Effect of Single Amino Acid Substitution on Oxidative Modifications of the Parkinson’s Disease-Related Protein, DJ-1

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Mutations in the gene encoding DJ-1 have been identified in patients with familial Parkinson’s disease (PD) and are thought to inactivate a neuroprotective function. Oxidation of the sulfhydryl group to a sulfenic acid on cysteine residue C106 of DJ-1 yields the “2O” form, a variant of the protein with enhanced neuroprotective function. We hypothesized that some familial mutations disrupt DJ-1 activity by interfering with conversion of the protein to the 2O form. To address this hypothesis, we developed a novel quantitative mass spectrometry approach to measure relative changes in oxidation at specific sites in mutant DJ-1 as compared with the wild-type protein. Treatment of recombinant wild-type DJ-1 with a 10-fold molar excess of H2O2 resulted in a robust oxidation of C106 to the sulfenic acid, whereas this modification was not detected in a sample of the familial PD mutant M26I exposed to identical conditions. Methionine oxidized isoforms of wild-type DJ-1 were depleted, presumably as a result of misfolding and aggregation, under conditions that normally promote conversion of the protein to the 2O form. These data suggest that the M26I familial substitution and methionine oxidation characteristic of sporadic PD may disrupt DJ-1 function by disfavoring a site-specific modification required for optimal neuroprotective activity. Our findings indicate that a single amino acid substitution can markedly alter a protein’s ability to undergo oxidative modification, and they imply that stimulating the conversion of DJ-1 to the 2O form may be therapeutically beneficial in familial or sporadic PD. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.010892, 1–15, 2012.

Parkinson’s disease (PD)1 is a neurodegenerative disorder characterized by muscular rigidity, slowness of voluntary movement, poor balance, and resting tremor (1). These symptoms are caused by the death of neurons in a region of the midbrain called the substantia nigra. The neurons that survive in this region exhibit a defect in complex I of the mitochondrial electron transport chain and show signs of oxidative damage (2–4). In addition, surviving neurons contain characteristic cytosolic inclusions named “Lewy bodies” that are enriched with aggregated forms of the presynaptic protein α-synuclein (aSyn) (5). It is hypothesized that reactive oxygen species (ROS) accumulate as a result of mitochondrial impairment and contribute to neurodegeneration by causing the oxidation of lipid, proteins, and DNA (2–4). A buildup of ROS may promote the formation of harmful aSyn aggregates in the brains of PD patients (6–8). ROS accumulation is thought to occur preferentially in nigral dopaminergic neurons because of the catabolism and auto-oxidation of dopamine, reactions that result in the generation of H2O2 (2, 9). In addition, an abundance of iron in the substantia nigra promotes the decomposition of H2O2 to OH· by Fenton chemistry (10).

A number of patients with familial, early-onset, recessive PD have been found to harbor loss-of-function mutations (e.g. M26I, E64D, A104T, D149A, E163K, and L166P) in the gene encoding DJ-1, a protein that is abundant throughout the central nervous system (11, 12). Dysfunction of wild-type DJ-1 as a result of destabilizing oxidative modifications is also thought to play a role in more common sporadic forms of PD (13–15). DJ-1 has been reported to suppress neurodegeneration in cellular and animal models by activating antioxidant responses (16–21), up-regulating or carrying out a molecular chaperone function (20–24), and/or inducing prosurvival signaling responses (12, 19, 25). DJ-1 is a homodimer (subunit molecular weight = 20 kDa) for which there is evidence that cysteine 106 (C106) in the subunit sequence is readily oxidized to the sulfenic acid under oxidizing conditions, yielding the “2O” form of the protein (14, 16, 17, 26). Structural studies indicate that C106 is located in a pocket at the interface...
between the DJ-1 subunits that is lined with polar residues from both subunits, and oxidation of C106 to the sulfinic acid is facilitated by structure(s) within the pocket (16, 27–29). The design of this pocket suggests that controlled oxidation of DJ-1 at position 106 is advantageous for optimal DJ-1 function. Consistent with this idea, the oxidation of C106 to the 2O form is apparently critical for the ability of DJ-1 to translocate to mitochondria (16), inhibit mitochondrial fragmentation (30–32), or suppress fibril formation by recombinant aSyn (24). In contrast, overoxidation of DJ-1 leading to the conversion of C106 to the sulfonic acid (“3O”) form results in thermodynamic instability (15) and a loss of chaperone function (24).

Data from various groups indicate that L166P has a pronounced protein folding defect, resulting in impaired homodimer formation, rapid protein turnover, and a high propensity to form large protein complexes (33–35). Clearly, pronounced structural defects lead to the compromised functionality of L166P. Substitutions at other locations destabilize the structure of DJ-1 to a lesser extent (15, 36, 37), raising questions about why they are functionally diminished. As one possibility, we hypothesized that some familial DJ-1 mutants may have altered abilities to undergo key oxidative modifications, namely, (1) a decreased propensity to undergo oxidation at position 106 to yield the 2O form, and/or (2) an increased susceptibility to undergo oxidative modifications with potentially deleterious effects on DJ-1 function at other sites in the polypeptide chain. To address this hypothesis, we examined the impact of one familial substitution (the M26I mutation) on the ability of DJ-1 to undergo oxidation at C106 and other residues in the amino acid sequence. Using a novel mass spectrometry approach, we quantified site-specific oxidative modifications of M26I as compared with wild-type DJ-1 after treatment with different amounts of H₂O₂. Our results indicate that the M26I substitution has a profound disruptive effect on the ability of DJ-1 to undergo oxidation at C106.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise specified, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Isopropylthiogalactoside (IPTG) was purchased from Gold Biotechnologies (St. Louis, MO). PreScission™ Protease was obtained from GE Healthcare (Piscataway, NJ). The bicheninonic acid (BCA) protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). Immobilized trypsin and tris (2-carboxyethyl) phosphine (TCEP) were purchased as Pierce products from Thermofisher Scientific (Rockford, IL). Amicon Ultra-0.5 ml (Ultracel YM-10) and Amicon Ultra-4 (Ultracel-3) centrifugal filters and H₂O₂ were purchased from Millipore (Billerica, MA). Trifluoroacetic acid and high-performance liquid chromatography (HPLC) grade CH₃CN were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Materials for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (immobilized pH gradient (IPG) strips, sample rehydration buffer, Criterion XT 12% Bis-Tris precast gels, XT MOPS 2D PAGE running buffer, protein standards) were obtained from Bio-Rad (Hercules, CA). Urea was obtained from Mallinckrodt Laboratories (Phillipsburg, NJ), and agarose was purchased from Invitrogen (Carlsbad, CA).

**Preparation of Bacterial Expression Constructs**—Human wild-type DJ-1 and the familial mutant M26I were expressed as N-terminal glutathione-S-transferase (GST) fusions. A construct encoding GST-DJ-1_M26I in the pGEX-6P1 vector (courtesy of Dr. Soumya Ray, Brigham and Women’s Hospital (38)) was converted to a new construct encoding GST-DJ-1_M26I by PCR using the QuikChange method (Stratagene, La Jolla, CA). The sequence of the DJ-1-encoding insert in each construct was verified using an Applied Biosystems (Foster City, CA; ABI 3700) DNA sequencer.

**Purification of Recombinant DJ-1**—Wild-type and mutant DJ-1 were purified as described (38). Cells of the BL21(DE3) strain of *Escherichia coli* were transformed with each pGEX-6P-1 GST-DJ-1 construct by electroporation. The transformed cells were grown to an OD₆₀₀ of 0.4–0.6 in LB plus ampicillin (100 μg/ml) at 37 °C, and IPTG was added to a final concentration of 1 mM. The cells were grown under inducing conditions for 18 h at 18 °C, harvested by centrifugation, and resuspended in buffer G (25 mM K₃PO₄, pH 8.0, 500 mM KCl). The cells were lysed by incubation on ice in the presence of lysozyme (1 mg/ml, 30 min) followed by passage through a French pressure cell (p.s.i. > 1000). After centrifugation (20,000 × g, 20 min), the supernatant was applied to a GSTPrep FF column (GE Healthcare), from which GST-DJ-1 was eluted in 250 mM Tris HCl, pH 8.0, 500 mM NaCl, and 0.3% [w/v] reduced glutathione. Fractions most highly enriched with GST-DJ-1 were identified by SDS-PAGE with Coomassie Blue staining and pooled. The pooled fractions were dialyzed against buffer G plus diithiothreitol (DTT) (0.25 mM) to remove excess reduced glutathione. The overall protein concentration was determined with a BCA protein assay kit, and the fusion protein was cleaved with PreScission™ Protease (16 h, 4 °C, 1 unit protease per 133 μg DJ-1). Untagged DJ-1 was separated from uncleaved GST-DJ-1, free GST, and residual protease (which contains an uncleavable GST tag) by elution from a GSTPrep FF column equilibrated with buffer G. Fractions most highly enriched with DJ-1 were identified by SDS-PAGE with Coomassie Blue staining and pooled. The final protein sample (estimated purity, 95%) was supplemented with glycerol (5%, [v/v]) and DTT (2–3 mM), and aliquots were frozen at −80 °C. For all of the analyses outlined below, the concentration of recombinant DJ-1 was estimated using the BCA assay and verified with quantitative amino acid analysis (Purdue University Proteomics Core).

**Controlled Oxidation of DJ-1**—Aliquots of purified DJ-1 (wild-type or M26I) were dialyzed against 10 mM Tris at pH 8.0 to remove reductant, and the protein was treated with a 10-fold or 500-fold molar excess of H₂O₂ for 1 h at 22 °C. These conditions were previously found to be suitable to convert DJ-1 to the 2O or 3O form, respectively (24). Excess H₂O₂ was removed from each sample by exchanging the protein into fresh buffer (10 mM Tris at pH 8.0) using Amicon Ultra-4 centrifugal filters (molecular weight cutoff, 3 kDa).

**Two-dimensional Polyacrylamide Gel Electrophoresis**—Changes in the isoelectric point (pI) of DJ-1 following oxidation in the presence of H₂O₂ as outlined above were monitored via 2D-PAGE (16). Protein aliquots were dialyzed overnight against PBS. An aliquot of the protein (15 μg) was mixed with sample rehydration buffer (8 μl urea, 2% [w/v] 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 50 mM DTT, 0.2% [w/v] BioLyte 3/10 ampholyte, 0.001% [w/v] bromphenol blue) and recombinant aSyn as an internal standard (predicted pI = 4.67) and Bio-Rad protein standards in a total volume of 185 μl. The solution was added to an 11 cm IPG strip with a pH range of 4 to 7. Mineral oil was added to the top of the IPG strip to reduce evaporation during electrophoresis. The IPG strip was actively rehydrated at 20 °C for 12 h and subjected to isoelectric focusing in a Protean isoelectric focusing (IEF) Cell (Bio-Rad) with the following voltage parameters: step one, 250 V for 15 min; step two, 8000 V for 2.5 h; step three, 8000 V for 4.4 h. The IPG strip was reduced
with 2% [w/v] DTT in equilibration buffer (6 M urea, 0.375 M Tris HCl, pH 8.8, 2% [w/v] SDS, 20% [v/v] glycerol) for 15 min, followed by alkylation with 2.5% [w/v] iodoacetamide for 15 min. The IPG strip was loaded onto the top of a Criterion XT 12% Bis-Tris pre-cast gel (Bio-Rad) and sealed with 0.5% [w/v] agarose in 1 × 1 XT MOPS buffer to ensure an even transfer of protein. The second-dimension gel was stained with Coomassie Blue and analyzed with a Typhoon Imaging System. Approximate pI values were determined by calibration with the aSyn internal standard and the ends of the IPG strip.

Intensities of spots on 2D-PAGE gels were quantitated using Image J software (National Institutes of Health). Each spot was selected using a rectangular selection tool. The dimensions of the selected rectangular area were kept constant through the analysis of all spots on multiple 2D-PAGE gels. Each selected spot was displayed as a rectangular area were kept constant through the analysis of all spots

In order to be considered a correct match.

Circular Dichroism—Secondary structure analyses of recombinant DJ-1 were carried out via far-ultraviolet circular dichroism (far-UV CD) as described (15). Protein solutions were dialyzed against 10 mM KPi, pH 7.0, 150 mM NaCl. Aliquots of the protein (0.4 ml, 0.06 mg/ml) were introduced into a 0.2 cm quartz cuvette and analyzed using a J810 spectropolarimeter (JASCO, Easton, MD). Full spectra were collected at 20 °C with a bandwidth of 2 nm, a response time of 1 s, and a data pitch of 1 nm. Molar ellipticity was calculated using the equation:

\[
\theta = \frac{m \cdot d \cdot \phi}{10 \cdot d \cdot c}
\]

where \( \theta \) is the molar ellipticity, \( m \) is the mean residual weight (g/mol), \( \phi \) is the ellipticity, \( d \) is the pathlength (cm), and \( c \) is the protein concentration (g/L).

Sedimentation-Equilibrium—Sedimentation-equilibrium runs were conducted at 22 °C in a Beckman XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) with absorbance optics, as described (15), using the methods of Laue and Stafford (42). Prior to ultracentrifugation, protein samples were dialyzed against 50 mM Tris HCl, pH 7.0, 200 mM NaCl, 4 °C, 48 h, with a change in buffer after 24 h. Aliquots (110 μl) of the protein solution (0.8–2.8 mg/ml) were loaded into six-sector charcoal filled epon (CFE) sample cells, allowing three concentrations to be run simultaneously. Runs were performed at a minimum of three different speeds (2.0 × 10⁻⁴–2.4 × 10⁴ rpm), and each speed was maintained until there was no significant change in scans of r²/2 versus absorbance taken 2 h apart to ensure that equilibrium was achieved. Sedimentation-equilibrium data were evaluated using the program NONLIN, which employs a nonlinear least squares curve-fitting algorithm described by Johnson et al. (43). The program allows for analysis of both single and multiple data files and can be fit to models containing up to four associating species, depending upon which parameters are permitted to vary during the fitting routine. The protein’s partial specific volume and the solvent density were estimated using the Sednterp program (44).
Effect of Amino Acid Substitution on DJ-1 Oxidation

Fig. 1. Schematic overview of the strategy used to quantitate oxidative modifications of DJ-1. Wild-type DJ-1 and M26I were untreated or exposed to a 10- or 500-fold molar excess of H2O2. The proteins were digested with trypsin, and the tryptic peptides were labeled by trypsin-catalyzed 18O/16O labeling. The 16O- and 18O-labeled peptides were mixed and analyzed by nanoscale LC/MS/MS.

Pymol Analysis—Electrostatic and hydrogen bonding interactions involving H115 were identified with PyMol software using the “find polar contacts” command. Distance measurements were carried out in the “Editing Mode” using the commands _dist(pk1), (pk2) in which pk1 = atom 1, and pk2 = atom 2. To identify structural changes we compared the following pair of proteins: 1SOA.pdb versus 1P5F.pdb and 2RK4.pdb versus 1P5F.pdb. Each pair was uploaded on the Pymol Viewer and superimposed using the align molecule command. 1P5F.pdb, wild type DJ-1 unoxidized (28); 1SOA.pdb, oxidized wild type DJ-1 (16); 2RK4.pdb, M26I DJ-1 (unoxidized) (36).

RESULTS

Strategy Overview—The primary objectives of this study were (1) to develop a mass spectrometry method for quantitative measurement of fold changes in DJ-1 oxidation at specific sites under different oxidizing conditions, and (2) to use this method to determine the impact of the M26I substitution on DJ-1 oxidation at C106 and other residues. To accomplish these goals, we implemented a strategy that combines oxidation of recombinant wild-type DJ-1 or M26I (via treatment with a 10- or 500-fold molar excess of H2O2), 18O labeling, and liquid chromatography (LC)-MS analysis (Fig. 1). Sequence-specific oxidative modifications were then quantitated by measuring the ratio of 18O- to 16O-labeled peptides. Next, the mass spectrometry data were validated by characterizing wild-type DJ-1 and M26I in terms of their propensities to undergo C106 oxidation via 2D-PAGE. Finally, to better understand why wild-type DJ-1 and M26I exhibit different fold changes in oxidation, the two proteins were compared in terms of their secondary and quaternary structures in both the untreated and 2O forms.

Oxidative Modifications of Wild-type DJ-1 and M26I (Mass Spectrometry)—Approximately 6400 spectra were obtained for each analysis. The following post-translational modifications were identified and quantified: (1) oxidation of two cysteine residues to the sulfonic acid (C106) or the sulfonic acid (C53, C106); (2) oxidation of four methionine residues (M17, M26, M133, and M134) to the sulfoxide; and (3) oxidation of a histidine residue (H115) to asparagine. Representative mass spectrometry data that enabled us to identify these modifications are described in detail below (Supplemental Table S1).

Identification of Oxidized Cysteine Residues—Treatment of DJ-1 with H2O2 resulted in a dose-dependent oxidation of the protein’s cysteine residues to the sulfenic or sulfonic acid form. In Fig. 2 we show the MS/MS spectrum of peptide 99KGLIAAICAGPTALLAHEIGFGSK122 derived from wild-type DJ-1 following oxidation with a 10-fold molar excess of H2O2. The “y” and “b” ion peaks in the spectrum were assigned labels corresponding to their m/z values. The mass difference between the b(8) ion (m/z = 802.464 Da) and the b(7) ion (m/z = 667.47 Da) was equal to the mass of a cysteine sulfonic acid (134.99 Da), suggesting that residue C106 had undergone oxidation to the 2O form under these conditions. In supplemental Fig. S1, we show the MS/MS spectrum of peptide 99KGLIAAICAGPTALLAHEIGFGSK122 derived from wild-type DJ-1 after oxidation with a 500-fold molar excess of H2O2. In this case the mass difference between b(8) (m/z = 818.44 Da) and b(7) (m/z = 677.49 Da) was equal to the mass of a cysteine sulfonic acid (134.99 Da), suggesting that residue C106 had undergone oxidation to the 2O form under these conditions. Similarly, the MS/MS spectrum of peptide 46DVVIAICPDASLEDAKK163 derived from M26I DJ-1 following treatment with a 500-fold molar excess of H2O2 clearly revealed a difference between b(5) (m/z = 578.249 Da) and b(4) (m/z = 427.255 Da) equal to the mass of cysteine sulfonic acid (supplemental Fig. S2), suggesting that C53 was oxidized to the 3O form upon exposure to the high dose of peroxide.

Identification of Oxidized Methionine Residues—The identification of oxidized methionine residues is more complex because methionine sulfoxide (MetSO) undergoes a characteristic neutral loss of methanesulfenic acid (CH3SOH, 63.998 Da) during collision-induced dissociation (45, 46). The Mascot search engine accounts for this neutral loss when assigning peaks in the MS/MS spectrum. In Fig. 3 we show the MS/MS spectrum of peptide 13GAEEMETVIPVDVMR27 derived from wild-type DJ-1 following oxidation with a 500-fold molar ex-
cess of H₂O₂. The mass difference between y(11) (\(m/z = 1193.697\) Da after the neutral loss of two CH₃SOH groups) and y(10) (\(m/z = 1110.631\) Da after the neutral loss of one CH₃SOH group) was equal to the mass of methionine sulfoxide minus the neutral loss mass (83 Da). Additionally, the mass difference between y(2) (\(m/z = 258.105\) Da after the neutral loss of CH₃SOH) and y(1) (\(m/z = 175.106\) Da) was equal to the mass of methionine sulfoxide minus the neutral loss mass (83 Da). These results indicate that both methionine residues in the parent peptide (M17 and M26) were oxidized to methionine sulfoxide under these H₂O₂ treatment conditions. Additional MS/MS data revealed the presence of methionine sulfoxide at positions 133 (supplemental Fig. S3) and 134 (supplemental Fig. S4) of peptide 131DKMMNGGMYTYSENRVEK148 derived from wild-type DJ-1 oxidized with a 500-fold molar excess of H₂O₂.

Identification of the Oxidation of Histidine to Asparagine—Histidine residues can be oxidized to asparagine as a major product (47), resulting in a mass difference of –23.01. The mascot search engine detected the presence of asparagine in place of H₁₁₅ based on the decrease in the mass of peptide 99KGLIAAICAGPTALLAHEIGFGSK₁₂₂ derived from wild-type DJ-1 (but not M₂₆I) oxidized with a 10- or 500-fold molar excess of H₂O₂.

Quantitation of Oxidative Modifications—In the next phase of our study we determined relative levels of post-translationally modified DJ-1 peptides generated in the presence of a low or high concentration of H₂O₂ via quantitative analysis of the MS peak intensities. The method involved dividing the peak intensity of each ¹₈O-labeled peptide from H₂O₂-treated wild-type DJ-1 or M₂₆I by the peak intensity of the identical ¹₆O-labeled peptide derived from the untreated protein. Reproducible data from three separate labeling experiments revealed remarkable differences in relative levels of cysteine-modified peptides when comparing wild-type DJ-1 and M₂₆I exposed to a 10- or 500-fold molar excess of H₂O₂ (Table I). These differences are summarized as follows: (1) wild-type DJ-1 exhibited a marked increase in the level of C₁₀₆ sulfenic acid.
acid following incubation with a 10-fold molar excess of H$_2$O$_2$, whereas the 2O form of C106 was not detected in a sample of M26I exposed to identical oxidizing conditions; (2) the wild-type protein (but not M26I) exhibited a higher relative level of C106 sulfinic acid after exposure to a 10-fold molar excess of H$_2$O$_2$ compared with a 500-fold molar excess of the peroxide; (3) wild-type DJ-1 exhibited a dramatically higher relative level of C106 sulfonic acid compared with M26I after incubation with a 500-fold molar excess of H$_2$O$_2$ whereas the 3O form of C106 was essentially undetectable in samples of wild-type DJ-1 or M26I exposed to a 10-fold molar excess of the peroxide; (4) M26I (but not wild-type DJ-1) exhibited an increase in the level of C53 sulfonic acid following exposure to a 500-fold molar excess of H$_2$O$_2$ (whereas this modification didn’t appear in any other forms of the protein); and (5) wild-type DJ-1 (but not M26I) exhibited an increase in the level of

FIG. 3. MS/MS analysis of peptide $^{13}$GAEMETVIPVDVMR$^{27}$ in wild-type DJ-1 oxidized with a 500-fold molar excess of H$_2$O$_2$. (Top) MS/MS spectrum with all of the y and b ions assigned. The y and b ion map is superimposed on the peptide sequence. (Bottom) Both methionine residues (M17 and M26) are oxidized to methionine sulfoxide according to the Mascot identification. The difference between the masses of y2 (after the neutral loss of one CH$_3$SOH group, mass = 63.998 Da) and y1 equals 83 Da. The difference between the masses of y11 (after the neutral loss of two CH$_3$SOH groups, total mass = 127.996 Da) and y10 (after the neutral loss of one CH$_3$SOH group, mass = 63.998 Da) equals 83.06 Da. Addition of the neutral loss mass to the 83 Da value obtained for y2-y1 and y11-y10 yields the mass of methionine sulfoxide.

| Oxidative modification | MS ratio$^a$ | 10-fold molar excess H$_2$O$_2$ | 500-fold molar excess H$_2$O$_2$ |
|------------------------|-------------|-------------------------------|-------------------------------|
|                        |             | wild-type DJ-1 | M26I | wild-type DJ-1 | M26I | wild-type DJ-1 | M26I |
|                        | average | S.E. | average | S.E. or range | average | S.E. or range | average | S.E. or range |
| C53 sulfonic acid      | ND$^b$     | NA$^c$           | ND$^b$                  | NA$^c$                  | ND$^b$             | NA$^c$             | ND$^b$             | NA$^c$             |
| C106 sulfonic acid     | 12         | 3               | ND$^b$                  | NA$^c$                  | 5.1              | 4.9–6.5            | 4.0               | 3.4–4.7            |
| C106 sulfuric acid     | ND$^b$     | NA$^c$           | ND$^b$                  | NA$^c$                  | 30.3$^c$            | 0.9               | 4.1$^c$             | 0.4               |
| H115 asparagine        | 1.95       | 0.03            | ND$^b$                  | NA$^c$                  | 2.1              | 0.7               | ND$^b$             | NA$^c$             |

$^a$ MS ratios were determined by dividing the peak intensity of each $^{18}$O-labeled peptide derived from H$_2$O$_2$-treated DJ-1 by the peak intensity of the identical $^{16}$O-labeled peptide derived from untreated (control) protein. $n = 2$ or 3.

$^b$ ND, not detected; NA, not applicable.

$^c$ p value <0.01, Student’s t test. Detailed results for the MS ratios for the three experiments can be found in supplemental Table S2.
H2O2. The untreated forms of both variants consisted of two was either untreated or treated with a 10-fold molar excess of using 2D-PAGE as an independent method. Each DJ-1 variant DJ-1 and M26I to undergo C106 oxidation to the 2O form. In the next phase of our study, we validated the mass spectrom-etry data by assessing the relative propensities of wild-type PAGE)—
The results outlined above suggested that M26I has a reduced propensity to undergo oxidation to the 2O form. In general, quantitative analysis of methionine oxidized peptides revealed greater variability across replicate runs and less pronounced differences between wild-type DJ-1 and M26I. Specifically, the following trends were noted (Table II): (1) wild-type DJ-1 exhibited a decrease in the level of a peptide containing methionine sulfoxide at positions 17 and 26 after incubation with a 10-fold molar excess of H2O2; (2) wild-type DJ-1 and M26I exhibited a decrease in the level of a peptide with methionine sulfoxide at position 133 after incubation with a 10-fold molar excess of H2O2; and (3) wild-type DJ-1 and M26I exhibited a decrease in the level of a peptide with methionine sulfoxide at position 134 after incubation with a 10-fold molar excess of H2O2.

**Oxidative Modifications of Wild-type DJ-1 and M26I (2D-PAGE)—** The results outlined above suggested that M26I has a reduced propensity to undergo oxidation to the 2O form. In the next phase of our study, we validated the mass spectrometry data by assessing the relative propensities of wild-type DJ-1 and M26I to undergo C106 oxidation to the 2O form using 2D-PAGE as an independent method. Each DJ-1 variant was either untreated or treated with a 10-fold molar excess of H2O2. The untreated forms of both variants consisted of two major species with estimated pI values of 5.9 and 6.2, similar to pl values previously assigned to the 2O and unoxidized DJ-1 isoforms, respectively (Fig. 4A, panels i and ii) (16). Data reported by several groups indicate that DJ-1 exists as multiple (> 2) species spanning a range of pl values in cell culture and in brain tissue (16, 48, 49), presumably because the protein undergoes various modifications (both oxidative and nonoxidative) in addition to the conversion to the 2O form observed here. We confirmed that the DJ-1 isoforms with pl values of 5.9 and 6.2 most likely correspond to the 2O and unoxidized species (respectively) by showing that the C106A mutant, which cannot undergo oxidation to the 2O form, only appears as the more basic species on a 2D-PAGE gel (data not shown). After exposure to H2O2 the wild-type protein consisted almost entirely of the lower pl species, whereas M26I was only partially converted to this acidic isoform (Fig. 4A, panels iii and iv). Quantitative analysis of spot intensities from replicate 2D-PAGE runs revealed a significant increase in the relative level of the 2O form in a sample of wild-type DJ-1, but not M26I, after peroxide treatment (Fig. 4B). From these data, we inferred that the mutant protein has a lower propensity to undergo oxidation at C106 upon treatment with a 10-fold molar excess of H2O2.

**Secondary and Quaternary Structure of Wild-type DJ-1 and M26I—** Next, we examined whether wild-type DJ-1 and M26I differed in their ability to undergo oxidation to the 2O form as a result of differences in their protein folds. We considered
that M26I might have a decreased oxidation propensity at C106 because of (1) a local perturbation of structural elements in proximity to the cysteine residue, or (2) a more global perturbation (unfolding) of the mutant protein. Both types of perturbations would be expected to interfere with conversion to the 2O form by destabilizing the “C106 pocket,” which has polar residues in a configuration that favors C106 oxidation in the wild-type protein (16, 27–29). To address whether the overall fold of M26I was markedly different from that of wild-type DJ-1, we analyzed both proteins by far-UV CD and ultracentrifugation, two approaches that report on the protein’s secondary and quaternary structures, respectively (15).

CD spectra recorded for unoxidized wild-type DJ-1 and M26I (Fig. 5) displayed broad negative ellipticity from 208 to 222 nm, consistent with a mixed α/β structure. The relative magnitude of the signals recorded for the two variants showed some variability, presumably because of differences in the extent to which the proteins were oxidized during purification (24). Although the spectrum of M26I occasionally overlapped closely with that of the wild-type protein, in most cases the mutant exhibited an overall reduction in negative ellipticity from 208 to 222 nm (Figs. 5A, 5B). Both variants exhibited a slightly reduced signal after treatment with a 10-fold molar excess of H2O2, suggesting that oxidation led to a small decrease in overall secondary structure (Figs. 5C, 5D).

Collectively, these results suggested that (1) unoxidized wild-type DJ-1 and M26I have similar secondary and quaternary structures, although M26I forms a less stable homodimer; (2) oxidation of wild-type DJ-1 in the presence of a 10-fold molar excess of H2O2, a treatment that results in selective oxidation of C106 to the sulfinic acid without affecting other oxidizable residues (24), does not alter the protein’s quaternary structure or stability; and (3) oxidation of M26I in the presence of a 10-fold molar excess of H2O2 promotes higher order self-assembly.

DISCUSSION

Several loss-of-function mutations in the gene encoding DJ-1 have been identified in patients with familial PD (11, 12). One mutation (L166P) abrogates DJ-1 function by destabilizing the native homodimer, thus promoting aggregation or
turnover of the protein (33–35). In contrast, other mutations have a less pronounced effect on DJ-1 stability, and it is unclear how they perturb DJ-1 function. In this study we addressed the hypothesis that the M26I familial mutant exhibits reduced protective activity as a result of (1) a decreased ability to undergo oxidation to the functionally important 2O form (16, 24, 30), and/or (2) an increased propensity to undergo oxidative modifications with potentially disruptive effects on DJ-1 function at other sites in the polypeptide chain.

Our experimental approach consisted of comparing recombinant wild-type DJ-1 and M26I in terms of fold changes in oxidation at C106 and other sites following treatment with a 10- or 500-fold molar excess of H$_2$O$_2$. This approach was reasonable because our focus was to compare the two DJ-1 variants in terms of their intrinsic propensities to undergo oxidation. Accordingly, we considered it important to avoid complicating factors that might mask or offset these intrinsic propensities, including effects of protein-protein interactions in a cellular or animal model. To quantitate fold changes in oxidation at each site, we developed a new method involving LC/MS/MS analysis of a mixture of $^{16}$O- and $^{18}$O-labeled peptides derived from untreated and H$_2$O$_2$-treated DJ-1, respectively. To our knowledge, $^{18}$O labeling technology has not been applied previously to quantitative analysis of oxidative protein modifications. An advantage of the $^{18}$O-labeling method is that it can be used to quantitate multiple oxidative modifications simultaneously, whereas other approaches enable quantitation of only a single type of modification (e.g. cysteine oxidation (50–55) and carbonylation (56)). While this manuscript was being written, Pimenova et.al (57) described a method to simultaneously measure multiple oxidative protein modifications using isobaric tags for relative and absolute quantitation labeling technology. A potential drawback of labels such as isobaric tags for relative and absolute quantitation is that these reagents can produce unpredicted fragmentation patterns which confound the interpretation of MS data (58). In contrast, the $^{18}$O-labeling approach described here avoids this pitfall because facile incorporation of the $^{18}$O
Effects of the M26I Substitution on C106 Oxidation—Oxidative modifications of DJ-1 have been characterized by mass spectrometry in a number of earlier studies (13, 15, 24, 26). Here we describe fold changes in oxidation at specific sites deduced from quantitative MS data. We found that residue C106 of wild-type DJ-1 was preferentially oxidized to the sulfenic or sulfonic acid upon treatment of the protein with a 10- or 500-fold molar excess of H$_2$O$_2$, consistent with previous findings (24). Wild-type DJ-1 (but not the M26I mutant) exhibited an increase in the level of C106 sulfenic acid after exposure to a 10-fold molar excess of H$_2$O$_2$. On the basis of semiquantitative 2D PAGE data we confirmed that wild-type DJ-1 had a greater propensity to convert to the 2O form under these oxidizing conditions.

From these observations, we infer that the M26I substitution alters the structure of DJ-1 in a way that decreases the reactivity of C106 with H$_2$O$_2$. A previous study showed that C106 is more readily oxidized than the single cysteine residue of bovine serum albumin (26), presumably because the “active site” pocket surrounding C106 contains a number of acidic and basic residues (including the protonated carboxylic acid sidechain of E18) that are suitably positioned to promote the transfer of two oxygen atoms to the cysteine sidechain (27–29). The M26I mutation may induce a conformational change that disrupts the configuration of residues in this pocket.

### Table III

| Variant | Best-fit model     | Molecular weight (Da) | $K_d$         |
|---------|--------------------|-----------------------|---------------|
| WT 2O   | monomer-dimer      | 37,980                | $6 \pm 1 \times 10^{-8}$ (M) |
| WT     | monomer-dimer      | 38,060                | $3.6 \pm 2 \times 10^{-8}$ (M) |
| M26I 2O | monomer-tetramer   | 28,769                | $4.2 \pm 0.6 \times 10^{-17}$ (M$^2$) |
| M26I   | monomer-dimer      | 30,386                | $6 \pm 2 \times 10^{-7}$ (M) |
Consistent with this idea, analysis of M26I by two-dimensional NMR revealed chemical shift changes in charged residues that may play a role in modulating C106 oxidation (37). In addition, the M26I substitution was shown by x-ray crystallography to induce a packing defect in the interior of the protein (36). Moreover, we found that C53 was oxidized to the sulfonic acid upon exposure of M26I (but not wild-type DJ-1) to a 500-fold molar excess of \( \text{H}_2\text{O}_2 \). C53 is located at the subunit interface (22, 29, 59, 60) and is less readily oxidized than C106 in the wild-type protein (16, 24). Therefore, increased oxidation of C53 in M26I may reflect a weakening of intersubunit contacts in the mutant homodimer. In support of this idea, our sedimentation data revealed that M26I has a slightly higher dimer dissociation constant than the wild-type protein. In turn, a structural perturbation at the dimer interface could contribute to the impaired ability of M26I to convert to the 2O form, given that the active site pocket surrounding C106 consists of residues from both subunits. Importantly, our far-UV CD and sedimentation data indicated that unoxidized wild-type DJ-1 and M26I have similar secondary and quaternary structures, consistent with previous reports that the M26I mutation has little impact on the global fold of the protein (36, 37). In an earlier report we presented evidence that M26I had substantially reduced secondary structure compared with wild-type DJ-1, even without exposure to \( \text{H}_2\text{O}_2 \) (15). However, in contrast to the experiments with untagged DJ-1 summarized here, the previous study was carried out using N-terminally histidine-tagged variants, and we have found that the presence of the histidine tag promotes oxidative modifications that induce M26I misfolding and aggregation (Hulleman and Rochet, unpublished observations).

In addition to having conformational properties that potentially disfavor C106 oxidation, M26I may incur a loss of stability upon oxidation by \( \text{H}_2\text{O}_2 \). In turn, this loss of stability may initially disfavor C106 oxidation, M26I may incur a loss of stability modifications that induce M26I misfolding and aggregation upon oxidation to the 3O form (15, 36, 37), resulting in a diminished recovery of the mutant protein prior to MS analysis (as outlined above in the case of M26I oxidized to the 2O form).

Based on the results presented herein and data reported by Canet-Avielé et al. (16), we would predict that M26I should have a decreased ability to localize to mitochondria in oxidatively stressed cells. Surprisingly, however, the Cookson group reported data showing the opposite trend—namely, that M26I has a greater propensity to relocalize to mitochondria in oxidatively stressed cells than the wild-type protein (61). Therefore, we infer that oxidation to the 2O form may not always be a key regulatory event that determines whether wild-type or mutant forms of DJ-1 localize to mitochondria (i.e. because M26I, despite its reduced propensity to convert to the 2O form, has a strong ability to localize to mitochondria). Consistent with this inference, another group has reported that a significant level of DJ-1 is associated with mitochondria in cultured cells and animals even in the absence of oxidative stress (62).

The data presented here, together with observations that oxidation of DJ-1 to the 2O form is necessary for inhibition of aSyn fibril formation (24), suggest that M26I may have a reduced ability to suppress aSyn fibrilization compared with the wild-type protein. In support of this idea, a recent study revealed that M26I pretreated with \( \text{H}_2\text{O}_2 \) inhibited aSyn fibril formation less efficiently than the oxidized wild-type protein (38). Additional evidence suggests that the oxidation of DJ-1 at position 106 is necessary for protection against toxicity related to complex I inhibition (Liu and Rochet, unpublished data) (30). Accordingly, we infer that the decreased propensity of M26I to undergo conversion to the 2O form as reported here may result in various functional defects in familial PD patients, including a reduced ability of the protein to carry out a chaperone function in cytosolic and/or mitochondrial compartments.

**Effects of the M26I Substitution on H115 Conversion to Asparagine—Treatment of wild-type DJ-1 (but not M26I) with a 10- or 500-fold molar excess of \( \text{H}_2\text{O}_2 \) led to the conversion of H115 to asparagine. We interpret this result to mean that H115 oxidation to asparagine is (1) disfavored by conformational rearrangements resulting from the M26I substitution, and/or (2) promoted by structural changes resulting from C106 oxidation in wild-type DJ-1. A comparison of the x-ray structures of the wild-type protein (unoxidized and 2O) and M26I (unoxidized) using PyMol software revealed similar residue configurations near H115 in all three structures (Fig. 8), although crystal lattice packing may have played a role in stabilizing the protein conformation in this region. NMR analysis revealed only very minor chemical shift changes between wild-type DJ-1 and M26I at position 115 (37). Thus, we favor...
the second interpretation outlined above, namely, that the wild-type protein more readily undergoes H-to-N conversion at position 115 because of its greater propensity to become oxidized at position 106. Because H115 is strictly conserved among eukaryotic DJ-1 variants (63), we predict that oxidation of this residue to asparagine could have functional consequences. PyMol analysis revealed that replacement of H115 with asparagine only subtly perturbed interactions with nearby residues in the structure of wild-type 2O DJ-1 (Fig. 8).

Effects of Methionine Oxidation on DJ-1 Stability—The data in Table II revealed that peptides having undergone methionine oxidation at positions 17, 26, 133, or 134 were depleted in samples of wild-type DJ-1 exposed to a 10-fold molar excess of H2O2 compared with untreated samples. These results imply that DJ-1 isoforms with methionine sulfoxide at these sites have a high propensity to undergo misfolding and aggregation on further oxidation, and are thus lost from H2O2-treated samples prior to MS analysis. Consistent with this interpretation, all four methionine residues are highly conserved among vertebrate forms of DJ-1 (63), suggesting that the structural elements encompassing these residues are intolerant of chemical modifications. Accordingly, oxidation of these methionine residues may cause a partial loss of DJ-1 stability, thereby predisposing the protein to unfold and aggregate following additional modifications.

Because M26 is located near the subunit interface of DJ-1, oxidation at this site may destabilize the wild-type homodimer. This prediction is supported by evidence presented here and in other reports (15, 36, 37) that the M26I substitution elicits conformational changes that in turn may cause a decrease in dimer stability. Interestingly, MS analysis of DJ-1 from human postmortem brain samples reveals that methionine is oxidized to methionine sulfoxide at positions 17, 26, 133, and 134 in PD patients, whereas methionine sulfoxide is only detected at positions 17 and 26 in age-matched controls (13). These findings indicate that the oxidation of each methionine residue occurs under physiological conditions. Moreover, the abundance of DJ-1 isoforms with methionine sulf oxide at these sites may increase as the activity of MsrA, an enzyme that repairs methionine sulfoxide, decreases with age (64). An age-dependent decrease in MsrA activity coupled with increases in methionine oxidation of DJ-1 could contribute to the increased risk of PD with aging (14, 65). Importantly, DJ-1 isoforms with methionine sulfoxide at positions 133 and 134 are detected in the post-mortem brains of sporadic PD patients, but not age-matched controls (13). Our findings suggest that DJ-1 dysfunction, resulting from misfolding and aggregation of the methionine-oxidized protein under conditions that normally favor oxidation to the functionally relevant 2O form, may contribute to neurodegeneration in sporadic PD.

The more pronounced loss of methionine-oxidized M26I compared with methionine-oxidized wild-type DJ-1 in the presence of a 10-fold molar excess of H2O2 is consistent with
the observation that M26I is less stable than the wild-type protein (15, 36, 37). Thus, the combined destabilizing effects of the M26I substitution and methionine oxidation may result in greater DJ-1 misfolding and aggregation (and a more pronounced loss of function) upon further oxidation than the milder destabilizing effect of methionine oxidation alone. In turn, the enhanced loss of function expected of methionine-oxidized M26I compared with methionine-oxidized wild-type DJ-1 may contribute to the earlier onset of disease in familial PD patients with the M26I mutation.

Conclusion—In summary, we have exploited the unique power of mass spectrometry and proteomic technology to determine the impact of a familial mutation on the propensity of DJ-1 to undergo oxidation at specific sites. We find that the M26I substitution interferes with the ability of the protein to undergo oxidation to the 2O form, a functionally important modification. This effect apparently involves subtle conformational perturbations that may alter the geometry of the active site pocket surrounding C106 rather than a global unfolding of the protein. These observations lay the groundwork for investigating whether other DJ-1 mutants without a global folding site pocket surrounding C106 rather than a global unfolding of the protein. Our findings also suggest that the mild oxidation that normally results in functional enhancement of wild-type DJ-1 can cause destabilization and aggregation of DJ-1 isoforms having undergone initial stages of oxidative damage, including methionine oxidation characteristic of sporadic PD. Importantly, our approach of studying recombinant DJ-1 variants in an isolated protein (15, 36, 37) has enabled us to characterize mutant and oxidized forms of the protein in terms of their intrinsic propensities to undergo a functionally relevant oxidative modification. We note that these intrinsic propensities are likely modulated by additional factors (e.g. protein-protein interactions) in the complex environment of the brain, and thus our findings set the stage for future research aimed at understanding the role of potential in vivo modulatory factors.

The results reported here represent new evidence that DJ-1 dysfunction resulting from a mutation or oxidative damage may contribute to neurodegeneration in familial or sporadic PD. Our findings also imply that the function of wild-type or mutant DJ-1 may be enhanced by small molecules which promote conversion to the 2O form by (1) selectively binding the C106 sulfenic acid (66), or (2) stabilizing structural motifs destabilized by methionine oxidation. Such compounds may be useful therapeutic agents to enhance DJ-1 function and alleviate neurodegeneration in PD patients.

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This article contains supplemental Figs. S1 to S4 and Tables S1 to S3.

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