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Impact of Autosomal Recessive Juvenile Parkinson's Disease Mutations on the Structure and Interactions of the Parkin Ubiquitin-like Domain

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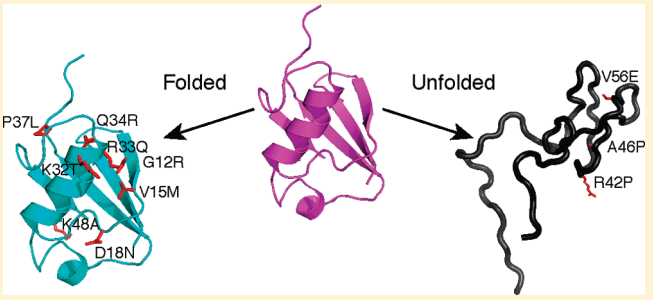
Supporting Information

ABSTRACT: Autosomal recessive juvenile parkinsonism (ARJP) is an early onset familial form of Parkinson's disease. Approximately 50% of all ARJP cases are attributed to mutations in the gene *park2*, coding for the protein parkin. Parkin is a multidomain E3 ubiquitin ligase with six distinct domains including an N-terminal ubiquitin-like (Ubl) domain. In this work we examined the structure, stability, and interactions of the parkin Ubl domain containing most ARJP causative mutations. Using NMR spectroscopy we show that the Ubl domain proteins containing the ARJP substitutions G12R, D18N, K32T, R33Q, P37L, and K48A retained a similar three-dimensional fold as the Ubl domain, while at least one other (V15M) had altered packing. Four substitutions (A31D, R42P, A46P, and V56E) result in poor folding of the domain, while one protein (T55I) showed evidence of heterogeneity and aggregation. Further, of the substitutions that maintained their three-dimensional fold, we found that four of these (V15M, K32T, R33Q, and P37L) lead to impaired function due to decreased ability to interact with the 19S regulatory subunit S5a. Three substitutions (G12R, D18N, and Q34R) with an uncertain role in the disease did not alter the three-dimensional fold or S5a interaction. This work provides the first extensive characterization of the structural effects of causative mutations within the ubiquitin-like domain in ARJP.



Autosomal recessive juvenile parkinsonism (ARJP) is an early onset familial form of Parkinson's disease. The distinguishing features of ARJP from the more prevalent idiopathic form of the disease include its earlier onset age, slower disease course, occasional dystonia, and long-lasting response to low doses of L-dopa. ARJP is thought to result from mutations in one or more genes, including the gene that encodes the E3 ubiquitin ligase parkin,[1] accounting for approximately 50% of all cases. Parkin, as with all E3 ligases, is an important enzyme in the ubiquitin-mediated proteolysis pathway. It has been shown to covalently link ubiquitin to potential substrates such as Eps15,[2,3] CDCrel-1,[4] α-synuclein,[5] synphilin-1,[6] AIMP2,[7] and FBP-1.[8] However, confirmation of some of these substrates and their functional outcomes remains controversial.[9,10] More recently, parkin has been shown to have an important role in mitochondrial function. Parkin ubiquitination of mitochondrial proteins serves to recruit and assemble the autophagy machinery to clear impaired mitochondria.[11] Interestingly, the activity of complex I of the mitochondrial respiratory chain is decreased in the substantia nigra and other tissues in Parkinson's disease patients. Further, several complex I inhibitors reproduce key features of Parkinson's disease such as loss of dopaminergic neurons and motor deficits.[12,13]

Parkin is a multidomain E3 ubiquitin ligase consisting of 465 amino acids with five distinct domains: an N-terminal ubiquitin-like (Ubl) domain followed by a unique parkin-specific domain (UPD), a RING0 domain, and two C-terminal RING domains separated by an in-between RING (IBR) domain.[14] The N-terminal Ubl domain has been shown to be essential for the ligase activity of parkin since deletion or mutation of this domain results in impaired E3 ligase activity.[5,15] The Ubl domain has also been shown to participate in a variety of protein interactions. For example, binding of the Ubl domain to the S5a proteasomal subunit has been suggested to position parkin and its bound substrates near the degradation machinery.[16] Alternatively, the parkin Ubl domain has been shown to interact with the endocytotic protein, Eps15[2,3,16], in order to facilitate Eps15 ubiquitination used in the Akt signaling cascade.

Early onset PD has been linked to homozygous and compound heterozygous mutations in the parkin gene. However, heterozygous mutations in parkin also exist and are more controversial[17] including some that are likely polymorphisms. Approximately 95 nonstop, missense mutations are associated with ARJP (see http://www.molgen.ua.ac.be/PDmutDB and http://www.hgmd.org) and are located throughout the parkin gene resulting in single residue substitutions in the coded protein

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(for a summary see refs 18 and 19). This includes at least 20 substitutions at 16 different positions in the Ubl domain. Some disease state mutations localized to the Ubl domain have been shown to decrease the stability of the full-length parkin protein *in vivo*, although conflicting evidence has been presented for the effects of the mutations on E3 ligase activity.[15,20,21] To date, it has been difficult to distinguish whether the observations arise from substitutions causing structural or folding alterations in parkin or are a result of disrupted protein interactions with E2 ubiquitin conjugating enzymes, potential substrates, or the S5a proteasomal subunit. One reason for this uncertainty has been the inability to purify parkin, its constitutive domains, and especially proteins containing disease-causing mutations on a large scale suitable for biophysical studies. To date, only the parkin Ubl[16,22] and IBR[23] domains have been purified and their three-dimensional structures determined. While some mutant forms of the parkin IBR domain have been isolated and characterized,[23] similar approaches have not been completed for the Ubl domain mutant containing proteins. To date, only two substitutions (K48A, R42P) have been structurally examined in the Ubl domain leaving the majority of the disease-related substitutions uncharacterized.[3,24] Further, while a large number of mutations in the RING domains have been examined for interactions with different E2 enzymes [25] and ubiquitination,[20,25−27] similar comprehensive studies have not been completed for the Ubl domain.

In this work we have examined proteins carrying ARJP disease-associated mutations at 13 of the possible 16 positions in the parkin Ubl domain in order to identify the impact of these substitutions on the Ubl domain structure, stability, and interactions with the S5a proteasome. Our work provides evidence that several ARJP disease state mutations cause drastic alterations in the Ubl domain structure and/or stability, while others disrupt binding to the S5a proteasomal subunit. These observations provide two different mechanisms that ARJP-associated missense mutations in the parkin gene may contribute to the dysfunction of the protein in the ubiquitination pathway.

EXPERIMENTAL PROCEDURES

Cloning. The DNA fragment encoding the Ubl domain (residues 1−77) of human parkin was cloned into the *Nde*I and *Bam*HI sites of the pET44a vector (Novagen). The substitutions G12R, V15M, D18N, A31D, K32T, R33Q, Q34R, P37L, R42P, A46P, K48A, T55I, and V56E in the Ubl domain were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The DNA fragment encoding the UIMs (residues 196−309) of the human proteasomal S5a subunit was cloned into a pET21a vector (Novagen) modified to include a tobacco etch virus protease cleavage site before the six-residue histidine C-terminal tag. For expression with an in-frame N-terminal GB1 fusion, the DNA fragment encoding the Ubl domain (1−77) was cloned into the *Nhe*I and *Xho*I sites of the GEV1 vector,[28] a generous gift from Dr. M. Clore (NIDDK, Bethesda, MD). The V56E substituted version of the GB1-Ubl domain fusion was created using the QuikChange site-directed mutagenesis kit.

Protein Expression and Purification. The Ubl domain, GB1-Ubl domain, and all substituted proteins were overexpressed in the BL21(DE3) Codon PlusRIL *Escherichia coli* strain and purified as previously described using the same chromatographic methods.[24] The $S5a^{196-309}$ fragment containing both UIM regions was expressed with a six-residue histidine tag and purified using a Ni-NTA FPLC affinity column (GE Healthcare) followed by size exclusion chromatography. Ubiquitin from *Saccharomyces cerevisiae* was expressed from a pET3a vector as previously described.[29] The integrity of all proteins was confirmed by electrospray ionization mass spectrometry (UWO Biological Mass Spectrometry Laboratory).

NMR Spectroscopy. All NMR experiments were performed on a 600 MHz Varian Inova spectrometer equipped with either a ^{13}C-enhanced triple resonance cold probe with *z*-gradients or an *xyz* gradient, triple resonance probe (Biomolecular NMR Facility, UWO). ^{1}H chemical shifts were referenced directly to internal DSS at 0 ppm. Sensitivity-enhanced $^{1}H-^{15}N$ HSQC spectra[30] were recorded at 25 °C on ^{15}N-labeled proteins in 10 mM KH_2PO_4, 1 mM EDTA, 1 mM DTT, 30 μM DSS, and 10% D_2O at pH 7.0. All spectra were processed with NMRPipe[31] software using a 60° shifted, cosine-squared function in both ^{1}H and ^{15}N dimensions and analyzed using NMRView.[32]

Protein Unfolding Experiments. Unfolding experiments were monitored by circular dichroism (CD) spectropolarimetry using a Jasco J-810 instrument (Biomolecular Interactions and Conformations Facility, UWO). Spectra (190−250 nm) were initially measured at 5 °C using ten averaged scans for protein samples ranging in concentration from 20 to 80 μM in a 1 mm cell. Following this, the ellipticity at 222 nm was measured as a function of temperature between 5 and 95 °C using a 1 °C/min temperature gradient. Data were analyzed by plotting the observed ellipticity as a function of temperature and fitting for the melting point transition (T_m) and the enthalpy (ΔH_m) according to eq 1. During the fitting process a heat capacity (ΔC_p) of 3.1 kJ/(mol K) was used based on calculated accessible surface areas of the folded and unfolded Ubl domain.[33,34] The data were found to be insensitive to heat capacity as fits done using ΔC_p 0 kJ/(mol K) yielded near identical values for T_m and ΔH_m.

$$\Delta G = \Delta H_m\left(1 - \frac{T}{T_m}\right) + \Delta C_p\left[(T - T_m) - T\ln\left(\frac{T}{T_m}\right)\right] \quad (1)$$

The differences in stability ($\Delta\Delta G$) between substituted Ubl domain proteins and the wild-type protein were calculated using ΔH_m(wt), T_m(wt), and the differences in melting temperatures (ΔT_m) between the wild type and the appropriate substituted proteins[35] according to eq 2.

$$\Delta\Delta G = \Delta T_m\frac{\Delta H_m(\text{wt})}{T_m(\text{wt})} \quad (2)$$

Ubl Domain−S5a Binding Assays. Purified His-tagged $S5a^{196-309}$ was mixed with untagged Ubl domain, substituted Ubl domain proteins, or ubiquitin at a 1:2 molar ratio, respectively, in a total volume of 300 μL and placed on a rotating shaker at 4 °C for 1 h. The mixture was then loaded onto a Ni-NTA spin column (Qiagen) preequilibrated in binding buffer (20 mM sodium phosphate, 10 mM imidazole, 300 mM NaCl, pH 8). The column was washed twice with 600 μL of binding buffer and then eluted (20 mM sodium phosphate, 250 mM imidazole, 300 mM NaCl, pH 8). Elution samples were fractionated by electrophoresis on a tricine−polyacrylamide gel and stained with Coomassie brilliant blue dye. Protein concentrations were determined using the extinction coefficient method measured in guanidine hydrochloride.[36]

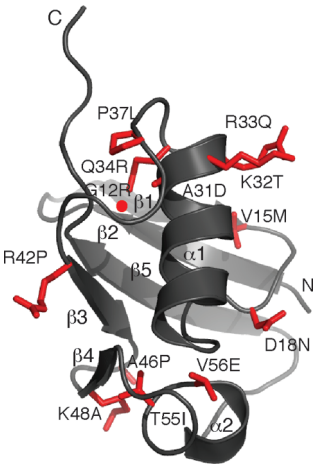

Figure 1. Ribbon drawing of the parkin Ubl domain illustrating the location of ARJP disease state substitutions. Side chains are indicated in red and labeled with the ARJP causative substitution. This figure was produced using the program PyMOL[48] using the human parkin Ubl domain structure (Protein Data Bank 1IYF).[16]

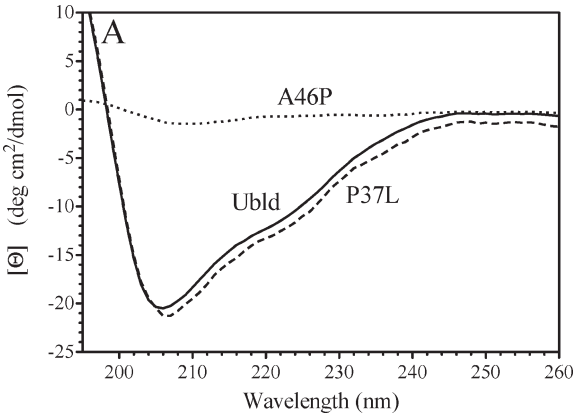

Figure 2. Folding comparison of substituted Ubl domains using CD spectropolarimetry. The spectrum of the wild-type parkin Ubl domain is shown (—) compared to the Ubld^{P37L} protein (- - -) that maintained a similar fold. The spectrum of the Ubld^{A46P} protein (· · ·) which had little secondary structure and was shown by mass spectrometry to be sensitive to proteolysis is also shown. All spectra were collected using 20−80 μM protein in 10 mM KH_2PO_4, 1 mM EDTA, and 1 mM DTT, pH 7.0 at 5 °C.

RESULTS

The three-dimensional structure of the parkin Ubl domain comprises a β-grasp fold (Figure 1) containing a five-strand β-sheet (β1, H11−V15; β2, I2−F7; β3, R42−F45; β4, K48−L50; and β5, Q64−V70) and two α-helices (α1, S22-Q34; α2, V56-D60).[16] This structure is characteristic of other ubiquitin-like domains such as hPLIC-2 and hHR23a[37,38] and ubiquitin.[39] Despite these structural similarities, the parkin Ubl domain is nearly 11 kJ/mol less stable than ubiquitin.[24] The lower stability of the parkin Ubl domain may be an inherent property of the domain or might indicate that intramolecular interactions with other domains in the protein are needed to enhance its stability. Therefore, missense mutations in the Ubl domain could contribute to its improper folding, instability that would interfere with the overall structure of parkin or compromise its interactions with other proteins. To determine the effects of ARJP mutations on the structure and stability of the parkin Ubl domain, we used site-directed mutagenesis to create 13 of the possible substituted proteins. NMR spectroscopy was used to assess how each of these single residue substitutions might affect the overall structure of the proteins. Thermal denaturation, as measured by CD spectropolarimetry, was used to identify the change in stability caused by the substitutions. Both of these methods were used to determine whether structural alterations might render the Ubl domain ineffective for interaction with the proteasomal S5a subunit. The substitutions investigated covered most secondary structure regions of the Ubl domain including G12R (Ubld^{G12R}) and V15 M (Ubld^{V15M}) in β1; R42P (Ubld^{R42P}) in β3; K48A (Ubld^{K48A}) in β4; A31D (Ubld^{A31D}), K32T (Ubld^{K32T}), R33Q (Ubld^{R33Q}), and Q34R (Ubld^{Q34R}) in β1; V56E (Ubld^{V56E}) in β2; and D18N (Ubld^{D18N}), P37L (Ubld^{P37L}), A46P (Ubld^{A46P}), and T55I (Ubld^{T55I}) in loop regions (Figure 1). The ARJP substitution Ubld^{R42P} [40] and another reported,[41] but not confirmed (Ubld^{K48A}), have been previously described[24] and are included here for completeness. The proteins covered 13 of the 16 sites for ARJP mutations. The only substitutions not assessed were at the initiating methionine position (M1L) and S10N and W54R that were reported after this work was started.

Most Ubl domain substituted proteins with the exception of Ubld^{A31D}, Ubld^{R42P}, Ubld^{A46P}, and Ubld^{V56E} expressed well and produced yields comparable to that of the wild-type protein. The Ubld^{A31D} protein expressed at very low levels, could not be purified to homogeneity, and was not further investigated in these studies. The Ubld^{A46P} and Ubld^{V56E} proteins were expressed, but more than 90% of the protein was found in the insoluble fraction of the cell lysate as determined from Coomassie stained gels (data not shown). Attempts to purify these proteins showed wide variations in heterogeneity and susceptibility to proteolysis as determined by mass spectrometry. The Ubld^{T55I} protein was expressed and could be purified intact, but preliminary NMR spectra showed a heterogeneous population of peaks with different line widths. The behaviors of Ubld^{A31D}, Ubld^{A46P}, Ubld^{T55I}, and Ubld^{V56E} are consistent with poorly folded protein structures that are sensitive to degradation and/or aggregation, similar to that observed for Ubld^{R42P}. To circumvent this issue, we selected a candidate protein (Ubld^{V56E}) to express as a fusion protein linked to the B1 domain from immunoglobulin G (GB1), similar to that described previously for Ubld^{R42P}.[24] The resulting protein GB1-Ubld^{V56E} could be expressed to high levels and purified to homogeneity for structural characterization.

Parkin R42P, A46P, and V56E Substitutions Cause Ubl Domain Unfolding. CD spectropolarimetry and NMR spectroscopy were used to determine whether any significant changes to the secondary and tertiary structures of the substituted proteins occurred compared to the wild-type Ubl domain. The CD spectrum of the wild-type Ubl domain at 5 °C had well-defined minima near 208 and 225 nm (Figure 2) consistent with its three-dimensional structure, which contains approximately 21% α-helix and 43% β-sheet. A comparison of the CD spectra for Ubld^{P37L} (Figure 2) and several other substituted proteins (Ubld^{G12R}, Ubld^{V15M}, Ubld^{D18N}, Ubld^{K32T}, Ubld^{R33Q}, Ubld^{Q34R}, Ubld^{K48A}) with the wild-type Ubl domain showed less than 5% difference between the spectra, indicating that the secondary structure of these Ubl domain proteins was very similar to the normal protein at this temperature. A CD spectrum of Ubld^{A46P}

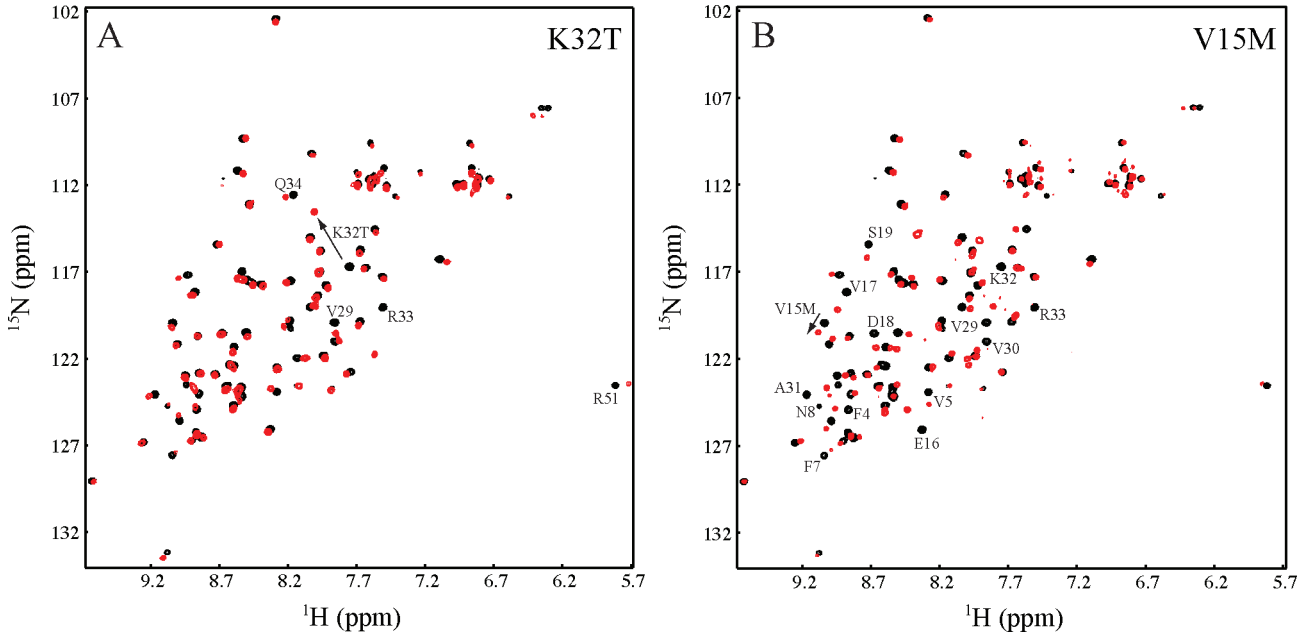

Figure 3. ^{1}H−^{15}N HSQC spectra of parkin Ubl domain and substituted proteins resulting from ARJP mutations. Superposition of spectra for the parkin Ubl domain (black) with (A) Ubld^{K32T} (red) and (B) Ubld^{V15M} (red). In each panel selected residue assignments are shown that have the largest chemical shift differences from the wild-type Ubl domain. An arrow is used to indicate the positions of peaks for the substituted residue in the wild-type and substituted proteins.

(Figure 2) showed very poor signal from α-helix or β-sheet structure indicating this protein was likely unfolded. Analysis of this sample using mass spectrometry subsequent to CD measurement revealed that a portion of the protein had undergone proteolysis.

Each of the Ubl domain substituted proteins was examined by comparing their ^{1}H−^{15}N HSQC spectrum with the wild-type parkin Ubl domain spectrum (Figure 3). It was expected that a benign single site substitution should have only local effects on the spectrum reflecting the amino acid change whereas a greater disruption in structure would be reflected in a large number of spectral changes, especially for residues remote from the substitution. The ^{1}H−^{15}N HSQC spectra for the Ubld^{G12R}, Ubld^{V15M}, Ubld^{D18N}, Ubld^{K32T}, Ubld^{R33Q}, Ubld^{Q34R}, Ubld^{P37L}, and Ubld^{K48A} proteins (Figure 3 and Figure S1 of Supporting Information) all had well-dispersed peaks and similar spectral patterns to the wild-type Ubl domain spectrum. In several cases (Ubld^{D18N}, Ubld^{K32T}, Ubld^{R33Q}, Ubld^{P37L}, and Ubld^{K48A}) the single amino acid substitutions resulted in minimal changes in the NMR spectrum. For example, in the Ubld^{K32T} protein (Figure 3A) the appearance of a new peak for T32 and a large shift of the neighboring R33 occurred while the remainder of the spectrum was unaffected. This is consistent with a minor environmental change around position 32 with little alteration in structure. In contrast, the ^{1}H−^{15}N HSQC spectrum for Ubld^{V15M} resulted in changes in chemical shift to the adjacent residues E16, V17, and D18 but also to V29 and V30 in the α1 helix and several residues (F4, V5, F7) in β2. In the Ubl domain structure, V15 packs against V29 and V30 and has backbone contacts with M1−V3 near the N-terminus, indicating the V15M substitution likely affected the packing between these residues. A similar observation was noted for the Q34R substitution.

Since the Ubld^{V56E} protein could not be purified in sufficient levels and was susceptible to proteolysis, the protein was linked

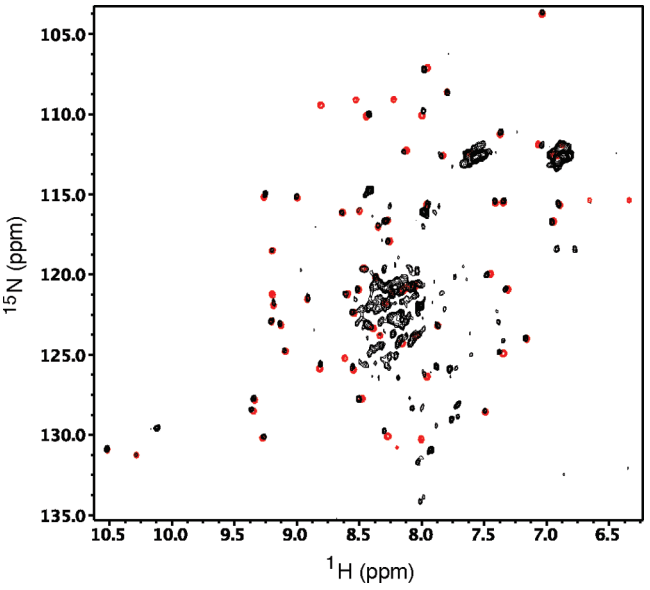

Figure 4. ^{1}H−^{15}N HSQC spectra of GB1-Ubld^{V56E} showing the unfolded nature of Ubld^{V56E}. The spectrum of the GB1-Ubld^{V56E} protein is plotted in black contours while the isolated GB1 protein is shown in red contours. The spectra were collected at 600 MHz in 10 mM KH_2PO_4, 1 mM EDTA, and 1 mM DTT, pH 7.0 at 25 °C.

to the B1 domain from immunoglobulin G (GB1; GB1-Ubld^{V56E}) to improve these properties. The resulting ^{1}H−^{15}N HSQC spectrum (Figure 4) displayed a population of well-dispersed peaks that were in excellent agreement with those observed in the ^{1}H−^{15}N HSQC spectrum of the isolated GB1 domain.[28] This indicated that the GB1 domain maintained its native fold having little interaction with the Ubld^{V56E} portion of the construct. The remaining peaks

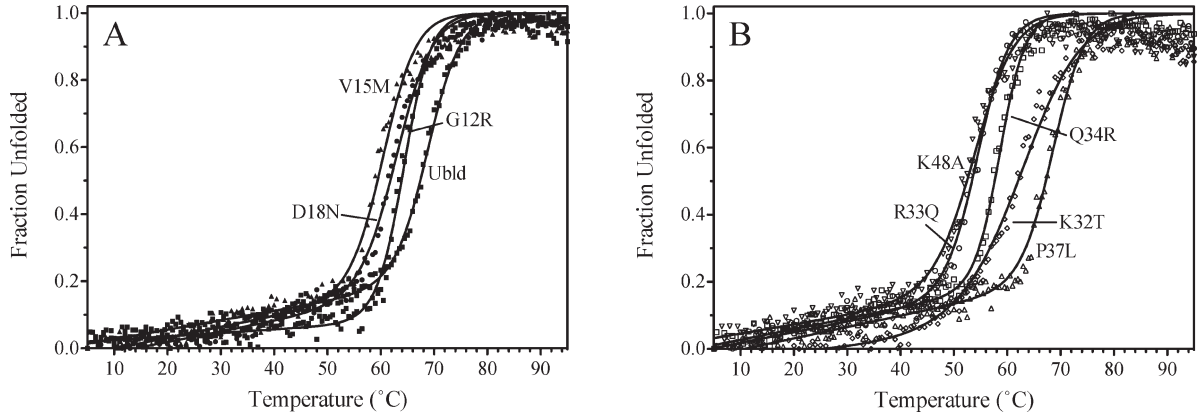

Figure 5. Thermal unfolding curves for the parkin Ubl domain and substituted proteins resulting from ARJP disease state mutations. Data were collected using CD spectropolarimetry between 5 and 95 °C using a 1 °C/min temperature gradient. The ellipticity at 222 nm was plotted as fraction unfolded and fit according to equations described in Experimental Procedures. All samples were comprised of 20−80 μM protein in 10 mM KH_2PO_4, 1 mM EDTA, and 1 mM DTT, pH 7.0.

arising from the Ubld^{V56E} protein had little resemblance to the $^{1}H-^{15}N$ HSQC spectrum of the wild-type Ubl domain or most of the substituted proteins (Figure 3). The nondisperse nature of these peaks and their tight clustering between 8.0 and 8.5 ppm in the ^{1}H dimension indicated the Ubld^{V56E} protein was unfolded. Subsequent to the NMR experiments, size exclusion chromatography showed the elution volume of GB1-Ubld^{V56E} was much smaller than expected for a 16 kDa protein, consistent with its unfolded structure. A similar observation has been made previously for Ubld^{R42P}.[24] Overall, these results indicate that the Ubl domain proteins containing the ARJP mutations G12R, D18N, K32T, R33Q, P37L, and K48A retain a similar three-dimensional fold as the wild-type domain, while at least two others (Ubld^{V15M}, Ubld^{Q34R}) have evidence of altered packing. In contrast, three substitutions (R42P, A46P, and V56E) result in gross structural differences due to unfolding of the domain.

Folded ARJP Substituted Proteins Lead to Differential Ubl Domain Stability. The substituted proteins (Ubld^{G12R}, Ubld^{V15M}, Ubld^{D18N}, Ubld^{K32T}, Ubld^{R33Q}, Ubld^{Q34R}, Ubld^{P37L}, Ubld^{K48A}) that exhibited a folded structure were examined to determine if the substitutions had altered the stabilities of the proteins. This was done using thermal unfolding of each protein and monitoring the change in ellipticity at 222 nm as a function of temperature. Initial spectra were recorded at 5 °C where the CD spectra of the selected substituted proteins were similar to the wild-type protein (Figure 2). Most of the melting curves for native parkin Ubl domain and the ARJP substituted proteins showed smooth sigmoidal transitions between folded and unfolded species indicative of a two-state unfolding process (Figure 5). Ubld^{D18N} and Ubld^{K32T} had shallower slopes that might indicate some deviation from this model. The data were fit using eq 1 to determine the midpoint of the transition (T_m) and the enthalpy (ΔH_m). The data showed that the parkin Ubl domain had a melting temperature (T_m) near 63 °C (Table 1). There were three ARJP-substituted proteins that melted at higher temperatures (Ubld^{G12R}, Ubld^{D18N}, Ubld^{P37L}) and five substituted Ubl domain proteins that unfolded at lower temperatures including Ubld^{R33Q} and Ubld^{K48A} that melted nearly 10 °C lower than the wild-type protein. The difference in stabilities was calculated using the method of Becktel and Schellman using the ΔH_m for the wild type and the T_m for each protein.[35] These results generally followed the order of the melting stabilities indicating that Ubld^{D18N} was the most stable protein and Ubld^{K48A} had the poorest stability.

Table 1. Thermodynamic Parameters for Substituted Parkin Ubl Proteins

protein	T_m (°C)[a]	ΔH_m (kJ/mol)[b]	$\Delta\Delta G$ (kJ/mol)[c]
parkin Ubl domain	62.7 ± 0.1	246.8 ± 9.5	
G12R	64.0 ± 0.1	271.2 ± 16.2	−1.0
V15M	60.2 ± 0.1	246.2 ± 7.9	1.8
D18N	68.7 ± 0.1	336.7 ± 14.9	−4.4
K32T	61.9 ± 0.2	117.1 ± 7.6	0.6
R33Q	54.1 ± 0.1	198.9 ± 8.5	6.3
Q34R	58.4 ± 0.1	267.3 ± 8.0	3.2
P37L	68.0 ± 0.1	223.5 ± 7.1	−3.9
K48A	53.2 ± 0.2	179.2 ± 8.4	7.0

[a] Determined from nonlinear fit using ΔC_p = 3.1 kJ mol^{-1} K^{-1} according to eq 1. [b] Calculated from the linear fit near the T_m of ln $K = \Delta S/R - \Delta H/R(1/T)$. [c] Estimated using eq 2 and T_m, ΔH_m for parkin Ubl domain wild-type protein.

ARJP-Substituted Proteins Compromise S5a Interaction. The interaction of the parkin Ubl domain with the 19S regulatory subunit S5a was studied using residues 196−309 of the 377 amino acid protein. This fragment (S5a$^{196-309}$) could be expressed and purified with a six-residue histidine tag and possesses ubiquitin interacting motifs (UIMs) shown previously to interact with the parkin Ubl domain.[16] To assess the impact of different ARJP substitutions on the interaction of the Ubl domain with S5a$^{196-309}$, affinity pull-down experiments were employed. Figure 6 shows that His-tagged S5a$^{196-309}$ bound to Ni-NTA beads successfully pulled down untagged parkin Ubl domain. The S5a fragment also bound similarly to Ubld^{G12R}, Ubld^{D18N}, and Ubld^{Q34R}, indicating these substitutions are not involved at the parkin Ubl domain−S5a interface. However, S5a$^{196-309}$ interactions with the parkin- substituted proteins Ubld^{V15M}, Ubld^{K32T}, Ubld^{R33Q}, and Ubld^{P37L} were notably weaker than wild-type Ubl domain, similar to previous observations with the Ubld^{K48A} protein.[3] Since NMR experiments show some of these proteins (Ubld^{K32T}, Ubld^{R33Q}, and Ubld^{P37L}) retain a similar three-dimensional fold as the parkin Ubl domain, this indicates the substitutions likely interfere with formation of a proper parkin

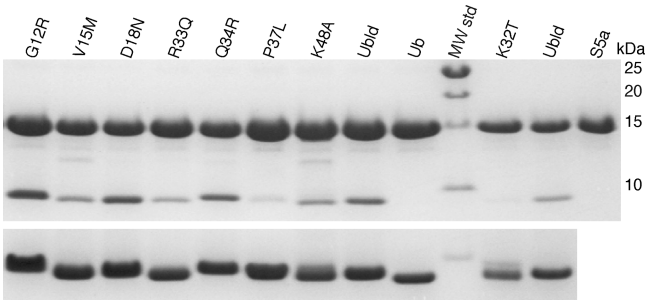

Figure 6. ARJP substitutions in the Ubl domain disrupt S5a interactions. His_6-$S5a^{196-309}$ was incubated with 2 molar equiv of the parkin Ubl domain, substituted protein, or ubiquitin for 1 h and then loaded onto a Ni-NTA spin column. Following elution the samples were fractionated on a 16.5% tricine gel and stained with Coomassie dye. The top panel shows the bound proteins eluted with His_6-$S5a^{196-309}$ as follows: $Ubld^{G12R}$ (lane 1), $Ubld^{V15M}$ (lane 2), $Ubld^{D18N}$ (lane 3), $Ubld^{R33Q}$ (lane 4), $Ubld^{Q34R}$ (lane 5), $Ubld^{P37L}$ (lane 6), $Ubld^{K48A}$ (lane 7), Ubl domain (lane 8), ubiquitin (lane 9), and His_6-$S5a^{196-309}$ alone (lane 13). Lower quantities of $Ubld^{K32T}$ (lane 11) were used in the assay (although at the same molar ratio as the other mutants) so parkin Ubl domain was assayed again at this concentration (lane 12). Molecular weight markers are shown in lane 10. The lower panel illustrates the pure proteins as used in the pull-down assay and correspond to the same labels.

Ubl domain−S5a interface. As expected, little or no interaction between $S5a^{196-309}$ and ubiquitin was observed.

DISCUSSION

Of the 13 substituted proteins we examined, five of them ($Ubld^{A31D}$, $Ubld^{R42P}$, $Ubld^{A46P}$, $Ubld^{T55I}$, and $Ubld^{V56E}$) showed significant structural alterations. For the $Ubld^{A31D}$ protein, its low level of expression in bacteria compared to wild type is evidence for a poorly folded protein with decreased stability that may be subject to proteolysis. Similarly, $Ubld^{A46P}$ showed little secondary or tertiary structure and was easily degraded. The $Ubld^{T55I}$ protein showed evidence of heterogeneity and aggregation in NMR spectra. In previous studies we used an N-terminal GB1 tag to stabilize and protect the Ubl domain proteins from degradation. Both $Ubld^{V56E}$ and $Ubld^{R42P}$ had $^1H-^{15}N$ HSQC spectra consistent with an unfolded state for the Ubl domain portion of the protein. In the parkin Ubl domain, V56 is 95% buried near the N-terminus of the short α2 helix and participates in hydrophobic interactions with the side chains of M1, V17, and L61. Molecular dynamics experiments suggest that substitution of this hydrophobic residue with a charged glutamic acid residue would disrupt these interactions.[42] Similarly, A46 is located in the loop region between β3 and β4, and the substitution of alanine to proline would disrupt its hydrogen bonding to I66 in β5. Further, the addition of proline at this position could restrict the flexibility of the loop. As shown previously for $Ubld^{R42P}$, disruption of hydrogen bonding within the β-sheet regions can lead to unfolding of the entire domain.[24]

The eight proteins that contained substitutions but formed folded domains showed differential interactions with the tandem UIM region from the S5a subunit, proposed to allow for efficient delivery of polyubiquitinated cargo to the proteasome for degradation. The $Ubld^{K48A}$ substitution resulted in decreased binding with S5a consistent with previous results showing this residue is involved in the interaction with the UIM region(s) from either S5a or Eps15.[3] In contrast, the $Ubld^{V15M}$, $Ubld^{K32T}$, $Ubld^{R33Q}$, and $Ubld^{P37L}$ proteins showed diminished binding compared to wild-type Ubl domain even though none of these residues lies on the binding interface required for S5a interaction. Three of the substitutions ($Ubld^{V15M}$, $Ubld^{K32T}$, $Ubld^{R33Q}$) lead to decreased stability of the Ubl domain. It is noted that V15, K32, and R33 all interact with F13 in sheet β1, a residue affected by S5a binding. The decreased stability of the Ubl domain carrying these substitutions suggests that some modification of the S5a binding site has occurred. It is not clear why decreased S5a binding to the $Ubld^{P37L}$ protein occurs as this substitution leads to increased domain stability. The similarity of S5a binding to $Ubld^{G12R}$ and $Ubld^{D18N}$ compared to wild-type Ubl domain was in agreement with their maintenance of three-dimensional fold and similar or increased stabilities. Although G12 is affected by the interaction with the UIMs from Eps15, neither substitution is at the interaction surface for S5a.[3]

The downstream effects of some interactions of the parkin Ubl domain have been validated *in vitro* and *in vivo*. It was recently shown that the interaction of the parkin Ubl domain with the regulatory particle of the proteasome activates the 26S proteasome.[43] This interaction provides a direct link between the cellular ubiquitination machinery and the regulation of the degradation machinery in the cell. The upregulation of proteasome activity was also shown in *Drosophila* where a K71P substitution (corresponding to R42P here) showed no regulation of proteasome activity, further indicating that a correctly folded Ubl domain is essential to the function of parkin *in vivo*.

Several ARJP substitutions in the Ubl domain shown to cause unfolding ($Ubld^{R42P}$, $Ubld^{V56E}$) or large decreases in stability ($Ubld^{R33Q}$, $Ubld^{K48A}$) alter the behavior of the full-length protein *in vivo*. In work done by Henn et al.[15] ARJP substitutions including R33Q, R42P, K48A, and V56E resulted in decreased stability of the full-length protein in N2a cells. This effect was shown to be a result of an increased degradation of parkin by the 26S proteasome. Another interaction where substitutions in the Ubl domain have been shown to affect its function is in the parkin interaction with the SH3 domain of endophilin-A1.[44] It was observed that *in vitro* parkin ubiquitinated endophilin-A1. The Ubl domain with the reported ARJP causative substitutions R42P and K48A was shown to disrupt this interaction, providing a new link between mutations in parkin and disruption to proteins in synaptic transmission.

These results clearly demonstrate there are multiple outcomes of the different ARJP disease-state mutations in the parkin Ubl domain. Some of these substitutions (A31D, R42P, A46P, T55I, V56E) result in disruption of the domain fold. Others, while maintaining the three-dimensional fold of the Ubl domain, have defects in interactions with the S5a subunit including K48A that has not been clearly linked to ARJP. Further, one must consider the outcomes of three of the substitutions (G12R, D18N, Q34R) that have little effect on the three-dimensional fold or S5a interaction. The corresponding mutations in the parkin gene are observed in the heterozygous state[17,45−47] and could simply be a result of a polymorphism that has little impact on the protein function. Alternatively, it is possible that some of the disease-state residues in the parkin Ubl domain could be stabilized or are important for other essential, unidentified interactions with the remaining portions of the protein. Further studies will be needed to clarify the roles of these residues in parkin function and in ARJP.

■ ASSOCIATED CONTENT

Supporting Information. $^{1}H-^{15}N$ HSQC spectra of substituted parkin Ubl domain proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ARJP, autosomal recessive juvenile parkinsonism; Ubl, ubiquitin-like; Ub, ubiquitin; UIM, ubiquitin-interacting motif; Ni-NTA, nickel nitrilotriacetic acid; HSQC, heteronuclear single-quantum coherence.

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