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RESEARCH PAPER

Engineered Protein Nanopore for Real-time Monitoring Single-molecule Reaction Between Cadmium Ion and Glutathione

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Abstract: The complexation of glutathione (GSH) with divalent cadmium ion has been used as a typical model for investigating the coordination chemistry of sulfydryl-containing peptides and heavy metal ions, which is essential to understand the mechanism of intracellular cadmium detoxification. In this study, the single-molecule reaction between GSH molecule and Cd^{2+} ion was monitored in real time by a nanoreactor that formed by a mutant $(M113R)_7$ α-hemolysin (αHL) protein nanopore equipped with a novel per-6-quaternary ammonium-*β*-cyclodextrin (*p*-QABCD). The reaction pathways, intermediates, and products could be recognized by analyzing the current fluctuations. The reaction between Cd^{2+} and GSH was highly dependent on solution pH value. $Cd(GSH)_2$ was the only final product at pH 7.4, while both $Cd(GSH)_2$ and $Cd_2(GSH)_2$ were present at pH 9.0. The formation of $Cd_2(GSH)_2$ follows two possible pathways: (1) one Cd^{2+} ion first coordinates with the thiol group of two GSH molecules to form $Cd(GSH)_2$, and then the second Cd^{2+} ion quickly incorporates with the deprotonated amino group of $Cd(GSH)_2$ to produce $Cd_2(GSH)_2$; (2) two Cd^{2+} ions separately coordinate with the thiol and deprotonated amino group of one GSH molecule to yield $Cd_2(GSH)_1$, and the second GSH molecule binds Cd^{2+} ions quickly to form $Cd_2(GSH)_2$. The free-labeling and free-modifying method for monitoring single-molecule chemical reaction was simple and sensitive, which would be important to further understand intracellular mechanisms of detoxification of heavy metals. This work greatly expands the research field of single-molecule nanopore technique.

Key Words: Nanopore; Single-molecule detection; Per-6-quaternary ammonium-*β*-cyclodextrin; Glutathione; Cadmium ion

1 Introduction

Cadmium, a non-essential element in human body, may damage normal physiological function of enzymes in organs such as liver and kidney by coordinating with sulfhydryl group or amino group of protein, disturb the calcium metabolism in cells, leading to disorder of the bone process or normal bone metabolism, and reduce the activity of antioxidant enzyme to generate oxidative damage of organisms[1,2]. Glutathione (GSH, *γ*-*L*-glutamyl-*L*-cysteinyl-glycine), a sulfydryl-containing tripeptide, is ubiquitous in cells and plays an important role in detoxification owing to the affinity of thiol group for heavy metals[3–7]. The investigation of the complexation between GSH molecules and Cd^{2+} ions is essential to understand the mechanism of intracellular cadmium detoxification.

The complexation between metal ions and sulfydryl-containing peptides, including binding sites, the structure, and the constitute of coordinating products has been extensively investigated[8–18]. The main research methods include nuclear magnetic resonance (NMR)[8,9], absorption spectroscopy[10], potentiometric titration[11], fluorescence[12], cyclic voltammetry (CV)[13,14], infrared spectrometry[15,16], differential pulse polarography (DPP)[17] combined with multivariate curve analysis, *etc*. The acidity coefficient (pK_a) of various functional groups of GSH are different[18].

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pK_{a1}(–COOH) = 2.12, pK_{a2}(–COOH) = 3.59, pK_{a3}(–NH_2) = 8.75, pK_{a4}(–SH) = 9.65. So, the pH-controlled deprotonation rate of GSH could cause the exposure of various coordination sites, which eventually results in different modes of coordination between Cd^{2+} and GSH.

However, the reaction pathways, intermediates, and products cannot be recognized directly and synchronously by the existing methods. Therefore, it is vital to develop a simple, sensitive, and real-time detection method for monitoring the reaction process between Cd^{2+} and GSH, especially at single-molecule level.

Nanopore sensor has been widely used in different fields including single-molecule detection, single-molecule chemical reaction, and DNA sequencing. The research objects mainly include metal ions, small organic molecules, DNA, and others[19–23]. Nanopore single molecule analysis technique is based on the change of ionic current when the interaction of individual molecules with nanopores happens. The analyte can be quantified by the events frequency, and characterized by current amplitude and dwell time[24–26]. As a nanoreactor, nanopore sensors can investigate single-molecule chemistry reaction by recording the formation and cleavage process of covalent bonds. The short-lived intermediate generated during disulfide reduced by dithiothreitol[24], the reversible covalent-bond formation and cleavage between cysteine residue and organoarsenic (III) compound[25] and the interconversion of seven reaction components of arsenic (III) compounds and thiols substitution reactions have been observed[26]. But, these methods need the modification of nanopore with reactant, which is susceptible to the conformation of molecule. In this paper, a positively charged molecular adapter per-6-quaternary ammonium-*β*-cyclodextrin (*p*-QABCD) was lodged into the $(M113R)_7$ αHL nanopore as a nanoreactor and detector to monitor the reaction process between GSH molecules and Cd^{2+} ions sensitively. The final products and intermediates of Cd^{2+}-GSH with different stoichiometric ratios could be distinguished. Based on these results, the reaction pathways of the Cd^{2+}-GSH complexation at single-molecule level could be detected directly.

2 Experimental

2.1 Instruments and reagents

Axopatch 200B Patch-clamp amplifier (Axon Instruments, USA), 4040A function generator (BK Precision, USA), and 1440A A/D digital converter (Axon Instruments, USA) were used to record the current signals of nanopores.

1,2-Diphytanoyl-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids Company, USA. Pentane (> 99.7%) was purchased from Honeywell Burdick & Jackson Company, USA. Polytetrafluoroethylene membranes (Telfon) were purchased from Goodfollow Company, UK. Hexadecane (99%), cadmium chloride (≥ 98%), potassium chloride (≥ 99.0%), potassium hydroxide (≥ 85%), formic acid (≥ 95%), formaldehyde (≥ 36.0%), *N*,*N*-dimethylformamide (≥ 99%), tetrahydrofuran (≥ 99.9%), glutathione (GSH, 99%), and per-6-amino-*β*-cyclodextrin (> 98%) were purchased from Sigma-Aldrich. All aqueous solutions were prepared with ultrapure water (18.25 MΩ cm).

2.2 Synthesis of per-6-quaternary ammonium-*β*-cyclodextrin[27]

Per-6-amino-*β*-cyclodextrin (0.236 g) was mixed with formic acid (15.0 mL), formaldehyde (8.0 mL) and H_2O (15.0 mL), and stirred together under 70 °C for 18 h. After reaction, the mixture was evaporated and dried under vacuum to generate per-6-N$(CH_3)_2$-*β*-CD. Then, per-6-N$(CH_3)_2$-*β*-CD and KOH (0.015 g) were suspended in *N*,*N*-dimethyl-formamide before adding iodomethane (300 μL). The reaction mixture was stirred for 4 h under N_2 atmosphere at 4 °C and subsequently concentrated under reduced pressure to approximately 10.0 mL. The product was then precipitated by the addition of tetrahydrofuran (60.0 mL), washed with tetrahydrofuran several times, and dried under high vacuum to yield a white solid product. The product was identified with mass spectroscopy, ESI-MS: $[M]^{7+}$ (*m*/*z* 208.4).

2.3 Preparation of $(M113R)_7$ αHL protein nanopore[28]

The M113R αHL monomers were expressed in *Escherichia coli* BL-21(DE3)pLysS and purified by ultrafiltration centrifugation. Monomers were assembled into erythrocyte membrane and the heptamer protein was formed.

2.4 Experiment method[29]

A bilayer of 2-diphytanoylphosphatidylcholine was formed over a 130–150 μm aperture in a Teflon septum that divided a planar bilayer chamber into two compartments, each compartment contained 1 M KCl and was buffered with 10 mM Tris-HCl (pH 7.4 or pH 9.0). The aperture was pretreated by pentane-hexadecane (99:1, *V*/*V*) to keep the surface hydrophobic. The $(M113R)_7$ αHL protein was added to the chamber (*cis*) which was connected to ground and inserted to the lipid bilayer. The final concentration of the αHL protein in chamber was 0.05–0.30 ng mL^{-1}. Once the successful insertion of a single $(M113R)_7$ pore occurred, the system formed a conducting loop by applying voltage to the other chamber (*trans* chamber). Current fluctuations was recorded with a patch clamp amplifier, filtered with a built-in four-pole Bessel filter at 3 kHz, and sampled at 100 kHz by a computer equipped with a Digidata converter. Single-channel event amplitude and dwell time were processed and analyzed by Clampfit 10.5 and Origin 8.0 software. Mean dwell time values and the event frequency $f_{(1/ron)}$ were obtained