9 ± 0.3 ms (in 57% w/w sucrose solution). Effective volume was also determined by imaging of sub-resolution gold nano-rods. The calculated volume was found to be similar to the values obtained with above two approaches using ATTO655. Next, we looked into the diffusion time scales of labeled proteins and the rates of the bimolecular reaction between the labeled azurin and redox agents. These bimolecular reactions have very small activation barriers and are diffusion controlled reactions with rates of $k_d = 10^9$-10^{10} $M^{-1}s^{-1}$ in aqueous solution at room temperature. A rough estimate of the rate in sucrose solution was made using Smoluchowski's principle and it can reach values of 10^6 $M^{-1}s^{-1}$ in high viscous liquid. Then we briefly discussed the photoinduced processes of the dye using Jablonski diagram. At last, an overview was given on the thermodynamics of photoinduced electron transfer (PET) and a brief review was presented on the major contributions in understanding PET reactions in metalloproteins like azurin.

Chapter 3 presents a detailed investigation of the products of the labeling reaction of Zinc azurin variants with the fluorescent dye ATTO655. First, we investigated the effects of viscosity on the translational diffusion times of labeled Zn-azurin in the absence of any redox chemicals. The diffusion time of the labeled molecule became larger with increase in sucrose concentration in the buffer [200 µs in pure buffer (0% sucrose) and 12 ms in 57% (w/w) sucrose solution]. Then FCS experiments were performed to understand the behavior of the labeled products under redox conditions. We investigated the PET reactions using two species: one labeled at the N-terminus and another one labeled at Lys122 position. As Zn-azurin is inactive, only intermolecular ET reactions were observed between the label and the redox chemicals. Oxidizing agents had no effect on the autocorrelation functions of labeled Zn-azurin and could be fit with the FCS equation containing only diffusion term. But, the ACFs under reducing conditions could be fit with FCS equation containing diffusion and $G_1(\tau)$ terms and the analysis showed that the forward reaction rate was linearly dependent on the reductant concentrations corresponding to the one-electron reduction of the label by the reductant e.g. sodium ascorbate, potassium hexacyanoferrate (II). It indicates that the reducing agents caused blinking of the dye. This bimolecular ET reaction rate between the label and the redox chemicals was found to be smaller ~10^7-10^8 $M^{-1}s^{-1}$ compared to diffusion-controlled reaction rates in pure buffer (10^9 $M^{-1}s^{-1}$) supporting the fact that these reactions are slow in 57% (w/w) sucrose solution. The oxidation of the reduced label back to the non-reduced form appears to consistent with the idea that dissolved oxygen is responsible for the back oxidation of the label as oxygen is present in the sucrose solution in large excess and its concentration will not change appreciably over the duration of the experiment.

A detailed investigation of the products of the labeling reaction of wild type Cu-azurin with the fluorescent dye ATTO655 has been presented in **Chapter 4**. Fluorescence correlation spectroscopy was performed to understand the behavior of the labeled products under redox

conditions. In this work, we have also tried to understand the PET reaction using two species: one labeled at the N-terminus and another one labeled at Lys122 position. In the former, only blinking of the dye or intermolecular ET reaction was observed between the dye and reducing agents. Oxidizing chemicals had no effect. In Lys122 labeled Cu-azurin, a microsecond ET dynamics was observed under redox conditions and the reaction was found to be independent of redox concentrations. This reaction has been referred as intramolecular ET reaction between the dye and redox center. Two different mechanisms are believed to be involved in ET events. Applying Marcus theory of ET transfer and using the experimentally observed forward and backward rates, the values of reorganization energies under redox conditions were calculated. Using k_1^f =4.8×10^4 s^{-1} and k_1^b =7.5×10^2 s^{-1} under oxidizing conditions, one obtains λ= 0.75 eV. Under reducing conditions, λ= 1.16 eV was obtained using the experimental values k_2^f = 4.2×10^6 s^{-1} and k_2^b = 1.3×10^4 s^{-1} The main conclusion from the present work is that no PET reactions with amino acids in the protein are observed, but PET to the metal is observed when Cu occupies the active site, and the label is attached close enough to the metal center (at Lys122). In the end, a covalent pathway model for ET reaction between Cu and label has been proposed to support the experimental data.

Similar to previous chapters 3 and 4**, Chapter 5** also represents a detailed investigation on photoinduced ET reactions in the labeled products of K122S and K122Q CuAzurin variants under redox conditions. In this work, we have tried to understand the ET reaction using a specific species where the label is assumed to be attached on a surface lysine. In case of these variants, no extra decay was observed in FCS curves and the time scale of ET reaction is sub-millisecond range. Slower ET rates revealed that the labeling position on the protein surface must be at least > 20 Å away from the copper center and the label is attached possibly to other lysine residues e.g. Lys 24/Lys27/Lys128 on the protein surface. Then, we calculated the reorganization energies of ET transfer under redox conditions for the labeled species using the experimentally obtained forward and backward rate constants and applying Marcus theory. In this chapter, we have also shown how the rate of PET between label and redox site may vary with the position of the label on the protein surface. As the ET parameters (driving forces, reorganization energies) have been determined experimentally for a number of cases the variation of the rates with distance were calculated and it became of interest to see how these rates compare to FRET promoted deactivation of the excited labels. Our calculation showed that inverse PET rate reaches the millisecond range for Cu-label distances between 15-20 Å, depending on driving force and reorganization energy, whereas the FRET rate showed a much shallower distance dependence. As no detailed structural information is available for the positions of the labels in K122S and K122Q-ATTO655 labeled species, we proposed the “organic glass” model of Moser and Dutton to calculate the ET rate through a protein.