Formation of a Stabilized Cysteine Sulfinic Acid Is Critical for the Mitochondrial Function of the Parkinsonism Protein DJ-1*

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The formation of cysteine-sulfinic acid has recently become appreciated as a modification that links protein function to cellular oxidative status. Human DJ-1, a protein associated with inherited parkinsonism, readily forms cysteine-sulfinic acid at a conserved cysteine residue (Cys106 in human DJ-1). Mutation of Cys106 causes the protein to lose its normal protective function in cell culture and model organisms. However, it is unknown whether the loss of DJ-1 protective function in these mutants is due to the absence of Cys106 oxidation or the absence of the cysteine residue itself. To address this question, we designed a series of substitutions at a proximal glutamic acid residue (Glu18) in human DJ-1 that alter the oxidative propensity of Cys106 through changes in hydrogen bonding. We show that two mutations, E18N and E18Q, allow Cys106 to be oxidized to Cys106-sulfinic acid under mild conditions. In contrast, the E18D mutation stabilizes a cysteine-sulfenic acid that is readily reduced to the thiol in solution and in vivo. We show that E18N and E18Q can both partially substitute for wild-type DJ-1 using mitochondrial fission and cell viability assays. In contrast, the oxidatively impaired E18D mutant behaves as an inactive C106A mutant and fails to protect cells. We therefore conclude that formation of Cys106-sulfinic acid is a key modification that regulates the protective function of DJ-1.

Reactive cysteine residues are susceptible to a variety of covalent modifications that are increasingly recognized as a major means of regulating the activities of many proteins (1). Cysteine forms three different species by the direct addition of oxygen; cysteine-sulfenic (-SOH), -sulfinic (-SO2H), and -sulfonic (-SO3H) acid. Because cysteine can be oxidized to three distinct species, each with different structural and chemical properties, cysteine oxidation is a versatile way for reactive oxygen species (ROS)4 to alter the activity of a protein. Of the three oxidation products of cysteine, only cysteine-sulfenic acid is readily reduced to the thiol under physiological conditions. However, enzymes that catalyze the ATP-dependent reduction of over-oxidized peroxiredoxins containing cysteine-sulfenic acid to cysteine have been discovered and characterized (2, 3). With reversibility comes the potential for cysteine-sulfinic acid modifications to modulate the function of various target proteins in a redox-dependent manner. Therefore, at least in some proteins, cysteine-sulfenic acid should be regarded as a post-translational modification rather than simply a type of protein damage.

As expected, many of the proteins that are modified by cysteine oxidation are involved in the oxidative stress response or in the maintenance of cellular redox homeostasis. Of these proteins, DJ-1 has special importance in understanding the role of regulatory cysteine oxidation in neuronal survival. Loss of function mutations in DJ-1 are a rare cause of early onset recessive parkinsonism (4, 5), although the exact function of DJ-1 is unclear. The protein is part of the large DJ-1 superfamily with evolutionarily conserved members in bacteria, fungi, plants, and animals (6, 7). A number of activities have been proposed for human DJ-1, including a weak peroxiredoxin-like activity (8), a chaperone activity (9, 10), and translational (11, 12) and transcriptional regulation (13, 14).

The best-established aspect of DJ-1 function is its ability to respond to oxidative stress. DJ-1 is modified under oxidative stress both in vitro and in vivo by oxidation of a very highly conserved cysteine residue (Cys106 in human DJ-1) to form a cysteine-sulfinic acid (Cys106-SO2OH) (15). Several studies have shown that of the three cysteine residues in human DJ-1, Cys106 is the most prone to oxidative modification (8, 16, 17). In addition, Cys106 has a low pKa value of 5.4 and therefore exists almost exclusively as the highly reactive cysteine thiolate anion at physiological pH (18). Replacement of Cys106 with other

4 The abbreviations used are: ROS, reactive oxygen species; ANOVA, analysis of variance; DTT, dithiothreitol; FRAP, fluorescence recovery after photobleaching; LC-MS/MS, liquid chromatography-mass spectrometry; MEF, mouse embryonic fibroblast; VDAC1, voltage-dependent anion channel 1; WT, wild type.
Cysteine Oxidation in DJ-1 Function

amino acids in DJ-1 results in a loss of protective activity against oxidative stressors in a number of systems (15, 19, 20).

We have therefore previously suggested that formation of cysteine-sulfenic acid is required for DJ-1 to exert its protective effects (15). However, the substitution of the equivalent cysteine residue in *Drosophila melanogaster* DJ-1 with aspartic acid (C104D in *Drosophila*) inactivates DJ-1, suggesting that the simple addition of a negatively charged residue at this position is insufficient to support DJ-1 function (21). As a consequence of the need for direct mutation of Cys106 in these studies, it is unclear whether the cysteine residue itself or its oxidation to a sulfenic acid is critical for the protective activity of DJ-1.

We have previously shown using x-ray crystallography that Cys\(^{106}\)-SO\(_2\) interacts with nearby residues, most notably the highly conserved Glu\(^{18}\) residue (15). In reduced DJ-1, the carboxylic acid side chain of Glu\(^{18}\) is protonated and donates a hydrogen bond to Cys\(^{106}\), which depresses the thiol pK\(_a\) value (18). Therefore, we hypothesized that modifying the environment around the side chain of Cys\(^{106}\) could decouple the oxidation propensity and pK\(_a\) of Cys\(^{106}\) without changing the cysteine residue itself. In the present study, we have tested this approach by characterizing the effects of Glu\(^{18}\) mutations on the oxidative propensity and cytoprotective activity of Cys\(^{106}\). Our results show that the formation of Cys\(^{106}\)-SO\(_2\) is critical for DJ-1 to protect cells against mitochondrial damage. In addition, this targeted mutagenesis strategy could be used to manipulate the oxidation state of other cysteine redox-regulated proteins of known structure.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Wild-type and mutant DJ-1 variants were cloned between the Ndel and XhoI sites of the bacterial expression vector pET21a and expressed in BL21(DE3) *Escherichia coli* (Novagen). All proteins were expressed with a noncleavable C-terminal His\(_6\) tag (vector-derived sequence LEHHHHHHH) for purification by metal affinity Ni\(^{2+}\)-nitrilotriacetic acid chromatography. Bacteria were grown in LB medium supplemented with 100 \(\mu\)g/ml ampicillin at 37 °C with shaking. Once the A\(_{600}\) of the culture reached 0.5–0.7, it was equilibrated at 20 °C for 3 h prior to induction of protein expression by the addition of 0.1 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside. The induced culture was incubated at 20 °C with shaking overnight and harvested by centrifugation. Cell pellets were stored at −80 °C until needed.

Recombinant His\(_6\)-tagged proteins were purified using Ni\(^{2+}\)-nitrilotriacetic acid His-Select resin (Sigma). Eluted DJ-1 protein was dialyzed against storage buffer (25 mM HEPES, pH 7.5, 100 mM KCl, 1 mM dithiothreitol (DTT)), loaded on an equilibrated High Q anion exchange column, and collected in the flow-through, since contaminants bind to the anion exchange resin under these conditions. Purified DJ-1 was concentrated to 1 mM (\(c_{280} = 4000 \text{ M}^{-1} \text{ cm}^{-1}\)) and ran as a single band on overloaded Coomassie-stained SDS-PAGE. The purified protein was supplemented with 2 mM DTT, snap-frozen on liquid nitrogen, and stored at −80 °C.

**Hydrogen Peroxide Titration of DJ-1**—The in vitro oxidative susceptibility of Cys\(^{106}\) in DJ-1 was assayed by titration with several molar ratios of H\(_2\)O\(_2\) to protein monomer. Thawed DJ-1 was rapidly exchanged into extensively degassed nanopure water using a centrifugal spin column containing P6-DG desalting resin (Bio-Rad). Control experiments using degassed buffered solutions (10 mM potassium phosphate, pH 7.4) instead of water showed similar oxidative behavior of DJ-1 but gave noisier mass spectrometry data due to the presence of buffer salts. Freshly diluted H\(_2\)O\(_2\) was added to DJ-1 in molar ratios of 0:1, 0.5:1, 1:1, 2.5:1, 5:1, 7.5:1, and 10:1 H\(_2\)O\(_2\)/protein monomer and incubated on ice for 30 min. Excess H\(_2\)O\(_2\) was removed using a P6-DG centrifugal desalting column, and the protein samples were immediately supplemented with 5 mM DTT, frozen on liquid nitrogen, and stored at −80 °C. DTT, which cannot reduce cysteine-sulfenic acid, was added to ensure that the sample did not further oxidize during sample handling for mass spectrometric analysis. Previous results have indicated that only Cys\(^{106}\) is oxidized by these conditions *in vitro* (10).

**Mass Spectrometry of Oxidized DJ-1**—Intact DJ-1 protein was analyzed by liquid chromatography-mass spectrometry (LC-MS/MS) with a 4000 Q-trap mass spectrometer (ABSS) using a turbo ion spray source probe at the University of Nebraska Redox Biology Center Mass Spectrometry Core Facility. Protein samples (20 \(\mu\)l) were loaded onto a C18 reverse phase column using a PE 200 Autosampler. A SCL-10A high performance liquid chromatography system (Shimadzu) was used for room temperature gradient elution at a flow rate of 100 ml/min in 5 min by using a linear gradient from 0.3% formic acid in water (Solvent A) to 0.3% formic acid in acetonitrile (Solvent B). The elution time for DJ-1 was about 4 min. Data were acquired and processed using Analyst 1.4.1 software in Q1 (quadrupole one)-positive ion mode, and the m/z range of 880–1120 atomic mass units was scanned in 4 s. The total run time for each sample was 10 min. The molecular mass of protein was generated from several multiply charged peaks using the Bayesian Protein Reconstruct option in BioAnalyst 1.4 software. For all experiments, only two species were observed: the reduced protein and an adduct at +32 atomic mass units, corresponding to the Cys\(^{106}\)-SO\(_2\) form of DJ-1.

**Crystallization, Data Collection, and Processing**—For all crystallization experiments, DJ-1 at 1 mM (20 mg/ml) in storage buffer was crystallized using the hanging drop vapor diffusion method with drops containing 2 \(\mu\)l of protein and 2 \(\mu\)l of reservoir solution. Crystals of E18Q DJ-1 in space group P2\(_1\) were grown in 2–5 days at room temperature using a reservoir solution of 30% polyethylene glycol 400, 50 mM HEPES, pH 7.5, 125 mM sodium citrate. Crystals of E18Q DJ-1 in space group P3\(_2\) were grown from 1.3–1.5 M sodium citrate, 50 mM HEPES, pH 7.5. For crystals of E18Q DJ-1, the 30% polyethylene glycol 400 in the mother liquor was sufficient for cryoprotection. E18Q DJ-1 crystals were cryoprotected in 2.4 M sodium malonate, pH 7.0 (22). All crystals were transferred to nylon loops and cryocooled by direct immersion into liquid nitrogen.

Diffraction data were collected at the Advanced Photon Source, BioCARS beamline 14BM-C using 13.776 keV (0.9 Å) incident x-rays and an ADSC Q315 detector. Single crystals maintained at 100 K were used for the collection of each data set, and both data sets were collected in separate high and low resolution passes with differing exposure times, oscillation...
Cysteine Oxidation in DJ-1 Function

| TABLE 1  | Data collection and refinement statistics | Oxidized E18D | Oxidized E18Q |
|----------|----------------------------------------|--------------|---------------|
| Resolution (Å) | 30.120 | 30.115 |
| No. of reflections | 75,731 | 85,756 |
| Rmerge (Rwork) for Fhkl > 4σ(Fhkl) (%) | 12.2; 11.0 | 11.6; 10.0 |
| Rmerge (Rwork) for Fhkl > 4σ(Fhkl) (%) | 15.5; 14.2 | 14.5; 13.6 |
| Root mean square deviations | | |
| Bond lengths (Å) | 0.014 | 0.015 |
| Bond angle 1–3 distances (Å) | 0.030 | 0.030 |

Values in parentheses are for the highest resolution shell.

Immunocytochemistry and Western Blotting—Mitochondrial fractions were prepared using a commercially available mitochondrial isolation kit (Pierce) as directed. Mitochondrial fractions were then stripped of all loosely associated proteins using 20 μM sodium carbonate in HEPEs buffer for 30 min on ice, followed by ultracentrifugation at 60,000 × g for 30 min. Subcellular fractions were Western blotted and probed with a V5-specific antibody (Invitrogen) to visualize transfected DJ-1. Enrichment of mitochondria was confirmed by simultaneously propping the same blots with monoclonal antibodies to the voltage-dependent anion channel (VDAC1; Calbiochem clone 31HL, 1:4000) and to cytosolic β-actin (clone AC-15, 1:5000; Sigma). Immunostaining for tagged DJ-1 was performed as described (30). Paraquat and rotenone were purchased from Sigma.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed as previously described (15). Samples were obtained from human M17 neuroblastoma cells that were transiently transfected with various DJ-1 Glu18 mutants. For all DJ-1 constructs, stop codons introduced immediately following the V5 tag were used to avoid changes in pl due to the His6 tag. Immobiline DryStrips (11 cm) with linear separation in the pH range 4–7 (GE Healthcare) were used for isoelectric focusing.

Fluorescence Recovery after Photobleaching (FRAP) Measurement of Mitochondrial Fragmentation—FRAP was performed using 20 μM sodium carbonate in HEPEs buffer for 30 min on ice, followed by ultracentrifugation at 60,000 × g for 30 min. Subcellular fractions were Western blotted and probed with a V5-specific antibody (Invitrogen) to visualize transfected DJ-1. Enrichment of mitochondria was confirmed by simultaneously propping the same blots with monoclonal antibodies to the voltage-dependent anion channel (VDAC1; Calbiochem clone 31HL, 1:4000) and to cytosolic β-actin (clone AC-15, 1:5000; Sigma). Immunostaining for tagged DJ-1 was performed as described (30). Paraquat and rotenone were purchased from Sigma.

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FRAP), \((\text{NSPB}_t - \text{background})/\text{NSPB}_i\), where NSPB is the nonspecific photobleaching, the subscript \(t\) refers to the signal at time \(t\) and the subscript \(i\) refers to the initial signal before photobleaching.

Cell Viability—M17 cells were seeded onto glass coverslips and transiently transfected with V5-tagged DJ-1 variants using Lipofectamine 2000 (Invitrogen) for 48 h and then either left untreated or exposed to rotenone (200 nM) for 24 h. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.1% Triton X-100, and stained using monoclonal antibody to V5 (1:500; Invitrogen), followed by anti-mouse IgG conjugated to Alexa-Fluor488 (Molecular Probes). Nuclei were counterstained with Hoechst 33342 (Roche Applied Science), and coverslips were mounted using ProLong gold (Molecular Probes). For each experiment, three randomly selected microscope fields (between 26 and 75 cells/field) were counted by an observer blind to the DJ-1 transfection status of the cells. Each experiment was then repeated three times, and statistical analysis was performed on the combined results. Cell viability was expressed as the percentage of transfected (V5-positive) cells that had intact nuclei compared with all transfected cells. For base line viability in the same cultures, we counted three fields of untransfected cells for base line viability in the same cultures, we counted three fields of untransfected cells in the same way, where viability was expressed as percentage of visible nuclei per field.

RESULTS
Substitutions at Residue 18 Alter the Oxidation Propensity of Cys\(^{106}\)—Three substitutions were made at residue 18 in human DJ-1 for this study; E18Q, E18D, and E18N. The 1.15 Å resolution crystal structure of oxidized E18Q DJ-1 superimposes nearly perfectly (Ca root mean square deviation = 0.08 Å) with wild-type DJ-1 (Protein Data Bank code 1SOA). In addition, like the wild-type protein (15), E18Q DJ-1 oxidizes during crystal growth to form Cys\(^{106}\)-SO\(^2-\) (Fig. 1B). There are a few small structural differences between E18Q and wild-type DJ-1 near the site of mutation, the most notable being the lengthening of the hydrogen bond between residue 18 and Cys\(^{106}\)-SO\(^2-\) to 2.71 Å (Fig. 1C). In wild-type DJ-1, the 2.47-Å hydrogen bond between the protonated Glu\(^{18}\) carboxylic acid side chain and Cys\(^{106}\)-SO\(^2-\) is an unusually short and presumably a very strong interaction (15). The 0.24-Å increase in hydrogen bond length in E18Q Cys\(^{106}\)-SO\(^2-\)
Cysteine Oxidation in DJ-1 Function

DJ-1 is accommodated by the correlated displacements of Cys106-SO2/H11002 and Gln18 away from each other (Fig. 1C). The E18Q substitution is very structurally conservative, altered oxidation for Cys106 in E18Q (see below) can be attributed solely to the changes in the hydrogen bond between residue 18 and Cys106.

The crystal structure of oxidized E18N was determined in a previous study and showed that Cys106 is rapidly oxidized to the sulfenic acid, even when these crystals were grown from solutions containing 10 mM DTT that favored reduced Cys106 in wild-type DJ-1 (18). Asn18 was discretely disordered in this structure but made a similar set of hydrogen bonds with the sulfinic acid, even when these crystals were grown from solutions containing 10 mM DTT that favored reduced Cys106.

Effects of Glu18 Substitutions on Cys106 Oxidation in Vitro and in Vivo— The ability of each of the mutant DJ-1 proteins to oxidize at Cys106 in solution was determined using controlled titration of hydrogen peroxide and electrospray mass spectrometry. Each mutant oxidizes less robustly than wild-type DJ-1 at higher peroxide ratios; however, E18N DJ-1 is ~50% oxidized to Cys106-SO2 at all molar ratios of H2O2/DJ-1, including the control with no added H2O2 (Fig. 2A). Multiple preparations of E18N display this behavior, even when the protein is freshly purified and immediately used, consistent with difficulties encountered in purifying and crystallizing reduced E18N DJ-1. E18Q DJ-1 shows a mild impairment of Cys106 oxidation at higher peroxide ratios in solution (Fig. 2A), although

FIGURE 2. Substitutions at position 18 of DJ-1 impact Cys106-SO2 formation. A, oxidation of Cys106 in vitro to Cys106-SO2. Mass spectrometry was used to monitor the oxidation of DJ-1 as a function of hydrogen peroxide concentration in solution. The fraction of protein oxidized was calculated as a ratio of the integrated area of the oxidized protein peak to the total area of both the oxidized and reduced peaks. A comparison of the oxidation curves of these proteins shows that every substitution at position 18 results in diminished oxidation compared with the wild-type protein, although the extent of this diminution varies among the three substitutions. E18D abolishes the ability of Cys106 to be oxidized to cysteine-sulfenic acid, and E18N oxidizes very easily at low H2O2 levels. B, oxidation of DJ-1 in vivo. Human M17 neuroblastoma cells were transfected with V5-tagged versions of the indicated DJ-1 mutants (wild type [WT], E18N, E18Q, E18D, and C106A, from top to bottom) and exposed to 300 μM paraquat for 24 h. Protein extracts were separated on two-dimensional gels and blotted for DJ-1. Estimated pI values for each isoform are indicated above the blots. Images are representative of duplicate experiments for each construct.

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Cysteine Oxidation in DJ-1 Function

at low molar ratios of H₂O₂/DJ-1 (0.5:1), Cys¹⁰⁶-SO₂⁻ formation was similar to that of wild-type protein.

In contrast, mass spectrometry of E18D DJ-1 shows no oxidized protein, even at a 10:1 molar ratio of H₂O₂/DJ-1 (Fig. 2A). Because E18D DJ-1 forms the easily reduced Cys¹⁰⁶-SO⁻ species, we propose that the DTQ quenching step in the oxidation reaction (see “Experimental Procedures”) may reduce the Cys¹⁰⁶-SO⁻ observed in the crystal structure of E18D DJ-1 (Fig. 1D). Therefore, the E18D substitution stabilizes an easily reduced oxidation product at Cys¹⁰⁶ that is expected to revert to the thiol in the reducing environment of the cytoplasm.

To evaluate the oxidation of these DJ-1 variants in an in vitro system, we transfected the same DJ-1 mutants into M17 neuroblastoma cells, subjected them to oxidative stress using 300 μM paraquat, and monitored DJ-1 mobility on two-dimensional gels. Using V5-tagged DJ-1 to facilitate electrophoretic separation from endogenous DJ-1, we observed a pattern of multiple pI isoforms that is similar to that found for the endogenous protein (15) but with a pI increase of ~0.4 units due to the V5 tag on DJ-1. Wild-type DJ-1 showed two major isoforms and other minor oxidized species (Fig. 2B). E18N and E18Q DJ-1 showed a greater amount of the 6.2 pI isoform (corresponding to the endogenous DJ-1 pI of 5.8) compared with the wild-type protein, suggesting increased sulfenic acid formation in these mutants. In contrast, both E18D and C106A showed primarily a single unoxidized pI isoform, with a small proportion of protein at pI = 6.5 (corresponding to the endogenous 6.1 isoform). These data support the in vitro finding that E18D is largely resistant to Cys¹⁰⁶ oxidation, whereas E18N and E18Q are somewhat more sensitive to oxidation than wild-type DJ-1.

Relative Cytosolic and Mitochondrial Pools of DJ-1 Are Influenced by Cys¹⁰⁶ Modification—DJ-1 is present in nuclear, cytoplasmic, and mitochondrial pools (8). In previous studies, DJ-1 has been shown to protect against mitochondrial damage, which is a major source of oxidative stress in eukaryotic cells (15, 21). We have previously suggested that the mitochondrial pool of DJ-1 responds to oxidative stress and that the oxidative response of DJ-1 correlates with recruitment of the protein to the mitochondria (15), although this has not been confirmed in other studies (34). Therefore, we sought to establish how mutation of Glu¹⁸ impacted recruitment of DJ-1 to mitochondria. We transfected DJ-1 variants into M17 neuroblastoma cells and exposed these cells to the oxidant paraquat at a concentration (300 μM) that did not affect cell viability. Using subcellular fractionation, we saw that a pool of wild-type DJ-1 was present in mitochondria and the amount of mitochondrial pools increased under oxidative stress (Fig. 3A).

We confirmed the mitochondrial enrichment of these fractionated samples by Western blot for VDAC1 and β-actin as markers for the mitochondria and cytosol, respectively (Fig. 3A). Relative mitochondrial enrichment of all samples was consistent between DJ-1 variants both in the presence or absence of oxidative stress. However, there is some residual contamination of mitochondrial fractions with the abundant

FIGURE 3. DJ-1 mutants are present in mitochondrial and cytosolic pools. A, M17 neuroblastoma cells were transfected with V5-tagged WT (lane 1), E18N (lane 2), E18Q (lane 3), E18D (lane 4), C53A (lane 5), or C106A (lane 6) DJ-1 variants. Cells were also subjected to oxidative stress by exposure to 300 μM paraquat (PQ) for 24 h as indicated. Cytosolic fractions (left, top two blots) or mitochondrial proteins retained after carbonate extraction (right, top two blots) were probed for V5-DJ-1. Mitochondrial enrichment was confirmed by simultaneously reprobing the same blots with monoclonal antibodies to VDAC1 and to cytosolic β-actin, as indicated. B, subcellular localization of the V5-tagged DJ-1 variants was verified using transiently transfected M17 neuroblastoma cells that were stained for V5 (green) and mitochondria using Mitotracker (red). Upper panels, untreated cells; middle panels, cells treated with 300 μM paraquat (PQ); lower panels, cells treated with 100 nM rotenone. The scale bar in the lower right panel of the merged images represents 10 μm and applies to all. To show mitochondrial morphology, a higher magnification view of the boxed areas of the rotenone-treated cells is shown in black and white below each set. Although cells transfected with WT, E18N, or E18Q DJ-1 maintained elongated and connected mitochondria in the presence of rotenone, cells transfected with E18D or C106A DJ-1 showed mitochondrial fragmentation.
enhanced by Cys106 oxidation, although oxidation of this residue indicates that mitochondrial localization of DJ-1 is supporting these observations (35). Considered together, these results are consistent with the hypothesis that mitochondrial localization of DJ-1 is required for protection against mitochondrial fragmentation and cell death—Oxidative and mitochondrial stressors can result in fragmentation of mitochondria due to stimulation of mitochondrial fission. The above experiments examining mitochondrial localization of DJ-1 provide some evidence that cells expressing wild-type but not E18D or C106A DJ-1 might protect against mitochondrial fission, presumably as a consequence of lowering cellular oxidative stress. Therefore, we investigated the ability of wild-type and Glu18 DJ-1 mutants to diminish mitochondrial fragmentation using FRAP of mitochondrially directed yellow fluorescent protein, which recovers well after photobleaching if the mitochondria are highly connected. We used DJ-1 knock-out MEFs for these experiments to avoid potential interference from endogenous DJ-1 protein. DJ-1 knock-out MEFs have grossly normal mitochondria (Fig. 4A) but lower FRAP mobile fraction values, indicating reduced mitochondrial connectivity (Fig. 4B and C). Mitochondrial connectivity was rescued by stable re-expression of human wild-type DJ-1, showing that fragmentation is due only to the absence of DJ-1 in these MEFs.

We used the FRAP assay to assess the ability of Glu18 mutations to increase mitochondrial connectivity using transient DJ-1 transfections of these MEFs. As before, wild-type DJ-1 improved FRAP recovery compared with empty vector (Fig. 4D). The Glu18 mutations showed variable ability to rescue mitochondrial localization of DJ-1 in the presence of Cys106 oxidation, although oxidation of this residue is not an absolute requirement for DJ-1 to associate with the mitochondria.

We confirmed the mitochondrial localization of DJ-1 using confocal microscopy (Fig. 3B). Exposure of cells to oxidative stress induced by exposure to either 300 μM paraquat or 100 nM rotenone caused an accumulation of wild-type DJ-1 in mitochondria. E18N and E18Q DJ-1 were clearly associated with mitochondria under basal conditions and remained so under oxidative stress. In contrast, E18D and C106A DJ-1 remained largely cytoplasmic under all conditions tested. In addition, during these experiments, we saw that treatment with rotenone and, to a lesser extent, paraquat was associated with a shorter, fragmented mitochondrial morphology (enlarged black and white images in Fig. 3B).

Cys106-Sulfenic Acid Formation Is Required for Protection against Mitochondrial Fragmentation and Cell Death—Oxidative and mitochondrial stressors can result in fragmentation of mitochondria due to stimulation of mitochondrial fission. The above experiments examining mitochondrial localization of DJ-1 provide some evidence that cells expressing wild-type but not E18D or C106A DJ-1 might protect against mitochondrial fission, presumably as a consequence of lowering cellular oxidative stress. Therefore, we investigated the ability of wild-type and Glu18 DJ-1 mutants to diminish mitochondrial fragmentation using FRAP of mitochondrially directed yellow fluorescent protein, which recovers well after photobleaching if the mitochondria are highly connected. We used DJ-1 knock-out MEFs for these experiments to avoid potential interference from endogenous DJ-1 protein. DJ-1 knock-out MEFs have grossly normal mitochondria (Fig. 4A) but lower FRAP mobile fraction values, indicating reduced mitochondrial connectivity (Fig. 4B and C). Mitochondrial connectivity was rescued by stable re-expression of human wild-type DJ-1, showing that fragmentation is due only to the absence of DJ-1 in these MEFs.

We used the FRAP assay to assess the ability of Glu18 mutations to increase mitochondrial connectivity using transient DJ-1 transfections of these MEFs. As before, wild-type DJ-1 improved FRAP recovery compared with empty vector (Fig. 4D). The Glu18 mutations showed variable ability to rescue the lack of endogenous DJ-1 in this assay, with E18N and E18Q being closer to wild-type DJ-1 than vector alone. In contrast, E18D (or C106A) DJ-1 transfectants did not rescue mitochondrial fragmentation and actually had a slightly lower mobile fraction value than vector alone. These results indicate that the Glu18 variants that support Cys106-SO2 formation (E18N and E18Q) can substitute for wild-type DJ-1, whereas those that do
Cysteine Oxidation in DJ-1 Function

FIGURE 5. DJ-1 Glu\textsuperscript{18} substitutions affect cellular resistance to rotenone-induced toxicity. A, nuclear morphology as a marker of rotenone-induced loss of cell viability. M17 neuroblastoma cells were transiently transfected with V5-tagged WT, E18N, E18Q, E18D, or C106A DJ-1 constructs, as indicated, and either left untreated (upper panels) or exposed to 200 nM rotenone for 24 h (lower panels). Cells were stained for V5 (green) and counterstained with Hoechst 33342 (blue) and scored as having intact nuclei (arrowheads) or fragmented/shrunken nuclei (arrows). The insets show examples of nuclei from the blue channel at higher magnification. The scale bar in the bottom right panel represents 20 \mu m and applies to all images. B, cells were transfected as in A with WT (green), E18N (magenta), E18Q (blue), E18D (cyan), and C106A (red) DJ-1 variants. Cell viability is expressed as the percentage of transfected (V5-positive) cells that had intact nuclei compared with all transfected cells. Each box plot represents data from three randomly selected microscope fields (between 26 and 75 cells/field) counted in each of three independent experiments for an overall \( n = 9 \) per construct. Horizontal lines, median values; boxes, upper and lower quartiles; range bars, the range of percentage viabilities for all fields counted. The dotted line represents mean viability counted in untransfected cells from the same cultures, with the shaded box indicating one S.D. value (84.6 \pm 2.7\% viability, \( n = 9 \) fields, mean of 28 cells/field). Differences were analyzed comparing the indicated untreated and rotenone-treated cells for the same construct; \( * \), \( p < 0.05 \) by one-way ANOVA (\( p < 0.001 \) overall) with Newman-Kuel's post hoc tests. \( m \), not significant.

not support sulfinic acid formation at Cys\textsuperscript{106} (E18D and C106A) are inactive.

Several studies have reported that DJ-1 can protect cells against mitochondrial toxins in a variety of \textit{in vitro} and \textit{in vivo} models (15, 20). We have shown previously that human dopaminergic M17 neuroblastoma cells show enhanced resistance to complex I inhibitors (15) when transfected with wild-type DJ-1 but not the oxidation-deficient C106A mutant. We investigated the ability of Glu\textsuperscript{18} DJ-1 variants to protect against rotenone-induced cell death using nuclear morphology as a measure of cell viability. M17 neuroblastoma cells that were transiently transfected with wild-type or Glu\textsuperscript{18} mutant DJ-1 were stained, visually assayed, and classified as either having intact rounded nuclei or having a shrunken and fragmented nuclear morphology (Fig. 5A). Using these criteria, the basal viability of untransfected, untreated cells was 84.6 \pm 2.7\% (mean \( \pm \) S.D., \( n = 9 \) fields). We counted transfected cells for all DJ-1 variants in the presence or absence of rotenone and saw statistically significant differences in viability across all groups (\( p < 0.0001 \) by one-way ANOVA; \( n = 9 \) groups per group combined from three independent experiments). Cells transfected with wild-type DJ-1 had a viability similar to that of untransfected cells without treatment (85.1 \pm 7.9\%). Cell viability was slightly decreased to 77.0 \pm 6.8\% after exposure to 200 nM rotenone for 24 h, although this difference did not reach statistical significance (\( p > 0.05 \) by Student-Newman-Kuell’s post hoc test comparing untreated versus treated wild-type DJ-1 transfected cells). Similarly, E18N DJ-1 did not affect basal cell viability (84.5 \pm 5.5\%), and cells transfected with E18N displayed greater resistance to rotenone toxicity than wild-type DJ-1 (82.8 \pm 5.1\%, \( p > 0.05 \) compared with untreated by post hoc tests, \( n = 9 \)). E18Q DJ-1 had a moderate effect on basal cell viability (77.3 \pm 10.3\%), and rotenone decreased viability further, to 67.6 \pm 8.2\% (\( p < 0.05 \) compared with untreated by post hoc tests, \( n = 9 \)). Interestingly, the two oxidation-impaired DJ-1 mutants, E18D and C106A, had negative effects on basal cell viability, which were decreased to 66.5 \pm 6.5 and 70.8 \pm 4.4\%, respectively. Furthermore, rotenone treatment had a statistically significant (\( p < 0.05 \) by post hoc test comparing untreated and treated cells for each variant, \( n = 9 \)) effect on viability for E18D and C106A DJ-1, which were 57.5 \pm 7.1 and 61.5 \pm 6.4\%, respectively. These results show that although wild-type and E18N DJ-1 are capable of protecting cells against rotenone-induced toxicity, the E18D and C106A mutants cannot and are therefore both loss of function variants.

DISCUSSION

In this study, we have examined the role that the specific formation of a cysteine-sulfenic acid has on the mitochondrial and cellular protective function of DJ-1, a protein involved in certain forms of rare familial parkinsonism. Our results show that the ability of Cys\textsuperscript{106} to be oxidized to cysteine-sulfenic acid is required for the protective activity of DJ-1. Importantly, by altering the dominant oxidation state of Cys\textsuperscript{106} without mutating Cys\textsuperscript{106} itself, we have been able to decouple the effect of Cys\textsuperscript{106} oxidation from other possible roles of this residue. Therefore, we can use this approach to independently interrogate the relative importance of Cys\textsuperscript{106} oxidation for the function of DJ-1. The use of engineered substitutions to modulate the redox state of important cysteine residues may be applicable to other systems that contain readily oxidized cysteine residues and where the structure of the protein is known.

As suggested previously (15, 18, 36) and as the current data confirm, the pocket around Cys\textsuperscript{106} in human DJ-1 contains a number of residues that modulate Cys\textsuperscript{106} reactivity toward reactive oxygen species. Glu\textsuperscript{18} forms a hydrogen bond with Cys\textsuperscript{106}-SO\textsubscript{2}- that is important for the stabilization of the modified residue. Interestingly, both Glu\textsuperscript{18} and Cys\textsuperscript{106} are very highly conserved in members of the DJ-1 family. This includes ancient homologues, such as YajL from \textit{E. coli} and YDR533c from \textit{Saccharomyces cer-
Cysteine Oxidation in DJ-1 Function

evisiae, where we have previously shown that the structurally equivalent cysteine residue is subject to similar oxidative modifications that are stabilized by a conserved glutamic acid (37, 38). Thus, Cys^{106} and Glu^{18} (or their equivalent residues in other DJ-1 superfAMILY proteins) are probably important for a conserved and ancient function of this protein family.

By mutating Glu^{18} to other residues, we were able to influence the ability of Cys^{106} to oxidize to sulfonic acid without changing the cysteine residue itself. Two variants (E18N and E18Q) allow sulfonic acid formation and, in fact, slightly enhance oxidation in a cellular context. In contrast, the moderately conservative E18D substitution, which shortens the side chain of residue 18 by a single methylene group, dramatically impairs Cys^{106}-SO_{2} formation and results in constitutively reduced Cys^{106} in the cellular environment. By comparing the behavior of E18D with C106A DJ-1, we can distinguish whether it is the cysteine residue per se or the cysteine-sulfonic acid that is important for DJ-1 function.

Our cell-based mitochondrial fission assay shows that although E18N and E18Q can functionally substitute for wild-type protein, neither C106A nor E18D can do so. The power of this approach is enhanced by the known pK_{a} values for Cys^{106} in each of these mutants (18). E18N and E18D DJ-1 have identical Cys^{106} pK_{a} values of 6.1 but very different oxidative and in vivo protective capabilities in the present study. Therefore, we conclude that it is the oxidative propensity rather than the nucleophilicity or general reactivity of Cys^{106} that is required for the mitochondrial protective activity of DJ-1.

We also confirmed that the ability of Glu^{18} to support oxidative modification of Cys^{106} is required for DJ-1 to protect cells against loss of viability due to exposure to the mitochondrial complex I inhibitor rotenone. This is consistent with our previous data (15) and similar results from other laboratories (21) but again emphasizes that the ability of Cys^{106} to undergo facile oxidative modification is the critical determinant for the cytoprotective effects of DJ-1. In these experiments, we also noted a generally detrimental effect of the oxidation-deficient DJ-1 variants on M17 neuroblastoma cell viability, which we speculate may represent a dominant negative effect resulting from the formation of functionally compromised DJ-1 heterodimers of E18D (or C106A) DJ-1 with endogenous wild-type protein.

In this study, a combination of the FRAP mitochondrial fission assay and rotenone-induced cellular toxicity was used because of the potential relevance of mitochondrial dysfunction to Parkinson disease, although it may also be important for other activities of DJ-1 in aerobic situations. Supporting a role for DJ-1 in the maintenance of redox homeostasis, a recent study has shown that DJ-1 deficiency is associated with increased generation of H_{2}O_{2} and diminished activity of mitochondrial iron-sulfur proteins (8). The loss of mitochondrial connectedness seen here in DJ-1-deficient MEFs is therefore probably related to increased oxidative stress, which is known to trigger mitochondrial fission (39). The observation that E18N and E18Q are largely mitochondrial suggests that this pool of protein contributes significantly to the observed effects on mitochondrial fission, supporting the results of a recent study that showed that intentionally targeting DJ-1 to the mitochondria enhances its protective activity (35). Furthermore, the ability of DJ-1 to be recruited to mitochondria is tightly correlated with its propensity for oxidation, confirming a previous suggestion that cysteine-sulfonic acid formation at Cys^{106} is important for mitochondrial localization (15).

The precise function of DJ-1 that protects cells against oxidative damage is unclear, and several previous studies have suggested different possible biochemical activities (8, 10–14). However, in the context of the oxidative stress response, a mitochondrial activity for DJ-1 is likely responsible for the protective effect of the protein. The ability of DJ-1 to scavenge reactive oxygen species could be important by itself, since this may decrease cellular levels of ROS. However, there are many more abundant antioxidant molecules with a greater ability to scavenge ROS in the cell. For example, cells have millimolar levels of glutathione under normal circumstances and low micromolar amounts of DJ-1. Furthermore, the observed second order rate constant of DJ-1 oxidation by H_{2}O_{2} in vitro is ~0.56 m^{-1} s^{-1}, or 10^{5} to 10^{6} lower than the better-characterized peroxiredoxins (8). In addition, the oxidation of glutathione and peroxiredoxins is reversible and thus can detoxify superstoichiometric quantities of ROS, whereas there is no known mechanism by which Cys^{106}-SO_{2} DJ-1 can be reduced. Therefore, since there are more efficient systems to remove ROS in the cell, sacrificial irreversible autooxidation of DJ-1 is unlikely to contribute significantly to its antioxidant status.

Furthermore, although a redox-sensitive chaperone activity toward α-synuclein has been demonstrated for Cys^{106}-SO_{2} DJ-1 (10), this does not account for the established protective role of DJ-1 homologues in nonvertebrate species, such as D. melanogaster, that lack an α-synuclein homologue but where the same cysteine oxidation has been shown to be important (21). We have proposed that DJ-1 may bind selectively to mitochondrial and antioxidant mRNA species (12), which is broadly consistent with the results presented here in supporting a major role for the mitochondrial pool of DJ-1 in protection against oxidative stress. This is formally difficult to assess, however, since DJ-1 oxidation influences its RNA binding activity, and thus mutations that affect Cys^{106} oxidation will also be predicted to influence RNA binding. Defining the activity of DJ-1 that is responsible for cellular protection against oxidative stress will require further studies that integrate structural, biochemical, cellular, and organismal approaches.

Overall, these data strengthen previous suggestions that oxidative stress and mitochondrial dysfunction are critical contributors to the etiology of various forms of parkinsonism. Furthermore, they identify cysteine-sulfenic acid as a critical post-translational modification that has powerful effects on DJ-1 function in vivo. Additional studies that use a similar strategy could be applied to other redox-sensitive proteins that contain oxidized cysteine residues.

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REFERENCES

1. Reddie, K. G., and Carroll, K. S. (2008) Curr. Opin. Chem. Biol. 12, 746–754
2. Biteau, B., Labarre, J., and Toledano, M. B. (2003) Nature 425, 980–984
3. Jonsson, T. I., Murray, M. S., Johnson, L. C., Poole, L. B., and Lowther, W. T. (2005) Biochemistry 44, 8634–8642
4. Annesi, G., Savettieri, G., Pugliese, P., D’Amelio, M., Tarantino, P., Ragonese, P., La Bella, V., Piccoli, T., Civitello, D., Annesi, F., Fierro, B., Piccoli, F., Arabia, G., Caracciolo, M., Ciró Candiano, I. C., and Quattrone, A. (2005) Ann. Neurol. 58, 803–807
5. Bonifati, V., Rizzu, P., van Baren, M. J., Schap, O., Breedveld, G. J., Krieger, E., Dekker, M. C., Squitieri, F., Ibáñez, P., Joosse, M., van Dongen, J. W., van Swieten, J. C., Brice, A., Meco, G., van Duijn, C. M., Oostra, B. A., and Heutink, P. (2003) Science 299, 256–259
6. Bandopadhyay, S., and Cookson, M. R. (2004) BMC Biol. Evol. 4, 6
7. Lucas, J. I., and Marin, I. (2007) Mol. Biol. Evol. 24, 551–561
8. Andres-Mateos, E., Perier, C., Zhang, L., Blanchard-Fillion, B., Greco, T. M., Thomas, B., Ko, H. S., Sasaki, M., Ischiropoulos, H., Przedborski, S., Dawson, T. M., and Dawson, V. L. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 14807–14812
9. Shendelman, S., Jonason, A., Martinat, C., Leete, T., and Abeliovich, A. (2004) PLoS Biol. 2, 1764–1773
10. Zhou, W., Zhu, M., Wilson, M. A., Petsko, G. A., and Fink, A. L. (2006) J. Mol. Biol. 356, 1036–1048
11. Hod, Y., Pentyala, S. N., Whyard, T. C., and El-Maghrabi, M. R. (1999) J. Cell Biochem. 72, 435–444
12. van der Brug, M. P., Blackinton, J., Chandran, J., Hao, L. Y., Lal, A., Mazan-Mamczarz, K., Martindale, J., Xie, C., Ahmad, R., Thomas, K. J., Bellina, A., Gibbs, J. R., Ding, J., Myers, A. J., Zhan, M., Cai, H., Bonini, N. M., Gorospe, M., and Cookson, M. R. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 10244–10249
13. Zhong, N., Kim, C. Y., Rizzu, P., Geula, C., Porter, D. R., Pothis, E. N., Squitieri, F., Heutink, P., and Xu, J. (2006) J. Biol. Chem. 281, 20940–20948
14. Zhou, W., and Freed, C. R. (2005) J. Biol. Chem. 280, 43130–43138
15. Canet-Aviles, R. M., Wilson, M. A., Miller, D. W., Ahmad, R., McLendon, C., Bandopadhyay, S., Baptista, M. J., Ringe, D., Petsko, G. A., and Cookson, M. R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9103–9108
16. Ito, G., Ariga, H., Nakagawa, Y., and Iwatsubo, T. (2006) Biochem. Biophys. Res. Commun. 339, 667–672
17. Kinumi, T., Kimata, J., Taira, T., Ariga, H., and Niki, E. (2004) Biochem. Biophys. Res. Commun. 317, 722–728
18. Witt, A. C., Lakshminarasimhan, M., Remington, B. C., Hasim, S., Pozharski, E., and Wilson, M. A. (2008) Biochemistry 47, 7430–7440
19. Aleyasin, H., Rousseaux, M. W., Phillips, M., Kim, R. H., Bland, R. J., Callaghan, S., Slack, R. S., During, M. J., Mak, T. W., and Park, D. S. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 18748–18753
20. Meulener, M., Whitworth, A. J., Armstrong-Gold, C. E., Rizzu, P., Heutink, P., Wes, P. D., Pallant, L. J., and Bonini, N. M. (2005) Curr. Biol. 15, 1572–1577
21. Meulener, M. C., Xu, K., Thomson, L., Thompson, L., Ischiropoulos, H., and Bonini, N. M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 12517–12522
22. Holyoak, T., Fenn, T. D., Wilson, M. A., Moulin, A. G., Ringe, D., and Petsko, G. A. (2003) Acta Crystallogr. Sect. D Biol. Crystallogr. 59, 2356–2358
23. Otnowinski, Z., and Minor, W. (1997) Methods Enzymol. 307–326
24. Sheldrick, G. M. (2008) Acta Crystallogr. A 64, 112–122
25. Brunger, A. T. (1992) Nature 355, 472–475
26. Wilson, M. A., Collins, J. L., Hod, Y., Ringe, D., and Petsko, G. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9256–9261
27. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132
28. Davis, I. W., Beaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Arendall, W. B., III, Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) Nucleic Acids Res. 35, W375–W383
29. Chandran, J. S., Lin, X., Zapata, A., Hōke, A., Shimoji, M., Moore, S. O., Galloway, M. P., Laird, F. M., Wong, P. C., Price, D. L., Bailey, K. R., Crawley, J. N., Shippenberg, T., and Cai, H. (2008) Neurobiol. Dis. 29, 505–514
30. Blackinton, J., Ahmad, R., Miller, D. W., van der Brug, M. P., Canet-Aviles, R. M., Hague, S. M., Kaleem, M., and Cookson, M. R. (2005) Brain Res. Mol. Brain Res. 134, 76–83
31. Karbowski, M., Norris, K. L., Cleland, M. M., Jeong, S. Y., and Youle, R. J. (2006) Nature 443, 658–662
32. Szabadkai, G., Simon, A. M., Bianchi, K., De Stefani, D., Leo, S., Wieckowski, M. R., and Rizzuto, R. (2006) Biochim. Biophys. Acta 1763, 442–449
33. Engb, R. A., and Huber, R. (1991) Acta Crystallogr. Sect. A 47, 392–400
34. Zhang, L., Shimoji, M., Thomas, B., Moore, D. J., Yu, S. W., Marupudi, N. J., Torp, R., Torgner, I. A., Ottersen, O. P., Dawson, T. M., and Dawson, V. L. (2005) Hum. Mol. Genet. 14, 2063–2073
35. Junn, E., Jiang, W. H., Zhao, X., Jeong, B. S., and Mouradian, M. M. (2008) J. Neurosci. Res. 87, 123–129
36. Wei, Y., Ringe, D., Wilson, M. A., and Ondrechen, M. (2007) PLoS Comput. Biol. 3, 120–126
37. Wilson, M. A., Ringe, D., and Petsko, G. A. (2005) J. Mol. Biol. 353, 678–691
38. Wilson, M. A., St Amour, C. V., Collins, J. L., Ringe, D., and Petsko, G. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1531–1536
39. Cheung, E., McBride, H. M., and Slack, R. S. (2007) Apoptosis 12, 979–992
40. Fenn, T., Ringe, D., and Petsko, G. A. (2003) J. Appl. Crystallogr. 36, 944–947