Crystal Structure of Filamentous Aggregates of Human DJ-1 Formed in an Inorganic Phosphate-dependent Manner*

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Mutations in the DJ-1 gene have been implicated in the autosomal recessive early onset parkinsonism. DJ-1 is a soluble dimeric protein with critical roles in response to oxidative stress and in neuronal maintenance. However, several lines of evidence suggest the existence of a nonfunctional aggregated form of DJ-1 in the brain of patients with some neurodegenerative diseases. Here, we show that inorganic phosphate, an important anion that exhibits elevated levels in patients with Parkinson disease, transforms DJ-1 into filamentous aggregates. According to the 2.4-Å crystal structure, DJ-1 dimers are linearly stacked through P(1)-mediated interactions to form protofilaments, which are then bundled into a filamentous assembly.

Human DJ-1 is a 189-amino acid, homo-dimeric protein. It was first identified as a novel candidate for the oncogene product that transformed mouse NIH3T3 cells in concert with activated ras (1). After this initial identification, DJ-1 has been shown to have various physiological implications. DJ-1 was characterized as a protein that regulates an RNA-protein interaction (2) and positively modulates the androgen receptor (3). DJ-1 is a dimeric protein with critical roles in response to oxidative stress. DJ-1 is a molecular chaperone that is activated in an oxidative cytoplasmic environment (11) that is activated in an oxidative cytoplasmic environment (11). Activated DJ-1 inhibits α-synuclein aggregation, the major component of Lewy bodies, which are the characteristic intracytoplasmic neuronal inclusions in PD and are closely associated with the progression of PD. DJ-1 also acts as a redox-sensitive negative regulator of apoptosis. DJ-1 contributes to the activation of the PI3K/Akt survival signaling pathway (13, 14) and blocks the Daxx-ASK1 proapoptotic pathway by sequestering Daxx from ASK1 (15). Studies using mice and Drosophila also suggest that DJ-1 plays critical roles in cell survival and response to oxidative stress (13, 16–20).

Structural alterations of DJ-1 are closely associated with its functions. Thus far, three structural modifications of the DJ-1 dimeric structure have been reported. First, DJ-1 is sumoylated at lysine residue 130 by PIASxα. Proper sumoylation is thought to be essential in ras-dependent cellular transformation, cell growth promotion, and anti-UV-induced apoptosis (21). Second, a cysteine residue (Cys106) of DJ-1 is oxidized to sulfenic acid under oxidative stress conditions (11, 22), which causes a relocation of DJ-1 to mitochondria. In addition, a C106A mutant fails to prevent cell death against oxidative stress, indicating the importance of oxidation at Cys106 for DJ-1 function (22, 23). Third, the solubility of DJ-1 is altered to form insoluble aggregates in brains of patients with neurodegenerative diseases (24–29). DJ-1 colocalizes with tau-positive inclusions over a range of neurodegenerative tauopathies, including Alzheimer disease, and with α-synuclein-positive glial inclusions in multiple system atrophy (24, 25, 29). In addition, insoluble DJ-1 is dramatically increased in brains of sporadic PD patients (28, 29). These findings suggest that abnormal aggregation of DJ-1 may be involved in the pathogenesis of multiple neurodegenerative diseases. Here, we report biochemical and structural investigations of DJ-1 aggregates formed in a P1-dependent manner.
Crystal Structure of DJ-1 Aggregates

EXPERIMENTAL PROCEDURES

Purification of DJ-1—The DJ-1 gene (encoding residues 1–189) was amplified, inserted into pET-21a (Novagen) and introduced into Escherichia coli strain B834 (DE3), and tDJ-1 was purified as described (11). For purification of pDJ-1, the cells were grown to an A600 of ~0.7 in Luria-Bertani medium containing 0.1 mg/ml ampicillin at 37 °C, and the expression of DJ-1 was induced by 1 mM isopropyl-β-D-thiogalactoside. After 4-h induction at 37 °C, the cells were harvested and resuspended in sodium phosphate buffers (pH 7.5) containing 1 mM dithiothreitol. The cells were disrupted by sonication, and the cell debris was discarded by centrifugation. The supernatants were loaded on a nickel-nitrilotriacetic acid column (Novagen) and eluted with sodium phosphate buffers containing 20 mM imidazole. The eluted fraction was loaded on a Q-Sepharose Fast Flow column (Amersham Biosciences) and eluted with sodium phosphate buffers (pH 7.5) containing 1 mM dithiothreitol.

Dynamic Light Scattering—Measurements were taken at 22 °C using a DynaPro instrument (Protein Solutions Inc.) equipped with a thermostatted cell. DJ-1 in 20 mM Tris buffer, pH 7.5 (tDJ-1), and DJ-1 in sodium phosphate, pH 7.5 (pDJ-1), at concentrations of 1 mg/ml were centrifuged at 14,000 rpm for 10 min at 4 °C. 60 μl of the supernatants were added to the cuvette, and the light scattering intensity was collected 30 times at an angle of 90° using a 10-s acquisition time. Data analyses were performed using the Dynapro V.5 software.

Circular Dichroism—CD experiments were performed on a Jasco J-810 spectropolarimeter using a 0.2-cm-path length cell, with a 1-nm bandwidth and a 4-s response time. Near-UV CD spectra were collected from 340 to 240 nm with a scan speed of 50 nm/min and 1-nm step resolution. Five individual scans were added and averaged.

Electron Microscopy—Purified pDJ-1 in a phosphate buffer condition was diluted and sampled 1 and 3 days after purification. pDJ-1 (0.6 mg/ml) was applied to carbon-coated grids and negatively stained with 2% uranyl acetate. A 2.4 Å data set containing 20% glycerol in the same mother liquor. A 2.4 Å data set was collected at beamline 5A of the Photon Factory (Table 1). The data were integrated and scaled using HKL2000. The crystal structure of DJ-1 was determined by molecular replacement using AMoRe. Refinement was done with a maximum likelihood algorithm implemented in CNS program (Table 1). Molecular graphic manipulations were performed with QUANTA software (Molecular Simulations Inc., San Diego, CA.). After completing the adjustment of side chains according to electron density, the model was subjected to a positional refinement, causing the R value to decrease to 27.2%. At this stage, water molecules were added using the X-solvate utility of QUANTA, and Pi was incorporated into the corresponding density. The subsequent refinement and manual retitling of model reduced R and Rfree values to 23.4 and 28.7%, respectively. The ideality of the model stereochemistry was verified by PROCHECK. The Ramachandran plot indicates 90.1% of non-glycine, and nonproline residues are in the most favored regions in the final model.

RESULTS AND DISCUSSION

Inorganic Phosphate Induces DJ-1 Aggregation—Despite the existence of DJ-1 aggregates in vivo, recombinant DJ-1 was known to be a dimeric protein (11, 30–33). During efforts to obtain aggregated DJ-1, we were aware that brain cells of PD, multiple system atrophy, and Alzheimer disease patients where DJ-1 aggregation has been observed also contain elevated levels of inorganic phosphate, a vital molecule involved in almost all metabolic processes (34, 35). High Pi levels in serum are directly related to some diseases (36). Thus, we tested sodium phosphate buffers of different concentrations were subjected to dynamic light scattering measurements. DJ-1 purified using sodium phosphate buffers (pDJ-1) exhibited a very interesting feature compared with DJ-1 purified using Tris buffers (tDJ-1). tDJ-1 showed a hydrodynamic radius (Rh) of 2.92 nm, representing the dimeric conformation with a molecular mass of 40 kDa, whereas pDJ-1 had an Rh of 41.04 nm, a clear indication of aggregation (Fig. 1A). The pDJ-1 proteins purified with sodium phosphate buffers of different concentrations were subjected to dynamic light scattering to determine the effect of pH, concentration on DJ-1 aggregation. Interestingly, DJ-1 aggregates with large Rh were detected over 2 mM sodium phosphate buffers,
which is a concentration comparable with the elevated $P_i$ levels in the brains of PD patients (34, 35). We also observed time dependence of DJ-1 aggregation in buffers of low $P_i$ concentration. Fresh pDJ-1 purified with 20–50 mM sodium phosphate buffers exhibited large $R_h$ values. In contrast, fresh pDJ-1 in 2–10 mM sodium phosphate buffers exhibited $R_h$ values in a time-dependent manner. For example, pDJ-1 purified with a 2 mM sodium phosphate buffer began to display large $R_h$ values 15 days after purification. It should be noted that tDJ-1 has the dimeric $R_h$ value even 15 days after purification.

The structural differences between tDJ-1 and pDJ-1 are also reflected in a near-UV CD spectrum (Fig. 1B). The near-UV CD spectrum of a protein provides a valuable fingerprint of the tertiary structure of proteins, which can be used to compare protein structures (37).

**FIGURE 1.** The size distribution histograms from dynamic light scattering experiments and CD spectra. A, histogram of tDJ-1, pDJ-1, and R48A. The table located below the size distribution histogram lists the number of peaks and their mean value (radius equal to $R_h$), polydispersity ($Polyd$), percentage of polydispersity ($%Polyd$), and estimated relative amount of mass (concentration) of each peak or species ($%Mass$). B, near-UV CD spectra of DJ-1 (○) in 20 mM Tris buffer and pDJ-1 (●) in 50 mM sodium phosphate buffer. The final CD spectra were obtained by subtracting the spectra of the buffers from the spectra of the samples. Protein solutions with an OD of 0.66 at 280 nm were used for CD experiments.

Within the protofilaments, DJ-1 dimers are piled in a rotational head-to-tail manner. The rotational axis of this conformation coincides with the protofilament axis (Fig. 3A), giving the protofilaments the appearance of circular cylinders when viewed from above (Fig. 4A). The top side of a DJ-1 dimer makes tight contact with the bottom side of the next dimer, displaying a $\sim 103^\circ$ ($720^\circ/7$) rotational difference (Fig. 3A). As a result, the structural information on DJ-1 filamentous aggregates. Thus, to obtain an atomic view of DJ-1 aggregates, we grew pDJ-1 crystals. The rod-shaped crystals of pDJ-1 exhibit a filamentous arrangement of DJ-1 molecules. Three DJ-1 dimers and one monomer, which are stacked, exist in an asymmetric unit. The monomer becomes a dimer via the 2-fold crystallographic symmetry. Consequently, continuous stacking of DJ-1 dimers is achieved, resulting in the formation of straight protofilaments (Fig. 3, A and B). Protofilaments are further packed in a side-by-side manner, organized in a filamentous assembly (Fig. 4A).
I7 dimer exhibits the same orientation as the I0 dimer after 720° rotation; hence, the seven (from I0 to I6) dimers constitute one longitudinal repeat unit of DJ-1 protofilaments (Fig. 3B). No detectable structural differences were observed among the dimers stacked in the protofilaments. In addition, the dimers in the protofilaments are nearly structurally identical to free DJ-1 dimers (11); their backbone atoms align with a root mean square deviation of 0.175 Å. The contacts between dimers in the protofilaments, hereafter referred to as “F contacts,” bury 1809 Å² of the solvent-accessible surface area of a DJ-1 dimer. A closer look at the F interfaces reveals their inherent 2-fold symmetry, as well as three major F contacts along the 2-fold axis: ion pairs, a hydrophobic core, and Pi-mediated contacts (Fig. 3, D and E). Because all of the F interfaces in protofilaments are identical, we describe only one F interface, which is located between the I0 and I1 dimers. Lys130 and Glu143 of the I0L monomer form ion pairs with Glu143 and Lys130 of the I1R monomer, respectively. Pro127 and Leu128 from the I0L monomer and I1R monomer, coupled with Pro184 from the I0R and I1L monomers, form the hydrophobic core. Two P_i, related by the 2-fold axis, take positions at the F interface, mediating extensive contact: one is located between the I0L and I1L monomers (LP_i), and the other is located between the I0R and I1R monomers (RP_i) (Fig. 3, C–E). LP_i directly forms polar interactions with the guanidium group of Arg48 and the amide nitrogen of Asn76 in the I0L monomer and makes a water-mediated hydrogen bond with the carbonyl oxygen of Gln180 in the I1L monomer. RP_i is coordinated in an identical fashion. Because the guanidium group of Arg48 participates in coordinating P_i (Fig. 3, C–E), its elimination would prevent the binding of P_i at the F interfaces, affecting the formation of filamentous aggregates. To establish the role of P_i as an inducer of filamentous aggregates, we generated a R48A mutant. The mutant exhibits an R₀ of 2.75 nm even in a high P_i concentration (Fig. 1A), pointing to the critical role of P_i in DJ-1 aggregation.

Protofilaments run parallel with one another in crystals. Although one protofilament is surrounded by six others, two of these do not actually contact the central protofilament (Fig. 4A, B).
bottom panel), thus displaying a parallelogram pattern when viewed from the top (Fig. 4A, top panel). The lateral interactions between protofilaments exhibit good geometric complementarities, such that prominence regions in protofilaments fit into grooves in adjacent protofilaments (Fig. 4B). The I_{0L} and I_{6L} monomers function as prominences, and the grooves participating in these lateral interactions are located both between the I_{1R} and I_{2L} monomers and between the I_{3R} and I_{1L} monomers (Fig. 3B). Consequently, four lateral contact sites are located in each longitudinal repeat unit of a protofilament. Each site in a longitudinal repeat unit interacts with one of the surrounding protofilaments, and thus each longitudinal repeat unit makes contact with four protofilaments. The lateral interface between a prominence and a groove is composed of two contacting parts. Because all the lateral interfaces are identical, for convenience we describe only one lateral interface between the I_{0L} prominence of a protofilament and the groove between the I_{3R} and I_{1L} monomers of an adjacent protofilament. One component of the interface is formed by the contact between the I_{0L} prominence and the I_{5L} monomer of the groove (Fig. 4), where Glu^{116} and His^{138} of I_{0L} form ion pairs with Arg^{98} and the carbonyl carbon of Ala^{63} of I_{5L} in the groove, respectively. The other interface component is formed by the contact between the I_{1R} prominence and the I_{4R} monomer of the groove (Fig. 4). The only interaction occurring in the second component is the ion pairing between Arg^{98} of I_{0L} and Glu^{64} of I_{4R} in the groove. Compared with the F contacts, lateral contacts are not extensive. Lateral interactions between a prominence and a groove bury only 357 Å² of the solvent-accessible surface. This nonextensive lateral contact would allow DJ-1 to be assembled into a multitude of different aggregates through a different arrangement of lateral interactions, in the same manner as other filamentous proteins that form crystalline, curved, or branched aggregates (38, 39).

Comparison between EM and Crystal Structure—Although detailed comparisons between the EM and crystal structure are not possible because of the low resolution of the EM, the overall nature of the crystal structure seems to be well reflected in the EM. Similar to the protofilament structure (Fig. 3B), the EM protofilaments resemble the anterior view of a human spine, i.e.
a row of small bones (Fig. 2, A and B). The dimensions (~60 × ~50 Å) of the objects that correspond to small bones in the spine are comparable with those of DJ-1 dimers, representing the stacking of DJ-1 dimers in the protofilaments (Fig. 3B). In addition, the contact between protofilaments, which is marked by an arrow in Fig. 2B), is reminiscent of the aforementioned lateral interactions, exhibiting geometric complementarity between prominences and grooves (Fig. 4B).

Concluding Remarks—DJ-1 is implicated in PD pathogenesis (7), and insoluble DJ-1 aggregates have been observed in brains of patients with neurodegenerative diseases (24–29). Because protein aggregates not only are diagnostic hallmarks but have also been implicated in the pathogenesis of neurodegenerative diseases (40), physicochemical information on in vitro DJ-1 aggregates bears potential for further understanding of the etiology of PD. Here, we describe a biochemical and structural investigation of in vitro DJ-1 aggregates whose formation is dependent only on P$_i$. Unfortunately, there have been no reports on the characterization of in vitro DJ-1 aggregates, which precludes direct comparison between in vitro aggregates and the P$_i$-induced DJ-1 aggregates presented in this study. Therefore, we cannot assert that in vitro DJ-1 aggregates share characteristics with the P$_i$-induced aggregates. However, considering the observation that recombinant proteins forming pathological aggregates in brains can assemble into aggregates in vitro that closely resemble corresponding in vivo aggregates (41), the physicochemical properties of the P$_i$-induced aggregates might provide some insights into those of in vivo DJ-1 aggregates.

The loss of DJ-1 function induced by mutations in the DJ-1 gene has been reported to lead to neurodegeneration (7). There is an emerging consensus that DJ-1 may provide protective roles against various stresses such as the oxidative damage involved in PD pathogenesis (22, 42–44). DJ-1 aggregation, which removes soluble DJ-1, would lead cells to lose functional DJ-1 that normally performs neuroprotective roles. As such, DJ-1 aggregation may be compatible with the loss-of-function pathogenic mechanism of DJ-1 (7). To determine the pathological role of the P$_i$-dependent DJ-1 aggregation, however, it is prerequisite to reveal the in vivo relationship between fragmentary observations of the P$_i$ level and the DJ-1 aggregation. The increased P$_i$ level in the brains of patients with neurodegenerative diseases (34, 35) shows that their physiological conditions can be thought to have been shifted to more suitable environments for DJ-1 aggregation. Nevertheless, because of the lack of any information on the cellular factors affecting protein aggregation, it is not certain that the observed DJ-1 aggregates in vivo (24–29) are correlated with the P$_i$ level. It should also be noted that the P$_i$-dependent DJ-1 aggregation is described here for the first time. Consequently, at present we cannot determine whether P$_i$ elevation induces DJ-1 aggregation in the brain, culminating in neurodegeneration. A future challenge is to address this issue.

Although the implication of the P$_i$-dependent DJ-1 aggregation in the pathogenesis of PD remains to be elucidated, the novel finding regarding the critical role of P$_i$ in DJ-1 aggregation and the detailed view of a DJ-1 filamentous assembly at atomic resolution extends our knowledge of molecular mechanisms behind protein aggregation and provides an important framework for future works in unveiling the physiological meaning of the P$_i$-dependent DJ-1 aggregation.

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