

Review

Single-molecule fluorescence studies from a bioinorganic perspective

Peng Chen *, Nesha May Andoy

Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14850, USA

Received 21 June 2007; received in revised form 6 August 2007; accepted 17 August 2007
Available online 23 August 2007

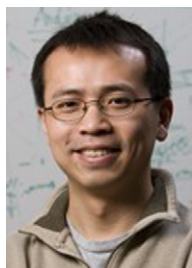
Contribution to the special issue in celebration of the 60th birthday of Professor Edward I. Solomon.

Abstract

In recent years, single-molecule methods have enabled many innovative studies in the life sciences, which generated unprecedented insights into the workings of many macromolecular machineries. Single-molecule studies of bioinorganic systems have been limited, however, even though bioinorganic chemistry represents one of the frontiers in the life sciences. With the hope to stimulate more interest in applying existing and developing new single-molecule methods to address compelling bioinorganic problems, this review discusses a few single-molecule fluorescence approaches that have been or can be employed to study the functions and dynamics of metalloproteins. We focus on their principles, features and generality, possible further bioinorganic applications, and experimental challenges. The fluorescence quenching via energy transfer approach has been used to study the O₂-binding of hemocyanin, the redox states of azurin, and the folding dynamics of cytochrome *c* at the single-molecule level. Possible future applications of this approach to single-molecule studies of metalloenzyme catalysis and metalloprotein folding are discussed. The fluorescence quenching via electron transfer approach can probe the subtle conformational dynamics of proteins, and its possible application to probe metalloprotein structural dynamics is discussed. More examples are presented in using single-molecule fluorescence resonance energy transfer to probe metallochaperone protein interactions and metalloregulator–DNA interactions on a single-molecule basis.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Single-molecule fluorescence spectroscopy; Bioinorganic chemistry; FRET; Fluorescence quenching via energy transfer; Fluorescence quenching via electron transfer; Oxygen binding; Redox; Metalloprotein folding; Conformational dynamics; Catalysis; Metallochaperones; Metalloregulators



Peng Chen grew up in Jiangsu, China, and received his B.S. from Nanjing University, China in 1997. After spending a year at University of California at San Diego with Prof. Yitzhak Tor, he moved to Stanford University to work with Prof. Edward I. Solomon. There he did his Ph.D. in bioinorganic and physical inorganic chemistry, focusing on spectroscopic studies of biologically relevant Cu sites involved in O₂ and N₂O activation. In 2004, he joined Prof. X. Sunney Xie's group at Harvard University for his postdoctoral research in single-molecule biophysics, and worked on tracking individual motor proteins inside living cells. He started his assistant professorship at Cornell University in 2005. His current research interests span from single-molecule studies of bioinorganic chemistry, including protein-protein interactions for metal trafficking and protein-DNA interactions for metal regulation, to

single-molecule imaging of nanoparticle catalysis. His work was recently recognized by a Camille and Henry Dreyfus New Faculty Award and a CAREER award from the National Science Foundation.



Nesha May Andoy obtained her B.S. in Chemistry at the University of the Philippines in 2001. She is currently a graduate student at Cornell University in the Department of Chemistry and Chemical Biology, working on single molecule protein-DNA interactions and bioinorganic enzymology in Prof. Peng Chen's lab.

* Corresponding author. Tel.: +1 607 254 8533; fax: +1 607 255 4137.
E-mail address: pc252@cornell.edu (P. Chen).

Contents

1. Introduction	810
2. Fluorescence quenching via energy transfer	811
2.1. Case 1. Oxygen binding of hemocyanin	811
2.2. Case 2. Redox of azurin	812
2.3. Case 3. Cytochrome <i>c</i> folding	813
2.4. Features and generality	813
2.5. Potential applications	814
2.6. Challenges	815
3. Fluorescence quenching via electron transfer	815
3.1. Conformational dynamics of flavin reductase (Fre)	815
3.2. Features and generality	816
3.3. Potential applications	816
3.4. Challenges	816
4. Other single-molecule bioinorganic studies	817
4.1. Metallochaperone protein interactions	817
4.2. Metalloregulator DNA interactions	817
5. Summary	817
Acknowledgments	818
References	818

1. Introduction

In recent years, single-molecule methods have enabled many innovative studies in the life sciences and proven powerful in unraveling the detailed workings of macromolecular machineries. These single-molecule measurements have resulted in unprecedented insights in many biological areas, such as molecular motors [1–4], nucleic acid enzymes [5–7], RNA activities [8–10], protein folding [11–13], enzymology [14–19], and gene expression [20,21]. New discoveries continue to emerge through single-molecule experiments.

Many features make the single-molecule approach appealing. It removes ensemble averaging so that heterogeneous behaviors of biomolecules can be revealed and subpopulations analyzed. More important, it allows us to visualize the actions of individual biomolecules in real time, which is particularly useful in capturing reactive intermediates and elucidating biochemical mechanisms. Experimental methods to investigate single biomolecules fall into three categories: optical (e.g., fluorescence and nonlinear optical microscopy), mechanical (e.g., optical tweezers, magnetic tweezers, scanning probe microscopy, and microfluidics), and electrical measurements (e.g., patch clamp and nanopores). All these methods allow real-time observation of dynamic processes of individual biomolecules. Many excellent reviews on the principles and applications of these single-molecule methods are available [3,4,22–35].

Single-molecule fluorescence techniques are perhaps the most popular because of their straightforward instrumentation and easy operation [3,22,24,25]. One can monitor the fluorescence intensity, spectrum, polarization, or lifetime of a biomolecule to investigate its molecular properties. Among these, following fluorescence intensities is the

most straightforward. Introducing exogenous fluorescent labels is a general strategy when the target biomolecules are not naturally fluorescent. Experimentally, there are three common practices for single-molecule fluorescence detection. First, experiments are done at low concentrations (10^{-9} – 10^{-12} M) to spatially separate the individual molecules so that each of them can be studied without interference from surrounding molecules. Second, fluorescence signal detection is confined to a small volume ($<10^{-15}$ L) to minimize the background noises for single-molecule sensitivity. Third, biomolecules are often immobilized so that a single molecule can be studied over time.

Two experimental setups are widely used for single-molecule fluorescence detection: total internal reflection (TIR) fluorescence microscopy and confocal fluorescence microscopy. The evanescent field from TIR confines the laser excitation to a thin layer (~ 50 – 300 nm), while the confocal scheme focuses the laser beam to a diffraction limited volume and uses a pinhole to confine the signal detection around the focus ($\sim 300 \times 300 \times 600$ nm³). The TIR fluorescence microscopy typically uses electron multiplying cameras as detectors and can image hundreds of molecules simultaneously; the time resolution is about milliseconds limited by the camera speed, although sub-millisecond imaging is possible with state-of-the-art hardware and exceptionally bright probes [36]. The confocal microscopy uses point detectors, such as single-photon avalanche photodiodes, and examines one molecule at a time; the time resolution can be up to microseconds for following the dynamic processes of biomolecules. For both TIR and confocal microscopy, multiple detection channels, such as different colors and polarizations, can be readily implemented.

So far, most biological applications of the single-molecule approach have been in biophysics, unraveling the

functions and mechanisms of many biomacromolecules in which the metal cofactors are either not needed or nonessential for the function. Very limited single-molecule studies have been reported that probe metal cofactors directly or investigate metal-cofactor related biological processes. Metal-containing biomolecules are the subjects of bioinorganic chemistry, one of the frontiers in the life sciences. With the hope to stimulate more interest in applying the existing and developing new single-molecule methods to address compelling bioinorganic problems, we discuss here the single-molecule fluorescence approaches that have been or can be employed to study metalloprotein function and dynamics. We focus on their principles, features and generality, possible further bioinorganic applications, and experimental challenges. The examples are grouped based on their experimental principles. The intrinsic fluorescence of Mg^{2+} -containing chlorophyll has been utilized widely to study light-harvesting complexes at the single-molecule level [37–44]. This subject is by itself worth an in-depth review, and thus is not included here. Besides single-molecule fluorescence methods, scanning probe microscopy has also been used to study metalloproteins at the single-molecule level. These studies have been reviewed [45–47] and are not discussed here because of the limited scope of this review.

This review is organized as follows: we first discuss a fluorescence resonance energy transfer (FRET) based approach (i.e., fluorescence quenching via energy transfer), which has been applied to study O_2 -binding/unbinding events, redox changes, and folding dynamics of metalloproteins. Next we describe the fluorescence quenching via electron transfer approach, which has not been applied but has the potential to investigate bioinorganic problems. We will then finish with brief discussions of the current single-molecule bioinorganic research projects in our research group.

2. Fluorescence quenching via energy transfer

FRET is widely applied in single-molecule fluorescence studies [24,48]. Governed by the Förster mechanism [49], the spectral overlap between the fluorescence spectrum of a donor molecule and the absorption spectrum of an acceptor can result in an efficient energy transfer from the donor to the acceptor. The energy transfer efficiency is dependent on the donor to acceptor distance, r , within the nanometer range (energy transfer efficiency = $1/[1 + (r/r_0)^6]$, where r_0 is the Förster radius of the donor–acceptor pair, a constant typically of a few nanometers [49,50]). The most general experimental design of applying FRET is to label biomolecules with a fluorescent donor–acceptor pair, and then monitor the fluorescence intensities of both the donor and the acceptor at the single-molecule level.

A variant of FRET, fluorescence quenching via energy transfer, uses only one fluorescent probe as a donor. The acceptor is nonfluorescent and acts as a quencher – it has strong absorption bands that overlap spectrally with the

fluorescence of the donor. This energy-transfer-caused quenching changes the donor fluorescence, from which the chemical state of the acceptor (i.e., quencher) and the distance between the donor and the quencher can be deduced. Erker, Basché and co-workers used this fluorescence quenching approach to probe O_2 -binding by the coupled binuclear copper protein hemocyanin [51–53]; Davis, Schmauder, Kuznetsova et al. used this approach to report the redox state of the blue copper protein azurin [54–57]; and Takahashi and co-workers used it to study the folding dynamics of cytochrome *c* [58], all at the single-molecule level. More details of these experiments are discussed below.

2.1. Case 1. Oxygen binding of hemocyanin

Hemocyanin is a respiratory protein and uses two copper atoms at its active site to bind oxygen reversibly [59,60]. The reduced, deoxygenated form of hemocyanin (deoxy-Hc) has the two copper atoms at the +1 oxidation state (Fig. 1a). Oxygen binding to deoxy-Hc leads to the formation of oxygenated hemocyanin (oxy-Hc), in which O_2 binds as peroxide to the two oxidized Cu^{2+} centers in a side-on bridging geometry. The Cu^{2+} –peroxide bonds in oxy-Hc are highly covalent, shown by the large mixing between the Cu *d* orbitals and the peroxide π^* orbitals in its lowest unoccupied molecular orbital (LUMO) (Fig. 1b, inset) [59]. This highly covalent interaction leads to two intense peroxide-to- Cu^{2+} charge transfer transitions in the absorption spectrum of oxy-Hc, one centered at ~ 600 nm and the other at ~ 350 nm, from peroxide out-of-plane π^* and in-plane π^*_σ to Cu^{2+} charge transfer transitions, respectively (Fig. 1b). In contrast, deoxy-Hc has no strong absorption features in the visible region because of the d^{10} electronic configuration of the two reduced Cu^{1+} centers.

Erker et al. utilized the spectral contrast between oxy-Hc and deoxy-Hc to differentiate the oxygenation state of hemocyanin at the single-molecule level [53]. To a lysine residue on the protein surface, they attached a fluorescent probe TAMRA, whose fluorescence spectrum has a large spectral overlap with the 600-nm absorption band of oxy-Hc (Fig. 1c). Because of this spectral overlap and the proximity of the probe to the binuclear copper active site of Hc, the fluorescence of TAMRA in the oxy-Hc form is largely quenched via energy transfer to the side-on Cu_2O_2 core [51,52]. In contrast, the TAMRA fluorescence is not quenched in the deoxy-Hc form because deoxy-Hc does not have strong absorption. Therefore, the TAMRA-labeled oxy-Hc molecules have lower fluorescence intensities than the TAMRA-labeled deoxy-Hc molecules. Erker et al. immobilized TAMRA-labeled hemocyanin molecules by adsorption on glass surface and measured the fluorescence intensity of each molecule of deoxygenated and oxygenated hemocyanin samples. Analyses of the single-molecule intensity distributions led to the differentiation of deoxy-Hc and oxy-Hc at the single-molecule level

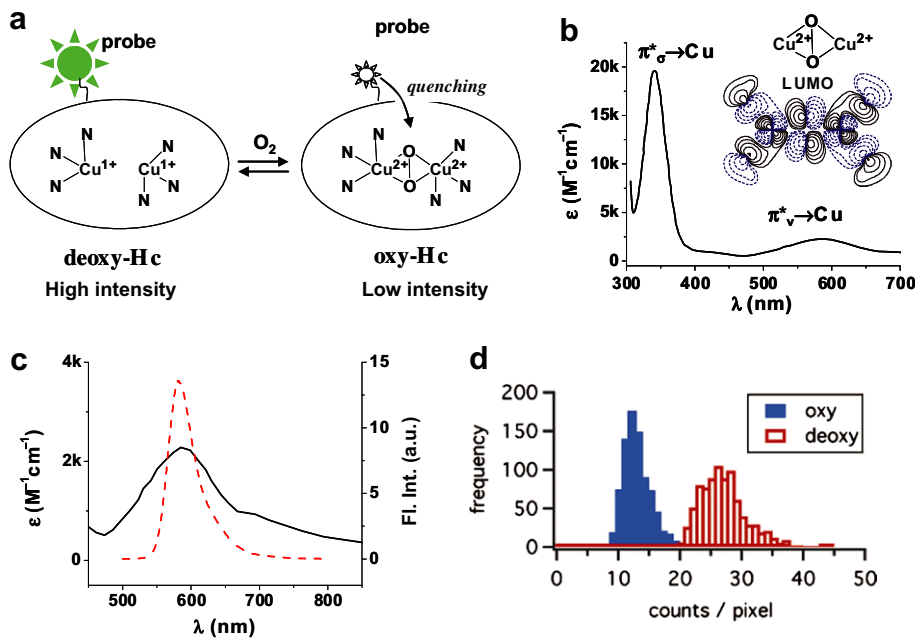


Fig. 1. (a) Fluorescence quenching via energy transfer to detect hemocyanin oxygenation state. (b) Absorption spectrum of oxy-Hc. Spectrum data taken with permission from Ref. [60]. Copyright 1996 American Chemical Society. Inset: contour plot of the lowest unoccupied molecule orbital (LUMO) of the side-on Cu_2O_2 core. (c) Spectral overlap between the absorption spectrum of oxy-Hc and the fluorescence spectrum of TAMRA. (d) Intensity histograms of single TAMRA-labeled hemocyanin molecules. Deoxy-Hc and oxy-Hc molecules have different fluorescence intensity distributions. Reproduced with permission from Ref. [53]. Copyright 2005 American Chemical Society.

(Fig. 1d). While still at the feasibility level and not targeted at the mechanism of protein functions, this study opened the possibility to study, in real time, the kinetics of oxygen binding/unbinding to a single hemocyanin molecule by following the fluorescence intensity of a TAMRA-labeled protein over time. Examining the possible time-dependent fluctuations of kinetic rates may offer information on the switching between the relaxed and tense states of hemocyanin, which are related to its cooperativity in O_2 -binding [60].

In principle, one could choose a probe whose fluorescence spectrum overlaps with the 350-nm absorption band of oxy-Hc. The fluorescence quenching via energy transfer would then be more effective, because the 350-nm band is much higher in intensity than the 600-nm band. In practice, detection of the single-molecule fluorescence in the UV region is technically challenging, however, and thus is not employed.

2.2. Case 2. Redox of azurin

Azurin is an electron transfer protein and contains a blue copper center that changes oxidation state between +1 and +2 (Fig. 2a) [60]. The reduced Cu^{1+} -azurin has no strong absorption bands in the visible region, while the oxidized Cu^{2+} -azurin has an intense cysteine \rightarrow Cu charge transfer band at ~ 630 nm in its absorption spectrum (Fig. 2b) [61], resulting from the highly covalent interaction between the Cu^{2+} and the sulfur atom of its cysteine ligand (Fig. 2b, inset) [62].

The spectral contrast between the reduced and the oxidized states of azurin makes it a good candidate for applying the fluorescence quenching via energy transfer approach for single-molecule detection. Schmauder et al. labeled azurin as an engineered cysteine residue with a fluorescent Cy5 probe [56], whose fluorescence spectrum has a large overlap with the absorption spectrum of the oxidized azurin (Fig. 2b) [50], and recorded the fluorescence intensity of Cy5-labeled azurin to report the copper oxidation state. They also immobilized the Cy5-labeled azurin molecules by adsorption on glass surface and measured the intensity distributions of single protein molecules at different redox conditions. They showed that the reduced Cy5-labeled azurin molecules have higher intensities than the oxidized ones, and the intensity distribution of single azurin molecules can be shifted between high and low values by reducing and oxidizing the samples (Fig. 2c). They further did a control experiment by substituting the copper atom at the active site with a redox-inactive Zn^{2+} ion. Zn^{2+} -azurin has no strong absorption features in the visible region due to its d^{10} electron configuration. Therefore, the intensity distribution of single Cy5-labeled Zn^{2+} -azurin molecules centers around a higher value consistently, regardless of the redox conditions of the solution (Fig. 2d). This study showed the possibility of detecting redox changes at a single metal center, which offers a way to study the kinetics of biological electron transfer at the single-molecule level. Coupling azurin to an analytical electrode and detecting its redox changes by fluorescence at the single-molecule level may provide increased sensitivity for

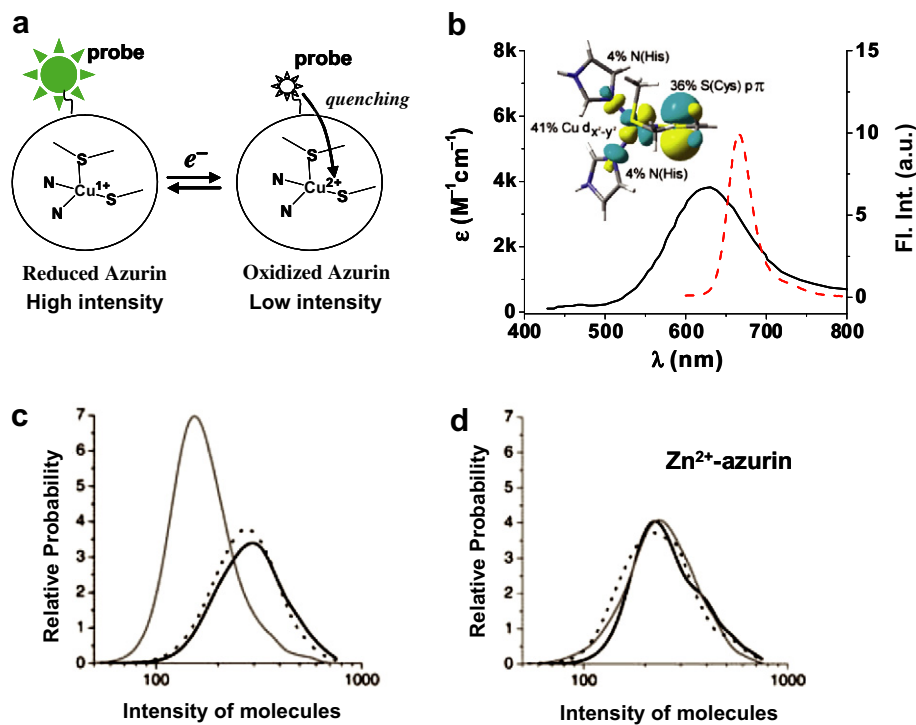


Fig. 2. (a) Fluorescence quenching via energy transfer to detect azurin redox states. (b) Absorption spectrum of oxidized Cu²⁺-azurin (adapted with permission from Ref. [61]. Copyright 1980 American Chemical Society) and the fluorescence spectrum of Cy5. Inset: the contour plot of the ground state wavefunction of the prototype blue copper center plastocyanin, showing the large mixing between the sulfur p orbital of the cysteine ligand and the copper d orbital. Adapted with permission from Ref. [62]. Copyright 2004 American Chemical Society. (c) Single-molecule intensity distributions for Cy5-labeled reduced azurin (black line), subsequently oxidized (gray line), and re-reduced (black dashed line). (d) Single-molecule intensity distributions for Cy5-labeled Zn²⁺-azurin at reducing conditions (black line), oxidizing conditions (gray line), and re-reducing conditions (black dashed line). Panels (c) and (d) adapted from Ref. [56] with permission. Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

sensing analytes that can undergo redox reactions with the protein [54].

2.3. Case 3. Cytochrome *c* folding

Cytochrome *c*, an electron transfer protein, has been used extensively as a model metalloprotein due to its availability, stability, and rich spectroscopic features [63]. Its heme center is a strong chromophore with broad UV–Vis absorption features and can act as an efficient quencher via energy transfer for an introduced fluorescent probe. Because the fluorescence quenching efficiency is distance dependent, the intensity of the probe reflects the distance between the probe and the heme center and thus will report the protein conformation when the protein folds or unfolds (Fig. 3a).

Takahashi and co-workers attached the fluorescent probe Alexa-532 to a C-terminal cysteine of yeast cytochrome *c*, and monitored the fluorescence intensity of Alexa-532 to investigate the folding dynamics of cytochrome *c* at the single-molecule level [58]. They developed a capillary-tube-based sample cell and a laser excitation scheme, so that single protein molecules can be monitored for ~ 100 ms without immobilization. They determined the intensity distributions of individual Alexa-532 labeled cytochrome *c* molecules at different concentrations of the

denaturant guanidium chloride (Gdm) (Fig. 3b). At 1.0 M Gdm, they observed two populations of cytochrome *c* molecules: one with lower fluorescence intensities and a narrow distribution width, and the other with higher intensities and a broad distribution width (Fig. 3b, bottom). These two populations were attributed to the intermediate and unfolded conformations of cytochrome *c*, respectively. At higher Gdm concentrations, the higher intensity population (i.e., the unfolded conformations) increases in probability (Fig. 3b). The broad width of the intensity distribution of the unfolded cytochrome *c* suggested its relatively slow conformational dynamics, which was supported by the correlation time (~ 15 ms) of the intensity autocorrelation functions of individual unfolded cytochrome *c* molecules (Fig. 3c). This study of cytochrome *c* folding exemplifies the prospects of single-molecule studies of metalloprotein folding dynamics, taking advantage of the spectroscopic properties of the metal cofactors.

2.4. Features and generality

The fluorescence quenching via energy transfer approach could be widely applicable for studying the metalloproteins. In principle, any metalloprotein that shows intense absorption properties can be targeted using

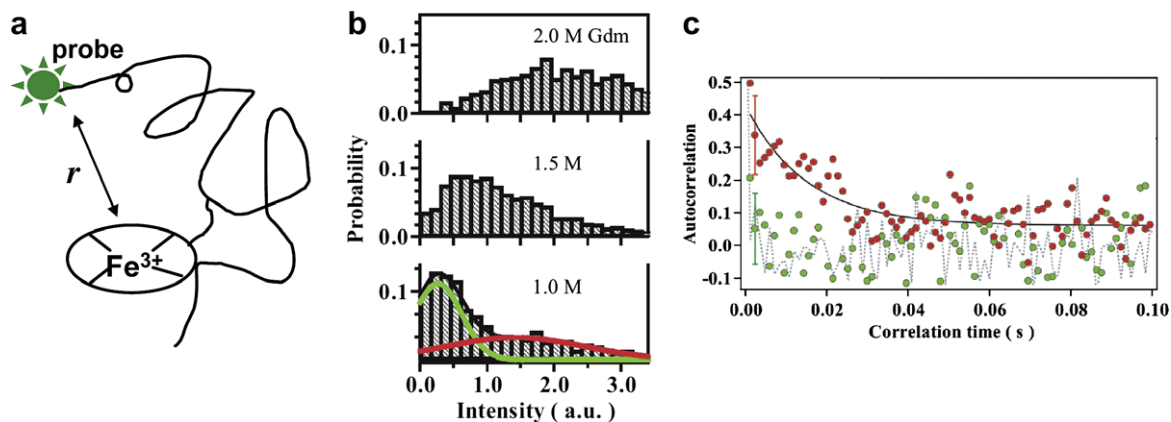


Fig. 3. (a) Fluorescence quenching via energy transfer to probe cytochrome *c* folding. The fluorescent probe Alexa-532 is attached to the protein C-terminal and its intensity is dependent on its distance to the heme center. (b) Fluorescence intensity histograms of single Alexa-532 labeled cytochrome *c* molecules at different guanidinium chloride (Gdm) concentrations. At 1.0 M Gdm, the distribution shows a low and a high intensity population, attributable to the intermediate and unfolded conformations of cytochrome *c*. At higher Gdm concentrations, the population of unfolded conformations dominates. (c) Intensity autocorrelation functions of single Alexa-532 labeled cytochrome *c* molecules in the presence of 1 M Gdm. The red circles are from data assigned to unfolded conformations of cytochrome *c* molecules and green circles from those of intermediate conformations. Solid line is an exponential fit with a time constant of ~ 15 ms. Panels (b) and (c) adapted with permission from Ref. [58]. Copyright 2007 National Academy of Sciences, USA.

this approach. Moreover, transition-metal-based chemistry often involves species that have intense ligand-to-metal charge transfer absorptions. These strong chromophoric species can be exploited as quenching centers for single-molecule fluorescence detection. As the fluorescence quenching targets directly the metal active site, the approach opens up new opportunities to study metal-based biochemical processes on a single-molecule basis.

The use of external fluorescent probes is also general. Site-specific labeling of proteins is readily achievable with many accessible labeling schemes including site-directed mutagenesis, GFP fusion, and unnatural amino acids [50,64]. Many fluorescent probes suitable for single-molecule detections are also available covering a wide spectral range [50].

2.5. Potential applications

One possible bioinorganic area to apply the fluorescence quenching via energy transfer approach is single-molecule metalloenzyme catalysis, examples of which are catalyses by binuclear copper enzymes, blue copper containing enzymes, and possibly mononuclear and binuclear non-heme iron enzymes [60]. Monitoring enzymatic reactions in real time at the single-molecule level provides a direct means to examine the contributions of conformational dynamics to enzyme activity that are challenging to study in ensemble measurements [14,15,17,65].

Tyrosinase and catechol oxidase are two binuclear copper enzymes that have active sites very similar to that of hemocyanin (Fig. 1a) [59,66]. Both enzymes interconvert between an oxy-form and a deoxy-form during catalysis that use oxygen to oxidize organic substrates. If attached at a distance close to the enzyme active site, a fluorescence probe (e.g., TAMRA) will undergo intensity fluctuations

between high and low levels, associated with the catalytic interconversions between deoxy- and oxy-forms. Following the fluorescence intensity of the probe at the single-molecule level could thus monitor the enzymatic turnovers of the enzyme in real time.

Many copper enzymes contain blue copper sites as electron transfer centers [60]. Applying the fluorescence quenching via energy transfer approach, as for azurin, could also enable study of their catalyses on a single-molecule basis. Possible target enzymes include nitrite reductase, ascorbate oxidase, laccase, and related multicopper oxidases [66,67]. All these enzymes contain blue copper centers that interconvert between an oxidized form, which has strong absorption bands, and a reduced form, which has no strong UV–Vis absorption. Looking at the catalyses of these metalloenzymes on a single-molecule basis may reveal new information on the coupling of electron transfer via the blue copper centers to the enzyme catalysis.

Another possible bioinorganic area to apply fluorescence quenching via energy transfer is single-molecule metalloprotein folding, exemplified by the cytochrome *c* folding discussed above. To understand how an unfolded protein evolves into its native structure is one of the major challenges in the post-genomic era. And the protein-folding problem is well suited for single-molecule studies because the folding process is intrinsically heterogeneous with different subpopulations and multiple pathways at many stages of folding. By removing ensemble averaging, single-molecule measurements could resolve subpopulations and heterogeneous folding pathways. The metal-containing cofactors of metalloenzymes introduce additional features into the folding energy landscapes. For example, they can act as nucleating points facilitating folding or as folding traps retarding folding [68,69]. Different redox states [70] and ligand binding properties [71] of metal centers in

metalloproteins also offer unique experimental opportunities for exploring various stages of folding. Taking cytochrome *c* folding as an example again, one can employ metal substitution and external ligand competition at the heme center, which are uniquely possible for metalloproteins, to perturb the folding dynamics and interrogate the folding process.

2.6. Challenges

Common to most fluorescence-based single-molecule methods, photobleaching limits the observation time window using the fluorescence quenching via energy transfer strategy. With a good oxygen scavenging system, a single fluorescent probe molecule can last for up to a few minutes before being photobleached [72]. In addition, this approach only obtains the fluorescence intensity from one probe; fluorescence intensity fluctuations due to probe photophysics, such as fluorescence blinking from inter-system crossing, can complicate the results and data analyses. Triplet quenchers, such as Trolox[®] [73], can reduce fluorescence blinking. Careful control experiments are always necessary.

In studying protein folding, the metal cofactor that acts as the fluorescence quencher is preferably bound to the protein covalently, as for cytochrome *c*. A noncovalently linked metal cofactor can dissociate from the peptide chain in the unfolded state, and the folding kinetics could be limited by the bimolecular association, which is slow at the 10^{-9} – 10^{-12} M concentrations used in typical single-molecule experiments. Confining the protein molecule spatially, for example using nanovesicle trapping [74,75], can achieve high effective local concentrations and circumvent this problem.

3. Fluorescence quenching via electron transfer

A molecule at excited states often has redox potentials different from that at its ground state, and photoinduced reduction (or oxidation) of a molecule via electron transfer with an external reductant (or oxidant) frequently occurs. For a fluorescent molecule, photoinduced electron transfer at an excited state can significantly shorten its fluorescence lifetime and quench its fluorescence intensity. This fluorescence quenching via electron transfer can be utilized to study biomolecules on a single-molecule basis. Xie and co-workers have used this single-molecule approach to probe the conformational dynamics of a flavin reductase [76]; Sauer and co-workers have incorporated this strategy into molecular beacons to detect nucleic acids [77,78]. Although this approach has not been used to study bioinorganic systems, we will use the flavin reductase study to exemplify the method and discuss its possible applications in the bioinorganic area.

3.1. Conformational dynamics of flavin reductase (Fre)

Fre binds tightly a naturally fluorescent flavin adenine dinucleotide (FAD) cofactor (Fig. 4a). Upon photoexcitation, the protein-bound FAD becomes reduced by a nearby tyrosine residue (Tyr³⁵) forming a transient charge-transferred state (Fig. 4b) [76]. The separated charges then quickly recombine, and the Fre–FAD complex returns to the ground state. The photoinduced electron transfer from Tyr³⁵ to FAD significantly shortens the fluorescence lifetime, γ^{-1} , of FAD ($\gamma^{-1} = 1/(k_r + k_{nr} + k_{ET}) \approx 1/k_{ET}$, when $k_{ET} \gg k_r$ and k_{nr} ; k_r , the radiative decay rate; k_{nr} , the non-radiative decay rate; k_{ET} , the electron transfer rate), which

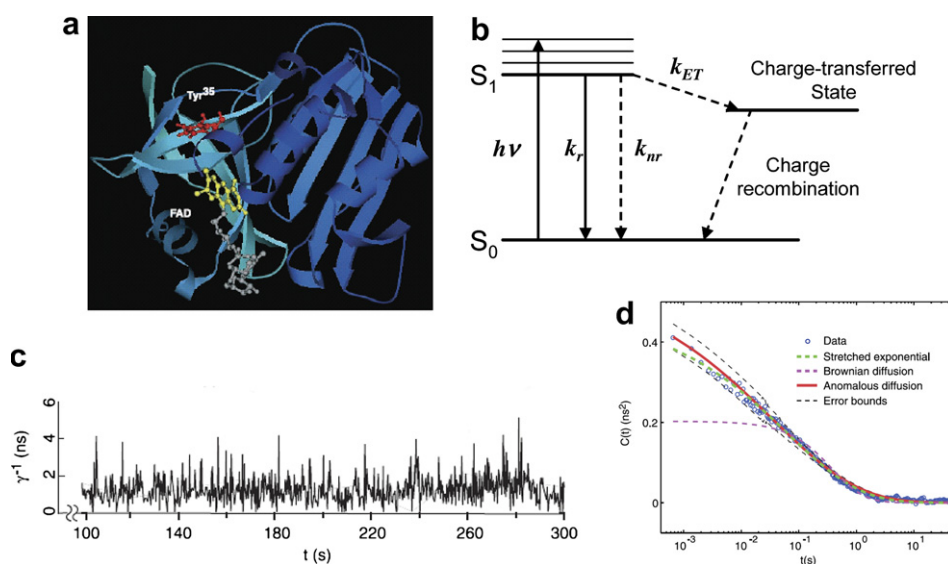


Fig. 4. (a) Structure of the Fre/FAD complex. The FAD and tyrosine 35 are highlighted. (b) Energy diagram and transition schemes for the fluorescence quenching via photo-induced electron transfer process. k_r : radiative decay rate; k_{nr} , all other non-radiative decay rate; k_{ET} , electron transfer rate. (c) Trajectory of the fluorescence lifetime of a Fre–FAD complex, indicating the fluctuations of the fluorescence lifetime. (d) Autocorrelation function of the fluorescence lifetime fluctuations of a single Fre–FAD complex and fits with different models. Panels (a), (c), and (d) from Ref. [76]. Reprinted with permission from AAAS.

leads to quenching of the FAD fluorescence. The electron transfer rate, k_{ET} , is exponentially dependent on and thus highly sensitive to the distance, r , between the electron donor and the acceptor ($k_{ET} = k_0 \exp(-\beta r)$, k_0 is a constant, $\beta \approx 1.4 \text{ \AA}^{-1}$ for electron transfer in proteins [79]). Fluctuations of r due to conformational dynamics of the protein will thus cause fluctuations of k_{ET} and of γ^{-1} . Consequently, monitoring the time-dependent changes of γ^{-1} of the FAD cofactor can probe the FAD–Tyr³⁵ distance fluctuations and the conformation dynamics of Fre on a single-molecule basis.

Xie and co-workers measured the real-time fluctuations of γ^{-1} of single Fre–FAD complex using a time-stamped photon-by-photon detection technique, which registers the arrival time of each emitted photon and the delay time between each detected fluorescence photon and the corresponding excitation laser pulse [76]. They found that γ^{-1} of FAD in a Fre–FAD complex fluctuates over time (Fig. 4c). Autocorrelation analysis indicates that the fluctuation of the fluorescence lifetime of a single Fre–FAD complex occurs at a broad range of timescales, from hundreds of microseconds to tens of seconds (Fig. 4d). The broad time range of fluctuation suggests the existence of multiple interconverting conformers of a Fre–FAD complex on a rugged energy landscape, and the interconversions can be described by an anomalous diffusion model (Fig. 4d). The existence of these interconverting conformers also relates to the fluctuating catalytic reactivity of the flavin enzyme cholesterol oxidase, reported in a previous study by Xie and co-workers [14].

3.2. Features and generality

Because the electron transfer rate decays rapidly over a few angstroms, the fluorescence quenching via electron transfer approach is sensitive to subtle distance changes on the angstrom scale. This distance range is complementary to that of the widely used FRET-based techniques, such as fluorescence quenching via energy transfer discussed in Section 2, which are effective in studying nanometer-scale distance changes. Many structural changes in biology, for example redox-induced geometry reorganizations of the metal active sites in electron transfer proteins, are in the angstrom range [60]. The fluorescence quenching via electron transfer approach offers an opportunity to study these types of structural dynamics at the single-molecule level.

The fluorescence quenching via electron transfer needs two redox-active centers: one as a quencher and the other as a probe that is also fluorescent. For the quencher, besides the organic redox-active groups (e.g., tyrosine, tryptophan, and guanine), metal active sites in biology have rich redox properties and cover a broad range of redox potentials [60]. For the probe, in addition to the naturally fluorescent cofactors such as FAD and FMN, one could label proteins externally with fluorescent probes that can undergo photoinduced electron transfer. With careful

experimental design, the fluorescence quenching via electron transfer approach could have applications in many systems.

3.3. Potential applications

One possible bioinorganic area to apply fluorescence quenching via electron transfer is the conformational dynamics of electron transfer proteins, in particular the structural dynamics around the metal active sites. Although typically small in amplitude, the structural changes associated with the redox of metal active sites contribute significantly to the reorganization energy term in determining the electron transfer rate. Therefore, conformational dynamics around the metal site can dramatically affect the electron transfer rate. It would not be unreasonable that conformational dynamics could modulate the electron transfer rate temporally and gate the electron transfer process. Using the fluorescence quenching via electron transfer approach, one could probe the timescale and amplitude of the conformational dynamics around the metal centers and reveal how these structural dynamics are coupled to and affect the electron transfer process.

Possible target systems include blue copper proteins (e.g., plastocyanin), iron–sulfur proteins (e.g., rubredoxin), and cytochromes (e.g., cytochrome *c*). Each of these proteins has two oxidation states: the reduced form of the protein can donate an electron for photoinduced reduction of a fluorescent probe, and the oxidized form of the protein can accept an electron for photoinduced oxidation. All these electron transfer proteins have variants covering a range of redox potentials so that one could select appropriate systems to match with the excited-state redox potential of a fluorescent probe to achieve photoinduced electron transfer.

3.4. Challenges

The fluorescence quenching via electron transfer approach is also limited by photobleaching and complicated by the intrinsic photophysics of the fluorescent probe, as mentioned previously. Competition with fluorescence quenching via energy transfer can also be a problem. For example, the oxidized blue copper centers, iron–sulfur centers, and cytochromes all have strong absorption features and can act as a quencher via energy transfer. Selecting fluorescent probes that have fluorescence emission well separated spectrally from the absorption spectra of the metalloproteins can circumvent this problem. Because the photoinduced electron transfer quenches the probe's fluorescence, the low detectable photon number and the shortened fluorescence lifetime of the probe are also challenges. High quantum yield (>30%) and fast response (<100 ps) detectors are needed to detect the weak fluorescence and resolve the short photon delay time in the photon-by-photon approach [76]. To use an externally introduced fluorescent probe to measure the angstrom scale distance changes,

the attachment of the probe is critical. The probe needs to be anchored to the protein rigidly so that its conformational flexibility around the attachment point will not overwhelm the measurements of the angstrom scale protein conformational dynamics. While there are many challenges in using the fluorescence quenching via electron transfer approach, we think that the rich redox properties provided by the metal centers in metalloproteins make it attractive to explore this approach for more applications and discoveries.

4. Other single-molecule bioinorganic studies

In the past two years, our lab has been applying and developing single-molecule fluorescence methods to study the dynamics and functions of metalloproteins involved in metal trafficking and metal regulation. Herein, we briefly describe our research efforts and readers are referred to the forthcoming references for details [80,81].

4.1. Metallochaperone protein interactions

We have recently used a nanovesicle trapping strategy in combination with single-molecule FRET methods to probe real-time transient metallochaperone–target protein interactions on a single-molecule basis [80]. Metallochaperones are transport proteins that mediate metal trafficking to their target proteins through specific protein–protein interactions, which are essential for the metal transfer process [82]. For copper chaperones, in particular the Atx1-family chaperones, the copper transfer is believed to be kinetically controlled [83,84]. Very limited information is available, however, on the kinetics of the copper transfer or the chaperone–target protein interactions. This is partly because of the transient nature of the protein interactions involved, which are challenging to study with ensemble-averaged measurements.

To provide the basis to understand the protein interaction mediated copper transfer, we set out to characterize the interaction dynamics between Hah1 and a single metal binding domain (the fourth domain, MBD4) of the Wilson disease protein (WDP). Hah1 is the human homologue of Atx1 and WDP is a target protein for Hah1 mediated copper transfer. We labeled Hah1 and MBD4 with fluorescent probes that form a FRET pair and used single-molecule FRET methods to monitor the Hah1–MBD4 interactions. Typical single-molecule experiments are done at 10^{-12} – 10^{-9} M concentrations and are limited to studies of strong interacting pairs. The Hah1–WDP interactions have dissociation constants in the μM regime and are, however, transient. We hence adopted a nanovesicle trapping strategy [11,74,75,85] to encapsulate a Hah1 and a MBD4 molecule in an 100-nm lipid vesicle, in which the effective concentration of a single molecule is $\sim 3 \mu\text{M}$. The total number of nanovesicles is kept low to maintain single-molecule detection conditions. The nanovesicles are then immobilized so that we could follow single pairs of Hah1–MBD4 mole-

cules over time. Because the immobilization is through the nanovesicles, nonspecific protein–glass surface interactions that are present in direct immobilization are eliminated. We were able to observe the transient Hah1–MBD4 interactions on a single-molecule basis, resolve and capture different protein interaction intermediates in real time, monitor the interconversion dynamics between intermediates directly, and quantify their interaction kinetics and thermodynamics.

4.2. Metalloregulator DNA interactions

Recently, we also developed a novel and generalizable method to probe metalloregulator–DNA interactions at the single-molecule level, using engineered DNA Holliday junctions as single-molecule reporters [81]. Metalloregulators respond to metal ion concentrations and regulate the transcription of genes for metal homeostasis [86]. The interactions between a metalloregulator and DNA differ in the absence and presence of the metal effector, and that difference determines whether gene transcription is suppressed or activated.

We utilized the structural dynamics of DNA Holliday junctions to probe the interaction with DNA of PbrR691, a Pb^{2+} -responsive MerR-family metalloregulator [81,87]. A DNA Holliday junction has two stacked conformations at a dynamic structural equilibrium, which can be followed easily with single-molecule FRET techniques [88]. We engineered a Holliday junction to encode the specific PbrR691-targeting DNA sequence. Upon PbrR691 binding to the specific sequence, the structural dynamics of the engineered Holliday junction is significantly perturbed, and thus it acts as a single-molecule reporter for PbrR691–DNA interactions. We characterized the kinetic, thermodynamic, and conformational changes of the structural dynamics of the engineered Holliday junction, and deduced how PbrR691 acts on DNA for its regulatory function in gene transcription.

5. Summary

The scope of single-molecule research is expanding rapidly in recent years, while single-molecule studies in bioinorganic chemistry has been very limited. Herein, we have reviewed a few single-molecule fluorescence studies of bioinorganic systems and discussed two types of approaches that have been or can be applied to probe the functions and dynamics of metalloproteins. We have also attempted to point out possible bioinorganic applications and associated challenges for the single-molecule fluorescence approaches discussed. The views here are certainly limited, and many more innovative approaches and applications are expected to emerge in the coming years. For example, semiconductor quantum dots have emerged as a new generation of fluorescent probes known for their exceptional brightness, high photostability, narrow emission bands, and broad excitation wavelength range [89,90]. Although

the large physical size (a few nanometers) and the fluorescence blinking of quantum dots present challenges for their applications, many single-molecule studies have appeared and future studies on bioinorganic systems are expected. It is the authors' hope that this limited review will stimulate more interests so that more researchers will venture into single-molecule bioinorganic chemistry, break new grounds, and make new discoveries.

Acknowledgments

We thank the National Science Foundation (CHE-0645392, CAREER Award) and the Camille and Henry Dreyfus Foundation (New Faculty Award) for funding our research in single-molecule bioinorganic chemistry.

References

- [1] A. Ishijima, T. Yanagida, *Trends Biochem. Sci.* 26 (2001) 438.
- [2] J.N. Forkey, M.E. Quinlan, Y.E. Goldman, *Prog. Biophys. Mol. Biol.* 74 (2000) 1.
- [3] E. Toprak, P.R. Selvin, *Annu. Rev. Biophys. Biomol. Struct.* 36 (2007) 349.
- [4] W.J. Greenleaf, M.T. Woodside, S.M. Block, *Annu. Rev. Biophys. Biomol. Struct.* 36 (2007) 171.
- [5] T. Ha, *Biochemistry* 43 (2004) 4055.
- [6] I. Amitani, R.J. Baskin, S.C. Kowalczykowski, *Mol. Cell* 23 (2006) 143.
- [7] T.T. Perkins, R.V. Dalal, P.G. Mitsis, S.M. Block, *Science* 301 (2003) 1914.
- [8] X. Zhuang, *Annu. Rev. Biophys. Biomol. Struct.* 34 (2005) 399.
- [9] J. Liphardt, B. Onoa, S.B. Smith, I. Tinoco Jr., C. Bustamante, *Science* 292 (2001) 733.
- [10] D. Rueda, G. Bokinsky, M.M. Rhodes, M.J. Rust, X. Zhuang, N.G. Walter, *Proc. Natl. Acad. Sci. USA* 101 (2004) 10066.
- [11] E. Rhoades, E. Gussakovsky, G. Haran, *Proc. Natl. Acad. Sci. USA* 100 (2003) 3197.
- [12] A.A. Deniz, T.A. Laurence, G.S. Beligere, M. Dahan, A.B. Martin, D.S. Chemla, P.E. Dawson, P.G. Schultz, S. Weiss, *Proc. Natl. Acad. Sci. USA* 97 (2000) 5179.
- [13] E.A. Lipman, B. Schuler, O. Bakajin, W.A. Eaton, *Science* 301 (2003) 1233.
- [14] H.P. Lu, L.Y. Xun, X.S. Xie, *Science* 282 (1998) 1877.
- [15] B.P. English, W. Min, A.M. van Oijen, K.T. Lee, G. Luo, Y. Sun, B.J. Cherayil, S.C. Kou, X.S. Xie, *Nat. Chem. Biol.* 3 (2006) 87.
- [16] L. Edman, R. Rigler, *Proc. Natl. Acad. Sci. USA* 97 (2000) 8266.
- [17] K. Velonia, O. Flomenbom, D. Loos, S. Masuo, M. Cotlet, Y. Engelborghs, J. Hofkens, A.E. Rowan, J. Klafter, R.J.M. Nolte, F.C. de Schryver, *Angew. Chem., Int. Ed.* 44 (2005) 560.
- [18] J.R. Brender, J. Dertouzos, D.P. Ballou, V. Massey, B.A. Palfey, B. Entsch, D.G. Steel, A. Gafni, *J. Am. Chem. Soc.* 127 (2005) 18171.
- [19] N.M. Antikainen, R.D. Smiley, S.J. Benkovic, G.G. Hammes, *Biochemistry* 44 (2005) 16835.
- [20] J. Yu, J. Xiao, X. Ren, K. Lao, X.S. Xie, *Science* 311 (2006) 1600.
- [21] L. Cai, N. Friedman, X.S. Xie, *Nature* 440 (2006) 358.
- [22] W.E. Moerner, D.P. Fromm, *Rev. Sci. Instrum.* 74 (2003) 3597.
- [23] X.S. Xie, J.K. Trautman, *Annu. Rev. Phys. Chem.* 49 (1998) 441.
- [24] X. Michalet, S. Weiss, M. Jaeger, *Chem. Rev.* 106 (2006) 1785.
- [25] P.V. Cornish, T. Ha, *ACS Chem. Biol.* 2 (2007) 53.
- [26] G. Bokinsky, X. Zhuang, *Acc. Chem. Res.* 38 (2005) 566.
- [27] P.F. Barbara, A.J. Gesquiere, S.-J. Park, Y.J. Lee, *Acc. Chem. Res.* 38 (2005) 602.
- [28] T. Basche, W.E. Moerner, M. Orrit, U.P. Wild, *Single-Molecule Optical Detection, Imaging and Spectroscopy*, VCH Verlagsgesellschaft mbH, Weinheim, 1997.
- [29] C. Bustamante, J.C. Macosko, G.J.L. Wuite, *Nat. Rev. Mol. Cell Biol.* 1 (2000) 130.
- [30] A.M. van Oijen, *Biopolymers* 85 (2007) 144.
- [31] G. Charvin, T.R. Strick, D. Bensimon, V. Croquette, *Annu. Rev. Biophys. Biomol. Struct.* 34 (2005) 201.
- [32] S.A. Rosenberg, M.E. Quinlan, J.N. Forkey, Y.E. Goldman, *Acc. Chem. Res.* 38 (2005) 583.
- [33] P. Tinnefeld, M. Sauer, *Angew. Chem., Int. Ed.* 44 (2005) 2642.
- [34] C. Dekker, *Nat. Nanotechnol.* 2 (2007) 209.
- [35] B. Sakmann, E. Neher (Eds.), *Single-Channel Recording*, second ed., Springer, Berlin, 1995.
- [36] X. Nan, P.A. Sims, P. Chen, X.S. Xie, *J. Phys. Chem. B* 109 (2005) 24220.
- [37] R.J. Cogdell, A. Gall, J. Kohler, *Q. Rev. Biophys.* 39 (2006) 227.
- [38] Y. Saga, H. Tamiaki, *Cell Biochem. Biophys.* 40 (2004) 149.
- [39] D. Rutkauskas, R.J. Cogdell, R. van Grondelle, *Biochemistry* 45 (2006) 1082.
- [40] D. Loos, M. Cotlet, F. de Schryver, S. Habuchi, J. Hofkens, *Biophys. J.* 87 (2004) 2598.
- [41] W.P. de Ruijter, S. Oellerich, J.M. Segura, A.M. Lawless, M. Papiz, T.J. Aartsma, *Biophys. J.* 87 (2004) 3413.
- [42] C. Tietz, F. Jelezko, U. Gerken, S. Schuler, A. Schubert, H. Rogl, J. Wrachtrup, *Biophys. J.* 81 (2001) 556.
- [43] A.M. van Oijen, M. Ketelaars, J. Kohler, T.J. Aartsma, J. Schmidt, *Science* 285 (1999) 400.
- [44] M.A. Bopp, Y. Jia, L. Li, R.J. Cogdell, R.M. Hochastrasser, *Proc. Natl. Acad. Sci. USA* 94 (1997) 10630.
- [45] A.G. Hansen, J. Zhang, H.E.M. Christensen, A.C. Welinder, H. Wackerbarth, J. Ulstrup, *Isr. J. Chem.* 44 (2004) 89.
- [46] A. Alessandrini, S. Corni, P. Facci, *Phys. Chem. Chem. Phys.* 8 (2006) 4383.
- [47] B. Bonanni, L. Andolfi, A.R. Bizzarri, S. Cannistraro, *J. Phys. Chem. B* 111 (2007) 5062.
- [48] S. Myong, B.C. Stevens, T. Ha, *Structure* 14 (2006) 633.
- [49] B.W. van der Meer, G. Coker III, S.-Y.S. Chen, *Resonance Energy Transfer: Theory and Data*, VCH, New York, 1994.
- [50] R.P. Haugland, *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies*, Invitrogen Corp, 2005.
- [51] W. Erker, M. Lippitz, T. Basche, H. Decker, *Micron* 35 (2004) 111.
- [52] W. Erker, A. Schoen, T. Basche, H. Decker, *Biochem. Biophys. Res. Commun.* 324 (2004) 893.
- [53] W. Erker, S. Dorr, T. Basche, *J. Am. Chem. Soc.* 127 (2005) 14532.
- [54] J.J. Davis, H. Burgess, G. Zauner, S. Kuznetsova, J. Salverda, T. Aartsma, G.W. Canters, *J. Phys. Chem. B* 110 (2006) 20649.
- [55] R. Schmauder, S. Alagaratnam, C. Chan, T. Schmidt, G.W. Canters, T.J. Aartsma, *J. Biol. Inorg. Chem.* 10 (2005) 683.
- [56] R. Schmauder, F. Librizzi, G.W. Canters, T. Schmidt, T.J. Aartsma, *ChemPhysChem* 6 (2005) 1381.
- [57] S. Kuznetsova, G. Zauner, R. Schmauder, O.A. Mayboroda, A.M. Deelder, T.J. Aartsma, G.W. Canters, *Anal. Biochem.* 350 (2006) 52.
- [58] M. Kinoshita, K. Kamagata, A. Maeda, Y. Goto, T. Komatsuzaki, S. Takahashi, *Proc. Natl. Acad. Sci. USA* 104 (2007) 10453.
- [59] E.I. Solomon, P. Chen, M. Metz, S.-K. Lee, A.E. Palmer, *Angew. Chem., Int. Ed.* 40 (2001) 4570.
- [60] R.H. Holm, P. Kennepohl, E.I. Solomon, *Chem. Rev.* 96 (1996) 2239.
- [61] E.I. Solomon, J.W. Hare, D.M. Dooley, J.H. Dawson, P.J. Stephen, H.B. Gray, *J. Am. Chem. Soc.* 102 (1980) 168.
- [62] E.I. Solomon, R.K. Szilagy, S.D. George, L. Basomallick, *Chem. Rev.* 104 (2004) 419.
- [63] G.R. Moore, G.W. Pettigrew, *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects*, Springer, Berlin, 1990.
- [64] B.N.G. Giepmans, S.R. Adams, M.H. Ellisman, R.Y. Tsien, *Science* 312 (2006) 217.
- [65] O. Flomenbom, K. Velonia, D. Loos, S. Masuo, M. Cotlet, Y. Engelborghs, J. Hofkens, A.E. Rowan, R.J.M. Nolte, M. van der Auweraer, F.C. de Schryver, J. Klafter, *Proc. Natl. Acad. Sci. USA* 102 (2005) 2368.

- [66] E.I. Solomon, U.M. Sundaram, T.E. Machonkin, *Chem. Rev.* 96 (1996) 2563.
- [67] W.G. Zumft, *Microbiol. Mol. Biol. Rev.* 61 (1997) 533.
- [68] P. Wittung-Stafshede, *Acc. Chem. Res.* 35 (2002) 201.
- [69] D.R. Winkler, *Curr. Opin. Chem. Biol.* 8 (2004) 169.
- [70] J.R. Telford, P. Wittung-Stafshede, H.B. Gray, J.R. Winkler, *Acc. Chem. Res.* 31 (1998) 755.
- [71] J.R. Telford, F.A. Tezcan, H.B. Gray, J.R. Winkler, *Biochemistry* 38 (1999) 1944.
- [72] T. Ha, *Methods* 25 (2001) 78.
- [73] I. Rasnik, S.A. McKinney, T. Ha, *Nat. Meth.* 3 (2006) 891.
- [74] E. Boukobza, A. Sonnenfeld, G. Haran, *J. Phys. Chem. B* 105 (2001) 12165.
- [75] B. Okumus, T.J. Wilson, D.M.J. Lilley, T. Ha, *Biophys. J.* 87 (2004) 2798.
- [76] H. Yang, G. Luo, P. Karnchanaphanurach, T.-M. Louie, I. Rech, S. Cova, L. Xun, X.S. Xie, *Science* 302 (2003) 262.
- [77] J.-P. Knemeyer, N. Marme, M. Sauer, *Anal. Chem.* 72 (2000) 3717.
- [78] O. Piestert, H. Barsch, V. Buschmann, T. Heinlein, J.-P. Knemeyer, K.D. Weston, M. Sauer, *Nano Lett.* 3 (2003) 979.
- [79] H.B. Gray, J.R. Winkler, *Annu. Rev. Biochem.* 65 (1996) 537.
- [80] J.J. Benitez, A.M. Keller, P. Ochieng, L.A. Yatsunyk, D.L. Huffman, A.C. Rosenzweig, P. Chen, (2007) Submitted.
- [81] S.K. Sarkar, N.M. Andoy, J.J. Benitez, P.R. Chen, J.S. Kong, C. He, P. Chen, *J. Am. Chem. Soc.* 129 (2007) 12461.
- [82] D.L. Huffman, T.V. O'Halloran, *Annu. Rev. Biochem.* 70 (2001) 677.
- [83] D.L. Huffman, T.V. O'Halloran, *J. Biol. Chem.* 275 (2000) 18611.
- [84] L.A. Yatsunyk, A.C. Rosenzweig, *J. Biol. Chem.* 282 (2007) 8622.
- [85] I. Cisse, B. Okumus, C. Joo, T. Ha, *Proc. Natl. Acad. Sci. USA* 104 (2007) 12646.
- [86] T.V. O'Halloran, *Science* 261 (1993) 715.
- [87] P. Chen, B. Greenberg, S. Taghavi, C. Romano, D. van der Lelie, C. He, *Angew. Chem., Int. Ed.* 44 (2005) 2715.
- [88] S.A. McKinney, A.C. Declais, D.M.J. Lilley, T. Ha, *Nat. Struct. Biol.* 10 (2003) 93.
- [89] A.P. Alivisatos, *Science* 271 (1996) 933.
- [90] X. Michalet, F.F. Pinaud, L.A. Bentolila, J.M. Tsay, S. Doose, J.J. Li, G. Sundaresan, A.M. Wu, S.S. Gambhir, S. Weiss, *Science* 307 (2005) 538.