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## **Towards a single-molecule FRET study of Frauenfelder's nonexponential rebinding of CO in myoglobin**

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# Summary

Förster Resonance Energy Transfer (FRET), is a known technique in biotechnology to monitor dynamics, conformational changes, and kinetics of binding-unbinding of small molecules in proteins through measuring inter- and intramolecular distance changes at a scale of 1-10 nm. FRET is based on a dipole dipole interaction between two fluorescent dyes (donor and acceptor) resulting in non-radiatively energy transfer from excited state of the donor to an acceptor molecule which is reversely proportional to the six power of distance between donor and acceptor ( $R^{-6}$ ). In this thesis we focus on the Förster resonance energy transfer technique to study the kinetics of binding and dissociation of CO to and from myoglobin which has been fluorescently labeled (Chapters 3,4). FRET quenching is reported for an acceptor (ATTO575Q dye) and a donor (Azaoxa-triangulenium, ADOTA dye) doped in thin polymeric layers at both ensemble and single molecule level (Chapter 5).

## **The photodissociation of carboxymyoglobin (MbCO)**

Myoglobin (Mb) plays a vital role in transporting small molecules such as O<sub>2</sub>, CO, and NO. The mechanism of the reaction of these ligands with ferrous Mb (deoxy-Mb) is interesting because the binding is reversible and the system can be used as a prototype for more complex systems.

Early time-resolved experiments by Frauenfelder and coworkers on ensembles of myoglobin demonstrated that, at cryogenic temperatures, rebinding of CO to myoglobin's heme cofactor after flash photolysis shows a strongly stretched exponential behavior, that is not observed at room temperature. This low temperature behavior has been assigned to structural heterogeneity of myoglobin and different rebinding rates of CO, but no direct observation of this effect exists to date. The conclusion of these experiments was that the reaction rates of individual molecules there exhibit large spread.

The protein relaxations and fluctuations are nonexponential in time at room temperature, which points to the possibly collective nature of these motions. Although recently a few studies have focused on the variation of protein reaction rates by means of single-molecule experiments, the mechanism by which small molecules such as CO bind/unbind to the heme of myoglobin is less well understood. In particular insight in the kinetics of CO rebinding at single molecule level is missing. Our aim is to study the rebinding kinetics of CO through fluorescence quenching of a dye attached to the myoglobin and by using FRET.

## **Estimation of dissociation quantum yield of the Mb-CO bond**

In chapter 3, FRET technology is described which was used to determine the interaction between myoglobin and CO. In this system, donor emission (dye attached to the the myoglobin) is quenched through a non-fluorescent acceptor (myoglobin). The acceptor can be a different state of myoglobin for example deoxy-Mb in which CO is unbound from Mb, can act as a quencher and MbCO where CO is bound to Mb has no quenching effect or less. The weak bands beyond 700 nm, particularly Band III at 760 nm, in the deoxy-Mb absorption spectrum quenches the fluorescence of a deep red dye, whereas those bands beyond 700 nm are absent in the spectrum of MbCO.

It is well known that visible light in a wide spectral range, particularly  $400 < \lambda < 550$  nm, breaks the Mb-CO bond with high efficiency, which precludes FRET-based investigations of CO dissociation in this spectral region. We therefore developed a strategy involving the far-red spectral region,  $\lambda > 700$  nm. The challenge is that if red-light illumination of a labeled MbCO would break the Mb-CO bond with high efficiency, probing the absorption of MbCO with red light would make it impossible to monitor CO rebinding by fluorescence spectroscopy and FRET. For this reason, we wanted to obtain the quantum yield for photodissociation of the Mb-CO under red-light illumination ( $\lambda > 700$  nm). Three states of myoglobin, met-Mb (heme contains  $\text{Fe}^{3+}$ ), deoxy-Mb (heme contains  $\text{Fe}^{2+}$ ), and MbCO (heme contains  $\text{Fe}^{2+}$ ), have been prepared and optically characterized.

Experimentally the quantum yield of Mb-CO bond breaking is established by following the number of deoxy-Mb molecules that have not re-bound to CO at a given time after photodissociation. Because the recombination of CO to the heme is fast, it is essential to slow down the kinetics of recombination of the escaped CO molecule to the heme. CO molecules after photodissociation and escaping from the protein should be removed from the vicinity of the newly formed deoxy-Mb. We prevented CO recombination chemically by using  $\text{H}_2\text{O}_2$  as an oxidant which quickly converts the deoxy-Mb to another form that does not bind CO, for example met-Mb. The exact amount of MbCO converted to deoxy-Mb was measured by following the absorption spectrum of MbCO (using the absorption bands at 542 nm and 579 nm which are characteristic of MbCO). As the quantum yield of MbCO bond breaking by blue light is close to 1, we could use this photoreaction as a reference. By comparing the effect of MbCO illumination by a red LED ( $\lambda = 730$  nm) in the presence of  $\text{H}_2\text{O}_2$  with the effects of the blue LED illumination ( $\lambda = 450$  nm) we could estimate an upper bound quantum yield of MbCO photodissociation by red light ( $\lambda = 730$  nm) of less than 6%.

### **FRET study of CO binding to fluorescently labeled myoglobin**

Based on the estimated quantum yield of bond breaking in (see chapter 3), we concluded that illumination of MbCO with far-red light (700-800 nm) does not break the Mb-CO bond efficiently and the MbCO complex should be stable enough to monitor FRET from the far-red donor to the heme, with enough donor fluorescence photons emitted before dissociating MbCO during the FRET measurement. For the FRET experiment selection of the proper dye, position of labeling on the protein and the labelling method are crucial. For this purpose, not only an independent estimation of FRET efficiency is needed to characterize CO re-binding but also the development of quantitative methodologies for steady-state parameters of ligand binding is crucial. In chapter 4, we performed two different labelling methods: labelling at engineered sites and N-terminal labelling. Different dyes were tried (ATTO643, ATTO740, and Cy7) which absorb and emit in the red. Various labeling positions for example at position of serine 3 were realized by site-directed mutagenesis. During the FRET experiments we measured the fluorescence intensity of the dye and/or the fluorescence lifetime of labelled Met-Mb, Deoxy-Mb and MbCO. We could observe significant intensity differences for ATTO 740 labeled-deoxy-Mb and -MbCO which show that it is possible to distinguish these two states in a mixture.

### **Non-fluorescent quenching for FRET assay in ensemble and at the single molecule level in polymeric layer**

Single Molecule FRET (sm-FRET) may provide the distributions of experimental parameters such as excited state lifetime, fluorescent intensity, local environmental fluctuations, etc. Theodor Förster some 80 years ago predicted a stretched-exponential fluorescence intensity decay under ensemble conditions. This is related to a distribution of acceptors in the vicinity of each donor; the decay rate depends on the concentration of acceptor around the donor. These non-exponential kinetics arise from distributions of exponential steps. In chapter 5, we first tested the consistency of Förster's theory by studying an ensemble of the acceptors (ATTO575Q) around the donor (Azaoxa-triangulenium, ADOTA) for different concentrations of acceptor in a thin polymeric layer. The advantage of dye-doped polymer layer is that it allows for control of the dispersion of the dye molecules in the polymer films and prevents any type of quenching other than FRET. Our single-molecule study showed that histograms of decay rates of single ADOTA molecules are much more sensitive to heterogeneity than the average non-exponential decay. Each individual molecule exhibits a single-exponential fluorescence intensity decay, but the distribution of acceptor sites around each molecule produces a wide distribution of decay rates.

