Unraveling the mechanism of multicopper oxidases : from ensemble to single molecule

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Chapter 1

Introduction

Ever since the structural data of biological macromolecules became available, there has been consistent struggle to relate this new information to the existing spectroscopy, activity and theoretical descriptions of these proteins and to understand the evolution and/or to predict the role of yet uncharacterized gene products in this light. This Chapter serves to provide basic background and introduction to this thesis that primarily deals with understanding the structure–function relationship of a newly discovered blue copper protein from *Streptomyces coelicolor*. Scope and outline of each chapter has also been presented.

1.1 Motivation

The evolution of life on earth has been intimately linked to the great oxidation event that occurred around 2.7 billion years ago.[1,2] At this time, the cyanobacteria started to poison the environment by releasing a toxic gas during photosynthesis which we know today as O_2.[2] There were only two ways for the existing life forms to cope up: either to avoid it altogether or to adapt their machinery to live with it. Some followed the former path and could still survive under deep oceans which remained anoxic. However, for others, it soon emerged that the advantages of this toxin were far more than the disadvantages involved. This is because oxygenic respiration could provide a lot more energy per reaction step than fermentation or other respiratory pathways. The toxicity of O_2 is related to the reactive oxygen species (ROS)[2] which are produced during respiration and by partial reduction of O_2 by organic matter. Thus, a tight control on the production of ROS was essential for survival. Transition metals like Cu, Fe and others were incorporated into the existing machineries for these purposes which have existed and further evolved since. The research presented in this thesis focuses on understanding the mechanism of O_2 metabolism by the small laccase (SLAC), a multicopper protein, from *Streptomyces coelicolor*.

1.2 (Multi)copper Proteins

Copper is an essential dietary element owing to its crucial role in biological redox processes.[3] Cu proteins are involved in oxygen transport (hemocyanin), electron transfer (azurin), respiration (cytochrome–c oxidase), metal homeostasis (ceruloplasmin) to name but a few examples. The latter two processes involve controlled reduction of O_2 to H_2O. Cytochrome–c oxidase (CcO), a membrane bound protein in the mitochondria, utilizes NADPH as a reductant and ceruloplasmin (Cp) which is found in human sera, uses Fe(II) to do so. In addition to Cu ions, CcO also contains heme prosthetic groups which are involved in catalysis, but Cp utilizes only Cu ions to accomplish the same task. Cp belongs to the category of oxidoreductases (enzymes which catalyze redox reactions) termed as multicopper oxidases (MCO).[4] Other well–known enzymes which fall into this category are the laccases (Lc) and ascorbate oxidase (AO). All

MCO's catalyze the reduction of O_2 to H_2O without the release of ROS to solution, while oxidizing one or more molecules of co–substrate, which acts as a sacrificial electron donor. Most MCO's are blue in color and contain four Cu ions which are important for catalysis (Figure 1).

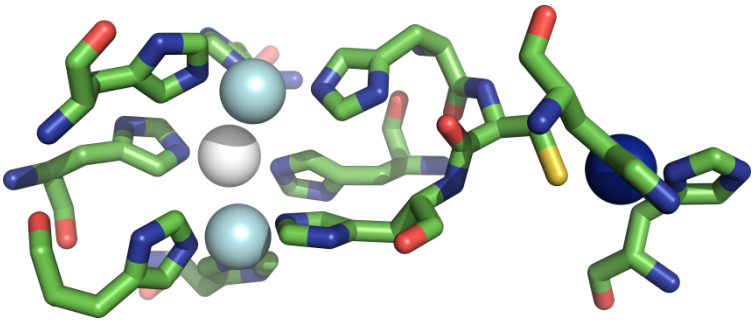

Figure 1: Active site of the MCO SLAC from *S. coelicolor* (pdb: 3CG8). Cu ions are shown as spheres (blue: T1 Cu; cyan: T3 Cu's; gray: T2 Cu). The coordinating residues are shown as sticks.

The blue color of MCO's is due to the presence of a Cu(II) ion (called type 1 (T1) Cu) with its unique coordination sphere. The T1 Cu is coordinated by two His and one Cys residue while the fourth ligand varies among different proteins. T1 Cu is characterized by its intense, $S_{Cys} \rightarrow Cu(II)$ charge transfer transition in the visible spectrum at ~600 nm and a narrow hyperfine splitting ($A_{\parallel}$ = (40–100) x 10^{-4} cm^{-1}) in the electron paramagnetic resonance (EPR) spectrum (Figure 2). This type of Cu is found in electron transfer (ET) proteins like azurin. It has a similar role in MCO's where the T1 Cu accepts electrons from reducing substrates, one at a time, and transfers them across a conserved HisCysHis pathway to the so called trinuclear Cu cluster (TNC) (Figure 1). The TNC is composed of the remaining three Cu(II) ions one of which is called type 2 (T2) Cu. T2 Cu does not contribute significantly to the absorption spectrum of the protein but has a large hyperfine splitting ($A_{\parallel}$ = (160–200) x 10^{-4} cm^{-1}) in the EPR spectrum (Figure 2). The other two Cu(II) ions at the TNC form a binuclear type 3 (T3) Cu pair bridged by a hydroxide anion. T3 Cu pair shows a band in the UV–region of the absorption spectrum around 330 nm (Figure 2). The two Cu's of the T3 Cu site are antiferromagnetically coupled and thus do

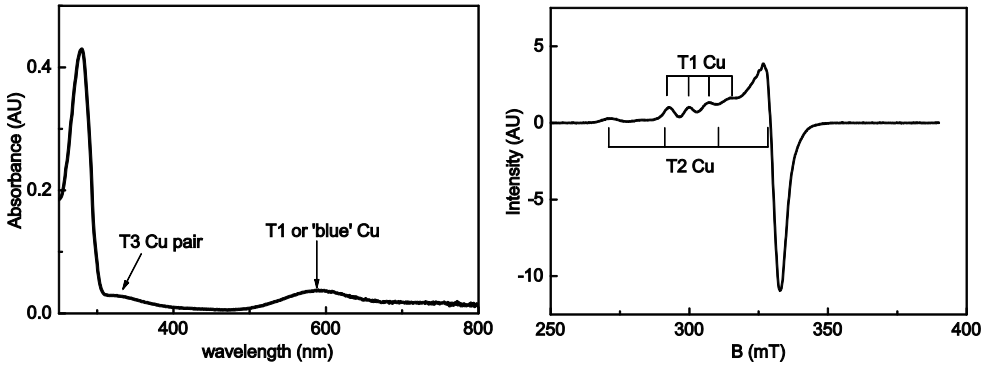

Figure 2: Typical (a) absorption spectrum and (b) EPR spectrum of the MCO SLAC from *S. coelicolor*.

not give rise to an EPR signal. This type of Cu pair is found in O_2 transport and O_2 activating proteins like hemocyanins and tyrosinases. In MCO's O_2 binds at the TNC, possibly at the T3 Cu site, and gets reduced to H_2O. The mechanism of O_2 binding and reduction to H_2O at the TNC has been subject of a long standing debate, which is discussed further in the forthcoming chapters.

1.3 Small Laccase

MCO's generally consist of three cupredoxin domains (3dMCO) which are arranged in three dimensional space such that the domains 1 and 3 fold back on each other where domain 3 contains the T1 Cu and the TNC is located at the interface of these two domains (Figure 3).[5–8] However, some exceptions do exist. Cp, for instance, consists of six, instead of three, cupredoxin domains where the TNC is located at the interface of domain 1 and domain 6 (Figure 3).[9] Domains 2, 4 and 6 contain three T1 Cu sites of which the one in domain 6, next to the TNC, is considered crucial for the enzyme activity.

Some years back, on the basis of a genome analysis, a new kind of MCO was identified and subsequently isolated from the culture of *Streptomyces coelicolor* which was different in shape and size from the MCO's known earlier.[10] The DNA sequence of this protein consisted of 1029 bases which accounts for 343 amino acids in the protein and an estimated molecular weight of 36.8 kDa. The protein was given the name 'small laccase' (SLAC), possessed the spectroscopic

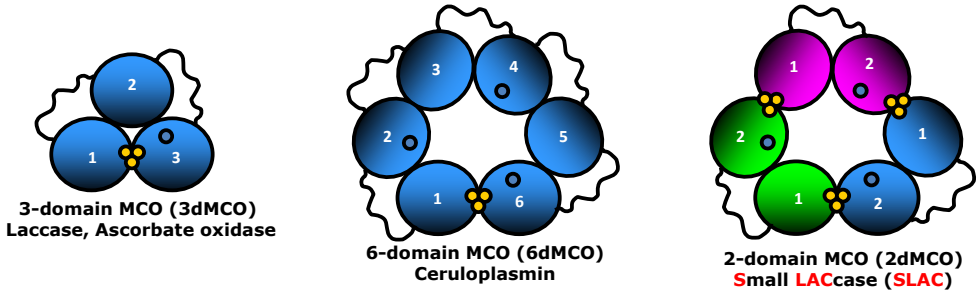

Figure 3: Cartoon representation of MCO's with different domain organization. The cupredoxin domains of same color belong to the same peptide chain and are also connected with a short loop. The Cu ions are shown as small spheres (blue: T1 Cu; yellow: TNC) within these domains.

features characteristic of MCO's and was found to be active as a trimer. Later the 3D structure of the protein revealed that SLAC is a homotrimer where each monomer consists of two cupredoxin domains.[11] The overall geometry appears very similar to Cp except that SLAC contains three T1 Cu's and three TNC's, and the six domains, two from each monomer, are not covalently linked (Figure 3). Moreover the TNC is situated between the N– and C–terminus of adjacent monomers unlike the 3dMCO's where the TNC is located between the N– and C–terminus of the same peptide chain. It was interesting to note that sequences similar to that of SLAC were identified in the genomes of many other organisms indicating the presence of such 2DMCO's in the *Streptomyces* genus. Phylogenetic analysis and evolutionary relationships of the blue copper proteins suggest that such two–domain multicopper blue proteins might have appeared earlier in the evolution of the conventional 3dMCO's like Lc's, AO and the six–domain Cp.[12–14] The research presented in this thesis was undertaken to understand the fundamental structure–function relationship of this new protein that may throw new light on the evolution of its family members.

1.4 Scope and outline of the work

Laccases are one of the well–studied members of the MCO family. Owing to their capability to oxidize a wide variety of substrates, they find use in the industry for textile dye finishing, waste treatment, food applications, biosensors and medicinal and asymmetric organic synthesis.[15] However, one of the major

upcoming applications of Lc's is in their capability to act as a cathode in a (bio)fuel cell where O_2 acts as the ultimate electron acceptor. SLAC is well suited for this application since there exists a recombinant expression system that can produce large quantities of protein which are easy to purify, and preliminary studies seem to suggest its superior performance over conventional Lc's.[16] Thus, it is important to understand the mechanism of O_2 reduction by SLAC and compare it to that of the well–studied Lc's.

The reaction of O_2 may take place with the four–electron reduced form of the protein.[17–19] This will prevent the formation of long lived ROS since the O_2 bound at the TNC can be completely reduced by $4e^-$ equivalents. However, the possibility of O_2 reacting with a two– or three–electron reduced form of the protein under turnover conditions cannot be excluded. Direct four–electron reduction of O_2 is unlikely. Instead, it is expected to occur in multiple steps (Figure 4).[17–19] There exists considerable evidence that the first step in this process is $2e^-$ reduction of O_2 to form peroxide intermediate (PI).[18,19] Since this intermediate is highly oxidizing, it may rapidly accept two more electrons to complete the four–electron reduction of O_2 while the enzyme is oxidized back to what is called as the native intermediate (NI). It is not clear whether the conversion of PI to NI takes place in two $1e^-$ transfer steps or a single $2e^-$ transfer step (see below). The NI is different from the resting form of enzyme in the way OH^- is bound at the TNC.[20] The multi–step process of O_2 reduction is fast, at least at room temperature, and it is very difficult to characterize the intermediates steps with conventional pre–steady–state kinetics techniques like stopped–flow. However, X–ray diffraction measurements of the crystals of multiple MCO's, often performed at cryogenic temperatures, have provided a snapshot of the O_2/ROS bound at the TNC.[21,22] Still, there is no clear evidence to support the idea that such intermediates exist during steady–state turnover. Therefore, to dissect the mechanism of O_2 reduction, enzyme forms have been prepared where the T1 Cu site is absent (T1D)[23] or has been replaced with a redox inert Hg(II) (T1Hg).[24] When fully reduced T1D or T1Hg Lc reacts with O_2, the reaction is stalled at the first $2e^-$ reduction stage, when a PI is formed, since there are not sufficient electrons (fully reduced protein has only 3 Cu's and thus can load a maximum of three electrons) to carry out complete reduction of O_2. It is

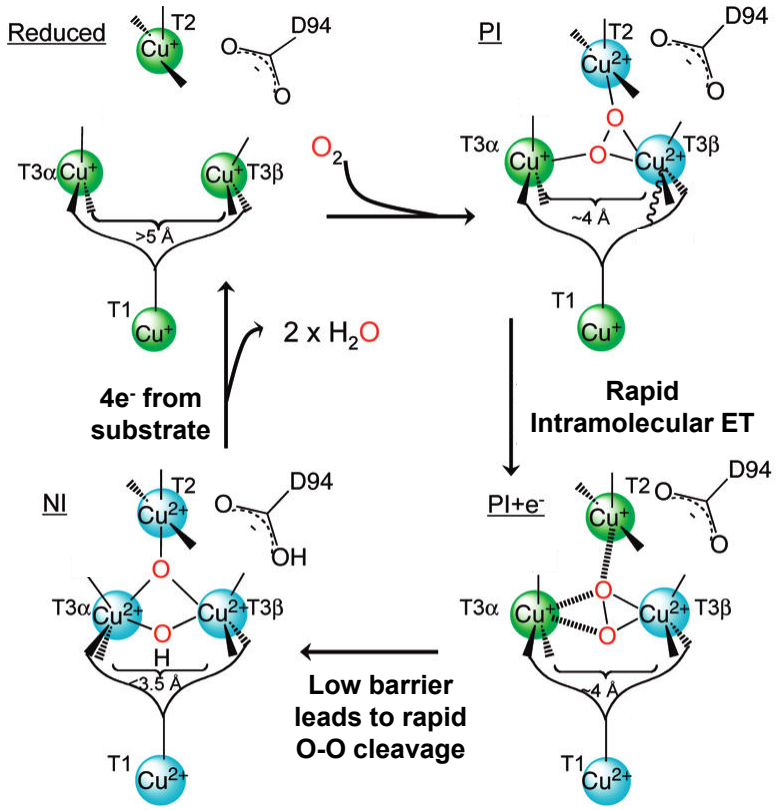

Figure 4: Possible mechanism of O_2 reduction by Fet3p, an MCO from *Saccharomyces cerevisiae*, as proposed by Solomon and coworkers. Reduced Cu's are depicted in green and oxidized Cu's in cyan. The T3 Cu's are inequivalent and are marked as T3α and T3β. The proposed mechanism may require further revisions and is not necessarily applicable to all other members of the MCO family. For more details, see the text. The figure is adapted from ref 28.

noteworthy that in spite of the protein having three–electrons, the reduction of O_2 doesn't go beyond the peroxide stage. There are some preliminary evidences in the literature which indicate that further one–electron reduction of PI may yield a molecule of water and an oxygen based radical (possibly hydroxyl) bound at the TNC.[25–27] However, there is a strong debate about the nature of the peroxide binding and the redox states of the different Cu ions in the PI. On multiple occasions, X–ray diffraction measurements have indicated a symmetric binding of the peroxide between the Cu ions of the T3 Cu pair but when

applying this technique as the only method of investigation, discrete assignment of the redox states of different Cu ions cannot be made.[21,22] On the other hand, mutagenesis experiments, spectroscopy and theoretical arguments indicate that the peroxide may also be coordinated to the T2 Cu.[28,29] The discrepancy could possibly arise because the above conclusions are each specific to a particular protein and, therefore, can't be generalized to all MCO's. Another possibility is that while the measurements on crystals are performed at cryogenic temperatures where natural movements of protein side chains are restricted, the measurements in solution are performed at ambient temperature allowing more motional freedom on atomic scale. It is beyond the scope of this thesis to review all possible mechanisms which have been proposed so far leading to the four–electron reduction of O_2. The interested reader is referred to the relevant literature.[17–19] However, because of its 3D–structural similarity with SLAC, it is appropriate to present here the mechanism proposed for ferrous oxidase (Fet3p) from *Saccharomyces cerevisiae*, which has been constructed on the basis of a variety of spectroscopic and kinetics measurements and theoretical considerations (Figure 4).[28] In the light of results obtained for other MCO's, the mechanism or the nature of intermediates presented in Figure 4 shall not be taken as a generalization for the entire 3dMCO family. In particular, it will be shown in Chapter 4 that some of the findings of our single molecule experiments may necessitate a revision of particular aspects of the scheme presented in Figure 4.

While the reaction of O_2 with fully reduced T1D Lc yields the PI, the similar reaction of T1D SLAC gives rise to a different kind of intermediate, a biradical intermediate, as demonstrated from the absorption and EPR spectroscopy.[30] **Chapter 2** of this thesis provides clear evidence about the nature of this intermediate and its possible role in the reduction of O_2 by SLAC. It appears that Tyr108 is a redox non–innocent residue which gets oxidized to form a radical when the TNC falls short of electrons, for example when the T1 Cu site is absent. Thus, the enzyme seems to adopt a rescue mechanism to prevent ROS formation and survive under conditions where there is an imbalance in the reducing and oxidizing co–substrates. Interestingly, this tyrosine residue is conserved across all the sequences of the purported 2dMCO's of this family and also in Cp.

For AO and many Lc's, it was also discovered that all four Cu ions are essential for the activity of the protein.[31] T2 Cu, for example, is coordinated by only two His residues and can be easily removed by using strong copper chelators and/or mild denaturing conditions.[31–34] Such a protein devoid of T2 Cu (T2D) does not possess any activity and neither can the fully reduced protein react with O_2 like the wt– or T1D forms of the protein. These findings together with a series of site–directed mutagenesis, kinetics and computational studies suggest that O_2 binds to the T2 Cu and one of the T3 Cu, referred to as T3Cuβ (Figure 4), instead of binding symmetrically to the two T3 Cu's as in hemocyanin.[28] This is because the coordination spheres of the two Cu's of the T3 site are slightly different leading to an asymmetry at the TNC. **Chapter 3** of this thesis aims to demonstrate another singularity of SLAC within the mechanism of O_2 reduction by this protein. The T2D form of SLAC only loses its activity partially, indicating that this copper is not a necessary requirement for the protein to function. Thus O_2 must bind to the T3 Cu pair, at least in SLAC. Interestingly, mutation of His102, one of the two histidine residues that coordinate to T2 Cu, abolishes enzyme activity almost completely, i.e. by 2–3 orders of magnitude, indicating the importance of this residue in the enzyme catalysis. However, it is still unclear how the protein containing only three equivalents of Cu's per monomer is capable of carrying out the four electron reduction of O_2 to H_2O.

Although the redox potentials of the Cu's at the TNC are very similar to that of T1 Cu, the TNC Cu's cannot be reduced directly by the substrate. T1 Cu, on the other hand, is directly reduced but doesn't interact or coordinate to O_2. This is why the protein lacking the T1 Cu site is inactive. Thus, the electrons which are accepted at the T1 Cu site must be transferred to the TNC, one at a time, before the TNC can react with O_2. This ET process must be fast and efficient so as to prevent formation of long lived ROS at the TNC which might pose a threat to the protein.

The vast majority of oxidoreductases, including the MCO's, uncouple the reduction and oxidation half reactions by using separate sites for them. The protein matrix takes care of ET and communication between the two sites. There has been extensive research on understanding the theory and mechanism of ET

kinetics and the role of the surrounding environment in this process. The interested reader is referred to the appropriate reviews to obtain an overview of ET mechanisms.[35,36] Conventional routes to follow the fast ET processes are to study pre–steady–state kinetics using rapid–mixing, flash quench/photolysis and pulse radiolysis. Usually when the ET rate is to be measured between two proteins (intermolecular ET) diffusing freely in solution, rapid–mixing techniques like stopped–flow, rapid–freeze–quench, chemical–quench are useful. To study the kinetics of intramolecular ET or ET between confined redox partners, the techniques that come handy are electrochemistry, flash quench/photolysis and pulse radiolysis.[37] However, there is a common limitation of all these techniques in that the ET process under investigation is measured under the pre–steady–state conditions. While such measurements continue to provide a wealth of information about the spectroscopy and nature of possible intermediates, not always does trapping of the intermediates under such conditions represent the series of events that occur in the turning over enzyme during steady–state. Electrochemistry is an exception here which sometimes allows investigation of steady–state kinetics of proteins immobilized on conducting electrodes, but the communication of protein with the electrode is rarely efficient and thus limits the study of the ET steps in question.[38]

Study of proteins at single molecule level has opened doors to investigate salient features of enzyme kinetics, including ET kinetics, that were inaccessible or very difficult to measure in bulk.[39–43] Recently, a Förster Resonance Energy Transfer (FRET) based approach was introduced, which provides fluorescence readout of the redox state of the protein or its specific cofactors.[44] Thus, the analyses of dwell times of a time trajectory of a single molecule provides information about the lifetimes of the respective 'on' and 'off' states which correspond to the rate of inter–conversion of two or more redox states involved in the process. The advantages of this approach are at least two–fold. First, it allows the real–time observation of individual ET events as the molecule is turning over under steady–state, and secondly, it allows studying the kinetics of individual molecules and unraveling the heterogeneity that exists across many molecules which is otherwise averaged out in an ensemble measurement. **Chapter 4** of this thesis makes use of the above approach to study kinetics of ET between the T1 and

TNC sites of SLAC. SLAC variants were prepared for site–specific labeling and immobilization on transparent supports to minimize the heterogeneity in sample preparation. It has been argued that the distribution of ET rates across many molecules corresponds to a disorder of activation energy in the ensemble. It remains to be seen whether this disorder is truly a random phenomenon or a systematic control that nature imposes over the active site to address different requirements in different environments by one and the same gene product.

1.5 References

[1] Bekker, A.; Holland, H. D.; Wang, P. L.; Rumble, D., 3rd; Stein, H. J.; Hannah, J. L.; Coetzee, L. L.; Beukes, N. J. *Nature* **2004**, *427*, 117.

[2] Halliwell, B.; Gutteridge, J. *Free Radicals in Biology and Medicine*; OUP Oxford, 2007.

[3] Karlin, K. D.; Tyeklár, Z. *Bioinorganic chemistry of copper*; Chapman & Hall, 1993.

[4] Messerschmidt, A. *Multi-copper Oxidases*; World Scientific, 1997.

[5] Messerschmidt, A.; Ladenstein, R.; Huber, R.; Bolognesi, M.; Avigliano, L.; Petruzzelli, R.; Rossi, A.; Finazzi-Agro, A. *J. Mol. Biol.* **1992**, *224*, 179.

[6] Taylor, A. B.; Stoj, C. S.; Ziegler, L.; Kosman, D. J.; Hart, P. J. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 15459.

[7] Roberts, S. A.; Weichsel, A.; Grass, G.; Thakali, K.; Hazzard, J. T.; Tollin, G.; Rensing, C.; Montfort, W. R. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 2766.

[8] Hakulinen, N.; Kiiskinen, L. L.; Kruus, K.; Saloheimo, M.; Paananen, A.; Koivula, A.; Rouvinen, J. *Nat. Struct. Biol.* **2002**, *9*, 601.

[9] Bento, I.; Peixoto, C.; Zaitsev, V. N.; Lindley, P. F. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2007**, *63*, 240.

[10] Machczynski, M. C.; Vijgenboom, E.; Samyn, B.; Canters, G. W. *Protein Sci.* **2004**, *13*, 2388.

[11] Skalova, T.; Dohnalek, J.; Ostergaard, L. H.; Ostergaard, P. R.; Kolenko, P.; Duskova, J.; Stepankova, A.; Hasek, J. *J. Mol. Biol.* **2009**, *385*, 1165.

[12] Dwulet, F. E.; Putnam, F. W. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **1981**, *78*, 2805.

[13] Nakamura, K.; Go, N. *Cell. Mol. Life Sci.* **2005**, *62*, 2050.

[14] Murphy, M. E. P.; Lindley, P. F.; Adman, E. T. *Protein Sci.* **1997**, *6*, 761.

[15] Xu, F.; Damhus, T.; Danielsen, S.; Østergaard, L. H. In *Modern Biooxidation*; Wiley–VCH Verlag GmbH & Co. KGaA: 2007, p 43.

[16] Wheeldon, I. R.; Gallaway, J. W.; Barton, S. C.; Banta, S. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 15275.

[17] Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. *Chem. Rev.* **1996**, *96*, 2563.

[18] Solomon, E. I.; Augustine, A. J.; Yoon, J. *Dalton Trans.* **2008**, 3921.

[19] Wherland, S.; Farver, O.; Pecht, I. *J. Biol. Inorg. Chem.* **2014**.

[20] Heppner, D. E.; Kjaergaard, C. H.; Solomon, E. I. *J. Am. Chem. Soc.* **2013**.

[21] Messerschmidt, A.; Luecke, H.; Huber, R. *J. Mol. Biol.* **1993**, *230*, 997.

[22] Ferraroni, M.; Matera, I.; Chernykh, A.; Kolomytseva, M.; Golovleva, L. A.; Scozzafava, A.; Briganti, F. *J. Inorg. Biochem.* **2012**, *111*, 203.

[23] Palmer, A. E.; Quintanar, L.; Severance, S.; Wang, T. P.; Kosman, D. J.; Solomon, E. I. *Biochemistry* **2002**, *41*, 6438.

[24] Cole, J. L.; Ballou, D. P.; Solomon, E. I. *J. Am. Chem. Soc.* **1991**, *113*, 8544.

[25] Andréasson, L. E.; Brändén, R.; Malsmtröm, B. G.; Vänngård, T. *FEBS Lett.* **1973**, *32*, 187.

[26] Aasa, R.; Brändén, R.; Deinum, J.; Malmström, B. G.; Reinhammar, B.; Vänngård, T. *FEBS Lett.* **1976**, *61*, 115.

[27] Branden, R.; Deinum, J. *Biochim. Biophys. Acta* **1978**, *524*, 297.

[28] Augustine, A. J.; Kjaergaard, C.; Qayyum, M.; Ziegler, L.; Kosman, D. J.; Hodgson, K. O.; Hedman, B.; Solomon, E. I. *J. Am. Chem. Soc.* **2010**, *132*, 6057.

[29] Solomon, E. I.; Ginsbach, J. W.; Heppner, D. E.; Kieber–Emmons, M. T.; Kjaergaard, C. H.; Smeets, P. J.; Tian, L.; Woertink, J. S. *Faraday Discuss.* **2011**, *148*, 11.

[30] Tepper, A. W. J. W.; Milikisyants, S.; Sottini, S.; Vijgenboom, E.; Groenen, E. J. J.; Canters, G. W. *J. Am. Chem. Soc.* **2009**, *131*, 11680.

[31] Malkin, R.; Malmstrom, B. G.; Vanngard, T. *Eur. J. Biochem.* **1969**, *7*, 253.

[32] Graziani, M. T.; Morpurgo, L.; Rotilio, G.; Mondovi, B. *FEBS Lett.* **1976**, *70*, 87.

[33] Avigliano, L.; Desideri, A.; Urbanelli, S.; Mondovi, B.; Marchesini, A. *FEBS Lett.* **1979**, *100*, 318.

[34] Morpurgo, L.; Savini, I.; Mondovi, B.; Avigliano, L. *J. Inorg. Biochem.* **1987**, *29*, 25.

[35] Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.

[36] Gray, H. B.; Winkler, J. R. *Q. Rev. Biophys.* **2003**, *36*, 341.

[37] Farver, O.; Pecht, I. *Coord. Chem. Rev.* **2011**, *255*, 757.

[38] Heering, H.; Canters, G. *Engineering the Bioelectronic Interface: Applications to Analyte Biosensing and Protein Detection; Davis, JJ, Ed* **2010**, 120.

[39] Lu, H. P.; Xun, L.; Xie, X. S. *Science* **1998**, *282*, 1877.

[40] English, B. P.; Min, W.; van Oijen, A. M.; Lee, K. T.; Luo, G.; Sun, H.; Cherayil, B. J.; Kou, S. C.; Xie, X. S. *Nat. Chem. Biol.* **2006**, *2*, 87.

[41] Kuznetsova, S.; Zauner, G.; Aartsma, T. J.; Engelkamp, H.; Hatzakis, N.; Rowan, A. E.; Nolte, R. J.; Christianen, P. C.; Canters, G. W. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 3250.

[42] Goldsmith, R. H.; Tabares, L. C.; Kostrz, D.; Dennison, C.; Aartsma, T. J.; Canters, G. W.; Moerner, W. E. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 17269.

[43] Tabares, L. C.; Kostrz, D.; Elmalk, A.; Andreoni, A.; Dennison, C.; Aartsma, T. J.; Canters, G. W. *Chem. Eur. J.* **2011**, *17*, 12015.

[44] Kuznetsova, S.; Zauner, G.; Schmauder, R.; Mayboroda, O. A.; Deelder, A. M.; Aartsma, T. J.; Canters, G. W. *Anal. Biochem.* **2006**, *350*, 52.