ACS OMEGA

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Cite This: ACS Omega 2019, 4, 9603−9614

http://pubs.acs.org/journal/acsodf

Polymerization of Oxidized DJ-1 via Noncovalent and Covalent Binding: Significance of Disulfide Bond Formation

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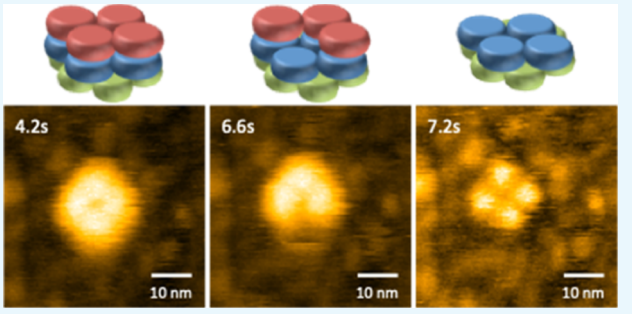
Supporting Information

ABSTRACT: The reactive cysteine residue at position 106 (Cys106) of DJ-1 is preferentially oxidized under oxidative stress, generating oxidized DJ-1 (oxDJ-1). Oxidation of Cys106 to sulfinic acid changes the biologic action of DJ-1 and increases its cytoprotective properties. The similar activation step is known in peroxiredoxins (Prxs), in which oxidation of reactive Cys to sulfinic acid induces polymerization of Prxs and changes its enzyme characteristic from peroxidase to molecular chaperone. In the present study, oxDJ-1 was prepared and its polymerization and related amino acid residues were investigated. We found that oxDJ-1 formed a characteristic polymer with disulfide bonds and with noncovalent and covalent binding other than disulfide. The physiological concentration of glutathione resolved the polymer form of oxDJ-1, and glutathionylation of other two Cys residues, such as Cys 46 and 53, was detected. Mutant analysis indicated the necessity not only of Cys106 but also of Cys46 for the polymer formation. The cellular experiment demonstrated that the electrophilic quinone treatment induced a high-molecular-weight complex containing oxDJ-1. Dynamic polymerization of oxDJ-1 with a ring and a stacked structure was observed by an atomic force microscope. Collectively, these results clearly demonstrated the characteristic polymer formation of oxDJ-1 with a disulfide bond and noncovalent and covalent binding other than disulfide, which might be related to the biologic function of oxDJ-1.



INTRODUCTION

DJ-1 is a member of the DJ-1/Hsp31/PfpI superfamily, and its function as a molecular chaperone has been reported.[1,2] An apparent molecular mass of the DJ-1 monomer is 22 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), forming a dimer in physiological condition. DJ-1 is a multifunctional protein that is involved in various physiological processes, including transcriptional regulation, signal transduction, and mitochondrial function.[3] DJ-1 is related to regulate the transcriptional activity of Nrf2 and p53 and to change glutathione (GSH) metabolism and the expression levels of heat-shock and uncoupling proteins.[4] DJ-1 is also identified as a regulator of the 20S proteasome.[5] DJ-1 plays a significant role in antioxidative defense; its loss of function increases the sensitivity of cell death induced by oxidative stress.[6] DJ-1 regulates signal mediators of oxidative stress, such as ASK1 and PTEN, via direct interactions with these proteins.[7] The function of DJ-1 as a redox-activated chaperone might account for these multiple interacting proteins and functions. DJ-1 is known as a product of *PARK7*, a causative gene of familial form of Parkinson's disease, and its loss of function with mutation is related to the onset of this neurodegenerative disease.[8]

DJ-1 possesses three cysteine residues at positions 46 (Cys46), 53 (Cys53), and 106 (Cys106), which play an important role in the biological function of DJ-1.[9] Cys106 is a critical residue for DJ-1 function, and several studies demonstrated that Cys106 substitution to Ser mutant lost its biological function, including its role in antioxidative defense.[6,9] Cys106 is a reactive cysteine with a depressed pK_a of 5.4, in which thiolate accepts a hydrogen bond from a protonated side chain of glutamate at position 18.[10] Cys106 is sequentially oxidized to cysteine sulfenic acid (Cys-SOH), cysteine sulfinic acid (Cys-SO_2H), and cysteine sulfonic acid

Received: February 4, 2019
Accepted: May 21, 2019
Published: June 3, 2019

DOI: 10.1021/acsomega.9b00324
ACS Omega 2019, 4, 9603−9614

(Cys-SO_3H).[11] DJ-1 oxidation is induced by several oxidative stimuli, such as hydrogen peroxide (H_2O_2), paraquat, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and 6-hydroxydopamine (6-OHDA).[11,12] It has been reported that 6-OHDA is rapidly oxidized by molecular oxygen to generate the superoxide anion, H_2O_2, and 2-hydroxy-5-(2-aminoethyl)-1,4-benzoquinone (*p*Q).[13] We have previously reported that the decrease of GSH induced by arylating *p*Q, which is oxidatively generated from 6-OHDA, is an important step for 6-OHDA-induced DJ-1 oxidation.[14] Cys-SO_2H can be oxidized to Cys-SO_3H; however, Cys106-SO_2H in oxidized DJ-1 is stable because of the surrounding amino acid residues.[15] The crystal structure of human DJ-1 revealed that Cys53 at the dimer interface is within 4 Å of each other, which makes a disulfide bond.[9,16] Further, the thiol residue of Cys53 is exposed to the outside and is covalently modified by dopamine-derived arylating quinone, which is generated in the presence of tyrosinase.[17] On the other hand, Cys46 plays a relevant role for the preservation of three-dimensional structure of DJ-1 and is not reactive against dopamine-derived quinones.[9,16,17]

To detect Cys106-oxidized DJ-1 (oxDJ-1) conveniently, we have prepared specific antibodies (Abs) against oxDJ-1 to allow oxDJ-1-specific western blotting, immunostaining, and enzyme-linked immunosorbent assay (ELISA) to be carried out.[18,19] Our recent data suggest that oxDJ-1 levels in the brain, heart, and skeletal muscle are high.[20] These regions have high mitochondrial loads in both mitochondrial mass and respiratory demand. The substantial connection between oxDJ-1 and mitochondrial function has been demonstrated.[15] In addition, Cys-SO_2H-driven mitochondrial localization is known to be important in the neuroprotective role of DJ-1.[16] We prepared an oxDJ-1 recombinant protein to use as an immunogen.[21] We found that the treatment of human DJ-1-expressed *Escherichia coli* with H_2O_2 resulted in the specific oxidation of Cys106 to Cys-SO_2H/-SO_3H, but not in irreversible oxidation of Cys46 and Cys53, which could be purified by His-tag-affinity chromatography. The recombinant oxDJ-1 protein was used for western blotting and ELISA; however, its structure was not characterized yet.

The Cys-SO_2H form of DJ-1 is postulated to be the active form of DJ-1 with increased chaperone activity.[9,22] These properties of DJ-1 have a similarity to peroxiredoxin (Prx), which is known to catalyze the removal of H_2O_2 and organic hydroperoxides.[23] The Prx family consists of two groups, that is, 1-Cys Prx and 2-Cys Prx, depending on the numbers of conserved Cys.[24] 2-Cys Prx catalytically reduces H_2O_2 to water using thioredoxin as a reductant. 2-Cys Prx has a reactive Cys residue to be oxidized; this oxidation causes the protein structure of Prx to shift from low-molecular-weight species to high-molecular-weight (HMW) polymer complexes.[25] This conformational change triggers a peroxidase-to-molecular chaperone functional switch. It has been known that the polymer formation of Prx is depending on the oxidation of Cys and that GSH, a cellular major reductant, promotes its structure changes from decamer to dimer via glutathionylation of Prx and concomitantly inactivates its molecular chaperone function.[26] In the case of DJ-1, oxidation-dependent increase of chaperone activity has been identified; however, the detailed protein structure and the role of Cys residues have not been elucidated.

In the present study, we characterized the three-dimensional structure of the oxDJ-1 recombinant protein, particularly the role of Cys residues and the polymer formation with covalent and noncovalent bonds. We also examined the formation of HMW oxDJ-1 in cellular experiments. Finally, we observed the dynamic polymer formation of oxDJ-1 using an atomic force microscope (AFM).

MATERIALS AND METHODS

Materials. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and H_2O_2 were purchased from Wako Pure Chemical Industries (Osaka, Japan). The reduced form of GSH, 6-OHDA, catalase, and anti-β-actin (AC-15) was obtained from Sigma-Aldrich (St Louis, MO). A protease inhibitor cocktail tablet was obtained from Nacalai Tesque (Kyoto, Japan). Ni-nitrilotriacetic acid (NTA) agarose was purchased from QIAGEN GmbH (Hilden, Germany). Benzoquinone (BzQ) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12, 11320-033) was obtained from Thermo Fisher Scientific (Waltham, MA), and fetal bovine serum (FBS, GPK0029) was purchased from HyClone (Logan, UT). SH-SY5Y cells were obtained from the American Tissue Type Collection (Manassas, VA). Other chemicals used were of the highest quality commercially available. Monoclonal antibodies against oxDJ-1 were prepared as previously described.[18]

Preparation of DJ-1 and oxDJ-1 Recombinant Protein.[18] Full-length human DJ-1 cDNA (570 bp, NM_007262) cloned into pEXP1-DEST was transformed into *E. coli* strain BL21(DE3)pLysS, and a fusion DJ-1 protein with a 6-His tag at the amino terminus was obtained. Until the absorbance value of the medium at 600 nm had reached 0.5, the bacterial culture was grown in the Luria-Bertani medium with 50 μg/mL ampicillin. Protein expression was induced by 0.5 mM IPTG addition. After 2 h, DJ-1 in the cells was oxidized by the addition of 150 mM H_2O_2 for 15 min at 37 °C. In the case of DJ-1 recombinant protein, H_2O_2 treatment was not conducted. These recombinant proteins were purified by using Ni-NTA agarose. The oxidation of Cys106 in purified proteins was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), described below. We observed by MS analysis that Cys106 of purified recombinant oxDJ-1 protein was fully oxidized to sulfonic acid. In addition, it was also confirmed that in oxDJ-1 recombinant protein, the unoxidized form of Cys106 was not detected.

SDS-PAGE for Western Blotting and Silver Staining. Protein samples were denatured in the SDS sample buffer [63 mM Tris-HCl (pH 6.8) containing 0.012% bromophenol blue, 5% sucrose, and 2% SDS] for 5 min at 95 °C (nonreduced condition). To reduce protein samples, 1% beta-mercaptoethanol (2-ME) was added to SDS sample buffer and then heat treatment was conducted. After separation of the samples by SDS-PAGE (12.5% gel), the proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA) for the western blot analysis. The membranes were blocked in a Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 (TBS-T) containing 5% skimmed milk powder (Snow Brand Milk Products, Tokyo, Japan), incubated with anti-oxDJ-1 mAb (clone M106, 1 μg/mL, ref 18), mouse anti-DJ-1 mAb (clone 3E8, 1 μg/mL, Medical & Biological Laboratories, Nagoya, Japan), or anti-β-actin mAb (AC-15) at 4 °C for 18 h, and incubated with HRP-conjugated secondary antibodies for at least 1 h. After the incubation with Immobilon Western (Millipore), the immunoreactivity was visualized with an LAS-

DOI: 10.1021/acsomega.9b00324
ACS Omega 2019, 4, 9603−9614

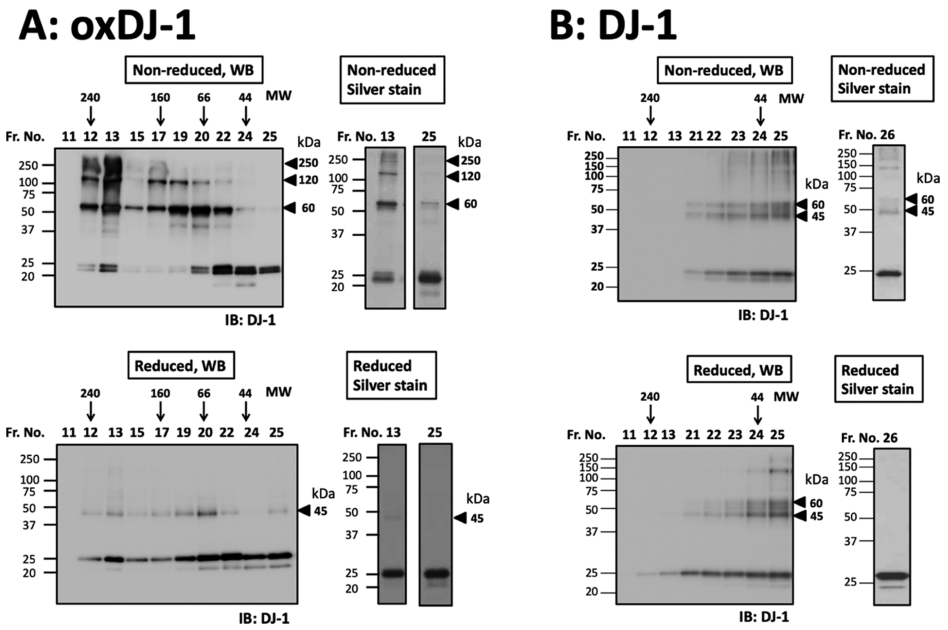


Figure 1. Polymer formation of oxidized DJ-1 via noncovalent and covalent binding. (A) Polymer formation of oxDJ-1 recombinant protein. Purified oxDJ-1 protein was separated by gel chromatography, and the oxDJ-1 content of each fraction was determined by using western blot analysis under nonreduced (upper panel) and reduced condition (lower panel). The fractions where each standard protein was eluted are indicated by arrows. The results of silver staining at Fr. 13 and 25 are shown in the right of each panel. These results suggest that detected HMW bands of oxDJ-1 at 60−250 kDa in Fr. 12 and 13 (nonreduced, WB) are formed via disulfide bonds between oxDJ-1 molecules and that the polymer form of oxDJ-1 at 160−240 kDa in Fr. 12 and 13 is formed via noncovalent bonds between HMW oxDJ-1 molecules. It is considered that the polymer form of oxDJ-1 is formed via both disulfide and noncovalent bonds. (B) Gel chromatography of DJ-1 recombinant protein. Purified DJ-1 protein was subjected to gel chromatography as described above. The polymer form of DJ-1 was not detected clearly at Fr. 12 and 13.

4000 luminescence imager (Fujifilm, Tokyo, Japan). For silver staining, the separated proteins were stained with the Dodeca Silver Stain Kit (Bio-Rad Laboratories, Hercules, CA).

Liquid Chromatography Coupled with Tandem MS (LC−MS/MS) Analysis. The oxidation of Cys in oxDJ-1 and DJ-1 recombinant proteins was evaluated by MALDI-TOF MS. Proteins were separated by SDS-PAGE and visualized by silver staining. The stained band was excised, treated with 25 mM ammonium bicarbonate (AB, pH 8.8) containing 50% acetonitrile, and then dehydrated with acetonitrile. The gel pieces were rehydrated in 25 mM AB solution-containing dithiothreitol (DTT) (10 mM) and alkylated with iodoacetamide (55 mM). To analyze the glutathionylation, DTT was removed. After dehydration with acetonitrile, the gel pieces were rehydrated on ice for 30 min in 50 mM AB solution-containing trypsin (sequence grade, 10 μg/mL, Promega) and digestion was performed for 18 h at 37 °C. The resulting peptides were extracted and concentrated with a SpeedVac (Thermo Electron). The samples were mixed with α-cyano-4-hydroxycinnamic acid and subjected to a MALDI-TOF MS 4800 Plus (AB SCIEX, Framingham, MA). Peptide samples for detailed sequence analysis were separated and spotted on a plate for MALDI-TOF MS analysis with a capillary liquid chromatography (LC) system coupled to an auto-spotter DiNa ASM-T-MaP (KYA TECH, Tokyo, Japan). UniProtKB/Swiss-Prot protein databases were used to identify peptide fragments. Data were processed with the ProteinPilot 3.0 software (AB SCIEX).

Gel Chromatography. AKTA prime plus (GE Healthcare) was used for the fractionation of DJ-1/oxDJ-1 recombinant proteins. Purified DJ-1/oxDJ-1 protein samples were fractionated by using a Sephacryl Column (HiPrep 16/60 Sephacryl Columns S-300 HR, GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl. To determine the molecular weight, following standard proteins were used: 240K, mouse catalase; 160K, bovine γ-globulins; 66K, bovine serum albumin (BSA); and 44K, ovalbumin.

Cell Culture and Treatment. Human neuroblastoma SH-SY5Y cells were routinely maintained in a D-MEM/F-12 medium containing 10% heat-inactivated FBS and antibiotics (0.05 U/mL penicillin, 0.05 mg/mL streptomycin; Invitrogen) at 37 °C in an atmosphere of CO_2 (5%) and air (95%). *para*-Quinone was prepared as described previously.[13] Indicated concentration of 6-OHDA was dissolved in D-MEM/F12 and reacted at 37 °C for 15 min in the presence of catalase. After the reaction, generated *p*Q was added to the cells. After the treatment, cells were resuspended in lysis buffer [50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 50 mM NaF, and 1 mM Na_3VO_4, and a protease inhibitor cocktail tablet] at 4 °C for 30 min. Cellular debris was removed by centrifugation. The bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL) was used for the determination of protein concentration with BSA as the standard.

Transfection of Small-Interfering RNA (siRNA). The human DJ-1-siRNA were designed and manufactured by Invitrogen, according to the current guidelines for effective knockdown by this method. The target sequences for DJ-1-siRNA (#1: 5′-AGCAGACUCAGAUAAAUUCUGUGCG-3′ and 5′-CGCACAGAAUUUAUCUGAGUCUGCU-3′, and #2: 5′-CCAGCUUCGAGUUUGCGCUUGCAAU-3′ and 5′-AUUGCAAGCGCAAACUCGAAGCUGG-3′) were used. The siRNAs were transfected into SH-SY5Y cells at a concentration of 100 pmol/10^5 cells by Lipofectamine RNAi MAX (Invitrogen) 3 days before further experiments.

DOI: 10.1021/acsomega.9b00324
ACS Omega 2019, 4, 9603−9614

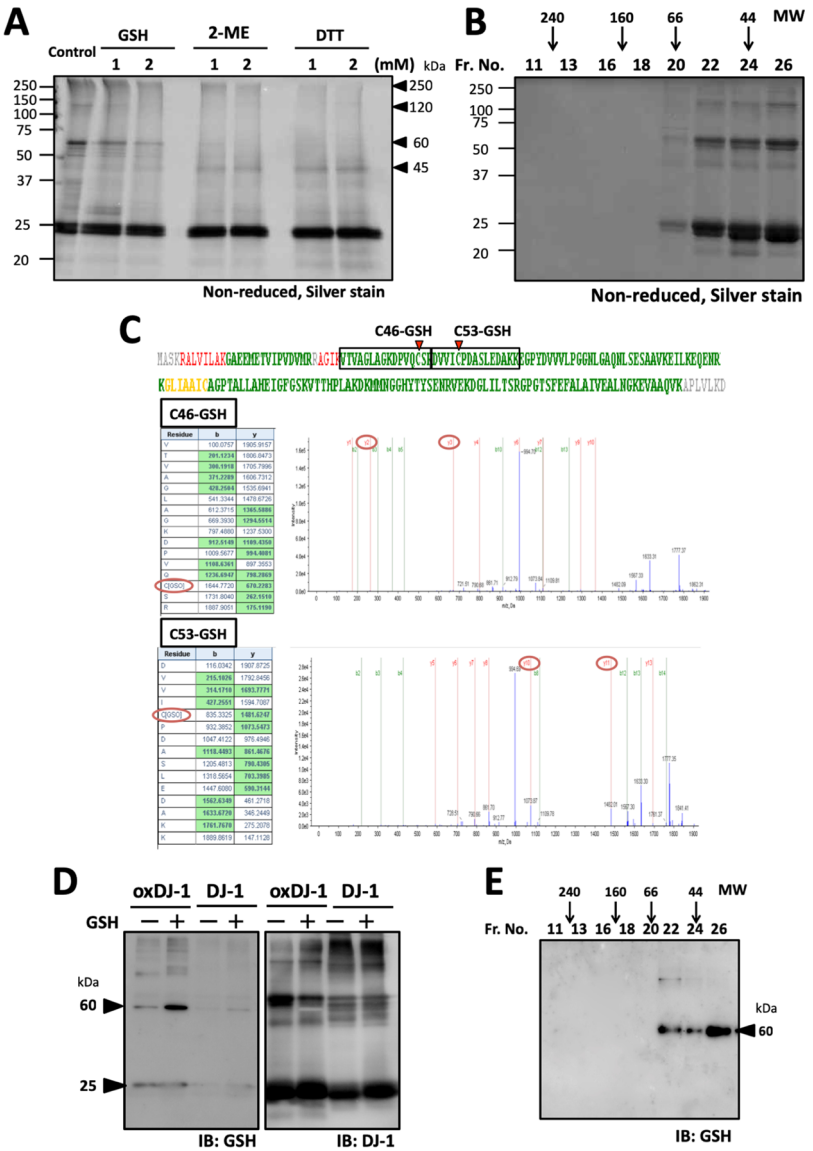


Figure 2. Depolymerization of oxidized DJ-1 by GSH. (A) Effects of reducing agent on oxDJ-1 polymerization. Reducing agents such as GSH, 2-ME, and DTT were added to oxDJ-1 recombinant proteins in tris-buffered saline (pH 7.4) and then incubated at 37 °C for 1 h. Protein samples were separated by SDS-PAGE under nonreducing condition and visualized by silver staining. We found that reducing agents could reduce HMW bands of oxDJ-1, particularly the 60 and 120 kDa band. (B,C) GSH-treated oxDJ-1 protein was separated by gel chromatography, and proteins in each fraction were detected by silver staining under nonreducing condition (B). Detected bands were subjected to in-gel digestion (C). Peptide samples were analyzed by MALDI-TOF MS. Identified peptide sequences with confidence intervals greater than 95% are shown in green. The sequences indicated by yellow and red correspond to confidence intervals higher than 50% and lower than 50%, respectively. The MS/MS spectrum of peptides predicted to contain Cys-GSH. We found glutathionylated Cys at positions 46 and 53 of oxDJ-1 at the 60 kDa band. (D) GSH-treated oxDJ-1 and DJ-1 proteins were subjected to western blot analysis using anti-GSH and anti-DJ-1 antibodies. Glutathionylation of oxDJ-1 at 60 kDa was detected. (E) GSH-treated oxDJ-1 was subjected to gel chromatography, and each fraction was subjected to western blotting using anti-GSH antibody. Glutathionylated oxDJ-1 was detected around dimer form.

High-Speed AFM (HS-AFM) Observation. AFM imaging of DJ-1 was performed in solution using a laboratory-built HS-AFM setup.[27−29] Each DJ-1 sample was deposited on a mica surface and incubated for 5−10 min. Unattached molecules were removed by washing with observation buffer (50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid−NaOH (pH 7.5) containing 100 mM NaCl). Imaging was carried out in the tapping mode, using small cantilevers BL-AC10DS-A2 or custom-made BL-AC7DS-KU4 (Olympus, Tokyo, Japan). The set-point amplitude was 80−90% of the free oscillation amplitude, and the cantilever-free oscillation amplitude was ∼1.5 nm. The imaging rate, scan size, and feedback parameters were optimized to enable visualization with a minimum tip force. The obtained HS-AFM image sequences were processed and analyzed by a custom software.

Statistical Analysis. Data are reported as mean ± standard deviation of at least three independent experiments. The statistical significance of the difference between the determinations was calculated by an analysis of variance using Tukey's test for multiple comparisons. The calculation method was described in each figure legend. Values of $p < 0.05$ were considered significant.

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Table 1. Summary of LC−MS/MS Analysis of Each oxDJ-1 Recombinant Protein[a]

	Cys	sequence	modification of Cys
oxDJ-1 (WT)	Cys46	DPVQCSR	carbamidomethyl
	Cys53	DVVICPDASLEDAKK	carbamidomethyl
	Cys106	GLIAAICAGPTALLAHEIGFGSK	dehydroalanine
		IAAICAGPTALLAHEIGFGSK	trioxidation
		KGLIAAICAGPTALLAHEIGFGSK	trioxidation
		GLIAAICAGPTALLAHEIGFGSK	trioxidation
oxDJ-1 (C106S)	Cys53	DVVICPDASLEDAKK	carbamidomethyl
	Cys106	GLIAAICAGPTALLAHEIGFGSK	serine
oxDJ-1 (C46/53S)	Cys53	DVVICPDASLEDAK	serine
	Cys106	GLIAAICAGPTALLAHEIGFGSK	trioxidation

[a]Prepared recombinant proteins were separated by SDS-PAGE under reduced condition and then were visualized by protein staining. The band corresponding to oxDJ-1 was subjected to in-gel digestion and MALDI-TOF MS analysis. Identified peptide sequences with confidence intervals greater than 95% are shown in the sequence column, and the position of Cys is indicated with an underline. Identified modification of Cys is shown in the modification of Cys column. Carbamidomethyl, carbamidomethyl Cys; dehydroalanine, the position of Cys was detected as dehydroalanine; trioxidation, cysteine sulfonic acid (Cys-SO_3H); serine, the position of Cys was detected as Ser.

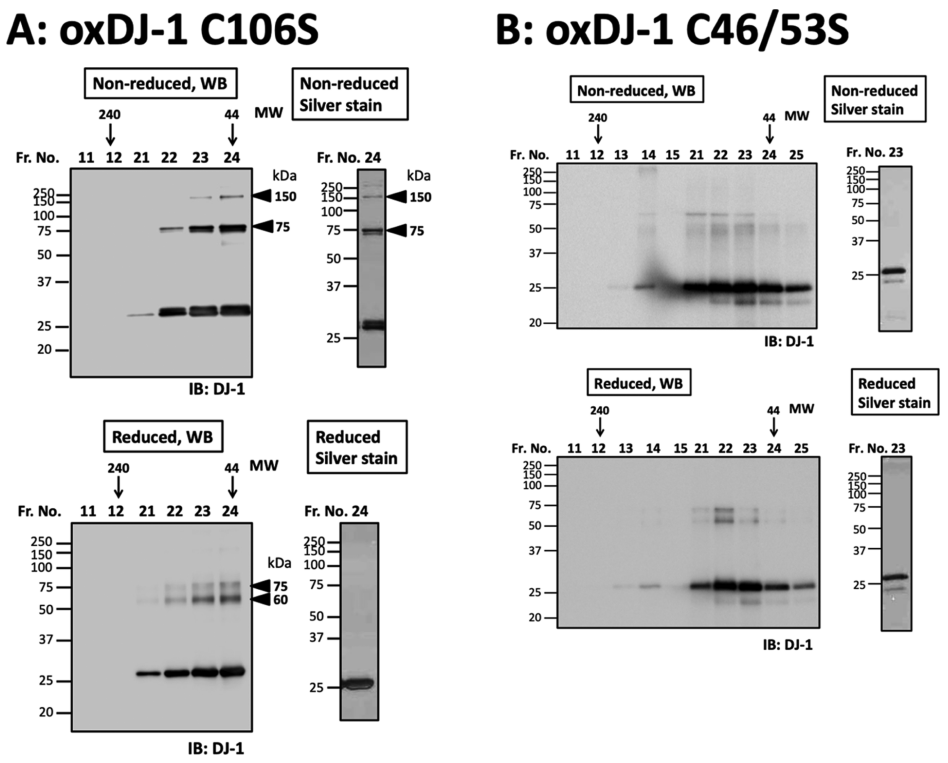

Figure 3. Role of cysteine residue for the polymerization of oxidized DJ-1. (A,B) Purified recombinant protein of oxidized DJ-1(C106S) (A) and oxidized DJ-1(C46/53S) (B) was separated by gel chromatography and protein content of each fraction was determined by using western blotting under nonreduced (upper panel) and reduced condition (lower panel), respectively. The fractions where each standard protein was eluted are indicated by arrows. The results of silver staining at indicated Fr. are shown in the right of each panel. The polymer form of oxDJ-1 (C106S) (A) and oxDJ-1 (C46/53S) (B) around Fr. 12 was not observed.

RESULTS

Formation of HMW Polymer in Oxidized DJ-1. On the basis of the previous studies showing the oxidation-dependent chaperone activity of DJ-1 and polymerization of Prxs, we determined the MW of oxDJ-1 under nondenaturing conditions. To prepare oxDJ-1 recombinant protein, *E. coli* expressing human DJ-1 was treated with H_2O_2, then cells were lysed, and oxDJ-1 recombinant protein was purified by Ni-NTA column chromatography, as described previously.[18] The oxidation of almost all Cys106 to Cys-SO_2H/SO_3H was confirmed by LC−MS/MS analysis. Next, oxDJ-1 protein was subjected to gel chromatography.[19] Proteins in each fraction were separated by SDS-PAGE under reduced and nonreduced conditions and then detected by anti-DJ-1 Ab. OxDJ-1 bands were detected not only in dimer at 44−66 kDa standard proteins of gel chromatography (Fr. 20−24) but also in the polymer at 160−240 kDa standard proteins of gel chromatography (Fr. 12−17) (Figure 1A). Fr. 11 was a void fraction. We found that under nonreduced condition, HMW bands of oxDJ-1 at 60, 120, and 250 kDa were detected in Fr. 12 and 13 by western blotting, whereas these oxDJ-1 bands were mostly detected at 25 kDa under reduced conditions (Figure 1A). These results suggest that detected HMW bands of oxDJ-1 at 60−250 kDa in Fr. 12 and 13 are formed via disulfide bonds

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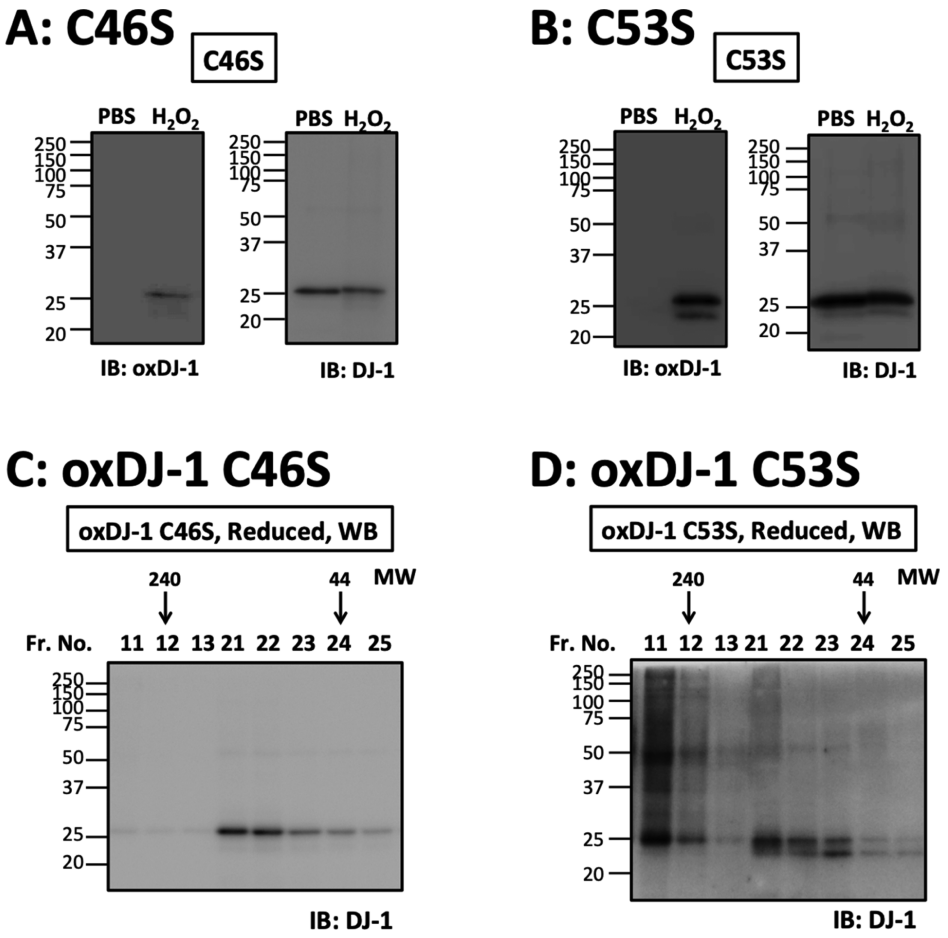

Figure 4. Substantial role of cysteine 46 residue for the polymerization of oxidized DJ-1. (A,B) Purified recombinant protein of oxidized DJ-1(C46S) (A) and oxidized DJ-1(C53S) (B) was subjected to western blot analysis using anti-DJ-1 and oxDJ-1 Ab, respectively. The oxidation of Cys106 of DJ-1 (C46S) (A) and DJ-1 (C53S) (B) to Cys-SO_2H/SO_3H by H_2O_2 treatment was confirmed by western blot analysis using oxDJ-1 Ab. (C,D) These recombinant proteins were further separated by gel chromatography and protein content of each fraction was determined by using western blot analysis under reduced condition. The fractions where each standard protein was eluted were indicated by arrows. We found that oxDJ-1(C53S) (D), but not oxDJ-1(C46S) (C), formed a polymer, which suggests the substantial role of Cys46 for the polymerization of oxDJ-1.

between oxDJ-1 molecules. Further, the polymer form of oxDJ-1 at 160−240 kDa in Fr. 12 and 13 is formed via noncovalent bonds between HMW oxDJ-1 molecules; therefore, it is considered that the polymer form of oxDJ-1 is formed via both disulfide and noncovalent bonds. In addition, a reducing agent-resistance band at 45 kDa was observed under reduced conditions, which suggested the presence of a covalent bond form of oxDJ-1 other than a disulfide bond (Figure 1A). These bands recognized by anti-DJ-1 Ab were detected by silver staining of proteins. The HMW bands of DJ-1 in western blotting were obvious rather than those in silver staining. A concentration-dependent study of H_2O_2 for DJ-1 oxidation suggests the concentration-dependent increase of HMW oxDJ-1 at 60 and 120 kDa, which approximately corresponds to dimer and tetramer (Supporting Information Figure S1).

In the case of DJ-1 recombinant protein, a dimer form with a disulfide and a covalent bond other than a disulfide bond were detected; however, the polymer form of DJ-1 was not detected clearly at Fr. 12 and 13 (Figure 1B). In the western blotting of native DJ-1 under both reducing and nonreducing conditions, HMW DJ-1 was slightly observed from Fr. 23 to Fr. 25, where were fractions of dimer or monomer in gel chromatography, which suggests the formation of HMW bands during the sample preparation for SDS-PAGE. Collectively, these results suggest that there is a characteristic polymeric form of oxDJ-1 with a disulfide bond and noncovalent and covalent binding other than a disulfide bond.

Depolymerization of oxDJ-1 by GSH and Glutathionylation of oxDJ-1. Next, we evaluated the effects of reducing agents, such as 2-ME, DTT, and GSH, on polymer formation of oxDJ-1. These reducing agents at 1 and 2 mM were added to oxDJ-1 recombinant protein in Tris-buffered saline, incubated at 37 °C for 1 h, and then subjected to SDS-PAGE under nonreduced conditions. The HMW bands of oxDJ-1 in silver staining were weak but obvious in the control lane (Figure 2A). Relatively strong reducing agents, such as 2-ME and DTT, could reduce HMW bands of oxDJ-1, particularly the 60 and 120 kDa band, whereas GSH at physiological intracellular concentrations slightly but obviously decreased HMW oxDJ-1 (Figure 2A). Further, we found the depolymerization of oxDJ-1 by GSH treatment in gel chromatography analysis (Figure 2B). To characterize the action of GSH on oxDJ-1, LC−MS/MS analysis was conducted for each band detected by protein staining. As a result, we found glutathionylated Cys at positions 46 and 53 of oxDJ-1 at the 60 kDa band (Figure 2C). Glutathionylation of oxDJ-1 at the 60 kDa band was also observed in western blot analysis using anti-GSH Ab, whereas glutathionylation was not

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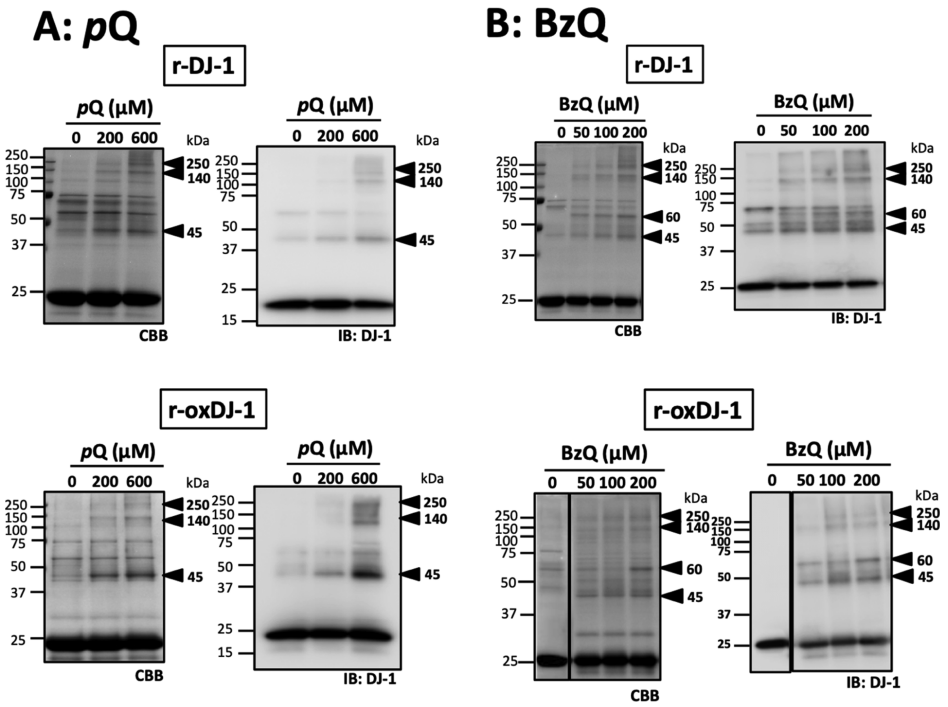

Figure 5. Formation of a covalently bound DJ-1 and oxDJ-1 by arylating quinones. (A,B) Purified recombinant DJ-1 (upper panel) and oxDJ-1 proteins (lower panel) were incubated with each concentration of arylating *para*-quinone (*p*Q) (A) and BzQ (B) at 37 °C for 1 h, respectively. After incubation, the formation of a covalently bound DJ-1 and oxDJ-1 with HMW was evaluated by SDS-PAGE and western blotting under reducing condition, respectively. We found that arylating quinone can form HMW DJ-1/oxDJ-1 with a covalent bond other than a disulfide bond.

observed in GSH-treated DJ-1 (Figure 2D). Glutathionylation of oxDJ-1 at 60 kDa band was completed during 1 h and was detected stably to 6 h at 37 °C (Supporting Information Figure S2). Glutathionylated oxDJ-1 was observed around the dimer form of oxDJ-1 in gel chromatography analysis (Figure 2E). Taken together, these results suggest that oxDJ-1 is depolymerized via the reduction of Cys46 and Cys53, implying the significant role of disulfide bond formation for the polymerization of oxDJ-1.

Substantial Role of Cys46 for the Polymerization of oxDJ-1. To determine the role of Cys residue of DJ-1 for the polymerization of oxDJ-1, we prepared mutant recombinant proteins, in which Cys was substituted to Ser. To confirm the role of irreversible oxidation at Cys106 on polymerization, *E. coli*-expressing mutant DJ-1 (C106S) was similarly treated with H_2O_2 and polymerization of purified oxDJ-1 (C106S) protein was analyzed by gel chromatography and western blotting. The LC−MS/MS analysis of oxDJ-1 (C106S) showed the mutation of Cys106 to Ser and reversible oxidation of Cys53 (Table 1). Cys46 of oxDJ-1 (C106S) was not detected (Table 1). Seventy-five and 150 kDa bands of HMW oxDJ-1 (C106S) were detected under nonreduced conditions and at 75 and 60 kDa bands under reduced conditions, respectively; however, the polymer form of oxDJ-1 (C106S) around Fr. 12 was not observed (Figure 3A). We further analyzed the polymerization of double mutant DJ-1 (C46/53S) treated with H_2O_2. Irreversible oxidation of Cys106 and mutation of Cys53 to Ser were confirmed by LC−MS/MS analysis, whereas Cys46 was not detected (Table 1). In gel chromatography analysis, obvious formation of polymer in oxDJ-1 (C46/53S) was not observed (Figure 3B).

We further analyzed polymer formation of a single mutant oxDJ-1 (C46S) and oxDJ-1 (C53S). Because expression levels of both single mutants were low and LC−MS/MS analysis of both mutants was difficult, we confirmed irreversible oxidation of Cys106 by specific Ab against oxDJ-1. We confirmed the oxidation of Cys106 of DJ-1 (C46S) (Figure 4A) and DJ-1 (C53S) (Figure 4B) to Cys-SO_2H/SO_3H by H_2O_2 treatment. These single mutants were subjected to the gel chromatography analysis. We found that oxDJ-1(C53S) (Figure 4D), but not oxDJ-1(C46S) (Figure 4C), formed a polymer, which suggests the substantial role of Cys46 for the polymerization of oxDJ-1.

Formation of a Covalently Bound oxDJ-1 by Arylating Quinones. As described above, oxDJ-1 formed a covalent bond dimer other than disulfide bond. To further get insight for the formation of a covalent bond oxDJ-1, we examined the effects of arylating quinone based on the previous report.[17] At first, purified DJ-1 and oxDJ-1 recombinant proteins were incubated with arylating *p*Q generated from 6-OHDA at 37 °C for 1 h and then subjected to SDS-PAGE and western blotting under reducing conditions to evaluate the formation of a covalent bond dimer. In both DJ-1 and oxDJ-1 recombinant proteins, we observed the increase of HMW bands with a covalent bond other than a disulfide bond by western blotting and protein staining (Figure 5A). It was notable that the formed HMW bands were relatively high in *p*Q-treated oxDJ-1 (Figure 5A). We also examined the effects of arylating BzQ on the formation of HMW bands in both DJ-1 and oxDJ-1 (Figure 5B). These results suggest that arylating quinone can form HMW DJ-1/oxDJ-1 with a covalent bond other than a disulfide bond.

The formation of HMW DJ-1/oxDJ-1 with a covalent bond by arylating quinone was evaluated in human neuroblastoma SH-SY5Y cells. Cells were treated with *p*Q and BzQ for 3 h at 37 °C, and then whole cell lysates were subjected to western blotting using anti-DJ-1 and anti-oxDJ-1 mAb under reduced conditions, respectively. An obvious HMW DJ-1 band with a

DOI: 10.1021/acsomega.9b00324
ACS Omega 2019, 4, 9603−9614

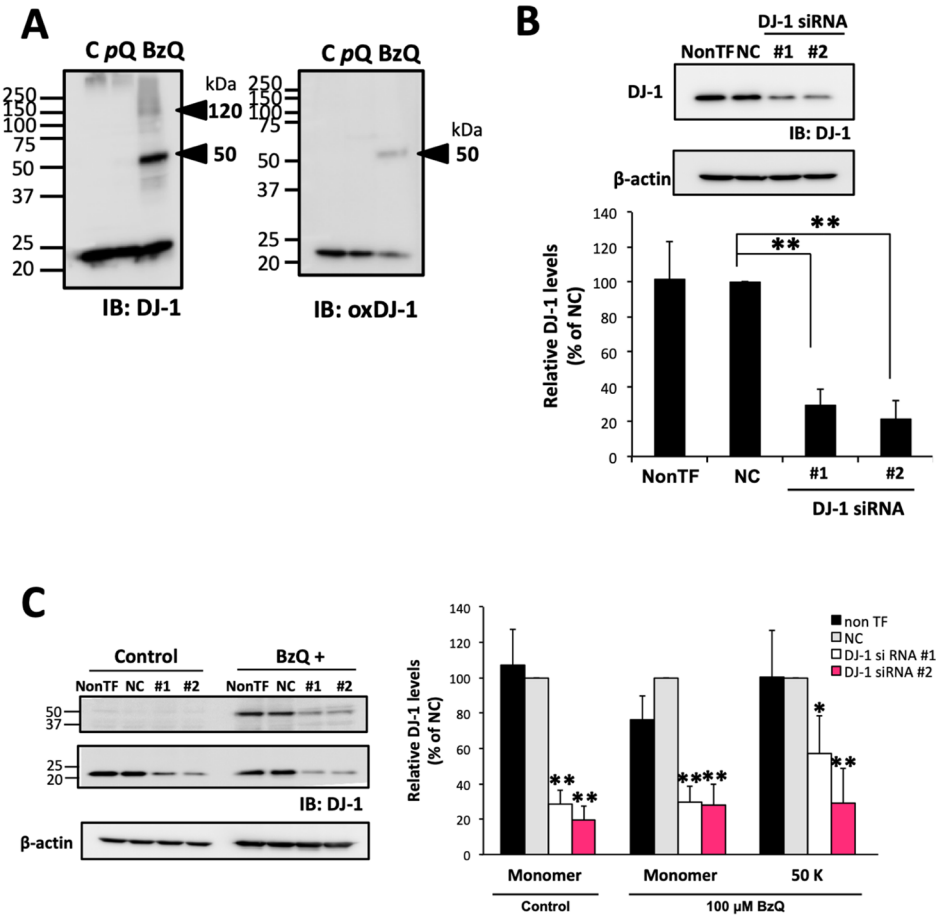

Figure 6. Formation of a covalently bound DJ-1 and oxDJ-1 by arylating quinones in SH-SY5Y cells. (A) Human neuroblastoma SH-SY5Y cells were treated with 600 μM *para*-quinone (*p*Q) and 100 μM BzQ for 3 h, and then whole cell lysates were subjected to western blotting under reducing condition. An obvious HMW DJ-1 band with a covalent bond, particularly at the 50 kDa band, was detected by western blot analysis using anti-DJ-1 and oxDJ-1 Ab. (B,C) Effect of DJ-1-siRNA on the formation of a covalently bound DJ-1 by BzQ exposure. SH-SY5Y cells were treated with DJ-1 siRNA #1 and #2 or nonspecific RNA (negative control, NC), and whole cell lysate was analyzed by western blotting. Non-TF: nontransfection. DJ-1 siRNA-treated cells were treated with 100 μM BzQ for 3 h. $^{**}P < 0.01$, $^{*}P < 0.05$, vs NC, Tukey-ANOVA. The specificity of the HMW band was further confirmed by DJ-1-deficient SH-SY5Y cells, which were prepared by a siRNA method.

covalent bond, particularly at the 50 kDa band, was detected (Figure 6A). OxDJ-1 blotting also showed that the HMW band at 50 kDa contains oxDJ-1 (Figure 6A). Quinone treatments were extended to 6 h, but no obvious change of HMW band levels was observed (Supporting Information Figure S3). A concentration-dependent study of BzQ suggests that the 50 kDa HMW band forms in a concentration-dependent manner from 50 μM BzQ treatment (Supporting Information Figure S4). The specificity of the HMW band was further confirmed by DJ-1-deficient SH-SY5Y cells, which were prepared by a siRNA method (Figure 6B). In nontransfected and negative control oligo-transfected cells, the formation of HMW DJ-1 at 50 kDa was observed by BzQ treatment, whereas this HMW band was significantly decreased in DJ-1 siRNA-treated cells (Figure 6C).

Atomic Force Microscope Observation of the Polymer Form of oxDJ-1. Finally, we investigated the structural characterization of DJ-1/oxDJ-1 recombinant protein by AFM, which can observe the structure of biological samples at a nanometer scale in a state close to the nature in various environments, including liquid.[30] In the AFM analysis of DJ-1 recombinant protein, we observed that DJ-1 protein had a uniform shape in general and partly had some high/large molecules (as indicated by arrows, Figure 7A). The enlarged AFM image showed that approximately 70−80% of the molecules had a cylindrical shape (as indicated by a dotted line, Figure 7A). It is thought that this AFM image of DJ-1 molecule was consistent with the reported X-ray crystal structure of the DJ-1 dimer. In addition, the remaining 20−30% was lower than this cylindrical molecule and diffusing vigorously (Supporting Information S5 Movie_DJ-1), which suggests the presence of a monomer form of DJ-1.

In the case of AFM analysis of oxDJ-1 recombinant protein, we found that the oxDJ-1 protein had a nonuniform shape with variations in molecular height compared to that of DJ-1 (Figure 7B). Focusing on large molecules, we found two distinct structures: a tetramer structure and a ring structure with an approximate hexamer (indicated by arrows, Figure 7B). The frequency of a tetramer was higher than the ring structure; these two forms were not observed in DJ-1, which suggests the oxidation-dependent formation. The polymer form of oxDJ-1 recombinant was fractionated by gel chromatography and subjected to AFM analysis; however, the AFM observation was similar and it appears that these polymer forms are partly broken by the procedure to bind oxDJ-1 protein to the mica surface. The further observation of the tetramer structure suggests several variants of the oxDJ-1 polymer form (Figure 7C). These possibilities are shown with

DOI: 10.1021/acsomega.9b00324
ACS Omega 2019, 4, 9603−9614

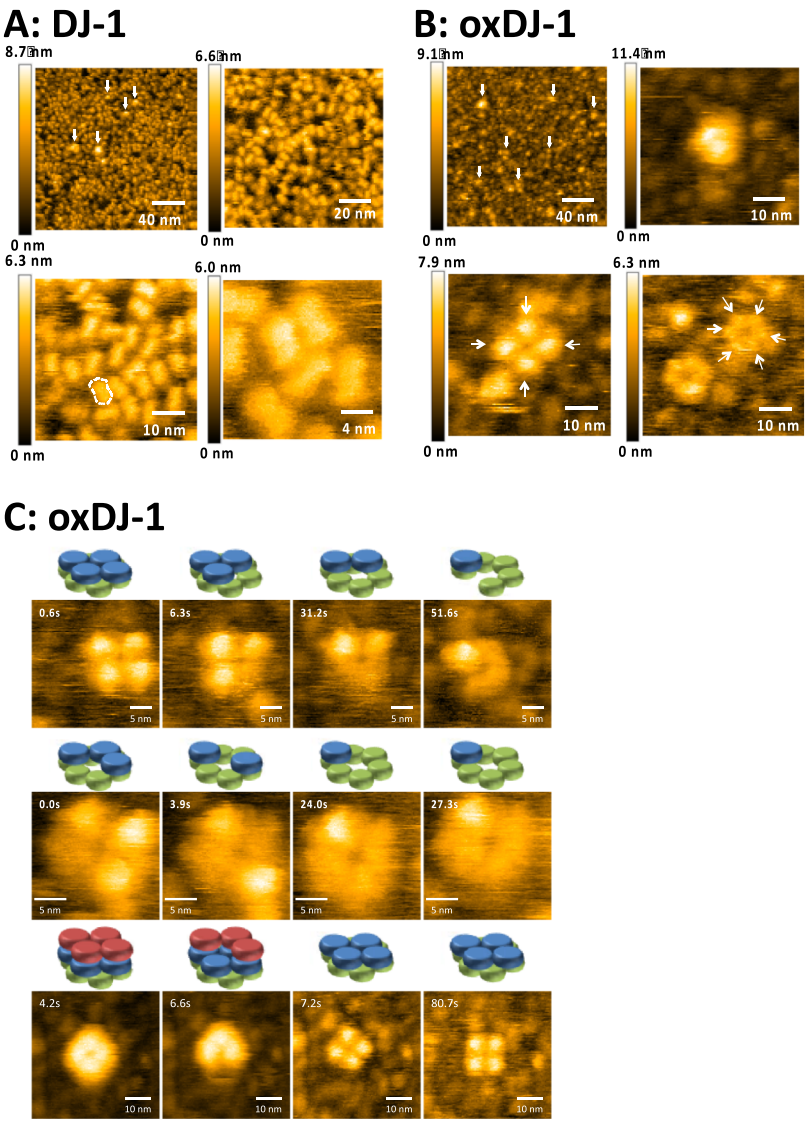

Figure 7. Atomic force microscopy observation of DJ-1 and oxidized DJ-1 recombinant proteins. (A) Purified DJ-1 recombinant protein was observed by AFM. Scale bars are shown in each picture. Some high/large molecules are indicated by each single arrow, and typical DJ-1 molecule with a cylindrical shape is indicated by a dotted line. (B) AFM image of purified oxDJ-1 recombinant protein is shown. Some high/large molecules are indicated by each single arrow. The enlarged AFM image of oxDJ-1 with a tetramer or the ring structure is indicated by the arrows. (C) Several variants of the oxDJ-1 polymer form are shown with a schematic diagram. Movies of these observations are shown in Supporting Information S6 and S7 Movie_oxDJ-1.

a schematic diagram. One is the tetramer or the ring under the tetramer (Figure 7C). During AFM observation, the tetramer was broken and then the ring structure and/or tetramer were appeared (Supporting Information S6 and S7 Movie_oxDJ-1). These observations suggest the dynamic properties of the oxDJ-1 polymer.

DISCUSSION

In the present study, we have revealed the formation of a polymer form of oxDJ-1 via several types of binding, such as disulfide bonds and noncovalent and covalent binding other than disulfide. The recombinant oxDJ-1 protein used in the present study was prepared by H_2O_2 treatment of human DJ-1-expressing *E. coli*.[18] The cells were lysed and subjected to a purification procedure. This oxidation method prepared Cys106-oxDJ-1 successfully, and Cys46 and Cys53 were not oxidized to Cys-SO_2H and/or Cys-SO_3H.[18,21,31] We observed that direct treatment of DJ-1 recombinant protein with H_2O_2 resulted in the random oxidation of all Cys residues,[18,21] which suggested the preferential oxidation of Cys106 in intracellular fluid. Polymer formation was not observed in the nonoxidized form of DJ-1, which indicated the significance of Cys106 oxidation. Further, the treatment of the polymer form of oxDJ-1 with reducing agents, such as GSH, dissolved its complex, which indicated the critical role of disulfide bond formation. It has been shown that the oxidation of Cys106 proceeded under GSH depletion;[14] therefore, the possibility was considered that severe deficiency of thiols generates the polymer form of oxDJ-1.

A physiological concentration of GSH could resolve the polymer form of oxDJ-1 and glutathionylation of Cys residue was observed in HMW oxDJ-1 by LC−MS/MS analysis and western blotting. Glutathionylated Cys46 and Cys53 were mainly observed in the covalently bound dimer form of oxDJ-1, and glutathionylation was not detected in the monomer

DOI: 10.1021/acsomega.9b00324
ACS Omega 2019, 4, 9603−9614

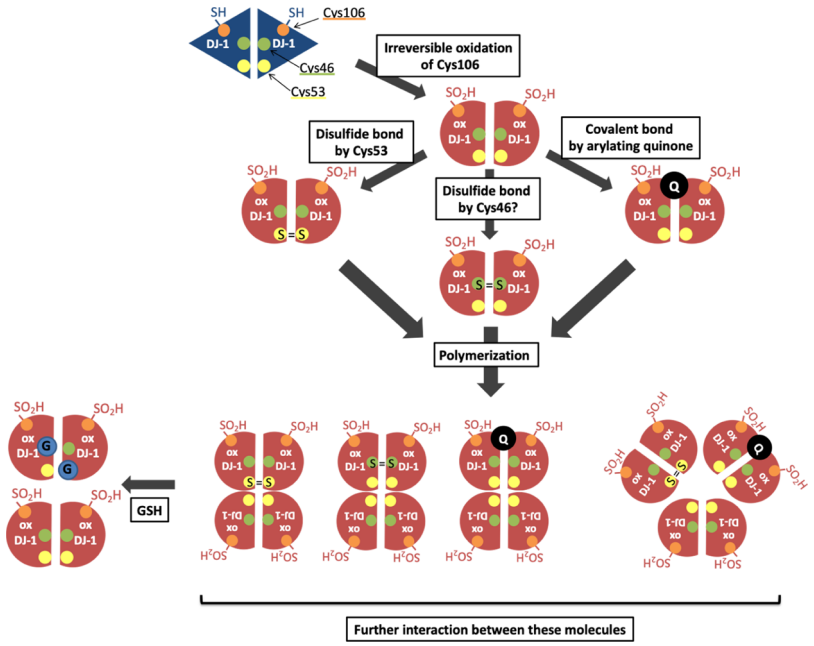

Figure 8. Polymerization and depolymerization of oxidized DJ-1. A possible scheme for the formation of polymer oxDJ-1 and its depolymerization by glutathionylation is shown.

form of oxDJ-1 under the denaturing condition of SDS-PAGE, which suggests that GSH bonded to Cys46 and Cys53 might be removed by further reaction with GSH. Cys46 and Cys53 are close to the dimerization interface.[16,32] Cys53 is present in the surface of DJ-1 and forms disulfide bonds between DJ-1 molecules, whereas Cys46 is located inside the protein and contributes to its stability in maintaining the basic structure of DJ-1.[9,33] Therefore, glutathionylation of both Cys46 and Cys53 in the covalently bound dimer form of oxDJ-1 shows that these Cys residues appeared on the oxDJ-1 surface and reacted with GSH, suggesting the change of three-dimensional structure of oxDJ-1. Multiple disulfide formation was suggested by the results of Figure 1A. Not only Cys53 but also Cys46 contribute to the disulfide bond formation of the oxDJ-1 polymer. Mutant analysis showed the indispensable role of Cys46 in polymer formation; that is, Cys46 appears to contribute to both protein stabilization and disulfide formation in oxDJ-1.

We found that electrophilic *p*Q and BzQ proceed to form HMW DJ-1 with covalent bonds other than disulfide bond in the in vitro experiment. These quinones could induce polymerization of both DJ-1 and oxDJ-1 recombinants, whereas it was found that oxDJ-1 preferentially formed HMW form compared with DJ-1. Electrophiles react not only with Cys but also with other nucleophilic amino acids such as Lys and His.[34,35] Although amino acid residue reacting with *p*Q and BzQ was not identified, there is a possibility that these electrophiles directly react with the polymer form of oxDJ-1 and stabilize its structure. It has been reported that DJ-1 is covalently modified by dopamine quinones and that Cys53 is the most reactive and forms a covalent dimer in SH-SY5Y cells, whereas Cys46 is not reactive.[17,36] In the cellular experiment in the present study, the covalently bound HMW DJ-1 was found to be formed by BzQ treatment. The band (50 kDa) was mainly detected as the HMW DJ-1/oxDJ-1 with a covalent bond in BzQ-treated SH-SY5Y-cells (Figure 6A), whereas 60 and 45 kDa bands were detected in BzQ-treated recombinant DJ-1/oxDJ-1 (Figure 5B). The former is the products from endogenous DJ-1/oxDJ-1 inside the cells, whereas the latter was generated by the direct reaction of DJ-1/oxDJ-1 recombinant protein with BzQ. It is considered that this experimental condition is one of the causes for the difference between these. Furthermore, the components of these experiments were different, and it is considered that the HMW DJ-1/oxDJ-1 with a covalent bond was formed with other proteins in BzQ-treated SH-SY5Y-cells. Western blot analysis using the oxDJ-1-specific antibody revealed that the HMW DJ-1 complex contained oxDJ-1. The oxDJ-1 monomer was obviously decreased by BzQ treatment, which suggested the preferential polymer formation of oxDJ-1.

AFM is a powerful tool for observing protein structures in solution;[30,37] thus, supporting observations were obtained for the biochemical analysis described above in the present study. In the DJ-1 recombinant protein, the dimer form was mainly observed, whereas several forms including polymer was observed in oxDJ-1 recombinant protein. The AFM observation showed the presence of tetramer and hexamer oxDJ-1, and some of these were stacked. It is also interesting to note that these polymers were dynamic and are changing during the observation. These observations also imply the stabilization of the oxDJ-1 polymer by covalent bindings, such as disulfide bonds. The polymer oxDJ-1 was fractionated by gel chromatography and subjected to AFM analysis; however, the observation was unchanged, which suggested that the fixation of proteins to the mica surface might affect observation.

In conclusion, this study clearly showed the formation of the polymer form of oxDJ-1 with disulfide bond and noncovalent and covalent binding other than disulfide. The possible model of polymerized oxDJ-1 is shown in Figure 8. These structural changes of DJ-1 with oxidation might relate to the physiological role of DJ-1 on redox chaperone and antioxidative defense.

DOI: 10.1021/acsomega.9b00324
ACS Omega 2019, 4, 9603−9614

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00324.

Concentration-dependent study of hydrogen peroxide for the formation of oxDJ-1 with high molecular weight; time- and concentration-dependent study for the glutathionylation of oxDJ-1; time- and concentration-dependent study for the formation of a covalently bound DJ-1 in SH-SY5Y cells; and concentration-dependent study for the formation of a covalently bound DJ-1 in SH-SY5Y cells (PDF)

Movie of AFM analysis of DJ-1 (MOV)

Movie of AFM analysis of oxDJ-1 (MOV)

Movie of AFM analysis of oxDJ-1 (MOV)

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Author Contributions

Y.S. designed the experiments. M.K., K.M., H.U., and A.K. performed the protein and cellular experiments and analyzed the corresponding results. T.H. and H.K. performed the AFM experiments and analyzed the results. Y.S. wrote the paper with H.K. and N.N.

Funding

This study was supported in part by part by the Michael J. Fox Foundation, JSPS KAKENHI grant number 25670084, and MEXT-Supported Program for the Strategic Research Foundation at Private Universities.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. T. Ando and Prof. N. Kodera (Kanazawa University) for providing us with experimental instruments. This work was supported by Kanazawa University CHOZEN project and World Premier International Research Center Initiative (WPI), MEXT, Japan.

■ ABBREVIATIONS

AFM, atomic force microscope; BzQ, benzoquinone; DTT, dithiothreitol; GSH, glutathione; H_2O_2, hydrogen peroxide; 2-ME, mercaptoethanol; oxDJ-1, Cys106-oxidized DJ-1; *p*Q, 2-hydroxy-5-(2-aminoethyl)-1,4-benzoquinone; 6-OHDA, 6-hydroxydopamine

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