Mutations in the PARK7/DJ-1 gene cause autosomal-recessive Parkinson's disease. In some patients the gene is deleted. The molecular basis of disease in patients with point mutations is less obvious. We have investigated the molecular properties of [L166P]DJ-1 and the novel variant [E64D]DJ-1. When transfected into non-neuronal and neuronal cell lines, steady-state expression levels of [L166P]DJ-1 were dramatically lower than wild-type [WT]DJ-1 and [E64D]DJ-1. Cycloheximide and pulse-chase experiments revealed that the decreased expression levels of [L166P]DJ-1 were because of accelerated protein turnover. Proteasomal degradation was not the major pathway of DJ-1 breakdown because treatment with the proteasome inhibitor MG-132 caused only minimal accumulation of DJ-1, even of the very unstable [L166P]DJ-1 mutant. Because of the structural resemblance of DJ-1 with bacterial cysteine proteases, we considered an autoproteolytic mechanism. However, neither pharmacological inhibition nor site-directed mutagenesis of the putative active site residue Cys-106 stabilized DJ-1. To gain further insight into the structural defects of DJ-1 mutants, human [WT]DJ-1 and both mutants were expressed in *Escherichia coli*. As in eukaryotic cells, expression levels of [L166P]DJ-1 were dramatically reduced compared with [WT]DJ-1 and [E64D]DJ-1. Circular dichroism spectrum revealed that the solution structures of [WT]DJ-1 and [E64D]DJ-1 are rich in β-strand and α-helix conformation. α-Helices were more susceptible to thermal denaturation than the β-sheet, and [WT]DJ-1 was more flexible in this regard than [E64D]DJ-1. Thus, structural defects of [E64D]DJ-1 only become apparent upon denaturing conditions, whereas the L166P mutation causes a drastic defect that leads to excessive degradation.

Although hereditary parkinsonism is very rare compared with sporadic Parkinson's disease (PD), the identification of PARK genes has greatly expanded the molecular understanding of the most common neurodegenerative movement disorders (1). The discovery of PARK1/α-SYNUCLEIN has led to the identification of α-synuclein fibrils as the neuropathological hallmark of PD, and the investigation of α-synuclein has provided important clues to the molecular mechanisms of PD (2, 3). Moreover, two enzymes involved in ubiquitin metabolism were found to be encoded by PARK2 (the ubiquitin ligase parkin) and PARK5 (ubiquitin C-terminal hydrolase-L1), suggesting that failure of the ubiquitin-proteasome system generally contributes to PD (4).

More recently, DJ-1 was identified to be the gene mutated in the PARK7 locus (5). Loss-of-function mutations of DJ-1 are compatible with the recessive inheritance of PARK7 (6). In the Dutch kindred, protein-coding sequences are deleted altogether (5). Complete loss of functional DJ-1 was also predicted for a young-onset PD patient with compound frameshift and splice mutations (7). It remains to be shown what physiological function of DJ-1 is depleted. Previous studies have implicated DJ-1 with tumor progression, RNA binding, male fertility, androgen receptor signaling, and cellular management of oxidative stress (8–14). Furthermore, there is structural similarity of DJ-1 with bacterial proteases and hydroperoxidases (15–18). However, the function of DJ-1 in the brain, where it is expressed at rather moderate levels (5, 8, 9), is completely unknown.

A second PARK7 family from Italy (19) was found to bear a point mutation in the DJ-1 gene, leading to replacement of leucine 166 with proline (5). The L166P mutation is predicted to break a characteristic α-helical fold in the DJ-1 structure. More recently, a second point mutation (E64D) was found in a small kindred of Turkish ancestry. The homoygous index patient had early-onset PD along with significant depletion of striatal dopamine receptors, as evidenced by the reduced 18F]FP-CIT uptake. Subclinical PD was indicated by reduction of [18F]FP-CIT positron emission in a homozygous sister, whereas a heterozygous brother had normal 18F]FP-CIT uptake. Five more siblings as well as the heterozygous mother were unaffected.

To elucidate the molecular basis of the defects of DJ-1 point mutations, the expression, processing, and turnover of WT and mutant DJ-1 were examined. Expression levels of mutant [L166P]DJ-1 were significantly reduced compared with [WT]DJ-1 (20, 21) as well as [E64D]DJ-1, both in prokaryotic and eukaryotic cells. Cycloheximide and pulse-chase experiments with transiently transfected human embryonic kidney HEK293 cells revealed that [L166P]DJ-1 was degraded much more rapidly than [WT]DJ-1 and [E64D]DJ-1. WT and mutant
DJ-1 minimally accumulated in the presence of MG-132, suggesting the existence of a major non-proteasomal DJ-1 degradation pathway. Because of structural resemblance of DJ-1 with Pyrococcus horikoshii PH1704 class cysteine proteases (17), we considered an autoproteolytic mechanism of [L166P]DJ-1 breakdown. However, neither pharmacological inhibition nor site-directed mutagenesis of the putative active site residue Thr 166, which is believed to stabilize DJ-1, prevented the accumulation of [L166P]DJ-1. Furthermore, we found that the solution structures of [WT]DJ-1 and [E64D]DJ-1 were rich in α-helices and β-sheets, but α-helical elements were more susceptible to thermal denaturation in [WT]DJ-1 than in [E64D]DJ-1. Thus, the L166P mutation enhances cellular breakdown and depletion of DJ-1, whereas the structural defects of [E64D]DJ-1 are more subtle and might become apparent only under stress conditions. We conclude that Parkinson's disease in PARK7 patients is caused by a depletion of functionally active DJ-1 protein.

MATERIALS AND METHODS

Antibodies—DJ-1 was probed with mouse monoclonal anti-V5 (Invitrogen), mouse monoclonal Penta-His (Qiagen, Hilden, Germany), and mouse monoclonal anti-DJ-1 (Medical & Biological Laboratories, Naganuma, Japan) and polyclonal antiserum against DJ-1 (gift of H. Ariga, Hokkaido University, Sapporo, Japan), followed by peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG, respectively (Sigma). Western blots were normalized by reprobing with anti-β-actin (Sigma). Efficacy of proteasome inhibitors was demonstrated with mouse monoclonal anti-β-catenin (Transduction Laboratories, Lexington, KY).

Site-directed Mutagenesis—[WT]DJ-1 and [L166P]DJ-1 (5' cDNAs were used as a template for two independent polymerase chain reactions (PCR) with the following primers: NcoI forward primer 5'-ATCTGGATCCGTCTTTAAAGATTTGACGAG-3' and the second PCR with 5'-AACAGTCGACCGTCTGGGCGGGG-3' and the second PCR with 5'-GGGCTTCGATCTGCAGGCTTTCT-3' and BamHI stop reverse primer 5'-ATCTGGATCCCGTAGAATCGAGACCGAG-3' and the second PCR with 5'-GGGCTTCGATCTGCAGGCTTTCT-3'. The two resulting PCR products were then used as templates for a PCR using NcoI forward (see above) and BamHI stop reverse primer (see above). The mutated PCR products were subcloned into pcDNA3.1/V5-His TOPO (Invitrogen) and sequenced (GATC Biotech). Generation of the [E64D]DJ-1 construct in the same vector was described elsewhere.

Eukaryotic DJ-1 Expression and Inhibitor Treatments—HEK293 cells, SV5Y cells, and MN9D cells were grown to near confluence and transiently transfected with eukaryotic expression constructs using LipofectAMINE 2000 in opti-minimal essential medium (Invitrogen) (DNA:lipid ratio 1:2.5). Two days after transfection, cells were treated with 100 μM cycloheximide (Sigma), 10 μM MG-132 (Calbiochem), 50 μM leupeptin (Roche Diagnostics), or directly lysed in 1% Triton X-100, 150 mM sodium chloride, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5) plus 10% glycerol, 3 mM dithiothreitol, 150 mM NaCl, 20 mM Tris (pH 8.0). DJ-1/HIS proteins eluted earlier than expected for globular proteins with molecular mass 21.3 kDa (low molecular weight gel filtration calibration markers were purchased from Amersham Biosciences), consistent with the dimeric structure of native DJ-1. Peak integrations of analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) runs revealed at least 90% purity of the DJ-1 preparations.

Expression of WT and Mutant DJ-1 in Eukaryotic Cells—To study the cellular expression of DJ-1, plasmids encoding [WT]DJ-1 and mutant DJ-1 with a C-terminal-fused V5/HIS tag were transfected into HEK293 cells. Two days after transfection, the expression levels relative to β-actin were found to be much lower for [L166P]DJ-1 than [WT]DJ-1 and [E64D]DJ-1, as evidenced from Western blots probed with anti-V5 (Fig. 1). Probing with polyclonal or monoclonal antibodies against DJ-1 revealed that the expression levels of transfected [WT]DJ-1 were comparable with endogenous DJ-1 levels, whereas [L166P]DJ-1 levels were dramatically reduced (Fig. 1). RT-PCR analysis of the transfected DJ-1 mRNAs revealed that all constructs were robustly expressed. Thus, [L166P]DJ-1 was lost at the protein level and not because of inefficient transcription or mRNA stability. [E64D]DJ-1 was expressed slightly less than [WT]DJ-1 and [E64D]DJ-1, but no polyclonal or monoclonal antibodies against DJ-1 were available for probing the Western blots. Consequently, the nucleotide sequence of the [E64D]DJ-1 protein was determined.

LONGER EXPRESSION OF HIGHER LEVELS OF DJ-1—Surprisingly, monoclonal 3E8 antibody against DJ-1 did not recognize the [E64D]/V5 tag was visualized by enhanced chemiluminescence.

Pulse-Chase Labeling—HEK293 cells were transiently transfected as above. One day after transfection, the cells were starved in methionine-free cell culture medium (Sigma) plus 1% penicillin/streptomycin and 1% γ-glutamine for one hour. They were then chased for 3 h with 75 μCi/ml of [35S]Met/[35S]Cys (Amersham Biosciences), rinsed, and chased with Dulbecco's modified Eagle's medium (PAA, Pasching, Austria) plus 1% penicillin/streptomycin, 1% γ-glutamine, 1 mM γ-methionine, and 10% fetal calf serum. The cells were lysed in 150 mM Tris-HCl buffer (pH 7.6) plus Complete protease inhibitor mixture. Immunoprecipitation was performed with anti-V5 and protein G-Sepharose (Amersham Biosciences). After denaturing 15% polyacrylamide gel electrophoresis, the gels were fixed, soaked in Amplify (Amersham Biosciences), and dried. The radioactively labeled proteins were visualized on BioMax film (Kodak, Rochester, New York).

RT-PCR Determination of DJ-1 mRNAs—Total RNA was extracted from transfected cells with Trizol (pQLab, Erlangen, Germany) and reverse-transcribed using superscript II and oligo(dT) primers (Invitrogen). The resulting cDNAs were PCR-amplified using Neol forward primer (see above) and BamHI V5 tag reverse primer (see below). Amplification rates were linear at 25 cycles, as visualized on ethidium bromide-stained agarose gels.

Bacterial DJ-1 Expression and Purification—[WT]DJ-1 and mutant DJ-1 cDNAs (see above) were amplified in polymerase chain reactions using the following primers: Neol forward primer (see above), BamHI V5 tag reverse primer 5'-ATCTGGATCCCTCAAAATCTGCGAGACG-3' and BamHI reverse primer 5'-ATCTGGATCCTGCTTATAAGAAACAGTGAGGCC-3'. After subcloning into pQE-60 (Qiagen), the prokaryotic expression constructs were used to transform E. coli BL21/RIL. All constructs were sequenced (GATC Biotech).

Bacterial cultures were induced with 100 μM isopropyl-β-D-thiogalacto-aside for 5 h. The pelleted bacteria were French-pressed into phosphate-buffered saline containing Complete protease inhibitors. Imidazole was added to a final concentration of 20 mM, and this lysate was loaded onto a nickel-nitriotriacetic acid Superflow column (Qiagen). The HIS-tagged DJ-1 proteins eluted in a linear gradient (20–500 mM) around 0.1 M imidazole as broad peaks that were concentrated in Centriprep-3 devices (Millipore). The concentrates were loaded onto a HiLoad Superdex 75 column (Amersham Biosciences) and eluted in 5% glycerol, 3 mM dithiothreitol, 150 mM NaCl, 20 mM Tris (pH 8.0). DJ-1/HIS proteins eluted earlier than expected for globular proteins with molecular mass 21.3 kDa (low molecular weight gel filtration calibration markers were purchased from Amersham Biosciences), consistent with the dimeric structure of native DJ-1. Peak integrations of analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) runs revealed at least 90% purity of the DJ-1 preparations.

Mass Spectrometry—[DJ-1] was diluted to 0.01–0.5 mg/ml with 0.1% trifluoroacetic acid in water. Two μl of this solution were mixed with 1 μl of sinapinic acid (Fluka) saturated in 0.4% trifluoroacetic acid in 30% acetonitrile as matrix. One μl was deposited on a stainless steel target plate. Matrix-assisted laser desorption ionization mass spectra were recorded in the linear mode on a Voyager DE STR mass spectrometer (Applied Biosystems) with 25,000 V accelerating voltage. 3–5 × 104 laser shots were collected from different positions and added.

CD Spectrometry—Protein solutions were scanned in a 0.1-cm quartz cuvette using a J-810 CD spectrophotometer (Jasco). Temperature in the measuring cell was gradually increased, and at defined temperature intervals CD spectra were taken from 300–190 nm. Secondary structure was calculated using the Jasco JWSSE-480 software (least squares method Yang).

RESULTS

Expression of WT and Mutant DJ-1 in Eukaryotic Cells—To study the cellular expression of DJ-1, plasmids encoding [WT]DJ-1 and mutant DJ-1 with a C-terminal-fused V5/HIS tag were transfected into HEK293 cells. Two days after transfection, the expression levels relative to β-actin were found to be much lower for [L166P]DJ-1 than [WT]DJ-1 and [E64D]DJ-1, as evidenced from Western blots probed with anti-V5 (Fig. 1). Probing with polyclonal or monoclonal antibodies against DJ-1 revealed that the expression levels of transfected [WT]DJ-1 were comparable with endogenous DJ-1 levels, whereas [L166P]DJ-1 levels were dramatically reduced (Fig. 1). RT-PCR analysis of the transfected DJ-1 mRNAs revealed that all constructs were robustly expressed. Thus, [L166P]DJ-1 was lost at the protein level and not because of inefficient transcription or mRNA stability. [E64D]DJ-1 was expressed slightly less than [WT]DJ-1. Surprisingly, monoclonal 3E8 anti-DJ-1 did not recognize the [E64D]/V5 tag was visualized by enhanced chemiluminescence.

Longer expression of higher levels of DJ-1—Surprisingly, monoclonal 3E8 antibody against DJ-1 did not recognize the [E64D]/V5 tag was visualized by enhanced chemiluminescence.

Turnover of WT and Mutant DJ-1 in HEK293 Cells—Despite the detection of similar mRNA levels, the steady-state levels of
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Fig. 1. Steady-state levels of WT and mutant DJ-1. HEK293 cells were lipofected in duplicate with 4 μg of plasmid encoding V5-tagged [WT]DJ-1 (lanes 1 and 2), [L166P]DJ-1 (lanes 3 and 4), or [E64D]DJ-1 (lanes 5 and 6). Two days after transfection cells were lysed, and 20 μg (50 μg in the case of [L166P]DJ-1 transfectants) of total cellular protein was electrophoresed in a denaturing 15% polyacrylamide gel and Western blotted. Transfected DJ-1/V5/HIS proteins were detected with anti-V5. In addition, polyclonal anti-DJ-1 and 3E8 monoclonal anti-DJ-1 recognized the endogenous DJ-1. Reprobing for β-actin confirmed equal loading, and RT-PCR with DJ-1/V5/HIS-specific primers revealed robust expression of all transgene constructs (lowest panel). Molecular mass standards are positioned to the right.

Mutant DJ-1 proteins were reduced. To test whether rapid degradation could account for the loss of mutant DJ-1 protein, pulse-chase experiments were performed. Transiently transfected HEK293 cells were pulse-labeled with a mixture of [35S]cysteine/methionine and chased with non-radioactive medium up to 24 h. Radiolabeled transfected DJ-1 proteins were immunoprecipitated with anti-V5. [WT]DJ-1 was found to be a very stable protein. There was practically no breakdown of [WT]DJ-1 for 6–8 h, with little further decay up to 24 h (Fig. 3A). [E64D]DJ-1 was an equally stable protein. In contrast, the degradation of [L166P]DJ-1 protein was dramatically accelerated. [L166P]DJ-1 was degraded within 4–8 h, although it was translated as efficiently as [WT]DJ-1 as evidenced by the comparable incorporation of [35S]-labeled amino acids at the 0-h chase time point. Thus, the dramatic reduction of [L166P]DJ-1 steady-state levels is not because of poor translation. Moreover, the 0-h chase time point showed only full-length [L166P]DJ-1. Both N-terminal-truncated [L166P]DJ-1 fragments ∆N1 and ∆N2 were cleaved from the full-length precursor protein within 4–6 h after translation, demonstrating that the N-terminal processing of [L166P]DJ-1/V5/HIS is a rapid event.

To confirm that the reduced steady-state expression of [L166P]DJ-1 was because of accelerated protein degradation and to measure the turnover of endogenous DJ-1, protein synthesis was blocked with cycloheximide and the decay of DJ-1 proteins was monitored over a time course of 12 h. Like the endogenous DJ-1, transfected [WT]DJ-1 and [E64D]DJ-1 remained fully stable for 6 h with little further decay (Fig. 3B). In striking contrast, full-length [L166P]DJ-1 immunoreactivity completely vanished within 4 h (Fig. 3B), but the N-terminal-truncated DJ-1 species ∆N1 and ∆N2 remained stable throughout the 12-h time course. Expression of mutant DJ-1 had no significant influence on the turnover of endogenous DJ-1.

Proteasomal and Autoproteolytic DJ-1 Degradation Pathways—Our results indicate that [WT]DJ-1 is a rather stable protein with relatively slow turnover. Indeed, blocking the major cellular protein degradation machinery (the proteasome) (22) with MG-132 did not lead to an accumulation of [WT]DJ-1 in transiently transfected HEK293 cells (Fig. 4). MG-132 treatment did not cause DJ-1 accumulation in stably transfected cells either (data not shown). Furthermore, accumulation of the endogenous DJ-1 was not found in MG-132-treated cells (Fig. 4). Likewise, the relatively stable mutant [E64D]DJ-1 did not accumulate in MG-132-treated HEK293 cells (Fig. 4). It has been proposed that the enhanced turnover of [L166P]DJ-1 was (at least in part) because of proteasomal degradation (20). However, MG-132 treatment only minimally increased the levels of [L166P]DJ-1, although the proteasome was effectively blocked in our system as evidenced by the massive accumulation of β-catenin, a protein that is rapidly turned over in the proteasome (23). Rather, the N-terminal-truncated [L166P]DJ-1 fragments ∆N1 and ∆N2 appeared to be stabilized upon proteasomal inhibition. Thus, there must exist proteolytic pathways for DJ-1 in addition to the proteasome.

To study alternative, non-proteasomal DJ-1 breakdown pathways, we inhibited cellular cytochrome proteases in HEK293 cells transiently transfected with DJ-1 with leupeptin. However, leupeptin treatment did not lead to significant accumulation of DJ-1 (Fig. 5A), although probing for co-transfected amyloid precursor protein revealed the expected accumulation of C-terminal stubs (not shown) because of efficient cytosine protease inhibition (24). Specifically, the highly unstable [L166P]DJ-1 protein was not stabilized by inhibition of cellular cytochrome proteases. Another cytochrome protease inhibitor, E64, did not stabilize DJ-1 either (results not shown).

To assess the autoproteolytic potential of DJ-1 more directly, we replaced the putative active-site cysteine 106 (17) with alanine by site-directed mutagenesis. When transiently transfected into HEK293 cells, the steady-state levels of [C106A]DJ-1 were not significantly elevated compared with [WT]DJ-1 (Fig. 5B). Likewise, introduction of the C106A active-site mutation did not stabilize [L166P]DJ-1. The steady-state expression levels of the double mutant [C106A/L166P]DJ-1 were as low as those of the single mutant [L166P]DJ-1 despite the strong expression of their mRNAs (Fig. 5B). Thus, the imperfectly conserved catalytic cleft around cysteine 106 of DJ-1, which is topologically equivalent to the catalytic cysteine residue of PH1704-type proteases, does not contribute to the rapid degradation of mutant [L166P]DJ-1. In other words, the fast breakdown of [L166P]DJ-1 is not because of derepressed self-digestion. Interestingly, however, the C106A mutation increased the appearance of a DJ-1 immunoreactive band that migrated at an apparent molecular mass expected for a dimer (Fig. 5B).
Bacterial Expression of WT and Mutant DJ-1—As in eukaryotic cells, the expression levels of [L166P]DJ-1 were greatly reduced compared with [WT]DJ-1. The dramatic reduction of [L166P]DJ-1 did not depend on the sequence of the C-terminal tag, because it was observed regardless of the presence or absence of a V5/HIS tag or with HIS6 fused directly onto the C terminus (Fig. 6, A and B). The low yield of [L166P]DJ-1 was not because of a toxic effect of mutant DJ-1, because growth curves of [WT]DJ-1-producing bacteria were identical to the ones for [L166P]DJ-1 bacteria (Fig. 6, C and D). In contrast to the [L166P]DJ-1 mutant protein, [E64D]DJ-1 was expressed at levels comparable with [WT]DJ-1, as assessed by immunoreactivity with antibodies against the C-terminal HIS tag and a polyclonal antiserum against DJ-1 (Fig. 6, A and B). As on Western blots prepared from eukaryotic cells, the [E64D]DJ-1 mutant protein expressed in bacteria was not detected with the 3E8 monoclonal anti-DJ-1 (Fig. 6, A and B).

Structural Dynamics of Purified Recombinant DJ-1—The CD spectrum of [WT]DJ-1 at 20 °C was indicative of a well-structured protein (Fig. 7B). The percentages of secondary structure elements calculated from the CD spectra of [WT]DJ-1 were 38 ± 3% a-helix, 32 ± 2% β-strand, 9 ± 3% β-turn, 23 ± 1% random coil (n = 3). The solution secondary structure
estimated here is well in accord with the crystal structure of DJ-1 (15–18). No significant changes in secondary structure elements were observed between 20–40 °C. Between 40–50 °C a marked loss of α-helical content occurred, concomitant with a transient gain of β conformation (Fig. 7C). Further raising the temperature beyond 60 °C led to a sharp increase of random coil conformation (Fig. 7C). At even higher temperatures the CD measurements became variable because of precipitation of the denatured DJ-1. Heat denaturation of [WT]DJ-1 was complete at 80 °C and irreversible; cooling back to 20 °C did not lead to recovery of the native CD spectrum (results not shown).

CD spectra of [E64D]DJ-1 recorded at 20 °C were very similar to those of [WT]DJ-1 (Fig. 7E), revealing 33 ± 3% α-helix, 36 ± 1% β-strand, 11 ± 1% β-turn, 21 ± 1% random coil (n = 4). Again, our secondary structure estimates for [E64D]DJ-1 in solution are in good agreement with the recently solved crystal structure of this mutant DJ-1.2 Increasing the temperature from 20 to 40 °C did not affect the structure of [E64D]DJ-1 as was the case for [WT]DJ-1. In fact, secondary structure of [E64D]DJ-1 was retained up to 50 °C. Between 50–60 °C there was a loss of α-helical structure concomitant with a parallel increase in random coil conformation (Fig. 7F). Protein precipitation started at 60 °C, and denaturation was complete at 80 °C. Thus, [E64D]DJ-1 had the same structure as [WT]DJ-1 up to 40 °C, but the α-helical structure elements of [E64D]DJ-1 required higher thermal energy to unfold than in [WT]DJ-1.

DISCUSSION

PARK7 mutations in the DJ-1 gene cause recessive PD (5). Affected members of the Dutch kindred have two alleles with a 14-kB deletion eliminating exons 1–5 of DJ-1 (5). Another PD patient with very early onset (24 years) was found to be a compound heterozygote with one nonsense DJ-1 allele (frameshift after Glu-18) and one allele with mutated splice acceptor site of intron 6 (7). These mutated DJ-1 alleles are highly unlikely to produce any functional DJ-1 at all. The deleterious effects of DJ-1 point mutations are less obvious. We have investigated the cellular and structural consequences of the Italian L166P mutation (5) and a novel Turkish E64D mutation.2

[L166P]DJ-1 was inherently unstable in cells of both eukaryotic and prokaryotic origin, indicating a complete loss of DJ-1 protein with the helix-breaking L166P mutation. Consistent with the recessive mode of inheritance, [L166P]DJ-1 did not affect the expression levels of endogenous DJ-1. Thus, affected homozygous members of the Italian PARK7 kindred do not express any DJ-1 protein (21) as is predicted for the patients of the Dutch kindred. We monitored DJ-1 protein decay in pulse-chase experiments as well as in the presence of the protein synthesis inhibitor cycloheximide and found that [WT]DJ-1 was very stable. Consequently, inhibition of the proteasome over an 11-h time course did not lead to an accumulation of the very stable [WT]DJ-1 protein. In contrast, the half-life time of [L166P]DJ-1 was dramatically reduced. The proteasome is the major cellular breakdown machinery to remove misfolded proteins (22). However, treatment with the proteasome inhibitor MG-132 poorly stabilized [L166P]DJ-1. Consistent with these findings, Miller et al. (20) showed only partial stabilization of [L166P]DJ-1/V5/HIS after 16 h of proteasomal inhibition in M17 neuroblastoma and COS-7 cells. Differences in cell type and slight culture conditions may account for our smaller effect of MG-132 on [L166P]DJ-1 stabilization. We have treated transiently transfected HEK293 cells with MG-132 for only 11–12 h because toxicity begins after this time, but we clearly demonstrate efficient proteasome inhibition by the massive accumulation of the proteasomal substrate β-catenin (23). Nevertheless, these studies show that the proteasome is not the single most important mediator of DJ-1 clearance. The relative increase of discrete N-terminal-truncated DJ-1 breakdown bands in [L166P]DJ-1-transfected HEK293 cells may be taken as an
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However, it remains to be shown whether N-terminal processing is a (patho)physiologically relevant pathway for endogenous DJ-1.

The crystal structure of DJ-1 is related to PH1704-like cysteine proteases. The catalytic center of these bacterial proteins contains a triadic cysteine-histidine-glutamate configuration that is partially conserved in the DJ-1 structure: the side chains of cysteine 106 and histidine 126 are located in equivalent positions as in PH1704. Although there is no glutamate side chain in the appropriate distance to histidine 126 to complete the catalytic triad of PH1704 in DJ-1, the sterically constrained cysteine 106 residue might be sufficiently reactive in a putative catalytic dyad. The putative catalytic cleft of DJ-1 is occluded by an extreme C-terminal helix-turn-helix motif that harbors the detrimental L166P mutation (17). This helix-breaking amino acid exchange might derepress the potential cysteine protease activity of DJ-1 and lead to autoproteolytic breakdown of [L166P]DJ-1. We have tested this hypothesis by inhibiting cellular cysteine proteases with leupeptin and E64, but neither WT nor mutant DJ-1 accumulated under these conditions. To investigate the potential cysteine protease activity of DJ-1 more directly, we have generated an “active site” mutant by replacing cysteine 106 with alanine. Consistent with the pharmacological suppression of cysteine proteases, the C106A mutation did not rescue the highly unstable [L166P]DJ-1. Although the exact proteolytic breakdown pathway(s) remains to be clarified, it can be concluded that the L166P mutation renders DJ-1 highly unstable, effectively leading to a loss of DJ-1 protein in the affected patients.

The consequences of the conservative E64D substitution were much subtler. Cellular steady-state expression levels and turnover rates of [E64D]DJ-1 were similar to [WT]DJ-1, and the native structures of purified recombinant [E64D]DJ-1 and [WT]DJ-1 were the same. The crystal structure of [E64D]DJ-1 revealed an intact DJ-1 dimer. Nevertheless, the epitope recognized by the monoclonal antibody 3E8 was completely lost on Western blots prepared from [E64D]DJ-1 expressing HEK293 cells as well as E. coli. Because bacteria very inefficiently phosphorylate recombinant proteins, it is unlikely that the 3E8 epitope is masked by phosphorylation near the Glu-64 residue, for example at Ser-57 and Tyr-67 that are predicted to be phosphorylated with probabilities of 0.997 and 0.899 (NetPhos 2.0 algorithm), respectively. Unfortunately, monoclonal 3E8 did not immunoprecipitate any DJ-1 species (data not shown), so binding to the native epitope could not be assessed. It will be interesting to determine whether the loss of the 3E8 epitope on Western blots reflects altered interaction with some putative binding partner(s), perhaps under cellular stress conditions (11). Alternatively, monoclonal 3E8 raised against full-length DJ-1 might recognize the amino acids around position 64 with extremely high specificity, distinguishing glutamate from aspartate based on a side chain that is longer by only one methylene group.

Moreover, α-helices in [E64D]DJ-1 required higher thermal energy for unfolding, possibly suggesting a more rigid structure compared with [WT]DJ-1. Honbou et al. (17) speculated that DJ-1 has cysteine protease activity that requires an activation step. According to this model, the C-terminal α-helices, which distinguish DJ-1 structurally from bacterial proteases such as PH1704, need to undergo a conformational change to relieve the putative active site from sterical blockage. In fact, the L166P mutation is predicted to have a deleterious effect on the integrity of this C-terminal α-helix configuration, but so far no one has been able to produce sufficient [L166P]DJ-1 protein for structure determination. It is interesting to note that in our thermal denaturation experiments, α-helix content of [WT]DJ-1/5/HIS was also observed in non-neuronal COS-7 cells (21).
decreased at 10 °C lower temperatures compared with [E64D]DJ-1. Our CD recordings do not allow the identification of the α-helices that unfold upon thermal denaturation. However, [WT]DJ-1 might have a greater structural flexibility allowing a regulated access to the putative active site, whereas the apparently more rigid [E64D]DJ-1 could be less well regulated.

We conclude that loss of functional DJ-1 protein contributes
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Fig. 7. Secondary structure analysis of purified recombinant DJ-1. [WT]DJ-1 (A–C) and [E64D]DJ-1 (D–F) were purified to homogeneity as verified by mass spectrometry (A and D). Major peaks were mono-ionized ([MH]+) and di-ionized ([MH]2+) as were dimeric ([M,H]2+) DJ-1 proteins. B and E, CD spectra of 2 μM [WT]DJ-1 (B) and 5 μM [E64D]DJ-1 (E) were recorded at increasing temperatures and molar residue ellipticity plotted against wavelength. Line colors correspond to the following temperatures: dark green, 20 °C; dark blue, 45 °C; red, 56 °C; yellow, 60 °C; light blue, 66 °C; pink, 70 °C; light green, 76 °C. C and F, secondary structure elements (diamonds, α-helix; squares, β-strand; triangles, random coil) were calculated from CD spectra and plotted against temperature. β-Turn was constantly around 10% and was omitted for reasons of clarity. Each data point represents the mean value of three ([WT]DJ-1 (C) and four ([E64D]DJ-1 (F) independent temperature scans, respectively. Error bars delineate S.E.

to the pathogenesis of PD. The L166P mutation causes a dramatic decrease of DJ-1 stability because of much accelerated protein degradation. The proteasome may contribute to L166P(DJ-1 breakdown, but major alternative proteolytic processing pathways remain to be identified. In contrast, the subtle effects of the conservative E64D substitution were only visible under denaturing conditions. It is possible that DJ-1 mutations such as E64D and also R98Q (7) predispose to PD in conjunction with additional risk factors.

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Differential Effects of Parkinson's Disease-associated Mutations on Stability and Folding of DJ-1

Karin Görner, Eve Holtorf, Sabine Odoy, Brigitte Nuscher, Ayako Yamamoto, Jörg T. Regula, Klaus Beyer, Christian Haass and Philipp J. Kahle

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