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

Sen, S.

Chapter 6

Outlook

6.1 Introduction

In the present thesis, a series of single molecule FCS experiments on fluorescently labeled azurin was presented. The aim of those experiments was to gain broader understanding in electron transfer processes in oxido-reductases. The FRET based approach makes it possible to probe the redox state of absorbing cofactors inside a protein by attaching a fluorescent dye to its surface. The beauty in our method is that it can allow a more or less free choice of (redox) enzyme to be studied. Therefore, one way to continue research described here would be to investigate the electron transfer reactions within metalloprotein complexes and monitor protein-protein interactions. Other way could be to extend the investigation on the ET reactions of azurin labeled with different classes of fluorescent dyes e.g. ATTO647N or ALEXA647.

6.2 Single molecule studies on azurin dimer

To investigate protein-protein electron transfer reactions(*1*)(*2*)(*3*), cross-linking methods were developed previously to "freeze" these transient complexes(*4*)(*5*)(*6*). A successful example is azurin dimer that was structurally characterized by our group(*7*). This well characterized construct has been chosen to investigate protein-protein ET reactions. Depending on the redox states of the copper centers in the dimer, this dimer can exist in three different redox states: oxidized [Cu(II)-Cu(II)], reduced [Cu(I)-Cu(I)]and half oxidized [Cu(II)-Cu(I)]. When the dimer is half oxidized, intramolecular ET can happen between two copper centers in a dimer. This ET exchange can affect the fluorescence of the attached label. This reaction has been schematically presented in Fig. 6.1. The analysis of ACF of the dimer under redox conditions will eventually show the intramolecular ET rate (Fig. 6.2). The results will confirm the potential of FCS in solution to investigate intramolecular ET reactions in metalloproteins containing more than one redox center. Protein-protein interactions in the naturally occurring redox chains can be the ultimate application of this technique.

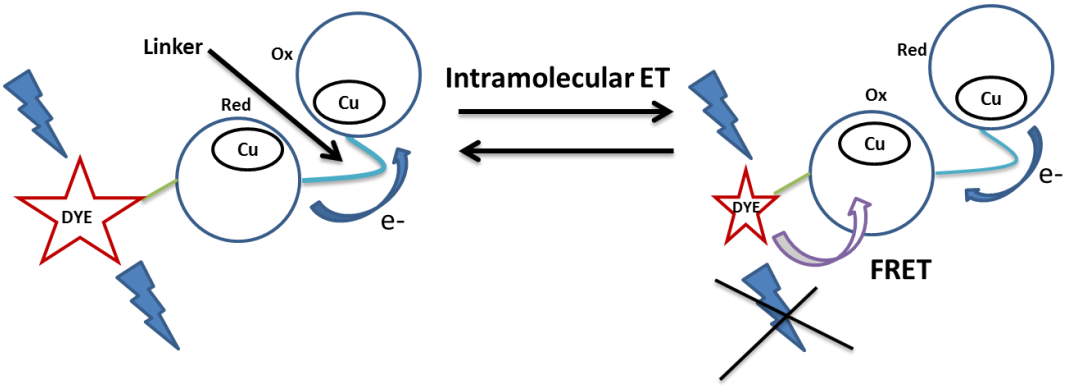

Figure 6.1: Schematic representation of the influence of the intramolecular ET reaction on the fluorescence emitted by the label attached to the half reduced/oxidized azurin dimer. Each monomer is depicted as a blue circle. Two monomers are connected by a light blue curved line representing BMME linker. On the left side: the dye is excited and its fluorescence is fully detected. On the right side: upon excitation, the fluorescence of the label is transferred to the oxidized copper due to FRET and the fluorescence intensity of the dye diminishes.

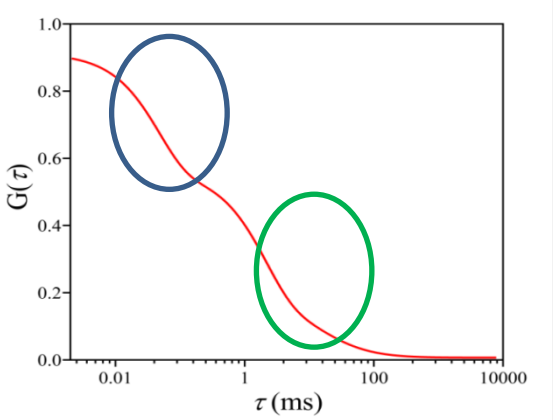

Figure 6.2: Example of an autocorrelation curve (red). The green circled region corresponds with the diffusion of the particle, the blue circled region corresponds with a reaction that is faster than diffusion. For azurin dimer, this blue circled region will represent the intramolecular ET reaction between two redox centers in the dimer.

Future plans

(Cu-Cu) BMME azurin dimer can be used to test whether the combination of FRET-based detection of the redox state with FCS is suitable to measure ET rates. Our group has previously characterized electron self-exchange reaction of the BMME dimer by NMR(*8*). Experimentally (FCS) obtained ET rates can be used to compare with the value reported in literature. This technique can also be extended to study the ET reaction between proteins containing multiple redox centers at physiological conditions. Two redox partners can be labeled with two labels with different colors and by means of either spectral or lifetime characteristics the redox behavior of each center can be investigated. It is possible to co-localize the protein-complex and then cross-correlating the signals by FRET technology to reveal the kinetics of ET processes between two partners. Single molecule experiments will eventually extend our understanding on protein-protein ET transfer complexes and open up the possibilities of the development of biosensors based redox proteins as ET rate can be crucial parameter in such systems.

6.3 Single molecule studies on azurin labeled with different classes of dyes

Our previous investigations on ATTO655 (oxazine dye) labeled copper azurin revealed that it is possible to separate and characterize individual labeled component using chromatography and mass spectroscopy techniques from a biological sample which is heterogeneous after labeling. In the case of azurin, we addressed the fluorescent behavior of those individual components at single molecule level (See chapter 3, 4 and 5). That work has enabled us to understand ET dynamics as a function of the position of the label on the protein surface. Specifically, the ET processes between the label ATTO655 and the copper center of the labeled azurin were demonstrated using FCS. Under certain redox conditions, we have been able to monitor intramolecular ET dynamics in the microsecond region between the label and the metal center in azurin.

New insights emerged from the observation of intramolecular ET dynamics at single molecule level. Subsequently further questions have appeared from the microsecond ET process detected from FCS curves. If single molecule detection for ET dynamics is possible for ATTO655-azurin system, would it be possible to encounter the same in other fluorophores conjugated with the protein? Will the time scale of electron transfer be similar for any azurin-dye bioconjugate?

In order to answer these questions, two commonly used and commercially available red-absorbing fluorophores of different classes, ATTO647N (carbo-rhodamine) and ALEXA647 (cyanine) were selected for single molecule applications (Fig 6.3). In this study, our first strategy is to characterize the products of the labeling reactions for the aforementioned two different classes of dyes and then extend our investigation into the properties of a specific labeled species under redox conditions using FCS. Specifically, K122 labeled copper azurin will be chosen to monitor the intramolecular ET dynamics. The redox properties of the azurin-dye solution can be manipulated using potassium hexacyanoferrate (III) and potassium hexacyanoferrate (II) or sodium ascorbate as previously described (See chapter 3-5 for details).

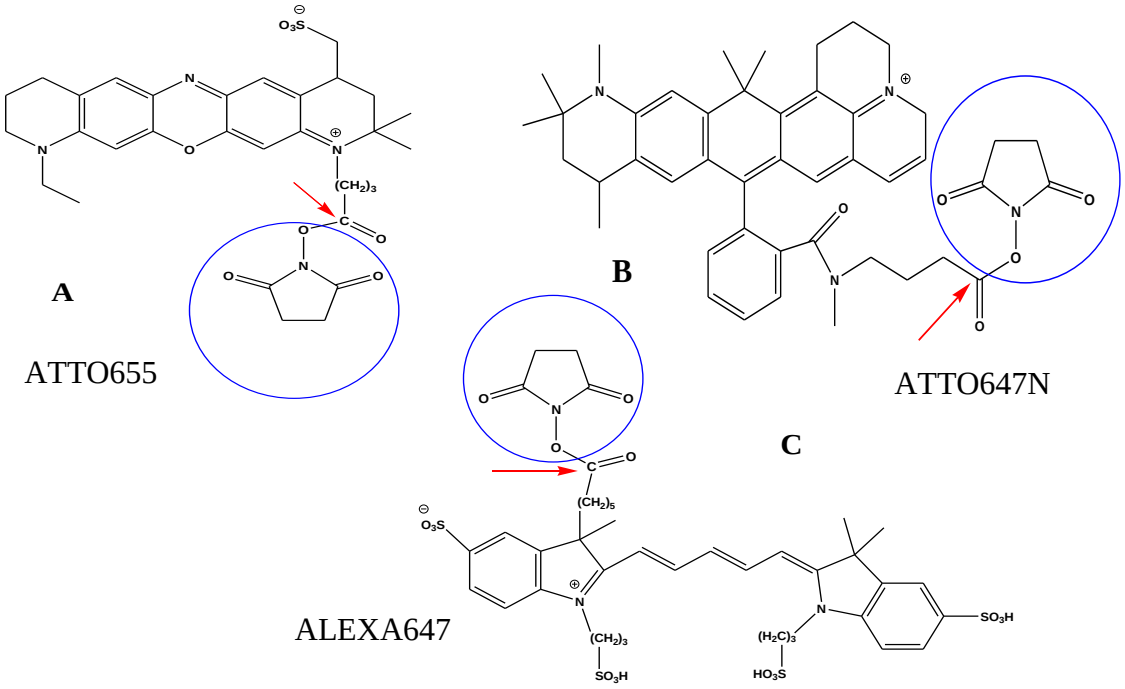

Figure 6.3: Chemical structures of three different classes of dyes: (A) ATTO 655 NHS (Oxazine class, overall charge on the dye = 0), (B) ATTO 647N NHS (Carbo-rhodamine group, overall charge on the dye = +1) and (C) ALEXA 647 NHS (Cyanine class, overall charge on the dye = -3). The blue circled region shows the NHS ester group of the individual dyes required for the labeling reaction with amino moieties on the protein surface. The red arrow shows the point of attachment of the protein with the dye.

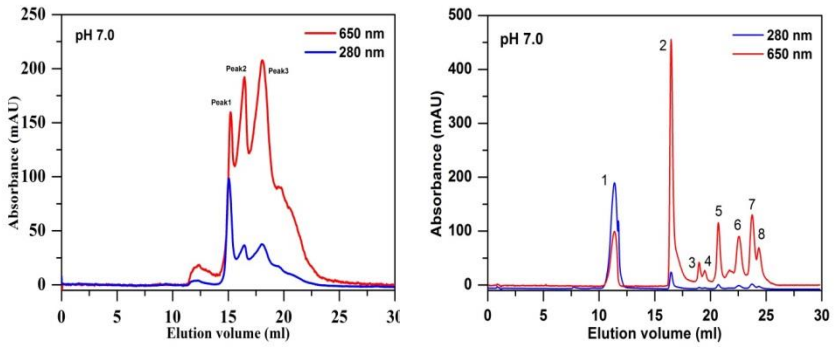

Figure 6.4: Anion exchange chromatography of the mixture obtained after labeling *wt* azurin with ATTO 647N (A) and ALEXA647 (B). The chromatogram shows the elution pattern with monitoring of two wavelengths: 280 nm (blue line), characteristic absorption of the protein; and 650 nm (red line), characteristic absorption of the label. The conditions used for the separations have been described in the main text.

Fluorescence switching experiments on the labeled species have suggested that fraction 3 of ATTO647N labeled Cu azurin and fraction 2 of ALEXA647 labeled Cu azurin contain the species where the label is attached to a lysine close to the copper center. Mass spectroscopic experiments confirmed the position (K122) of the label on the surface of azurin.

Future plans

Using the K122 labeled species, FCS experiments can be used to investigate the intramolecular ET reactions in Cu azurin labeled with different dyes. In parallel, it is also possible to monitor the redox state changes of a labeled azurin molecule after surface confinement with single molecule resolution. In these cases, we can address the kinetics of ET process between the dye (ATTO655, ATTO647N or ALEXA647) and the copper center as well as the blinking rates under different redox conditions. It is a known fact that the problem of nonspecific interactions between the labeled protein and the solid support or the receptor often makes the biochemical screening process troublesome, especially with fluorescently labeled substrates that have charged or hydrophobic functional groups. Specially, the hydrophobic interactions occur between proteins and the dye molecules, which make specific interactions e.g. ET process hard to ascertain. This work will demonstrate how redox potential, charge and the

hydrophobicity/lipophilicity of the dyes can influence the inter- and intramolecular ET dynamics. The excitation conditions and local environment can also be varied to investigate the effect of those on ET dynamics. This work may help in designing experiments and choosing suitable dyes for single molecule studies in case of metalloproteins.

References

(1) Teixeira, V. H.; Baptista, A. M.; Soares, C. M. Modeling electron transfer thermodynamics in protein complexes: interaction between two cytochromes c(3). *Biophys. J.* **2004**, *86*, 2773–85.

(2) Cardona, T.; Battchikova, N.; Zhang, P.; Stensjö, K.; Aro, E.-M.; Lindblad, P.; Magnuson, A. Electron transfer protein complexes in the thylakoid membranes of heterocysts from the cyanobacterium Nostoc punctiforme. *Biochim. Biophys. Acta* **2009**, *1787*, 252–63.

(3) Bashir, Q.; Scanu, S.; Ubbink, M. Dynamics in electron transfer protein complexes. *FEBS J.* **2011**, *278*, 1391–1400.

(4) Malatesta, F.; Antonini, G.; Nicoletti, F.; Giuffre, A.; D'Itri, E.; Sarti, P.; Brunori, M. Probing the high-affinity site of beef heart cytochrome c oxidase by cross-linking. *Biochem.J.* **1996**, *315*, 909–916.

(5) Hippler, M.; Drepper, F.; Haehnel, W.; Rochaix, J. D. The N-terminal domain of PsaF: precise recognition site for binding and fast electron transfer from cytochrome c6 and plastocyanin to photosystem I of Chlamydomonas reinhardtii. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 7339–44.

(6) Qin, L.; Kostić, N. M. Importance of protein rearrangement in the electron-transfer reaction between the physiological partners cytochrome f and plastocyanin. *Biochemistry* **1993**, *32*, 6073–80.

(7) van Amsterdam, I. M. C.; Ubbink, M.; Einsle, O.; Messerschmidt, A.; Merli, A.; Cavazzini, D.; Rossi, G. L.; Canters, G. W. Dramatic modulation of electron transfer in protein complexes by crosslinking. *Nat. Struct. Biol.* **2002**, *9*, 48–52.

(8) van Amsterdam, I. M. C.; Ubbink, M.; Einsle, O.; Messerschmidt, A.; Merli, A.; Cavazzini, D.; Rossi, G. L.; Canters, G. W. Dramatic modulation of electron transfer in protein complexes by crosslinking. *Nat. Struct. Biol.* **2002**, *9*, 48–52.