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Chapter 7

General conclusions and future prospects
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In the present work a series of single-molecule experiments on fluorescently labeled metal-containing proteins, was presented. The aim of the experiments was to gain a wider insight in electron-transfer processes. The methodology is based on a Förster Resonance Energy Transfer (FRET) approach that makes it possible to probe the redox state of absorbing cofactors inside a protein by attaching a fluorescent dye to its surface. It is shown here that this approach is suitable for single-molecule investigations both by FCS of diffusing molecules and SCM of trapped enzymes.

In Chapter 2, the products of the labeling reaction were investigated. It appears that labeling azurin with an amino reactive fluorescent label, ATTO 655, results in a mixture of species. This mixture can be separated into its components by means of ion exchange chromatography and the species can be analyzed by mass spectrometry. The experimental configuration used allows for concomitant determination of the stoichiometry, label-to-protein, and of the location of the label in the primary sequence. It was found that the label attaches preferentially to some sites: Lys122 is the most reactive, whereas Ala1 (N-terminus), Lys128 and Lys24 (or 27) show comparable reactivities. The reactivity of Lys122 is surprisingly high, due to its location in a hydrophobic region of the surface of the protein. Two main points have to be highlighted: by means of “one go” MS-experiments the label position could be established, avoiding the need of pre-digestion of the sample that is standard routine nowadays. Furthermore, what previously has been thought a drawback of using an amino reactive label, i.e. that it may attach to a multitude of surface positions, is shown here to be an advantage in the sense that it can be used to obtain a collection of species labeled in different positions in one single reaction step without the need of mutagenesis. The labeled products can be separated efficiently by ion exchange chromatography and the position of the label can be established in a straightforward manner by FTICR-MS spectrometry. Improvements in the separation, as well as application of methods other than ion exchange chromatography may reveal extremely useful for other proteins as well.

In Chapter 3 it is shown that by controlling the reaction conditions we can control the relative amount of each species obtained. The species isolated and characterized in Chapter 2 were used for single-molecule investigations, in particular FCS. The FCS behavior of the mixture is compared with the behavior of the single isolated species and the importance of knowing the exact composition of the sample is evident from the outcome of the experiments. An important finding is the observation of an electron-transfer reaction between the label and the copper site of azurin when the former is bound to Lys122. The proximity of Lys122 to the copper center makes it possible that either a direct through-bond electron tunneling or a
back folding of the label toward the surface of the protein allows for transfer of electrons. Theoretical calculations reveal that both possibilities may have to be taken into account. Further investigations may be reveal the exact contributions to the ET rate.

In Chapter 4 the electron-transfer reaction within a dimerized protein is measured by FCS. The (Cu-Cu) BMME azurin dimer was used to test whether the combination of the FRET-based detection of the redox state with FCS was suitable to measure such processes. The measured ET rate, $9.2 \times 10^3 \text{ s}^{-1}$, compares well with the value reported in literature, and thus confirms that FCS can be used for this purpose. Furthermore, the dimer may be considered as a paradigm for proteins containing multiple redox centers, as well as for complexes of redox partners. In fact the technique may be easily extended to the study of ET reactions between physiological partners. The use of multiple labels with different colors may be used to improve the applicability of the technique: two redox partners could be labeled each with a different label and by means of either spectral or lifetime characteristics the redox behavior of each could be monitored in solution. The co-localization of the proteins, and therefore the formation of a complex, may be followed by the simultaneous detection of both colors in the probe volume and cross-correlating the signals or by FRET technology; whereas dynamics in the fluorescence intensity of each label may be used to address the kinetics of the transfer of electrons between the two partners.

The results presented in Chapter 5 on the enzyme mechanism of the copper-containing blue nitrite reductase are very important to understand the details underlying the activity of the enzyme. Individual enzymes were investigated, during turnover conditions by means of SCM and the redox state of each molecule was monitored in time for few hundred molecules. The statical analysis of the data shows that the enzyme molecules are divided in two populations and each of them performs, apparently, catalysis at a different rate than the other. A comparison of these results with the proposed mechanism of action of the enzyme brought to light a new finding. The enzyme follows the random sequential mechanism as described from \textit{in-bulk} measurements but individual molecules seem to follow one of the two branches hypothesized in the mechanism for a continuus amount of time, as if a memory effect applies to the behavior of bNiR.

In the following chapter, Chapter 6, further experiments that allowed to monitor the enzyme for an extended period of time, compared with the configuration used in Chapter 5, were performed. The main aim of Chapter 6 was to explore the differences in the kinetic behavior and in the intramolecular electron-transfer properties of the enzyme as a function of substrate concentration and, more importantly, as a function of pH. The enzyme responds to the variation of pH as predicted by the postulated RSM. Further experiments along the lines elaborated in Chapter 6 may
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shed light on the question stated in Chapter 5: do the populations observed by SCM represent a static form of heterogeneity or are they in a dynamic equilibrium with each other?