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Conservation of Oxidative Protein Stabilization in an Insect Homologue of Parkinsonism-Associated Protein DJ-1

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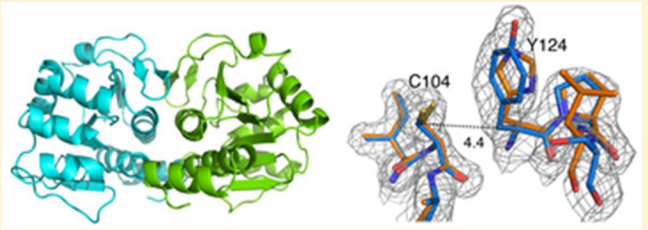
Supporting Information

ABSTRACT: DJ-1 is a conserved, disease-associated protein that protects against oxidative stress and mitochondrial damage in multiple organisms. Human DJ-1 contains a functionally essential cysteine residue (Cys106) whose oxidation is important for regulating protein function by an unknown mechanism. This residue is well-conserved in other DJ-1 homologues, including two (DJ-1α and DJ-1β) in *Drosophila melanogaster*. Because *D. melanogaster* is a powerful model system for studying DJ-1 function, we have determined the crystal structure and impact of cysteine oxidation on *Drosophila* DJ-1β. The structure of *D. melanogaster* DJ-1β is similar to that of human DJ-1, although two important residues in the human protein, Met26 and His126, are not conserved in DJ-1β. His126 in human DJ-1 is substituted with a tyrosine in DJ-1β, and this residue is not able to compose a putative catalytic dyad with Cys106 that was proposed to be important in the human protein. The reactive cysteine in DJ-1 is oxidized readily to the cysteine-sulfinic acid in both flies and humans, and this may regulate the cytoprotective function of the protein. We show that the oxidation of this conserved cysteine residue to its sulfinate form (Cys-SO_2^-) results in considerable thermal stabilization of both *Drosophila* DJ-1β and human DJ-1. Therefore, protein stabilization is one potential mechanism by which cysteine oxidation may regulate DJ-1 function in vivo. More generally, most close DJ-1 homologues are likely stabilized by cysteine-sulfinic acid formation but destabilized by further oxidation, suggesting that they are biphasically regulated by oxidative modification.



The DJ-1 superfamily is a large and functionally varied group of proteins with homologues in many organisms. Human DJ-1 has been independently shown to be an oncogene with an elevated level of expression in many types of cancer[1] and a cytoprotective protein whose deficiency is a rare cause of heritable parkinsonism.[2] DJ-1 enhances eukaryotic cell survival in response to various stressors, particularly oxidative stress and mitochondrial toxins.[3−6] Several activities have been demonstrated for DJ-1, including participation in multiple signaling pathways involving mitochondrial uncoupling proteins,[7] ASK1,[8,9] AKT/PI3K/PTEN,[10,11] JNK,[12,13] ERK,[14,15] p53,[16−19] NF-κB,[20] and Nrf2.[21] DJ-1 has also been proposed to act as a redox-sensitive chaperone that inhibits α-synuclein aggregation,[22,23] an RNA binding protein,[24] a regulator of glutathione synthesis,[25,26] and a transcriptional regulator.[27] Despite strong evidence of a role in stress response in eukaryotes and an abundance of proposed activities, the mechanistic details of DJ-1's function remain poorly understood.

Drosophila melanogaster provides a versatile animal model system for the study of neurodegenerative diseases, including parkinsonism.[10,28,29] *D. melanogaster* has two orthologs of human DJ-1, DJ-1α and DJ-1β, both of which have sequences ~50% identical with that of the human protein. These orthologs differ in tissue distribution, with DJ-1α being primarily expressed in the testes of male flies and DJ-1β being expressed in most tissues.[5,29,30] Although both DJ-1 orthologs appear to play a significant role in *Drosophila* biology, DJ-1β has the larger role in organismal defense against oxidative stress.[5,30] Flies lacking both DJ-1 homologues have reduced rates of survival when exposed to compounds that induce oxidative stress and display motor deficits without frank dopaminergic neurogeneration.[5,29−31] Consistent with results of several mammalian DJ-1 studies, the *Drosophila* DJ-1 homologues are important for the maintenance of proper mitochondrial function, which may be a key site of DJ-1 action.[30,32] Both proteins contain a highly conserved, oxidation-prone cysteine residue (Cys104 in DJ-1β and Cys106 in human DJ-1) that is critical for DJ-1's protective function against oxidative stress in *Drosophila* and several other model systems.[3,9,33] Furthermore, human DJ-1 can complement *Drosophila* DJ-1β knockout phenotypes, underscoring the similarity between the human and fly proteins.[33] Therefore, *D. melanogaster* provides a powerful and established animal model system for studying DJ-1 function.

Despite intensive study, a number of questions remain about the role of the conserved Cys106 residue (human residue numbering) in DJ-1 function. Multiple studies indicate that the oxidation of this residue to the sulfinate (Cys106-SO_2^-) is important for DJ-1 function in eukaryotes,[3,34−36] although the detailed mechanism by which oxidation regulates DJ-1 function

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is not fully understood. One model holds that modification of Cys106 allows DJ-1 to function as a redox sensor, implying that both reduced and oxidized forms of the residue may have functional significance and perhaps perform distinct cellular functions.[3,37] However, an unusual plant homologue of DJ-1 that lacks this cysteine residue is required for chloroplast development and thus viability, suggesting that some aspects of DJ-1 function may be redox-insensitive, at least in *Arabidopsis thaliana*.[38] Oxidation of human DJ-1 has been shown to destabilize the protein and is correlated with disease,[39,40] although it is unclear if this destabilization is due to the formation of the mildly oxidized Cys106-SO_2^- isoform, the more extensively oxidized Cys106-SO_3^- (sulfonate) modification, or other oxidative modifications in DJ-1.

In addition to its susceptibility to oxidative modification, reduced Cys106 itself might play a role in DJ-1 function. In human DJ-1, Cys106 has a low pK_a value of 5.4 and thus could serve as a catalytic nucleophile, although no definitive enzymatic activity for human DJ-1 has been demonstrated to date.[41] One proposed enzymatic activity for DJ-1 has been as a cysteine protease that uses a Cys106-His126 dyad in the active site.[42] However, His126 in human DJ-1 is not well-conserved in other homologues, including *D. melanogaster* DJ-1β (where it is a tyrosine), raising questions about the relevance of this proposed dyad for DJ-1 function. As human DJ-1 and *Drosophila* DJ-1β appear to have similar protective functions, it is unlikely that there is a large divergence in the molecular activity responsible for this function. Assessing the likelihood of an unconventional Cys104-Tyr124 catalytic dyad in DJ-1β has been hampered by the absence of an available structure for the *Drosophila* homologue.

We report the 2.0 Å resolution crystal structure of *D. melanogaster* DJ-1β. As expected, DJ-1β is structurally similar to human DJ-1, and most differences are confined to regions on the periphery of the protein. Tyr124 (equivalent to His126 in human DJ-1) is in a conformation that prohibits hydrogen bonding with Cys104 and thus is unlikely to compose an active site dyad that has been proposed to be important in the human protein. Therefore, if DJ-1 possesses an enzymatic activity, it is not likely to involve a dyad between the reactive cysteine and the histidine/tyrosine residue. The observed oxidation of Cys104 in DJ-1β to the cysteine-sulfinate thermally stabilizes the protein by 11.5 °C, similar to the case for human DJ-1. This substantial oxidative stabilization upon cysteine-sulfinic acid formation may contribute to the enhancement of DJ-1 function under oxidative stress conditions and is likely common to other DJ-1 homologues.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. *D. melanogaster* DJ-1β contains an N-terminal extension that is not found in many homologues of the protein and is a possible targeting sequence. Consequently, the mature coding sequence corresponding to the region of the protein that aligns well with other DJ-1 homologues (residues 19−205) was cloned between the NdeI and XhoI sites of bacterial expression vector pET15b. BL21(DE3) *Escherichia coli* were grown in Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin at 37 °C with shaking until the OD_{600} reached 0.5−0.7. The temperature of the culture was reduced to 20 °C and equilibrated for 2 h with shaking. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM to the culture, which was incubated at 20 °C with shaking overnight and harvested by centrifugation the next day. For some preparations, a same-day induction protocol in which protein expression was induced with 0.5 mM IPTG for 3−4 h at 37 °C was followed. Cell pellets were frozen and stored at −80 °C.

N-Terminally hexahistidine-tagged DJ-1β was purified using metal affinity chromatography with His-Select resin (Sigma). The cell pellet was thawed and then resuspended in extraction buffer [50 mM HEPES (pH 7.5), 300 mM NaCl, 10 mM imidazole, and 2 mM DTT], supplemented with 1 mg/mL lysozyme and incubated on ice for 30 min, and then sonicated to complete lysis. Centrifugally cleared lysate was mixed with His-Select resin (Sigma) in batch at 4 °C and then poured into a column and washed with 20 mM imidazole-supplemented extraction buffer until no protein was detected in the flow-through using Bradford reagent. The bound recombinant protein was eluted using 250 mM imidazole-supplemented extraction buffer. The N-terminal hexahistidine tag was removed by cleavage with 1 unit of thrombin per milligram of DJ-1β for 2 h at 22 °C followed by dialysis against storage buffer [25 mM HEPES (pH 7.5), 100 mM KCl, and 2 mM DTT] at 4 °C overnight. The protein was passed again over His-Select resin to remove any protein that retained the tag and then incubated at 4 °C with benzamidine-Sepharose resin to remove thrombin. Purified protein was concentrated using a centrifugal concentrator (Millipore) with a 10 kDa cutoff to 21 mg/mL as determined by the absorbance at 280 nm using a calculated extinction coefficient at 280 nm of 7500 M^{-1} cm^{-1}. The purified protein in storage buffer was snap-frozen in 50−100 μL aliquots on liquid nitrogen and stored at −80 °C.

Crystallization and Data Collection. DJ-1β (21 mg/mL) in storage buffer was crystallized using the sitting drop vapor diffusion method with drops containing 2 μL of protein and 2 μL of reservoir solution. Rhombohedrally shaped crystals of DJ-1β formed in space group $P3_221$ in 2 days at room temperature against a reservoir solution of 25% polyethylene glycol 4000, 0.2 M ammonium sulfate, and 0.1 M sodium acetate (pH 4.6). Crystals typically grew from a dense precipitate that was carefully removed using a nylon loop during crystal harvest. The crystals were cryoprotected by being serially transferred through reservoir solutions supplemented with ethylene glycol increasing in 5% increments to a final concentration of 25% (v/v). Cryoprotected crystals were cooled in liquid nitrogen.

Diffraction data were collected from a single crystal at 100 K at the Advanced Photon Source, GM/CA-CAT beamline 23 ID-D. The crystal was exposed to 12.66 keV (0.98 Å) X-rays for 2 s/deg of oscillation for a total of 180°, and the resulting diffraction data were recorded on a MARmosaic 300 CCD detector. Data were integrated and scaled using HKL2000,[43] and final data statistics for each data set are listed in Table 1.

Structure Determination, Refinement, and Validation. Phases for DJ-1β were determined by molecular replacement using the human DJ-1 dimer (PDB entry 2OR3)[41] as a search model in the CCP4[44] implementation of PHASER.[45] The initial model for DJ-1β was autobuilt using Phenix,[46] inspected and manually improved in COOT,[47] and subsequently refined in Refmac5.[48] Because the functional dimer of DJ-1β is present in the asymmetric unit, noncrystallographic symmetry restraints were applied to each monomer. Once maximum likelihood refinement of coordinates and isotropic displacement parameters had converged as judged by the absence of significant changes in the *R* factors or log likelihood gradient upon refinement, a translation-libration-screw (TLS) model with

Table 1. Data Collection and Refinement Statistics

Data Collection	
X-ray source	APS GM/CA 23ID-D
X-ray wavelength (Å)	0.98
space group	$P3_221$
cell dimensions, $a = b$, c (Å)	52.52, 227.17
no. of molecules in the asymmetric unit	2
Wilson B factor (Å^2)	26.9
resolution (Å)	75.7−2.0
R_{merge} [a,b]	0.098 (0.587)
$\langle I\rangle/\langle\sigma(I)\rangle$ [a]	21.1 (1.8)
completeness [a] (%)	97.3 (83.9)
redundancy [a]	8.5 (5.1)
Refinement	
PDB entry	4E08
program	Refmac5.6.0116
no. of unique reflections	23814
R_{work} [c], R_{free} [d] (%)	19.3, 24.6
average B factor (Å^2)	
chain A, chain B	30.8, 31.4
solvent	37.3
rmsd	
bond lengths (Å)	0.016
bond angles (deg)	1.503
Ramachandran plot [favored, allowed, forbidden (%)]	99.2, 99.7, 0.27

[a]Values in parentheses are for the highest-resolution shell (2.07−2.00 Å). [b]$R_{merge} = \sum_{hkl}\sum_i|I^i_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_i I^i_{hkl}$, where i is the ith observation of a reflection with indices h, k, and l and angle brackets indicate the average over all i observations. [c]$R_{work} = \sum_{hkl}|F^o_{hkl} - F^c_{hkl}|/\sum_{hkl}F^o_{hkl}$, where F^c_{hkl} is the calculated structure factor amplitude with indices h, k, and l and F^o_{hkl} is the observed structure factor amplitude with indices h, k, and l. [d]R_{free} is calculated as R_{work}, where the F^o_{hkl} values were taken from a test set comprising 5% of the data that were excluded from the refinement.

each monomer defined as a separate rigid group was refined.[49] The final model was validated using MolProbity[50] and COOT.[47] Final model statistics are listed in Table 1. Structural figures were made with PyMOL (Schrodinger).

Thermofluor Assay of DJ-1 Stability. Recombinant reduced human DJ-1 was purified and oxidized to the cysteine-sulfinate form as previously described.[34] We oxidized *Drosophila* DJ-1β to the cysteine-sulfinate form by desalting the purified protein into water using P6-DG resin (Bio-Rad), adding hydrogen peroxide (Fisher) to a final molar ratio of 7:1 with the monomeric protein, and incubating the sample on ice for 45 min. Unreacted peroxide was removed by centrifugal desalting using P6-DG resin. The oxidation states of both proteins were confirmed using electrospray mass spectrometry (see below).

The thermal stabilities of reduced and oxidized DJ-1 were measured using the thermofluor assay[51] in 25 mM HEPES (pH 7.5), 100 mM KCl, and 1 mM DTT. A 5000× stock of Sypro Orange in DMSO (Sigma-Aldrich) was added to protein (1−5 mg/mL) at concentrations ranging from 50× to 250× in optically clear polymerase chain reaction tube strips (Bio-Rad). The samples were heated from 20 to 95 °C at a rate of 2 °C/min, while fluorescence was excited at 490 nm and monitored at 575 nm using an iCycler iQ real-time thermal cycler (Bio-Rad). Data were plotted as the first derivative of fluorescence as a function of temperature, whose peak corresponds to the reported melting temperature (T_m). All measurements were taken in triplicate at multiple protein concentrations with representative data shown in Figure 5.

Mass Spectrometry Analysis of Oxidative Modifications in DJ-1. Protein samples were digested with endopeptidase GluC for 6 h according to the manufacturer's instructions (Promega) followed by digestion with trypsin (Roche) at 37 °C without prior protein reduction or alkylation. The tryptic peptides were dried in a SpeedVac (Savant) and then desalted and concentrated using a monolithic PepMap C18 trap column (300 μm inner diameter, 1 mm length, 5 μm particle size, 100 Å pore size) loaded at a flow rate of 300 nL/min. Eluted peptides were separated on a second C18 PepMap column (75 μm inner diameter, 15 cm length, 3 μm particle, 100 Å pore) with an acetonitrile/0.1% formic acid gradient. The tryptic peptides were subjected to LC−MS/MS analysis using an Ultimate 3000 Dionex MDLC system (Dionex Corp.) integrated with a nanospray source and an LCQ Fleet Ion Trap mass spectrometer (Thermo Finnigan). The LCQ Fleet mass spectrometer was operated with a nanospray voltage of 2 kV, a capillary temperature of 200 °C, and a full scan m/z range of 400−2000. The mass spectrometer was operated in data-dependent mode with four MS/MS spectra collected for every full scan, five microscan averaging for full and MS/MS scans, a three m/z unit isolation width, and 35% collision energy for collision-induced dissociation. Dynamic exclusion was enabled with an exclusion duration of 1 min. The MS/MS spectra were searched against sequences for human DJ-1 and *Drosophila* DJ-1β that had been imported into the IPI human protein database using MASCOT (version 2.2, Matrix Science). Database search criteria were as follows: GluC/trypsin cleavage (i.e., peptides cleaved at Lys, Arg, Asp, or Glu residues), two missed cleavages permitted, monoisotropic masses, variable modifications that included cysteine-sulfenic acid (Cys-SOH), cysteine-sulfinic acid (Cys-SO_2^-), cysteine-sulfonic acid (Cys-SO_3^-), and methionine sulfoxide, a peptide mass tolerance of 1.5 Da, and a MS/MS fragment ion tolerance of 1 Da.

RESULTS

Structure of *Drosophila* DJ-1β. The DJ-1β construct used in this work results in a 187-amino acid monomer (19 kDa), and the numbering used in this paper assigns the first amino acid in the protein to amino acid 19 in the deposited sequence (GenBank entry AAF57086.2, gil28381503; see Experimental Procedures). Electron density maps calculated after density modification of molecular replacement phases in Phenix allowed for the automatic placement of all residues in DJ-1β except for two amino acids each at the N- and C-termini, which are disordered. Like other close DJ-1 homologues, DJ-1β is a dimer with extensive intersubunit interactions, resulting in 1278 Å^2 of buried surface area per monomer at the dimer interface as calculated by the ePDB PISA webserver.[52] This amount of buried surface area is comparable to that of human DJ-1 (PDB entry 2OR3, 1349 Å^2), indicating that DJ-1β is also an obligate homodimer. The only Ramachandran outlier in the refined model is the conserved Cys104 residue, which is often found in marginal or unfavorable regions of Ramachandran space.

Structural Comparison of DJ-1β and Human DJ-1. The backbone structure of *Drosophila* DJ-1β is highly similar to that of human DJ-1 (PDB entry 2OR3) with a core Cα atom rmsd value for the dimers of 0.69 Å (Figure 1A). Differences between the two structures are most pronounced at the termini and in two solvent-exposed helical regions, including amino acids 58−64 and 125−134 (DJ-1β numbering). These regions also have

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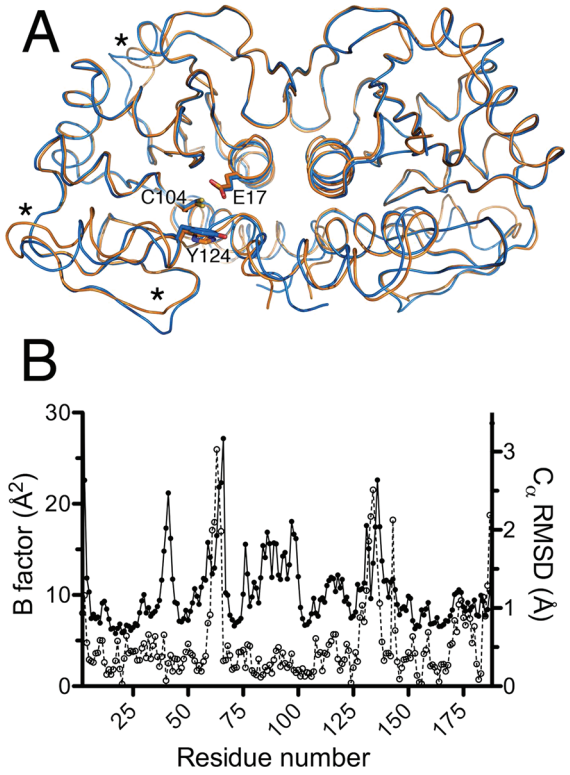

Figure 1. DJ-1β and human DJ-1 are structurally similar. (A) The physiologically relevant dimeric forms of human DJ-1 (orange) and *Drosophila* DJ-1β (blue) are superimposed, showing nearly identical backbone structures. The functionally significant residues Cys104 (Cys106), Glu17 (Glu18), and Tyr124 (His126) are shown (human DJ-1 residue numbering in parentheses). Regions with the largest variation between the human and fly proteins are marked with asterisks. (B) Areas of greatest structural variation between the human and *Drosophila* proteins correlate with elevated atomic displacement parameter values in the human protein. Filled circles represent the average equivalent isotropic *B* factors for the Cα atoms of human DJ-1 (PDB entry 2OR3), while empty circles represent the rmsd of the Cα atoms of the *Drosophila* and human DJ-1 crystal structures. The areas marked with asterisks in panel A correspond to the rmsd peaks from residues 60−70 and 125−150. In both plots, only data for chain A of the dimeric proteins are shown.

elevated atomic displacement parameters (ADPs) for Cα atoms in human DJ-1, suggesting that they are intrinsically flexible in close DJ-1 homologues (Figure 1B). However, the converse is not true: there are regions with elevated ADPs in human DJ-1 that do not correspond to regions with high Cα rmsd values for DJ-1β (Figure 1B), likely because of crystal packing contacts. Most amino acids that have been shown to be important for human DJ-1 function or have been identified in patients with DJ-1-related parkinsonism are conserved in DJ-1β, including Leu10, Glu18, Ala104, Cys106, Asp149, Glu163, and Leu166 (human DJ-1 numbering).

However, one position at which a functionally important residue in human DJ-1 is not conserved in *Drosophila* DJ-1β is Met26. The M26I mutation in human DJ-1 was identified in an Ashkenazi Jewish patient with early onset parkinsonism[53] and has been shown to be detrimental to DJ-1 function in cell culture.[9,54] Therefore, this clinical variant is likely a bona fide pathological mutation and not a polymorphism. The structures of DJ-1β and human DJ-1 are highly similar in this region (Figure 2), despite the Met to Leu substitution at position 25.

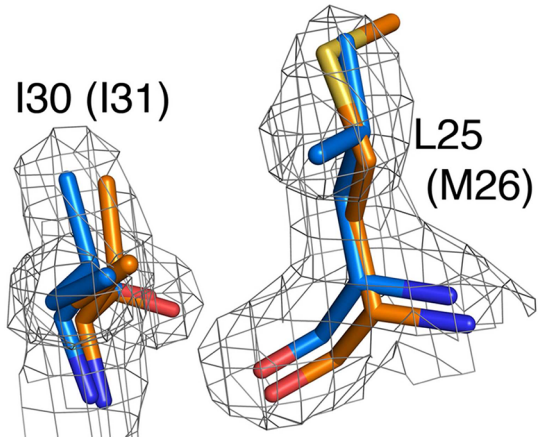

Figure 2. Parkinsonism-associated Met26 residue replaced with a structurally conservative leucine residue in DJ-1β. A stick representation of the superimposed structures of human DJ-1 (orange) and *Drosophila* DJ-1β (blue) shows that the substitution of leucine for methionine causes minimal structural disruption in that area, in contrast to packing defects created by the M26I mutation in human DJ-1. *Drosophila* DJ-1β numbering is shown with human residue numbering in parentheses.

Importantly, *Drosophila* DJ-1β does not exhibit the displaced I31 residue that is found in human M26I DJ-1,[55] indicating that the modest perturbation at this region in human M26I DJ-1 is a good candidate for the structural disruption that results in M26I DJ-1-linked pathogenesis.

Cys104 Is Reactive and Partially Oxidized to the Sulfinate in the Crystal. Nearly all members of the DJ-1 superfamily contain a cysteine residue at the juncture between a β-strand and an α-helix called the "nucleophile elbow" in the α/β-hydrolases. This cysteine serves a catalytic role in some members of the DJ-1 superfamily, including validated peptidases, glyoxalases, and isocyanide hydratases.[56−59] In the close homologues of human DJ-1, this residue is highly reactive and oxidation-prone, although no unambiguous enzymatic activity has been identified in these proteins. The environment surrounding Cys104 in DJ-1β is similar to that of human DJ-1, including the presence of a functionally important glutamic acid (Glu18 in human DJ-1 and Glu17 in *Drosophila* DJ-1β). The $2mF_o - DF_c$ electron density for Cys104 in DJ-1β is consistent with a predominantly reduced thiol(ate), although difference electron density indicates some minor amount of modification has occurred (Figure 3). The two peaks in the difference electron density are consistent with partial oxidation to the cysteine-sulfinate, which has been directly observed in the crystal structure of the human protein and inferred on the basis of acidic pI shifts for *Drosophila* DJ-1β. We note that these two difference electron density peaks could alternatively be modeled as a cysteine-sulfenic acid (Cys-SOH) sampling two alternative conformations. However, electrospray mass spectrometry of intentionally oxidized DJ-1β (see Experimental Procedures) confirms that the peptide containing Cys104 is oxidized to the cysteine-sulfinate in solution (Table 1 of the Supporting Information).

A Proposed Catalytic Dyad Is Absent in DJ-1β. The presence of a reactive cysteine suggests the possibility that DJ-1β may be an enzyme. Members of the PfpI and Hsp31 clades of the DJ-1 superfamily have the conserved reactive cysteine in a putative Cys-His-Glu/Asp catalytic triad in which the histidine immediately follows the cysteine residue.[60,61] Some

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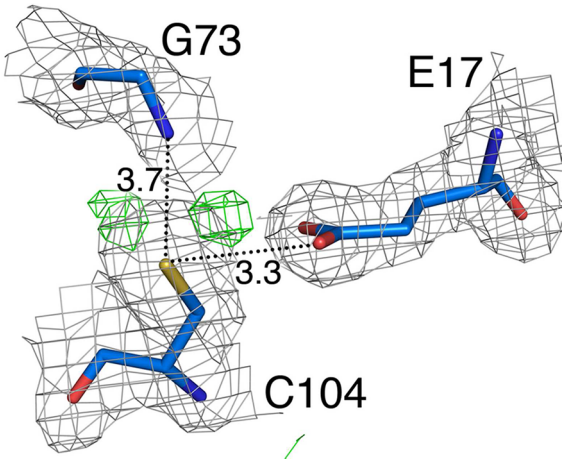

Figure 3. Cys104 is reactive and makes hydrogen bonds with several surrounding residues. The hydrogen bonding environment of Cys104 is shown, with $2mF_o - DF_c$ electron density (1σ) colored gray and $mF_o - DF_c$ difference electron density (3.5σ) colored green and red. The small positive peaks in difference electron density (green) around Cys104 indicate a minor amount of oxidative modification of the residue, likely to the cysteine-sulfinate. Dotted lines represent hydrogen bonds with distances in angstroms.

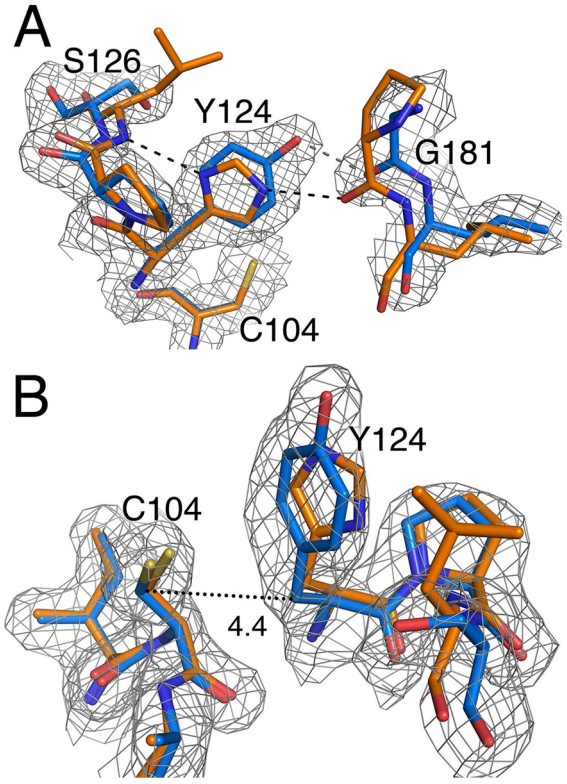

Figure 4. Putative cysteine-histidine dyad in human DJ-1 that is absent in the active site of DJ-1β. (A) Superimposed models for human DJ-1 (orange) and DJ-1β (blue) around His126 in human DJ-1. This residue was proposed to compose a dyad with Cys106, although it is substituted with a tyrosine in DJ-1β. The $2mF_o - DF_c$ electron density map calculated for DJ-1β at 2.0 Å resolution is contoured at 1σ and colored gray. (B) Putative active site region around the reactive Cys104 in DJ-1β. Although the histidine side chain in the human protein could reorient by ~180° to form a dyad with Cys106, the limited space (4.4 Å) between Cys104 and Tyr124 precludes rotational reorientation to create a Cys-Tyr dyad required for the protease model for DJ-1 function.

of these proteins are validated proteases or peptidases,[56,62] although others appear to lack this activity.[63,64] Early speculation about a function for human DJ-1 suggested that it might also be a protease, although a catalytic triad is absent in human DJ-1.[65] A different but nearby histidine residue (His126) in human DJ-1 was proposed to compose an alternative catalytic dyad with Cys106.[42] Because the imidazole side chain of His126 is not oriented properly to form a hydrogen bond with Cys106 in full-length DJ-1 (Figure 4A), it has been suggested that DJ-1 may be a pro-protein that is activated by the proteolytic cleavage of the C-terminal α-helix (residues 174−189) of DJ-1. This cleavage would allow rotation of the His126 side chain and completion of the dyad with Cys106, and truncated recombinant DJ-1 lacking this C-terminal peptide does have elevated proteolytic activity in vitro.[42] *Drosophila* DJ-1β has a tyrosine at the equivalent position (Tyr124), which is a less compelling candidate for a second member of a catalytic dyad. The crystal structure of DJ-1β shows that the aromatic ring of Tyr124 superimposes well with the imidazole ring of His126 in human DJ-1, which positions its phenolic oxygen away from Cys104 (Figure 4A). Because any functional dyad would require a hydrogen bond donor to interact with Cys104, this orientation of Tyr124 prohibits formation of a dyad with Cys104. The proposed removal of the C-terminal peptide of DJ-1β could result in rotation of Tyr124, but the size of the Tyr124 side chain and its preferred rotameric states prohibit a hydrogen bonding interaction with Cys104, which is only 4.4 Å from Tyr124 as measured between Cβ atoms (Figure 4B).

Oxidation of Cys106 to the Cysteine-Sulfinate Stabilizes DJ-1. The conserved reactive cysteine residue in DJ-1 is easily oxidized to cysteine-sulfinate in vitro[3] and in vivo.[36,66] Oxidative post-translational modifications can alter protein stability, which may have functional significance in this system. The in vitro stabilities of DJ-1β and human DJ-1 in their reduced and Cys-SO_2^- forms were determined using the thermofluor stability assay. Proteins were oxidized using hydrogen peroxide in vitro (see Experimental Procedures), and the oxidation state of the modified cysteine was confirmed using electrospray mass spectrometry (Table 1 of the Supporting Information). In both homologues, oxidation of the reactive cysteine to the sulfinate stabilized the proteins by ~12 °C, from 64.5 to 76.0 °C for *Drosophila* DJ-1β (Figure 5A) and from 64.0 to 77.3 °C for human DJ-1 (Figure 5B). We note that the melting temperature (T_m) for reduced human DJ-1 determined here (64.0 °C) agrees well with that determined for the same protein by differential scanning calorimetry (66.2 °C),[55] confirming that the increase in the quantum yield for Sypro Orange being measured in this assay is in fact due to binding of the fluorphore in the unfolded protein. The formation of three additional hydrogen bonds between Cys106-SO_2^- and surrounding residues is likely responsible for this stabilization, particularly the short, strong 2.5 Å hydrogen bond between Cys106-SO_2^- and the protonated side chain of Glu18 (Glu17 in DJ-1β) (Figure 3).[41]

DISCUSSION

The predicted coding sequence for *D. melanogaster* DJ-1β deposited with the NCBI (GenBank entry AAF57086.2, gil 28381503) is 205 amino acids long, including an 18-amino acid N-terminal extension that is absent in many other DJ-1 proteins

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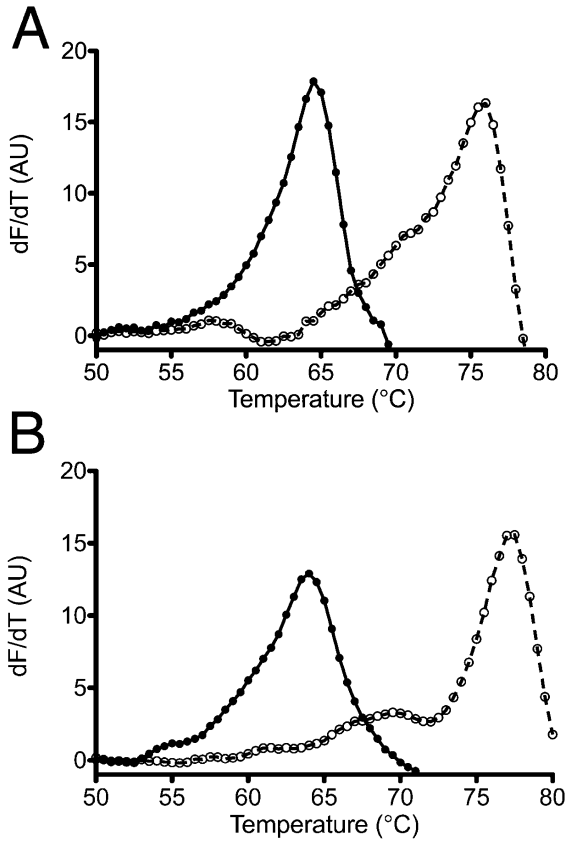

Figure 5. Cysteine-sulfinate formation stabilizes DJ-1. The thermofluor assay was used to monitor the thermal denaturation of reduced DJ-1 (●) or protein that was oxidized at the conserved cysteine residue to the cysteine-sulfinate (○). Data for *Drosophila* DJ-1β are shown in panel A and data for human DJ-1 in panel B. In both cases, oxidation increases the thermal stabilities of the proteins by ~12 °C.

from other organisms.[5] This region corresponds to either an incorrectly predicted (or alternative) translational start site or a targeting sequence. The TargetP 1.1 webserver[67] predicts that this extension is a possible mitochondrial localization sequence, but the level of confidence in this prediction is low (reliability class 4) and thus its significance uncertain. There is evidence that DJ-1β has a role in mitochondrial function,[32] and DJ-1β partially localizes to the mitochondria of *Drosophila* cells.[30] However, because this N-terminal region is not well conserved and native DJ-1β migrates via sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a molecular mass consistent with the absence of this peptide,[5] we have used a version of the protein starting from amino acid 19. However, it is possible that this N-terminal sequence is biologically significant and may warrant further investigation.

In human DJ-1, Met26 is a critically important residue whose mutation to isoleucine has been convincingly associated with loss of DJ-1-mediated cytoprotection and consequent parkinsonism.[9,53] Despite the importance of Met26 for human DJ-1 stability and function, it is poorly conserved in insect and bacterial DJ-1 homologues, where is it often substituted with a leucine, as in *D. melanogaster* DJ-1β. The crystal structure of *Drosophila* DJ-1β shows that the substitution of leucine for methionine at position 26 has little structural impact on the protein and is well-tolerated by surrounding residues. In contrast, the crystal structure of human M26I DJ-1 showed that placing a β-branched isoleucine side chain in this environment creates a steric conflict with Leu31 that can be relieved by an artificial M26L mutation.[55] This packing defect, although minor, was thought to contribute to the observed loss of stability and function of M26I DJ-1.[55] The fact that the previously designed M26L mutation in human DJ-1 relieves this steric conflict and corresponds closely with the native structure of functional *Drosphila* DJ-1β provides strong circumstantial evidence that the Ile26–Leu31 conflict in human M26I DJ-1 is responsible for loss of protein stability and, ultimately, pathogenesis. This structural effect may be indirectly responsible for the recently observed deficit in Cys106 oxidation in M26I DJ-1, which may compromise the protective function of this mutant protein.[35]

Drosophila DJ-1β has a reactive cysteine residue (Cys104) that has been shown to be critical for its function.[33] This residue is partially oxidized to the cysteine-sulfinate (Cys-SO_2^-) in the crystal, and this modification is stabilized by hydrogen bonding with surrounding residues, consistent with previous observations for the human protein. In particular, a previously identified hydrogen-bonded interaction between Glu18 and Cys106 is also seen in DJ-1β [Cys104 and Glu17 (Figure 3)]. In human DJ-1, the Glu18 side chain is protonated and donates a hydrogen bond to Cys106, depressing its pK_a and stabilizing the oxidized Cys106-SO_2^-.[41] The very similar orientation of these residues in DJ-1β suggests that Glu17 in DJ-1β is also likely a protonated carboxylic acid at physiological pH.

Initially on the basis of the structural similarity to the archaeal protease PfpI,[60] human DJ-1 has been considered a possible protease, although various studies have reported conflicting results about its in vitro proteolyitc activity.[22,42,68] DJ-1 lacks a canonical catalytic dyad or triad; however, His126 has been proposed to function as the second member of a catalytic dyad involving Cys106 that forms when the 15 C-terminal residues are cleaved from DJ-1.[42] The structure of *Drosophila* DJ-1β suggests that this mechanism is unlikely to be valid for homologues that contain a tyrosine at this postion, as the environment of this residue prohibits hydrogen bonding that is necessary for completion of the dyad. Moreover, the removal of the C-terminal portion of DJ-1β is not expected to alter this, as the bulky Tyr124 residue cannot sample a conformation that would permit dyad formation with Cys104 without major structural rearrangements in the protein. Consequently, a model requiring DJ-1 to be a zymogenic protease using a Cys-His/Tyr dyad[42] is inconsistent with the detailed structural features of DJ-1β. As the human and insect proteins appear to have similar cellular functions, it seems unlikely that human DJ-1 is a physiologically relevant protease. Importantly, this does not exclude other potential enzymatic activities for DJ-1.

Cys106 is subject to modification during oxidative stress, and its oxidation has been proposed to be an important post-translational modification that may allow DJ-1 to act as a cellular redox sensor[34] or to regulate interactions with its binding partners.[20,69] However, the detailed mechanism by which cysteine oxidation alters DJ-1 function is unclear, particularly because the crystal structures of the reduced and Cys106-SO_2^- proteins are essentially identical.[3,70] Our results show that both the human and insect proteins are substantially stabilized by oxidation of the reactive cysteine to the cysteine-sulfinate. The stabilization contrasts with many other proteins where cysteine oxidation to the sulfinate or sulfonate is associated with destabilization.[71,72] When considered in the context of previous evidence that oxidation of Cys106 in

human DJ-1 leads to protein destabilization,[39] our observations strongly suggest that it is the formation of the more highly oxidized cysteine-sulfonate (Cys106-SO_3^-) that disrupts DJ-1 stability. This is consistent with some previously proposed models[37,39] and indicates that DJ-1 stability is biphasically regulated by oxidative cysteine modification. Importantly, it is possible that Cys106-SO_3^- DJ-1 has a functional role, which has not been thoroughly investigated.

The melting temperatures of both reduced and oxidized DJ-1 from both species are well above physiological temperature. However, changes in thermal stability above the physiological range often correlate with changes in protein stability or half-life at 37 °C.[73] For example, the parkinsonian M26I mutation in DJ-1 has a modestly reduced melting temperature of ∼58 °C[39,55,74,75] in vitro but is unstable and poorly functional in a cell culture at 37 °C.[9,54] Furthermore, the engineered introduction of disulfide bonds in human DJ-1 both increases its melting temperature and enhances its chaperone activity against α-synuclein in vitro at 37 °C.[74] Although there are likely to be several mechanisms by which cysteine-sulfinic acid formation modulates DJ-1 activity, the stabilization of the protein by this oxidative modification may contribute to DJ-1's cytoprotective activity during oxidative stress. Moreover, because the residues that make hydrogen bonds to the cysteine-sulfinate in DJ-1 are well-conserved in close homologues, it is likely that the oxidative stabilization via cysteine-sulfinate formation is shared by many members of the DJ-1 family.

■ ASSOCIATED CONTENT

Supporting Information

Mass spectrometry analysis of oxidative modifications in proteolyzed reduced and hydrogen peroxide-oxidized human DJ-1 and *Drosophila* DJ-1β (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Refined model coordinates and experimental structure factors for *D. melanogaster* DJ-1β have been deposited in the Protein Data Bank as entry 4E08.

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Notes

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■ ABBREVIATIONS

ADP, atomic displacement parameters; CCD, charge-coupled device; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; LC−MS/MS, liquid chromotagraphy−tandem mass spectrometry; PDB, Protein Data Bank; rmsd, root-mean-square deviation; TLS, translation-libration-screw; UV, ultraviolet.

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