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Real-time detection of Cu^{2+} sequestration and release by immobilized apo-metallothioneins using SECM combined with SPR

Yinling Xin [a], Yu Gao [a], Jun Guo [a], Qian Chen [a], Juan Xiang [a,*], Feimeng Zhou [a,b,**]

[a] *Institute of Surface Analysis and Biosensing, School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, PR China*
[b] *Department of Chemistry and Biochemistry, California State University, Los Angeles, Los Angeles, CA 90032, USA*

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ABSTRACT

Scanning electrochemical microscopy (SECM) combined with surface plasmon resonance (SPR), SECM–SPR, was applied for real-time detection of the incorporation of Cu^{2+} by apo-metallothionein (apo-MT) immobilized on the SPR substrate and release of Cu^{2+} from surface-confined metallothionein (MT). Cu^{2+} anodically stripped from a Cu-coated SECM Au tip was sequestered by apo-MT upon its diffusion to the SPR substrate, and release of Cu^{2+} by MT was accomplished by generating protons via oxidation of hydroquinone at the tip. The high sensitivity of the SPR instrument is capable of following the structural and compositional changes of MT molecules during the metal sequestration and release processes. Due to the enhanced mass transfer rate at the SECM tip, the complication of mass transfer limitation on kinetic measurements, commonly encountered in flow injection SPR, is circumvented. The time-resolved SPR response reveals stepwise changes among three stable MT structures and allows the number of copper ions coordinated in each structure to be determined. The numbers of copper ions incorporated by each MT molecule in the three structures were determined to be 5, 9, and 12. This work expands the SECM–SPR approach to assessments of the dynamics and affinity of binding of small ions to surface-confined proteins and to studies of proteins that do not undergo facile electron transfer reactions.

1. Introduction

Unraveling the protein structure/function relationship is of immense biological importance (Zubay, 1998). Probing changes in protein structures induced by chemical and biological stimuli in real-time provides critical insight into their functions and properties. A number of spectroscopic techniques have been developed for monitoring structural changes of proteins *in vivo* and *in vitro*, such as infrared spectroscopy (Bulheller et al., 2007; Jackson and Mantsch, 1995; Schweitzer-Stenner, 2006), circular dichroism (CD) (Bulheller et al., 2007; Jackson and Mantsch, 1995; Schweitzer-Stenner, 2006), nuclear magnetic resonance (NMR) (Domon and Aebersold, 2006; Mittermaier and Kay, 2006) and X-ray crystallography (Schotte et al., 2003). However, many of the aforementioned techniques monitor changes either in solution or in the solid state and are not amenable for studies of rapid functional and dynamic changes of proteins at the solid/liquid interface. Furthermore, there are certain inherent limitations (e.g., relatively small signals in NMR and infrared spectra from protein samples present at low concentrations and the requirement of single crystals for X-ray crystallography).

Metal ion sequestration, release, and transfer by metallothioneins (MTs), a class of metalloproteins that play an important role in essential metal regulation and heavy metal detoxification, are dynamic cellular processes (Kagi and Kojima, 1987; Klaassen, 1997; Suzuki et al., 1993). The unique properties of MTs include low molecular weights (6000–7000 Da), abundant cysteine residues (~30% of the total amino acid residues), and a remarkable ability to coordinate a large number of transition metal ions in the form of metal-thiolate clusters. The structure of some mammalian MT forms has been determined by NMR (Kagi and Schaffer, 1988) and X-ray crystallography (Robbins et al., 1991) to contain two metal-binding domains. The N-terminal domain (β domain) comprises nine cysteine residues and the C-terminal domain (α domain) consists of 11 cysteines. All of the cysteines are involved in metal ion sequestration, transfer, and release (Fischer and Davie, 1998; Hamer, 1986; Mason and Jenkins, 1995). Studies of the MT/Cu ion interactions have been hampered by the lack of structures for X-ray

* Corresponding author.
** Corresponding author at: Institute of Surface Analysis and Biosensing, School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, PR China.
E-mail addresses: xiangj@mail.csu.edu.cn (J. Xiang), fzhou@calstatela.edu (F. Zhou).

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crystallography and complications given rise by the large electric quadrupole moment of Cu isotopes for NMR spectroscopy (Li and Weser, 1992). *In vitro* titrimetric assays of the binding between apo-MT and Cu^{2+} and spectroscopic studies of the interactions between Cu^{2+} and Cd- or Zn-containing MTs (Chen et al., 1996; Nielson et al., 1985; Stillman, 1995; Suzuki et al., 1993) have shown various binding stoichiometries, with Cu_{12}–MT being a stable structure. Reduction of Cu^{2+} to Cu^{+} was found to be accompanied by partial MT oxidation (oxidation of some cysteine residues) (Suzuki and Maitani, 1981; Vaher et al., 2001). In Cu_{12}–MT, the Cu^{+} ions are evenly distributed between the α and β domains, forming clusters of Cu_6S_9 and Cu_6S_{11} with trigonally coordinated Cu^{+}. Although titrations with UV–visible, CD, and luminescence spectroscopic detections are useful in deducing the stoichiometry of a stable metal complex with MT, their applications in determining the rates of metal ion binding and distribution could be hindered by the effective mass transfer (mixing) in a typical titrimetric experiment.

Surface plasmon resonance (SPR) (Homola, 2006; Rothenhäusler and Knoll, 1988; Wang et al., 2003; Yu et al., 2006) is an optical technique that can follow biological reactions at the solid/solution interface. SPR analyses are highly sensitive, allowing infinitesimal conformation/orientation changes of unmodified protein molecules to be monitored. When SPR is used in conjunction with electrochemistry, electrode reactions as diverse as pattern formation (Flatgen et al., 1995), ultrathin film reorganization (Yao et al., 2004), conformation changes of redox proteins (Zhai et al., 2007), and electropolymerization (Baba et al., 2003) can be studied. Recently, Szunerits et al. and our groups have successfully combined scanning electrochemical microscopy (SECM) with SPR for microarray fabrication and characterization (Szunerits et al., 2004) and studies of redox-induced thin film reorganization (Xiang et al., 2006), respectively. With this coupled technique (SECM–SPR), complications associated with the structural parameters of the substrate and/or solution/solid interface (Xiang et al., 2006) are largely mitigated. The SPR instrument we employed is capable of measuring film thickness variation as small as a few angstroms (Zhai et al., 2007) and DNA molecules as little as a few attomoles (Yao et al., 2006). However, two unique advantages of SECM–SPR, viz., the enhanced mass transfer within the SECM tip/substrate gap (Bard and Mirkin, 2001) and the quantitative induction of the changes at the SPR substrate by the SECM tip, were not fully explored in our previous work (Xiang et al., 2006). The former advantage is particularly noteworthy, since even in an optimized fluidic system SPR measurements of fast binding reactions are limited by the rate at which analyte molecules are transferred from the bulk solution to the substrate surface (Gervais and Jensen, 2006). Furthermore, the fact that the SPR measurement is essentially immune to certain interferences at the tip electrode used for fast kinetic studies (e.g., charging current during potential-step chronoamperometry (Bard and Faulkner, 2001) or possible contributions of short-lived adsorbates electrogenerated in fast scan cyclic voltammetry (Wightman and Wipf, 1990)) affords the possibility of extending the SECM–SPR operation from the steady-state feedback mode (Xiang et al., 2006) to transient measurements (i.e., time-of-flight or generation/collection experiments).

We describe herein the real-time SECM–SPR detection of copper ion sequestration by immobilized apo-MT and copper ion release by surface-confined MT. We show that not only did the coupled technique enable us to control the extent of metal binding, but also allowed the binding stoichiometry and dynamics to be quantitatively determined. Our work reveals that copper ion sequestration by apo-MT and copper ion release by MT are both stepwise processes, leading to three stable structures containing different numbers of metal ions. Since MTs in their native structure generally do not exhibit well-defined voltammograms at solid electrodes (Erk and Raspor, 2000; Rodriguez and Esteban, 2000; Sestakova and Mader, 2000; Song et al., 2001), our work expands the application of SECM–SPR from studies of redox proteins to proteins that do not undergo facile electron transfer reactions.

2. Materials and methods

2.1. Chemicals and materials

Metallothionein-2 (MT) isolated from rabbit liver was purchased from Hunan Lugu Biotechnology Co. (Changsha, China). *N*-Hydroxysulfosuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 11-mercaptoundecanoic acid (MUA) were acquired from Sigma (St. Louis, MO). Methyl viologen dichloride ($MVCl_2$) was obtained from Aldrich (Milwaukee, WI). High-purity HCl (99.999%) and NaCl were purchased from Alfa Aesar (Ward Hill, MA). Hydroquinone (HQ), $CdCl_2$, Na_2SO_4, and $CuSO_4$ (Beijing Chemical Reagent Co., Beijing, China) were all of AR grade and used as received. Deionized water with resistivity of 18.2 MΩ cm^{-1} was collected from a Millipore Simplicity 185 System (Millipore Co., Billerica, MA). MT, EDC (0.4 mol l^{-1}), NHS (0.1 mol l^{-1}) and HQ (10.0 mmol l^{-1}) solutions were freshly prepared in NaCl solution, and MUA (4.0 mmol l^{-1}) was dissolved in ethanol. Stock solutions of 1 mol l^{-1} HCl, 5.0 mmol l^{-1} $CuSO_4$ and 0.1 mol l^{-1} Na_2SO_4 solutions were made with deionized water. Glycine–HCl buffer (50 mmol l^{-1}, pH 2.0) was used to release cadmium and zinc ions from MT.

2.2. Instruments

The SECM–SPR apparatus and experimental procedure were reported previously (Xiang et al., 2006). The laser spot on the SPR substrate was kept relatively large (diameter ~700 μm) to attenuate the laser intensity so that potential denaturation of immobilized protein molecules was avoided. The bicell photodetector of the SPR instrument is capable of resolving angular shift less than 1×10^{-4} degrees and allows thermal and mechanical drifts to be corrected (Tao et al., 1999). The SECM probe was a 10 μm Au disk surrounded by a 500-μm-diameter glass layer. Flow injection SPR (FI-SPR) measurements were carried out with a BI-1000 SPR system (Biosensing Instrument, Tempe, AZ). The inlet of the flow cell was connected to a six-port Cheminert valve (Valco Inc., Houston, TX). For each measurement, the sample solution was preloaded into a 50-μl loop with a microsyringe (Hamilton, Reno, Nevada) and delivered to the flow cell by a Genie Plus syringe pump (Kent Scientific, Torrington, CT).

2.3. Procedures

2.3.1. SPR substrate preparation

Glass slides covered with a 50-nm-thick gold film and a 2-nm-thick Cr adhesive underlayer served as the SPR substrates. The substrate was annealed in a hydrogen flame to eliminate surface contamination. A MUA SAM was first attached to the substrate surface by immersing the gold film in 2.0 mmol l^{-1} MUA for 18 h. This was followed by covering the substrate with a mixture of 0.4 mol l^{-1} EDC and 0.1 mol l^{-1} NHS for 15 min and 80 μg ml^{-1} MT (50 μl each) for 3 h. Throughout the MT immobilization, the substrate was placed in a humidified chamber to avoid solvent evaporation. Before the MT attachment step, the surface was rinsed with phosphate buffer and water and dried with nitrogen.