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Electrochemical behavior of hemin binding with human centrin 3

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ABSTRACT

The electrochemical responses of human centrin 3 (HsCen3) binding with hemin were studied by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) using glassy carbon electrodes (GCEs). In CV, the formal potential ($E^{0'}$) of hemin with the addition of HsCen3 shifted from −0.51 to −0.36 V (versus saturated calomel electrode, SCE), indicating that a new species of hemin-HsCen3 had formed. Upon binding with HsCen3, the redox current of hemin in CV and DPV decreased significantly. Based on their titration curves, the association constant of HsCen3 with hemin was obtained with a logK of approximately 4, which was consistent with that obtained from spectroscopy. Combining UV–Vis, fluorescence emission, and electrochemical methods, His100 located on the α-helix between the two domains of HsCen3 was identified as the ligand binding residue of hemin. The protein binding-induced change in electrochemical signal was thus used to construct the diffusion coefficient ($D = 1.43 \times 10^{-7}$ cm^2/s), the charge-transfer coefficient ($\alpha = 0.49$), and electron transfer standard rate constant ($k_s = 2.54 \times 10^{-2}$ s^{-1}) in the presence or absence of HsCen3. The electrochemical investigation of hemin bound with HsCen3 may provide useful data for understanding the biological processes of calcium-binding protein.

1. Introduction

Centrin is a small (~20 kDa), acidic, calcium-binding protein that belongs to the EF-hand super-family. It is mainly located at the microtubule organizing center (MTOC), which plays an essential role in mitosis and meiosis [1]. The duplication of abnormal centrosomes (known as MTOC in animal) leads to chromosomal instability and ultimately results in cancer [2–5]; thus, extensive research has been focused on these functions. Centrins also play major roles in signal transduction [6,7], flagellar excision [8], cellular division [9], nuclear mRNA export [10], as well as regulation of fibroblast growth factor and fibroblast growth factor receptor [11]. Recently, accumulating data indicate that centrosomal abnormalities may be related to inflammation and oxidative stress [12–15]. Among the centrosomal proteins, centrin is thought to be sensitive to oxidative stress and be involved in centrosomal functioning abnormalities [16]. In addition, the protein calmodulin (CaM), a homolog of centrin, has been studied widely owing to its important roles in neurodegenerative disease, whose pathogenesis may originate from oxidative stress [4]. Thus, the relationship between centrosome amplification and oxidative stress has come into focus. The metabolism of hemin is known to participate in oxidative stress. Flavohemoglobin, a classical hemoprotein, has been reported to play a significant role in the oxidative stress response in *Saccharomyces cerevisiae* [17]. A heme binding protein of *Bartonella henselae* has also been found to be important in oxidative stress [18], as it can at least partly remove damage from oxidative stress. As an important oxidative stress factor, hemin produces significant levels of neurotoxic reactive oxygen species (ROS) by binding with different proteins in the cell [19]. Based on its role in oxidative stress, hemin binding with amyloid peptide was investigated using electron paramagnetic resonance (EPR) [19]. It can be inferred that hemin is involved in the oxidative stress in the form of hemin complexed with HsCen3. Therefore, reports regarding centrin binding with hemin may be of physiological significance. Due to the potential damage of ROS to macromolecules, assembly recruiting for the centrosome may be blocked, inducing abnormalities or defects in centrosome function [20,21].

The human genome contains three centrin isoforms, namely human centrin 1 (HsCen1), human centrin 2 (HsCen2), and human centrin 3 (HsCen3). Comparison of centrin sequences from different organisms indicated that there are two divergent subfamilies; HsCen1, HsCen2, and *Chlamydomonas* centrin (CrCen) belong to one branch, while HsCen3 and the yeast homolog Cdc31p belong to another branch [22]. Centrin from *Euplotes octocarinatus*, which has been studied extensively by our group, belongs to the HsCen3 branch. Evolutionarily, HsCen3 is closely related to *S. cerevisiae* CDC31, which localizes to the half bridge of the spindle pole body (SPB) and is involved in the duplication of SPB [16]. In contrast to HsCen1, which is mainly expressed in male germ cells, HsCen3 was shown to be expressed ubiquitously in somatic cells, and was localized in the distal lumen of centrioles and the procentriole bud [23,24]. HsCen3 shares 54% sequence identity with HsCen1 and HsCen2. Importantly, it contains two His residues (His48, located at the

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loop of EF-hand I, and His100, located at the α-helix linker between N-, C-terminal), two Tyr residues (Tyr47, located at the loop of EF-hand I, and Tyr73, located at the α-helix of EF-hand II) and one Trp residue (Trp93, located at the α-helix of EF-hand II) but no Cys residue. His and Tyr residues have been regarded as the most likely coordinating location of hemin (Fe^{3+} or Fe^{2+}) on proteins. Unlike HsCen1 or HsCen2, no phosphorylation site of protein kinase A has been observed at the C-terminal domain of HsCen3, which implies a unique centriole assembly pathway. To data, the precise functions of HsCen3 are remain poorly understood. Only a few studies have indicated that it can inhibit the catalytic activity of the centrosomal protein Mps1 at Thr676, a known site of T-loop auto-activation, and interferes with Mps1-dependent phosphorylation of HsCen2 [25].

From NMR and X-ray results of centrin or its truncates from different species, it can be inferred that HsCen3 may be composed of two structurally independent globular domains connected by a flexible linker. Each structural domain contains two helix-loop-helix (known as EF-hand) calcium-binding motifs that have the potential to bind two Ca^{2+} ions [26–28]. The readout of intracellular calcium signals must be very finely tuned to effect a rapid response to the transient and subtle (100-fold) variations in calcium ion (Ca^{2+}) concentrations that constitute the calcium signals [29,30].

To data, no available data regarding the role of HsCen3 in oxidative stress and the relationships of HsCen3 with the oxidative stress factor hemin have been reported. Identifying the exact impact of oxidative species on HsCen3 is suggested to be important for treating oxidative stress-related illnesses. Here, HsCen3 was constructed, expressed, and purified by biological engineering methods, and for the first time, the electrochemical responses of this protein to oxidative stress were explored. Using the electrochemical method of cyclic voltammetry (CV) and differential pulse voltammetry (DPV), binding of HsCen3 with hemin was investigated at neutral conditions. We also confirmed our findings by difference UV–Vis and fluorescence emission. In addition, the most plausible location of hemin on HsCen3 was predicated by molecular simulation. This result will shed light on the newly discovered function of centrin in diseases associated with oxidative stress.

2. Materials and methods

2.1. Reagents

N-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) and hemin were purchased from Sigma. And other chemicals utilized in protein purification are of analytical grade. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

Tryptone, yeast extract, ampicillin (Amp^r) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Amresco Ltd. Other biochemical reagents in construction, expression and purification of proteins were purchased from Trans Gene.

KOD-Plus-Mutagenesis Kit was purchased from TOYOBO CO., LTD. Life Science Department OSAKA JAPAN.

The peptide 82T-108K (TFEDFNEVVTDWILERDPHEEILKAFK) corresponding to the amino acid residues of HsCen3 was synthesized and verified by mass spectrometry by Minhao Wuhan.

2.2. Clone, expression and purification of human centrin 3

HsCen3 coding sequence was amplified from via polymerase chain reaction (PCR) using DNA polymerase, dNTPs, and primers of P_1-upper (GCCGGATCCATGAGTTTAGCTCTGAGAAGTG) as well as P_1-down (GCCGTCGACTTAAATGTCACCAGTCATAATAG). Using same enzymes of *Bam*HI and *Sal*I digesting PCR products and expression vector pGEX-6p-1, HsCen3 in the presence of ligation enzyme was sub-cloned onto vector forming recombinant plasmid of pGEX-6p-1-HsCen3. After verification by DNA sequence analysis, the recombinant plasmid was transferred into *E. col.* (DE3), which was incubated overnight at 25 °C. Protein synthesis was induced using IPTG (0.5 mM) for 10 h, at optical density of 0.6–0.8 (at 600 nm). And proteins were purified firstly as a GST fusion protein using glutathione sepharose 4FF in PBS buffer (KH_2PO_4 1.8, Na_2HPO_4 10, KCl 2.7 and NaCl 140, in mM). The GST fusion proteins were then cleaved by PreScission Protease (PPase) [31] and purified again through Sephadex G-75. The purity of the intermediate and final samples was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After purification, the proteins were concentrated and kept at −80 °C.

His100 on HsCen mutanted to Ala (HsCen3H100A) was achieved using KOD-Plus-Mutagenesis Kit. All reactions were carried by the manufacturer. Then, it was sub-cloned into the expression vector pGEX-6p-1. The recombinant plasmid of pGEX-6p-HsCen3H100A was constructed. After verification by DNA sequence analysis, the recombinant plasmid was transferred into *E. coli.* (DE3), expressed, and purified as described above.

2.3. Electrochemical experiments

The electrochemical measurements were carried out using a CHI 660C electrochemistry workstation (Shanghai CH Instruments, China). The electrochemical cell with volume of approximately 600 μL consists of a standard two-compartment glass cell with conventional three-electrode system. The side-arm including saturated calomel electrode (SCE) and reference electrode (RE) was connected to the working compartment via a Luggin capillary. Prior to each experiment, the glassy carbon electrodes (GCE) as working electrode were cleaned by polishing with an alumna-water slurry (high-purity Al_2O_3, particle size 0.3 and 0.05 μM, BDH) and were sonicated briefly, followed by thorough rising with water. The area of electrodes surface was calculated according to the Randle-Sevcik equation and diffusion coefficient of $K_3Fe(CN)_6$. The platinum gauze was used as counter-electrode (CE). The working solution was purged with oxygen-free nitrogen for at least 20 min prior to experiments and the nitrogen environment was maintained over the solution in the cell.

2.4. Spectra measurements

HP8453UV–Vis was used for absorbance data collection. The protein was diluted in 100 mM Hepes, (pH 7.4) and its concentration was determined spectroscopically. Using hemin as blank, hemin was titrated by HsCen3 at neutral conditions. To correct dilution during each titration and to normalize the results from different titrations, the UV–Vis spectra data were converted to absorptivity (A) by dividing the absorbance by the analytical concentration of hemin. Fluorescence emission spectra of HsCen3 were acquired from 290 nm to 500 nm with excitation at 295 nm. Both slit widths of excitation and emission were 5 nm. All fluorescence data were collected on Hitachi F-2500. Samples were prepared by gradually adding hemin solutions into the protein solutions. An equilibrium time of 5 min was used between each titration. Same methods to UV–Vis were used to deal with fluorescence emission data.

3. Results and discussion

3.1. Cyclic voltammetry (CV) of hemin and hemin/HsCen3 complex

In PBS buffer (pH 7.4), the cyclic voltammogram of hemin at a bare glassy carbon electrode (GCE) is shown in curve a (Fig. 1). Well-defined redox peaks of hemin appeared at −0.36 (E_{pa}) and −0.51 V (vs SCE) (E_{pc}), which may be the oxidation-reduction potentials of the Fe^{3+}/Fe^{2+} center on hemin/heme. Similar CV signals of hemin have appeared in different buffers and on different electrodes [32–34]. However, their anodic to cathodic peak separations were mostly ~−0.08 V (vs SCE). At neutral conditions, hemin may form a stable hexa-coordinate. Four nitrogen atoms on the porphyrin ring and two oxygen atoms from