On the Oligomeric State of DJ-1 Protein and Its Mutants Associated with Parkinson Disease

A COMBINED COMPUTATIONAL AND IN VITRO STUDY

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Mutations in the DJ-1 protein are present in patients suffering from familial Parkinson disease. Here we use computational methods and biological assays to investigate the relationship between DJ-1 missense mutations and the protein oligomeric state. Molecular dynamics calculations suggest that: (i) the structure of DJ-1 wild type (WT) in aqueous solution, in both oxidized and reduced forms, is similar to the crystal structure of the reduced form; (ii) the Parkinson disease-causing M26I variant is structurally similar to the WT, consistent with the experimental evidence showing the protein is a dimer as WT; (iii) R98Q is structurally similar to the WT, consistent with the fact that this is a physiological variant; and (iv) the L166P monomer rapidly evolves toward a conformation significantly different from WT, suggesting a change in its ability to oligomerize. Our combined computational and experimental approach is next used to identify a mutant (R28A) that, in contrast to L166P, destabilizes the dimer subunit-subunit interface without significantly changing secondary structure elements.

Parkinson disease (PD) is the second most common progressive neurodegenerative disorder, affecting 1–2% of all individuals above the age of 65 (1–12). PD is characterized by muscle rigidity, resting tremor, bradykinesia, and gait disturbance with dysequilibrium. The neuropathological hallmark in post mortem brains is the selective degeneration of specific subsets of mesencephalic dopaminergic cells and the formation of cytoplasmic aggregates called Lewy bodies.

The identification of genes associated with rare forms of early onset familial PD has provided crucial insights into the mechanism of the pathogenesis. Several loci have been mapped for monogenic forms of PD, and genes have been identified (1). By studying two families from genetically isolated communities, two mutations in the PARK7/DJ-1 gene were found to be associated with autosomal recessive early onset PD (13). DJ-1 encodes for a highly conserved, ubiquitously expressed, 189-amino acid-long protein (4) that is involved in multiple cellular processes including cell maturation, fertilization in rodents, and oncogenesis in humans. It is present in the nucleus, cytoplasm, mitochondria, and extracellular space of mammalian cells (14–17). Among several potential biochemical activities, DJ-1 has been proven to be a regulator of transcription with the promoter of tyrosine hydroxilase being one of its targets. Furthermore, DJ-1 may act as a redox-regulated chaperone (18). In vitro studies showed that the ectopic expression of DJ-1 protects cells from cell death induced by various toxic stimuli including oxidative stress (19, 20). DJ-1 is indeed an indicator of oxidative stress state in vivo since it is converted into pl variants in response to small amounts of reactive oxygen species. Interestingly, some pl isoforms are accumulated in PD post mortem brains (21, 22).

Several familial cases prove that DJ-1 loss in humans causes PD: (i) a frameshift and a splice mutation were found in a young onset PD patient (23); (ii) a Dutch family showed a large homozygous genomic deletion that removed 4 kb of promoter sequences and the first five exons of the gene (6); and (iii) additional PD cases have been described with truncating, splice site mutations and deletions.

To study the effects of the lack of a functional gene, DJ-1 knock-out mice and flies were generated. Although they did not show the death of dopaminergic neurons, increased vulnerabilities to neurotoxic agents were observed (24–27). Furthermore, embryonic stem cells deficient in DJ-1 and dopaminergic neurons derived in vitro from them displayed increased sensitivity to oxidative stress and/or proteasomal inhibition (28).

Interestingly, some PD families present missense mutations of DJ-1 in homozygous and/or heterogeneous forms (M26I, E64D, A104T, D149A, and L166P) (1, 6, 13, 23, 29, 30). How
these mutants change or abolish DJ-1 function is still a matter of debate.

X-ray crystallography (Fig. 1A) showed that WT DJ-1 in the reduced state (that is, in a state in which Cys and/or Met residues are not oxidized (3)) is a homodimer. Each monomer takes a flavodoxin-like Rossmann fold (3), containing seven parallel \( \beta \)-strands flanked by nine \( \alpha \)-helices. The subunit-subunit interface is stabilized by a large number of hydrophobic interactions, H-bonds, and salt bridges (supplemental Table S1). The dimeric state of the protein has been confirmed by both \textit{in vivo} and \textit{in vitro} investigations. It is believed that DJ-1 carries out its function exclusively in the dimeric state (31–34), because the formation of high molecular weight (HMW) oligomers and/or inefficient formation of dimeric structures may affect the stability of the protein and/or its affinity for molecular partners in the cell (2, 4, 5).

L166P and M26I are the most studied DJ-1 missense mutations (5, 7, 13, 34, 35). L166P is very unstable and its expression level, both in transfection studies and in patient lymphoblasts, is lower than WT. This suggests that L166P mutation may induce a loss of DJ-1 function (35–38). The mutant does not form a dimeric structure; instead, it may assemble in HMW oligomers (5, 33, 35). At the structural level, Leu166 is located in the middle of one of the helical regions of the protein \((\alpha 8\) helix in Figs. 1A and 2A); thus, its mutation to Pro breaks the helix (4, 13, 29, 35). Because the \(\alpha 8\) helix forms hydrophobic interactions with a series of residues located at the subunit-subunit interface (Val181, Lys182, and Leu187 of \(\beta 3\) \(\beta\)-sheet and the C terminus), such a mutation could affect the stability of the homodimer (4, 29, 36). However, this hypothesis has not yet been proven by \textit{in silico} and/or \textit{in vitro} analysis of the conformational changes in the mutant.

M26I does form dimers, but it is a matter of debate whether dimer formation occurs at the same rate as WT or less efficiently. Furthermore, the relevance of these differences, if any, for neurodegeneration is unclear (5, 7, 34). This mutation is located on \(\alpha 1\) helix at the subunit-subunit interface (Fig. 1A); therefore, it may affect subunit-subunit interactions. However, methionine is structurally and chemically very similar to isoleucine; they are similar in shape and volume, and they are both nonpolar residues. Thus, the interactions between M26I subunits may well be similar to those of the WT, although structural information for this mutant is lacking. Here we address these issues by computational approaches along with \textit{in vitro} assays.

Molecular dynamics (MD) simulations based on the DJ-1 WT x-ray structure are carried out on homodimeric and monomeric forms of the WT. An extensive MD study of DJ-1 in the oxidized state has been performed (Fig. 1B) as well as an analysis of the PD-causing mutations L166P and M26I. Comparison is also made with MD simulations of a physiological variant.
(R98Q), which is expected to alter neither the DJ-1 fold nor the oligomeric state (8).

Our calculations show that: (i) the reduced and oxidized WT dimers in aqueous solution are very similar to the reduced dimer in the solid state; (ii) M26I in the monomeric and dimeric states maintains completely the fold of the WT; (iii) R98Q is also similar to the WT, fully consistent with the fact that this is a physiological variant; and (iv) L166P causes local distortions at α8 helix (Fig. 2A) and at the surface-forming contacts in the dimeric structure. We conclude that this mutation might affect the stability of the dimer by altering the structure of its local environment. Unfortunately, at present, the stability differences (i.e. the free energy differences) between the WT and these mutants cannot be firmly established by calculations alone. However, our conclusions based on the calculations are corroborated by previous biological assays in vitro on the WT and its mutants (7, 29, 35). Furthermore, experiments carried out in this work confirm that L166P tends to form multimeric aggregates more than WT does (5, 33, 35).

Finally, our combined computational and experimental methodology is used to engineer a new mutation that, in contrast to L166P, does destabilize the subunit-subunit interactions without affecting the secondary structure elements of the protein. This mutation (R28A) is located at the subunit-subunit interface, and it causes the disruption of salt bridges and hydrophobic contacts at the interface.

MATERIALS AND METHODS

**Molecular Dynamics Simulations**—Dimeric and monomeric structural models of reduced and oxidized DJ-1 WT protein together with M26I, L166P, R98Q, and R28A were constructed based on the DJ-1 WT homodimer x-ray structure at a resolution of 1.95 Å (3).

In the oxidized form Cys and/or Met residues were assumed to be oxidized. We noticed that the shortest Cys-Cys distance (d[S-Cys53(A) and S-Cys53(B)] = 3.2 Å) is too large for the formation of disulfide bridges. Thus, the three cysteines present in the protein (Cys46, Cys53, and Cys106) are replaced by cysteine sulfonic acid (31) (Fig. 1A). The four methionines (Met17, Met26, Met133, and Met134; Fig. 1A) are oxidized to methionine sulfoxide (18, 22) or methionine sulfone (22). We further noticed that neither the cysteine residues nor the methionines interact directly with residues involved in the PD mutations. Several models were considered, based on the suggestion of Refs. 18, 22, and 31: (i) the protein with all the Cys residues oxidized and all the Met reduced (WT-OX_1); (ii) the protein with all the Cys oxidized and all the Met oxidized to methionine sulfoxide (WT-OX_2); and (iii) the protein with all the Cys oxidized, two methionines (Met26 and Met134) oxidized to methionine sulfoxide and the remaining two (Met17 and Met133) oxidized to methionine sulfone (WT-OX_3).

The point mutations were obtained by simple residue substitution, taking care that the substituted residue would not clash with the rest of the protein and that the χ1 and χ2 torsion angles would fall in the most energetically favorable regions (39, 40). The mutations in the dimeric structures were carried out for both subunits to obtain homodimers.

For this mutant, only the monomer was built (see “Results” for details).
The proteins were immersed in a water box with edges of \(\sim 69.0 \times 66.0 \times 69.0\) Å and \(81.0 \times 76.0 \times 94.0\) Å for the monomeric and dimeric forms, respectively. The solvent molecules were not included if the distance between any solvent atom and any protein atom was lower than the sum of their respective van der Waals’ radii. Two or three sodium counter ions were added to neutralize the systems. They were located in the regions of minimum electrostatic potential energy as calculated with the xleap program of the AMBER8 package (41, 42), namely close to Glu^{59} and Asp^{189}. Periodic boundary conditions were applied.

The AMBER99 (43, 44) and TIP3P (45) force fields were used for biomolecules with counter ions and water, respectively. The particle mesh Ewald method was applied to evaluate the long range electrostatic interactions (46–48). A cutoff of 8 Å was used for both the real part of the electrostatic interaction and the van der Waals’ nonbonded interaction evaluation. A time step of 2 fs was applied to propagate nuclear degrees of freedom. The SHAKE algorithm (49) was used to fix all bond lengths. The investigated models first underwent 10,000 steps of energy minimization. Then the systems were equilibrated at constant temperature and pressure for at least 0.5 ns. Subsequently, our models underwent MD simulations with constant temperature and volume for at least 10.0 ns. Constant temperature conditions were obtained by using a Langevin thermostat (50) at a target temperature of 300 K with a coupling coefficient of 5 ps\(^{-1}\). Constant pressure conditions were achieved with a Nosé-Hoover Langevin barostat (51, 52). For the barostat, an oscillation period of 200 fs was used, and the damping time scale used was 100 fs.

All of the calculations were performed using the NAMD program (53). The obtained results have been further analyzed using the Gromacs (54, 55) and VMD (56) packages.

**Computational Alanine Scanning**—Mutations destabilizing subunit-subunit interactions in the DJ-1 dimeric form were identified using Baker’s alanine scanning procedure (57, 58). This approach calculates the van der Waals’ and the electrostatic contributions to the free energy of binding. Binding energy hot spots are defined for residues at the subunit-subunit interface, whose Ala mutation causes a loss of free energy of the subunit-subunit interface greater or equal to 1 kcal/mol. The latter residues were defined as (57, 58): (i) residues that have at least one atom within a sphere with a 4-Å radius centered on an atom belonging to the other partner; (ii) residues that, upon dimer formation, become significantly buried, i.e. there is a significant increase in the number of C\(_\beta\) atoms located within an 8 Å sphere centered on the C\(_\beta\) atom of the analyzed residue (exposed 0–8, intermediate 9–14, and buried >14). The mutation causing the largest destabilization of the dimer (R28A) was selected for subsequent MD simulations according to the protocol described in the previous subsection.

**Preparation of DJ-1 R28A Mutant**—A first PCR amplification has been performed using pCDNA3-DJ1WT (kindly provided by P. Rizzu) as the template and the primers DJ1R28A F1 (cggtctacctgtctagagagggcagt) and FLAG DJ1 REV (gcgcgctctactatgactttgagaaaag). The purified PCR product served as the template for a second PCR amplification using the following primer: DJMET26 F2 (atatagaattcctccaaagagctctggtcatcctggtcagggcactcgtctagtctgtagagagggcagt) in combination with FLAG DJ1 REV to obtain the complete mutant sequence. After digestion with EcoRI and XbaI, a PCR fragment was ligated to a pCDNA3–2×FLAG vector, previously prepared in the laboratory.

**Cross-linking Assay**—Human HEK 293 cells were transiently transfected using the calcium phosphate method with pCDNA3–2×FLAG-DJ-1 WT, L166P, M26I, and R28A plasmids. 48 h after transfection the cells were extensively washed with phosphate-buffered saline and harvested in lysis buffer (0.5% Triton X-100 in phosphate-buffered saline) supplemented with 1× Complete protease inhibitor mixture (Roche Applied Science). The lysates were rotated at 4 °C for 30 min, and soluble cell lysates were obtained by centrifugation at 13,000 rpm for 30 min at 4 °C. Protein content in each lysate was determined by the Bradford method (Bio-Rad). Equal quantities of lysate (100 μg) were incubated with 5 mM disuccinimidyl suberate (Pierce) or Me\(_2\)SO control for 30 min at room temperature. The reaction was quenched by incubation with 50 mM Tris–HCl, pH 7.5, for 15 min at room temperature. The lysates (5 μg) were analyzed by Western blotting with an anti-FLAG antibody. Densitometric analysis was performed to quantify ratios between monomeric and dimeric DJ-1.

**Coimmunoprecipitation**—HEK 293 cells were transiently transfected with pCDNA3–2×FLAG and pCDNA3-HA vectors encoding DJ-1 WT, L166P, M26I, and R28A, and after 48 h, the cells were washed in phosphate-buffered saline and harvested in lysis buffer as previously described. Equivalent soluble lysates (0.9 mg) were immunoprecipitated with an anti-HA monoclonal antibody for 2 h at 4 °C, followed by incubation with Sepharose-protein A for 1 h at 4 °C. Bound proteins were eluted with Laemmli buffer and revealed by Western blotting with an anti-FLAG antibody.

**RESULTS**

**MD of the DJ-1 WT Protein**—Molecular dynamics simulations over an 11-ns time scale were used here to investigate the structure of the reduced and oxidized forms of the DJ-1 protein in aqueous solution. The simulations were performed for both the dimeric and monomeric forms of the reduced state and for the dimeric form of the oxidized state. In the latter state, either the Cys or the Met residues were assumed to be oxidized (22). The obtained results were used as a reference for comparison with the corresponding mutants.

In the reduced state, the root mean square deviation (RMSD) values fluctuated for the last 5 ns of the MD run around values of 1.8 ± 0.1 and 1.8 ± 0.2 Å for the dimer and the monomer, respectively (Fig. 1B, panel i, and Table 1). The S.D. value suggested that the monomer undergoes larger structural fluctuations than the dimer, pointing to a key stabilizing role of the subunit-subunit association. In particular, the association stabilized the flexible N- and C-terminal fragments. The distance values between C\(_n\) of the residues 1 and 189 fluctuated between 23 and 32 Å for the dimer, whereas for the monomeric form they were between 20 and 34 Å (Fig. 1B, panel ii). The average distance was 27.5 Å (Fig. 1B, panel ii) for both forms. The secondary structure found in the x-ray experiments was conserved in both cases. Most of the subunit-subunit hydrogen bonds,
along with the hydrophobic interactions and salt bridges, were conserved during the entire MD run (supplemental Table S1). However, a salt bridge between Asp$^{24}$ and Arg$^{48}$, not detected in the x-ray structure, was formed after a few hundred ps of dynamics. The average distance between the centers of mass of the DJ-1 subunits in the model structure was 28.1 ± 0.3 Å, whereas it was 27.9 Å in the x-ray structure of the DJ-1 protein (Fig. 1B, panel iii).

Several oxidized forms of the dimeric protein were considered, following previous work (18, 22, 31): (i) a form in which the cysteines are oxidized to cysteine sulfonic acid (WT-OX$_1$), (ii) a form in which the cysteines are oxidized to cysteine sulfonic acid and the methionines oxidized to methionine sulfone (WT-OX$_2$), and (iii) a form in which the cysteines are oxidized to cysteine sulfonic acid, two methionines (Met$^{26}$ and Met$^{133}$) oxidized to methionine sulfide and the remaining two (Met$^{17}$ and Met$^{133}$) oxidized to methionine sulfone (WT-OX$_3$) (22).

Our MD simulations showed that such forms are fairly similar to the reduced form: (i) the RMSD ranged between 1.4 and 1.7 Å (Table 1), to be compared with 1.8 ± 0.1 Å of the WT in the reduced state; (ii) the contacts at the subunit-subunit interface were rather similar to those of the DJ-1 reduced form in aqueous solution (supplemental Table S1); (iii) the MD-averaged distance between the centers of mass ranged between 28.0 and 29.1 Å (Fig. 1B), similar to the value of the reduced state (28.1 ± 0.3 Å); and (iv) the solvent-accessible surface area (SASA) (59, 60) for each subunit was similar to that of the reduced form (MD-averaged values ranging from 9,854 to 10,150 Å$^2$, to be compared with 10,160 Å$^2$ for the reduced state). We concluded that the oxidized and reduced forms of the DJ-1 protein in aqueous solution are very similar to the reduced form in the solid state.

**MD of PD-causing DJ-1 Mutations**—Here we focus on the most studied PD-linked mutations: M26I and L166P (5–7, 13, 29, 35, 36). Because the estimation of the free energy difference between the WT and these mutants in the dimeric and oligomeric states by MD is not possible at the present stage, we adopted a simple approach that can provide some indirect hints. First, we compared MD results on the mutant monomers to the monomeric WT in the reduced state$^7$ in aqueous solution. Within

### Table 1

| Mutant   | RMSD Average | RMSD S.D. | RMSD Final value | RMSF Average | RMSF S.D. | RMSF Final value |
|----------|--------------|-----------|------------------|--------------|-----------|------------------|
| WT       | 1.8          | 0.1       | 1.9              | 1.8          | 0.2       | 2.1              |
| WT-OX$_1$| 1.6          | 0.1       | 1.7              | 1.6          | 0.1       | 1.6              |
| WT-OX$_2$| 1.4          | 0.1       | 1.5              | 1.4          | 0.1       | 1.4              |
| WT-OX$_3$| 1.6          | 0.1       | 1.6              | 1.6          | 0.1       | 1.6              |
| L166P    | 1.7          | 0.1       | 1.7              | 2.0          | 0.2       | 1.9              |
| M26I     | 1.4          | 0.1       | 1.5              | 1.5          | 0.1       | 1.5              |
| R98Q     | 1.4          | 0.1       | 1.5              | 1.9          | 0.2       | 1.7              |
| R28A     | 2.5          | 0.1       | 1.8              | 1.8          | 0.1       | 1.9              |

$^7$We expect the mutant oxidized forms to be very similar to the reduced ones as shown by the MD simulations of the WT.

Along with the hydrophobic interactions and salt bridges, were conserved during the entire MD run (supplemental Table S1). However, a salt bridge between Asp$^{24}$ and Arg$^{48}$, not detected in the x-ray structure, was formed after a few hundred ps of dynamics. The average distance between the centers of mass of the DJ-1 subunits in the model structure was 28.1 ± 0.3 Å, whereas it was 27.9 Å in the x-ray structure of the DJ-1 protein (Fig. 1B, panel iii).

The RMSD of the M26I monomer turned out to be fairly similar to that of the WT in the monomeric state; it fluctuates around 1.9 Å over the last 5 ns, and the value at the end of the dynamics ranged between 1.6 and 2.5 Å (Table 1 and Fig. 3A). The secondary structural elements were fully maintained during the dynamics, and the overall fold was similar to the WT (Fig. 2B).

Next, we compared structural determinants of the protein surface involved in the dimerization with those of the WT: (i) the distance (D) between the centers of mass of the secondary structure elements located at the interface (α1 helix and β3 β-sheet in Fig. 1A) was similar to that of the WT (D = 15.0 ± 0.6 Å versus 14.8 ± 0.5 Å, respectively; Fig. 3B); and (ii) the root mean square fluctuation (RMSF) per residue for M26I was very similar to that of the WT (Fig. 4). The similarity between the WT and M26I can be rationalized observing that the mutation does not affect the intramolecular interactions (supplemental Table S2). The interactions formed by M26 in the WT were the same as those formed by Ile$^{26}$ in the mutant, because the methionine and isoleucine has similar volume and shape (61, 62) (supplemental Table S3). We concluded that the interface of the M26I mutant has a similar conformation to the WT and subsequently has a similar tendency to form dimers.

We then proceeded to investigate the dimeric structure of M26I. The RMSD value of M26I in the dimeric state was similar to that of the WT (Table 1 and supplemental Fig. S2), oscillating around 1.7 Å. The secondary structural elements were fully conserved during the molecular dynamics simulations. The nonbonded interactions at the subunit-subunit interface were also conserved during the MD run, analogously to the WT (supplemental Table S3). In addition, the centers of mass distance analysis showed that the distance between subunits in the mutant is similar to the distance between subunits obtained for the dimeric form of the WT (supplemental Fig. S2). The local interactions around residue 26 were also fully conserved (supplemental Table S2). Finally, the SASA for each subunit was similar to the WT (average values, 10,203 and 10,160 Å$^2$, respectively). Thus, both the monomeric and dimeric forms of M26I were similar to the WT.

L166P monomer showed a different behavior than the M26I monomer. Its RMSD exhibits larger fluctuations than in other mutants (Fig. 3A and Table 1). In addition, properties (i) and (ii) were different from that obtained for the WT: (i) the distance (D) between α1 helix and β3 β-sheet is smaller (D = 12.5 ± 0.4 and 14.8 ± 0.5 Å; Fig. 3B); and (ii) the RMSF of Asp$^{49}$ (located on the subunit surface involved in the dimerization process) is larger (Fig. 4). The discrepancies might have been caused, as already suggested (4, 36), by the fact that the replacement of Leu with Pro disrupted α8 helix, and therefore the local interactions of residue 166 were different (supplemental Table S2). Thus, our results suggested the lowered dimerization efficiency of the
L166P mutant may be due to structural differences in the monomer. These results were valid within the limits of the computational power of our analysis.

**MD of R98Q Physiological Variant**—Because it is a physiological variant of the DJ-1, R98Q was expected to alter none of the biochemical functions of DJ-1 (8). From a structural point of view, the replacement of Arg98, which is indeed exposed to the solvent, with a polar residue such as Gln should not dramatically affect the thermodynamic stability of the protein. To investigate the effect of such a mutation, we followed the same computational protocol as for the PD-causing mutations.

The mutant monomer showed a similar behavior to the WT form. Its RMSD did not show any large fluctuation compared with the WT (Fig. 3A and Table 1). In addition, properties (i) and (ii) were similar to those of the WT: (i) the distance (D) between α helix and β3 β-sheet was comparable with that of the WT (14.9 ± 0.5 and 14.8 ± 0.5 Å, respectively; Fig. 3B); and (ii) the RMSF showed a similar behavior to those of the WT and M26I mutation (Fig. 4). Furthermore, the RMSD value of the dimeric form was comparable with that of the WT dimer (Table 1 and supplemental Fig. S2), oscillating around 1.4 Å with small fluctuations after 5 ns. The behavior of this physiological variant was similar to that of the dimeric forms of the DJ-1 WT and of the M26I mutant (supplemental Table S3). The analysis of centers of mass distance showed that the DJ-1 variant behaves similarly to the M26I WT (supplemental Fig. S2). Finally, the SASA for each subunit was similar to that of the WT (average values, 10,040 and 10,160 Å², respectively). Within the limitations of our approach, we concluded that R98Q variation, like the M26I, affected neither the dimeric nor the monomeric structures of the WT.

**Computational Identification of a Dimer-incompetent DJ-1 Mutation**—Our calculations suggested that L166P is the only mutation among DJ-1 Protein and Its Mutants Associated with PD
those considered that may alter dimer stability by modifying the fold of the single subunits. These in turn might assemble differently than the WT to form HMW structures.

Here we attempted to identify a mutation that does affect stability by disrupting none of the secondary structure elements. We performed Baker’s computational alanine scanning procedure (57), which can quickly (albeit approximately) estimate changes in the interaction free energy ($\Delta \Delta G$) between the two subunits upon mutation of each interface residue with Ala. The largest $\Delta \Delta G$ value turned out to be associated with the R28A mutation (Table 2). This value is indeed twice as large as any other Ala substitutions presented in Table 2.

Following the MD protocol adopted for the other mutations, we investigated by MD simulation the structural properties of the R28A monomeric form, and we compared them with those of the WT monomer MD structure. The R28A monomer turned out to be similar to the WT; the RMSD value oscillated around 1.8 Å after 5 ns (Table 1 and Fig. 5A). In addition, properties (i) and (ii) were similar to those of the WT: (i) the distance ($D$) between the $\alpha$-1 helix and the $\beta\beta$-sheet was comparable with that of the WT (14.9 ± 0.5 and 14.8 ± 0.5 Å for the R28A mutant and the WT DJ-1; Fig. 3B); and (ii) the RMSF was similar to those of the WT and the M26I proteins (Fig. 4). The proteins fully maintained the secondary structure elements (Fig. 2B and supplemental Fig. S1). This was expected because the mutation is on the surface of the monomer.

We proceeded then to investigate the dimeric form of R28A by MD simulation (Fig. 5B). The mutation deeply destabilized the subunit-subunit interactions, and the mutant showed a completely different behavior compared with that of the WT protein: (i) The distance between the subunit centers of mass along with the SASA increased during the dynamics; at the end of the simulation it was much larger than those of the WT (Fig. 5). This suggested that the structure is evolving toward another minimum in which the subunit-subunit interaction is less strong than in the WT. Unfortunately, observing an eventual complete detachment of the two subunits is well beyond the present domain of applications of MD simulations; (ii) consistently, the RMSD value increased during the dynamics up to 3.0 Å (Fig. 5B), which is significantly larger than the final value found for the WT (1.9 Å, Table 1 and Fig. 5B). Thus, it is not correct to take the average values of the RMSD (the structure is still evolving). We then calculated for this mutant the average value of the RMSD and the S.D. of each subunit during the MD run. The values were 2.0 ± 0.5 Å for both subunits in the mutant, to be compared with 1.6 ± 0.1 and 1.7 ± 0.1 Å for each subunit of the WT dimer in the reduced form. This suggests that the R28A DJ-1 mutant is more flexible than the WT.

### TABLE 2
Computational alanine scanning-based (57, 58) free energies obtained for the DJ-1 dimeric structure taken from x-ray experiments (3)

| Residue | $\Delta \Delta G$ | kcal/mol |
|---------|------------------|---------|
| Met17   | 1.3              |         |
| Val20   | 1.3              |         |
| Arg27   | 1.6              |         |
| Arg28   | 2.9              |         |
| Val50   | 1.4              |         |
| Ile52   | 1.1              |         |
| Gly159  | 1.5              |         |

**FIGURE 5.** Comparison between selected properties of WT and R28A, plotted as a function of time. RMSD of backbone atoms of the WT (black) and R28A mutant (red) for monomers (A) and dimers (B). C, distance between the centers of mass (COM) of subunits of the WT and R28A dimers. D, solvent-accessible surface area for monomers (lower values) and dimers (larger values) of WT and R28A.
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**In Vitro Biochemical Assays**—To compare quantitatively the ability of PD-causing mutations to form dimers in vitro, we performed chemical cross-linking experiments. Human HEK 293 cells were transfected with FLAG-tagged DJ-1 constructs. Equal amounts of total protein (5 μg) were loaded on gel and immunblotted with anti-FLAG antibody. B, densitometric analysis of protein bands was performed using Adobe Photoshop 7.0 on two independent experiments. Relative expression was normalized to DJ-1 wt protein. C, HEK 293 cells were transfected as in A. Protein lysates were treated with chemical cross-linker disuccinimidyl suberate or with Me2SO as indicated. Normalized quantities of transfected proteins were loaded on gel. Monomer, dimer, and HMW forms of DJ-1 were visualized with anti-FLAG antibody. D, densitometric analysis of monomer and dimer bands was obtained by Adobe Photoshop 7.0 on two independent experiments. The dimer to monomer ratio was calculated and normalized to DJ-1 wt protein. WB, Western blot.

![FIGURE 6. In vitro analysis of DJ-1 dimer formation. A, HEK 293 cells were transiently transfected with FLAG-tagged DJ-1 constructs. Equal amounts of total protein (5 μg) were loaded on gel and immunblotted with anti-FLAG antibody. B, densitometric analysis of protein bands was performed using Adobe Photoshop 7.0 on two independent experiments. Relative expression was normalized to DJ-1 wt protein. C, HEK 293 cells were transfected as in A. Protein lysates were treated with chemical cross-linker disuccinimidyl suberate or with Me2SO as indicated. Normalized quantities of transfected proteins were loaded on gel. Monomer, dimer, and HMW forms of DJ-1 were visualized with anti-FLAG antibody. D, densitometric analysis of monomer and dimer bands was obtained by Adobe Photoshop 7.0 on two independent experiments. The dimer to monomer ratio was calculated and normalized to DJ-1 wt protein. WB, Western blot.](image)

DISCUSSION

Several mutations of the DJ-1 gene have been associated with familial cases of PD (2, 6, 13). Some of them clearly result in a loss of function because no DJ-1 protein is synthesized because of large deletions and/or splicing errors. The molecular basis of neurodegeneration induced by a group of DJ-1 missense mutations is less clear. A working hypothesis in the field is that DJ-1 carries out its function as a dimer and that missense mutations impair the quantity of the dimeric DJ-1 in the cell (2, 3, 34, 35). In addition, because oxidized DJ-1 has been found in post mortem brains of PD patients (22), it is of interest to determine the structural determinants of such modifications.

Here we have used MD simulations to assess the effects on DJ-1 protein structure of oxidative stress and of two missense mutations causing familial PD (L166P and M26I) (9) along with a physiologic variant (R98Q) (8).

Our MD simulations suggest that: (i) WT in solution is very similar to the x-ray structure in both reduced and oxidized states and in both monomeric and dimeric forms. (ii) The M26I monomer is structurally similar to the WT monomer, because the mutation does not disrupt intraprotein interactions of the WT (supplemental Table S2). Thus, this monomeric structure may assemble similarly to the WT to form dimers. (iii) The local conformation around Pro166 in the L166P monomer evolved toward a conformation significantly different from that of the WT. This might be due to the disruption of α8 helix, which in turn affects the conformations of the secondary structure elements at the subunit-subunit interface (α1, α9 helices, and β3 β-sheet). Thus, although Pro166 is not located at the subunit surface involved in the dimerization, it changes its shape by modifying some of the intraprotein interactions. This is shown by a comparison of structural determinants of the protein surface of the mutant with the WT (supplemental Table S2). The different conformation of L166P monomeric units might affect the ability of the mutant to assemble in dimeric and multimeric structures. Indeed, by gel filtration and other assays, L166P has been previously shown to be present mostly as an HMW complex that may contain either DJ-1 oligomers and/or aggregates tagged protein in HEK 293 cells. FLAG-tagged WT DJ-1 coimmunoprecipitated with HA-tagged protein, and similarly the FLAG-tagged M26I mutant coimmunoprecipitated with its HA-tagged version. In contrast, L166P was unable to do so. The R28A FLAG-tagged mutant could only partially immunoprecipitate with its HA-tagged form (data not shown).
with other proteins (5, 33, 35). This led to the hypothesis that L166P may have additional, unidentified, dominant-negative effects. Parkin, CHIP, and hsp70 were all able to interact with L166P as part of a large complex (34). Furthermore, Parkin interaction provoked the sequestration of DJ-1 mutants into insoluble fractions (34). By in vitro experiments, we confirmed previous data that the ectopic expression of L166P is very low and that dimer formation is very negligible. Interestingly, we detected the previously identified HMW complex that may be involved in abnormal oligomerization and protein/protein interactions (5, 33, 35). (iv) MD simulations showed that the dimeric structures of M26I and R98Q DJ-1s are similar to that of the WT, and they keep the subunit-subunit interactions intact. The dimeric structures turned out to be less flexible than the monomer, especially in the flexible N- and C-terminal fragments. This fact points to a stabilizing role for the subunit-subunit association.

These results for M26I are consistent with our in vitro assays, which showed that M26I has the same ability as WT to form dimers. Therefore, our combined computational and experimental approach, along with previous experimental work (5, 7), provided a coherent picture in which M26I assumes mostly a dimeric structure. The results for R98Q are consistent with the fact that this mutant is a physiological variant. We concluded that the PD-causing M26I mutation does not largely affect the stability of the dimer and does not destabilize it by changing the shape of the subunit-subunit contact surface.

To identify a mutation that decreases the affinity for the dimeric structure without changing the fold of the monomer, we next performed Baker’s computational alanine scanning procedure (57) and MD calculations. We find that R28A is indeed able to cause destabilization at the interface. Furthermore, the MD structure of the R28A monomer turns out to be similar to that of the WT monomer, as shown by the comparison of selected structural determinants (Figs. 3B and 4). This may be caused by the fact that Ala28 faces the solvent. However, the MD simulations point to the destabilization of the dimer as already observed in the time scale investigated (~10 ns).

The SASA for water and the distances between the centers of mass increased on passing from the WT to R28A (Fig. 5D). This is caused by the disruption of subunit-subunit interactions rather than a change of shape of the subunit surface. The computational results are consistent with in vitro experiments that showed R28A has a reduced, although not abolished, capacity to form homodimers as compared with DJ-1 WT. This effect is not caused by a reduced stability of the R28A mutant because the protein is expressed at even higher levels than the DJ-1 WT (Figs. 3 and 4).

Concluding Remarks—We have presented combined computational and experimental investigations of two PD-causing DJ-1 mutations: M26I and L166P. M26I affected neither the monomeric structure nor the dimeric one. Furthermore, our results show that the mutant has a similar tendency to form dimers as that of the WT.

The L166P mutation favors the formation of multimers against the dimeric structure (5, 34, 35). This is probably due to the different conformation of the monomeric subunit (3). The mutated residue is indeed located not at the subunit-subunit interface but rather on α helix. Thus, oligomer or monomer L166P may form in vivo and present a different pattern of protein-protein interactions than DJ-1 WT (Figs. 3 and 4). Although this issue cannot be firmly established with calculations alone, it is supported experimentally here and in the work of others (34). The relevance of these interactions will probably depend on the L166P protein level in vivo. It must be noted that to the best of our knowledge no investigation of DJ-1 L166P protein has been done in post mortem brains of PD patients carrying this mutation or in knock-in animal models. It will be interesting to investigate whether L166P in vivo is unstable or a component of HMW aggregates.

Our investigation was complemented by a search for mutations that decrease the affinity for the dimer without affecting the fold of the DJ-1 protein. In conclusion, this work presents MD simulations for L166P, M26I, and R28A DJ-1 protein mutants, giving an insight into their molecular structures and properties. This computational study is supported by biochemical in vitro data.

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