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Structural model of the dimeric Parkinson's protein LRRK2 reveals a compact architecture involving distant interdomain contacts

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Leucine-rich repeat kinase 2 (LRRK2) is a large, multidomain protein containing two catalytic domains: a Ras of complex proteins (Roc) G-domain and a kinase domain. Mutations associated with familial and sporadic Parkinson's disease (PD) have been identified in both catalytic domains, as well as in several of its multiple putative regulatory domains. Several of these mutations have been linked to increased kinase activity. Despite the role of LRRK2 in the pathogenesis of PD, little is known about its overall architecture and how PD-linked mutations alter its function and enzymatic activities. Here, we have modeled the 3D structure of dimeric, full-length LRRK2 by combining domain-based homology models with multiple experimental constraints provided by chemical cross-linking combined with mass spectrometry, negative-stain EM, and small-angle X-ray scattering. Our model reveals dimeric LRRK2 has a compact overall architecture with a tight, multidomain organization. Close contacts between the N-terminal ankyrin and C-terminal WD40 domains, and their proximity—together with the LRR domain—to the kinase domain suggest an intramolecular mechanism for LRRK2 kinase activity regulation. Overall, our studies provide, to our knowledge, the first structural framework for understanding the role of the different domains of full-length LRRK2 in the pathogenesis of PD.

LRRK2 | Parkinson's disease | structural modeling | EM | CL-MS

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease, and it is clinically characterized by movement impairment, bradykinesia, rigidity, and resting tremors. Pathologically, it is characterized by the progressive loss of dopaminergic neurons in the substantia nigra and the formation of Lewy bodies (1). Although the majority of cases are sporadic, mutations in the leucine-rich repeat kinase 2 (LRRK2) gene [*PARK8*; Online Mendelian Inheritance in Man (OMIM) no. 609007] have been unequivocally linked to late-onset autosomal dominant PD (2, 3).

Mutations in the *LRRK2* gene are found in 5–15% of families with autosomal dominant PD, making them the most common cause of Mendelian PD identified so far (4). Unfortunately, the biological function of LRRK2 and the mechanism by which it contributes to PD pathogenesis are not well understood. LRRK2 is a multidomain, 286-kDa protein exhibiting both GTPase and kinase activities (5–7) that belongs to the recently identified Roco protein family of G proteins. Members of this family have a Ras of complex proteins (Roc) G-domain and an adjacent conserved C-terminal of Roc (COR) dimerization domain in common (8, 9). Besides the enzymatic core region, LRRK2 contains

four predicted solenoid domains commonly involved in protein–protein interactions (10). These domains include the N-terminal ankyrin, armadillo, and namesake leucine-rich repeat (LRR) domains, along with a C-terminal WD40 domain. Various PD-associated mutations in LRRK2 have been shown to augment kinase activity (6, 7, 11, 12) and increase the phosphorylation of Rab proteins, recently identified as likely physiological substrates (12). In addition, LRRK2 mutations within the Roc domain have been associated with decreased LRRK2 GTPase activity (13, 14).

Significance

Leucine-rich repeat kinase 2 (LRRK2) represents a promising drug target for treatment and prevention of Parkinson's disease (PD), because mutations in LRRK2 are the most common cause of Mendelian forms of the disease. PD-associated LRRK2 variants show decreased GTPase and increased kinase activity. By integrating multiple experimental inputs provided by chemical cross-linking, small-angle X-ray scattering, and a negative-stain EM map, we present, to our knowledge, the first structural model of the full-length LRRK2 dimer. The model reveals a compact folding of the LRRK2 dimer with multiple domain–domain interactions that might be involved in the regulation of LRRK2 enzymatic properties.

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The authors declare no conflict of interest.

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Data deposition: The structure of the leucine-rich repeat domain of the *Chlorobium tepidum* Roco protein has been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 5IL7).

See Commentary on page 8346.

¹G.G., F. Raimondi, B.K.G., Y.G.-L., and E.D. contributed equally to this work.

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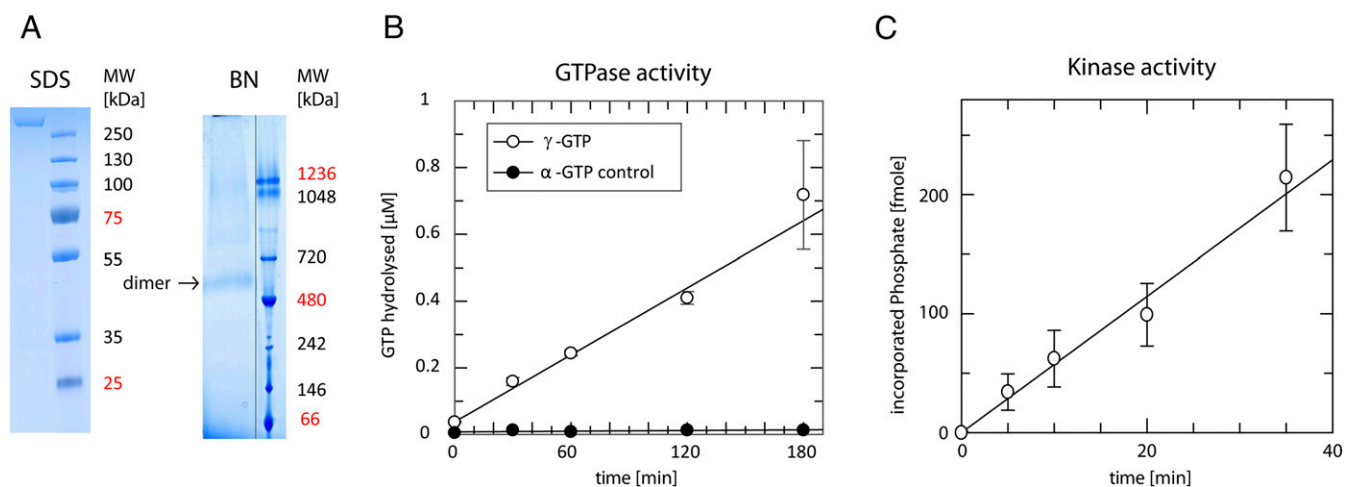


Fig. 1. (A, Left) SDS/PAGE to demonstrate the purity of LRRK2. Five percent of a typical batch (corresponding to ~ 600 cm² of HEK293T culture) was separated on a NuPage 4–12% gradient gel and stained with colloidal Coomassie. (Right) BN-PAGE (4–12% NativePage) analysis reveals that LRRK2 is predominantly dimeric after purification (colloidal Coomassie stain). MW, molecular weight. (B) Purified LRRK2 exhibits GTPase activity. The assay was performed with 0.1 μ M LRRK2 and either 10 μ M γ -GTP or 10 μ M α -GTP (specificity control) at 30 °C. The observed GTP hydrolysis rate is 0.03 min⁻¹ (± 0.0014) ($n = 3$ biological replicates with 2 technical replicates for each). (C) Kinase activity toward the synthetic LRRK2 substrate LRRKtide in the presence of GDP ($n = 3$ biological replicates with 2 technical replicates for each).

These results have prompted the development of highly potent and specific kinase inhibitors with K_i values in the low nanomolar range (15). However, the value of this therapeutic approach has been challenged by the observation that treatment with LRRK2 kinase inhibitors results in significant lung toxicity in primates (16). In addition, LRRK2 knockout rodent models display severe kidney phenotypes (17, 18). These results suggest that effective therapeutic intervention may require more subtle allosteric modulation of LRRK2 kinase activity, and highlight the critical importance of understanding the regulation and function of this enzyme. Here, we present a structural model of full-length, dimeric LRRK2 derived from an integrative structural modeling approach based on multiple experimental constraints provided by chemical cross-linking small-angle X-ray scattering (SAXS), as well as a 3D map of LRRK2 generated by negative-stain EM. The resulting model of LRRK2 reveals a compact architecture with close contacts of the C-terminal helix of the WD40 domain with the N-terminal ankyrin domain. These two domains, as well as the LRR domain, are in close proximity to the kinase domain, suggesting that they could play a potential role in modulating LRRK2 kinase activity.

Results

Purified LRRK2 Is an Active Dimer in Solution. To improve the yield and purity of LRRK2 necessary for structural studies, the expression and purification procedure for recombinant full-length LRRK2 was optimized from previously published protocols (19). Following transient transfection of HEK293T cells, N-terminal Strep/FLAG (SF)-tagged LRRK2 was purified via the tandem Strep-tag II moiety. The optimized protocol allowed a yield of ~ 100 – 150 μ g of LRRK2 at a purity $>98\%$ from confluent cells covering ~ 600 cm² (4×14 -cm dishes, 5 – 6×10^8 cells). The purity of the material is shown in Fig. 1A. In addition, the secondary structure of the protein was assessed by circular dichroism (CD) measurements in the far-UV region (250–190 nm). The CD spectra shown in Fig. S1 indicate that the protein is folded with a large fraction (52%) of α -helical secondary structure and just 9% of β -strand content. These data are in good agreement with estimations based on the final structural models of the LRRK2 dimer predicting 34–36% α -helical and 12–14% β -strand content. The purified LRRK2 was analyzed by dynamic light scattering (DLS) and Blue Native polyacrylamide gel electrophoresis (BN-PAGE) to evaluate purity and oligomeric state. DLS analysis revealed that LRRK2 is predominantly

dimeric in solution with an experimentally determined molecular mass of 581 kDa, which is in excellent agreement with the calculated mass of the SF-tagged LRRK2 dimer (native protein, 572 kDa; SF-tag fusion protein, 584 kDa) (Fig. S2). The BN-PAGE shows a prominent band with an apparent molecular mass corresponding to dimeric LRRK2 (Fig. 1A). Consistent with previous reports showing that LRRK2 is able to homodimerize (6, 20–23), these findings demonstrate that LRRK2 is a dimer in solution with no significant presence of higher oligomeric species under these conditions. In addition, no changes in the oligomeric state were visible when LRRK2 was purified in the presence of different G-nucleotides (Fig. S2), and biochemical analysis confirmed that the purified LRRK2 displays both GTPase and kinase activities (Fig. 1B and C).

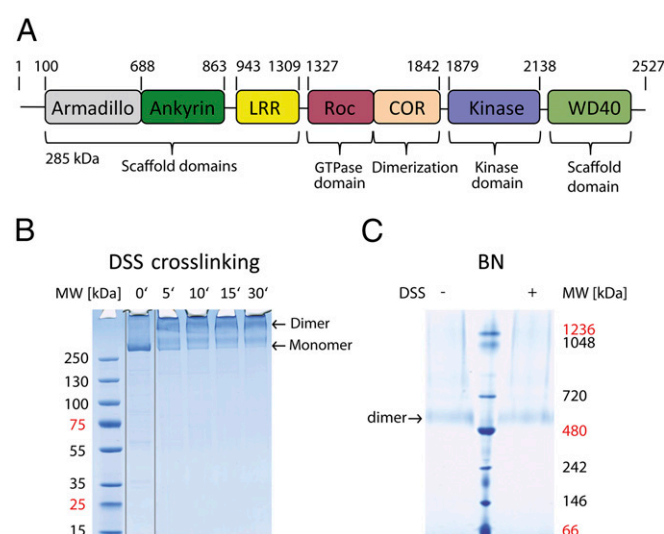


Fig. 2. (A) Schematic overview of the LRRK2 domain structure. (B) Time course for DSS cross-linking at room temperature (RT). Samples were separated on a NuPage 4–12% gradient gel and stained with colloidal Coomassie. (C) BN-PAGE analysis before and after 30 min of DSS cross-linking at RT (4–12% NativePage; colloidal Coomassie stain).

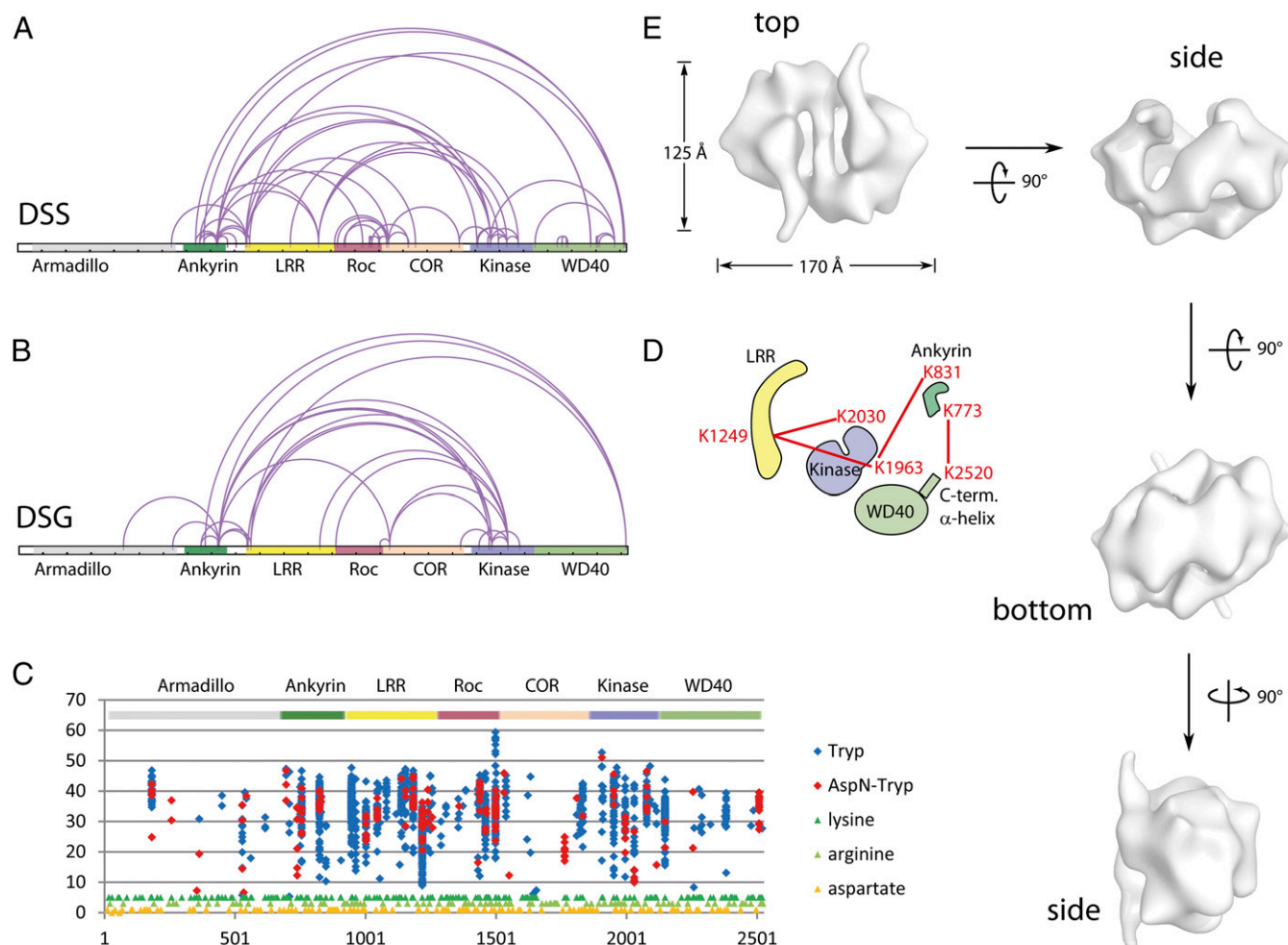


Fig. 3. (A and B) DSS and DSG cross-links mapped onto the LRRK2 sequence with domains indicated. The plots were generated with xiNET (65). (C) DSS-derivatized lysine residues (monolinks) plotted over the residue number in LRRK2 for two different proteolytic enzyme combinations: trypsin-only (Tryp) and AspN-trypsin (ApsN-Tryp). The distribution of theoretical cleavage sites (trypsin: lysine, arginine; AspN: aspartate) is represented by triangles at the bottom of the plot. (D) Representative cross-links between N- and C-terminal domains (N-terminal: ankyrin, LRR; C-terminal: WD40) and the kinase domain demonstrating a compact folding of LRRK2. (E) Three-dimensional reconstruction of LRRK2. Surface views of the symmetrical EM map of LRRK2 (rendered at a threshold corresponding to a molecular mass of 600 kDa).

Chemical Cross-Linking Coupled with Mass Spectrometry Reveals Intradomain Contacts Consistent with a Compact Architecture of LRRK2. Intramolecular chemical cross-linking is a powerful tool to analyze domain-domain interactions in complex proteins (24). We conducted a systematic cross-linking approach, combined with proteolytic cleavage and the subsequent identification of the cross-linked peptides by mass spectrometry (MS) to provide information about domain interactions in full-length LRRK2. We used the amine-specific/lysine-selective *N*-hydroxysuccinimide (NHS) esters disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG) with spacer arm lengths of 11.4 and 7.7 Å, respectively. As a first step, the conditions for selective cross-linking were optimized to favor cross-links over saturation of the accessible lysine side chains with monolinks of each cross-linking reagent. For the ensuing datasets, cross-linker/protein molar ratios of 50:1 and 25:1 were used, although the ratio of 25:1 gave the best results in terms of the formation of cross-links over monolinks; Fig. 2B shows a time course for DSS cross-linking. Using the optimized conditions, efficient cross-linking of the LRRK2 dimer without the appearance of higher aggregates was observed by BN-PAGE analysis (Fig. 2C). Notably, the cross-linking had no impact on the migration or intensity of LRRK2. After chemical cross-linking and before MS, the protein was subjected to proteolysis by different proteolytic enzymes

and their combinations, including AspN and trypsin, as well as LysC and GluC. The peptides were subsequently separated by size exclusion chromatography (SEC) to obtain fractions enriched in cross-linked peptide species. An overview of the chromatographic separation and fractions is shown in Fig. S3 A and B. Extracted tandem mass spectra (MS/MS) of the SEC fractions analyzed with xQuest/xProphet (25) demonstrate good enrichment of cross-linked species in the early fractions, in agreement with published observations (26). Based on these initial tests, we focused our subsequent analyses on fractions A2 to A5.

In its primary sequence, LRRK2 exhibits ~7% frequency of lysine residues. Lysine residues that were found to be modified by the reagent but without forming cross-links (monolinks) were also taken into account as an indicator of solvent accessibility. The frequencies represented by individual ID scores of monolink peptides were plotted versus LRRK2 residue number (Fig. 3C). The plot shows various hotspots of modification, indicating that these residues are highly accessible, and thus likely exposed to the solvent. Other regions of the protein, such as the COR domain, display reduced accessibility, in agreement with previous work showing that the COR domain of Roco proteins is involved in dimerization and is also likely surrounded by other domains in the full-length protein (27). Additional lysine residues with high accessibility to the cross-linking

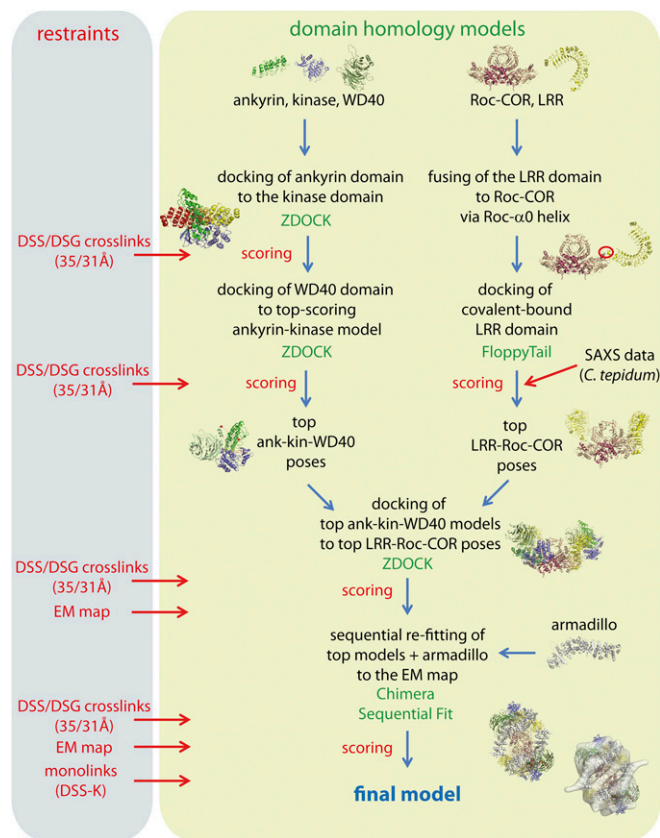


Fig. 4. Integrative modeling workflow.

reagent were observed in the neighborhood of known phosphorylation sites (19, 28). In contrast, the N terminus exhibits a low coverage with monolink peptides. This observation may be explained by the lower frequency of lysine residues in this part of the sequence, which leads to rather large peptide fragments that can remain undetected in our assay.

For the DSS cross-linking, the combined final dataset contains 64 different cross-links with a cut-off ID score of 28, a delta score (ΔS) < 0.95, and a false discovery rate (FDR) < 0.05 (Dataset S1). For DSG cross-links, 30 different cross-links matched these criteria (Dataset S2). A schematic overview of the found cross-links is given in Fig. 3A and B. Interestingly, the dataset revealed several long-range interdomain cross-links (by primary sequence), such as between the ankyrin domain and the outer C-terminal helix of the WD40 domain (i.e., between residues K773 and K2520). In addition, several contacts between the N-terminal ankyrin, as well as LRRs and the kinase domain (e.g., between residues K831 and K1963, as well as residues K1249 and K2030), were found, suggesting that these domains are in close proximity to each other (Fig. 3D).

The identification of various long-range interdomain cross-links and the interactions of the LRRK2 N-terminal part with the C terminus are consistent with a compact overall architecture of the dimeric LRRK2 holoenzyme.

Architecture of Dimeric LRRK2 Revealed by Negative-Stain Electron Microscopy. We analyzed LRRK2 by EM to generate a low-resolution 3D density map of the dimeric LRRK2 protein as input for an integrative structural modeling approach. We first imaged LRRK2 purified from brain tissue of BAC transgenic mice expressing wild-type 3 \times FLAG-tagged LRRK2 (29) embedded in negative stain to ensure sufficient contrast (30). Fig. S4B displays a representative field view of negatively stained LRRK2 showing

abundant elongated particles with a few protein aggregates. To ascertain the identity of the recorded particles and the oligomeric state they represent, we used gold markers in combination with antibody labeling (31). LRRK2 was first labeled with either monoclonal anti-FLAG or polyclonal anti-LRR antibodies, followed by the addition of 5 nm of gold-conjugated anti-IgG secondary antibody, and revealed particles marked with two gold moieties when imaged (Fig. S4C). Due to the fact that immunogold labeling relies on the concerted action of two antibodies, and is therefore relatively inefficient, a large proportion of particles were either labeled with just one nanogold moiety or unlabeled. The particles labeled with two nanogold moieties corresponded to LRRK2 dimers, in agreement with our DLS data, and had approximate dimensions of 125 \times 170 Å. We note that Civiero et al. (22) recently used a comparable labeling and imaging strategy on LRRK2 purified from HEK293 cells with a similar outcome.

Initially, we manually selected LRRK2 particles whose shape and dimensions resembled the dimeric particles identified by immunogold labeling, and obtained a dataset of \sim 2,600 particles that was subjected to reference-free alignment, classification, and 3D reconstruction by the cross-common lines method using EMAN (32). After the first refinement cycle, a clear twofold symmetry axis emerged, even though no geometrical constraints were imposed. A subsequent refinement imposing a twofold symmetry yielded an initial model at a resolution of \sim 33 Å. To improve the resolution of the map, \sim 127,000 single images of LRRK2 particles from an independent purification were automatically selected using projection matching with EMAN2 (33) and then subjected to iterative rounds of reference-free classification and heterogeneity analysis using 2D maximum likelihood analysis in Fourier space (2DMLF) (34). The dataset (51,000 particle images) corresponding to LRRK2 dimers was used for further refinement of the initial model. The reprojections of the final volume showed good agreement with experimental class averages, and the data displayed a comprehensive coverage of the Euler angular space. As calculated from the 0.5 value of the Fourier ring correlation (FRC) curve, the final resolution of the reconstruction was 22 Å (Fig. 3E and Fig. S4D). A comparison of EM maps reconstructed with and without imposing a twofold symmetry is shown in Fig. S5. The EM negative-stain map of dimeric LRRK2 suggests a compact globular architecture, where dimerization occurs via a single twofold rotation axis.

Determination of the LRRK2 Domain Assembly by Integrative Modeling. Seven unique domains have been identified from primary sequence of LRRK2, including three N-terminal repeat domains (armadillo, ankyrin, and LRR), followed by the RocCOR G-domain/dimerization interface, a kinase domain, and a C-terminal WD40 repeat (9). Single-domain crystal structures of the LRR (reported here), RocCOR, and kinase domains from LRRK2 homologs (e.g., a Roco protein from *Chlorobium tepidum*, Roco4 from *Dictyostelium discoideum*) are available and can be used in integrative modeling studies (35, 36). In addition, a structure of the human LRRK2 Roc domain has recently been published (37). Suitable templates to model the armadillo, ankyrin, and WD40 domains have been obtained from homologous proteins through the HHpred (38) homology detection and structure prediction server. The detailed analysis and refinement of the LRRK2 domain homology models are described in SI Materials and Methods. The domain borders, as well as the alignments used for the homology modeling of the single domains, are shown in Fig. 24 and Figs. S6–S9. The resulting domain borders are in agreement with previous work (39). For the determination of the quaternary domain assembly of the LRRK2 holoenzyme, a hierarchical and multiple-step integrative modeling approach was used. This modeling strategy was optimized by considering constraints provided by the cross-linking as well as SAXS data from

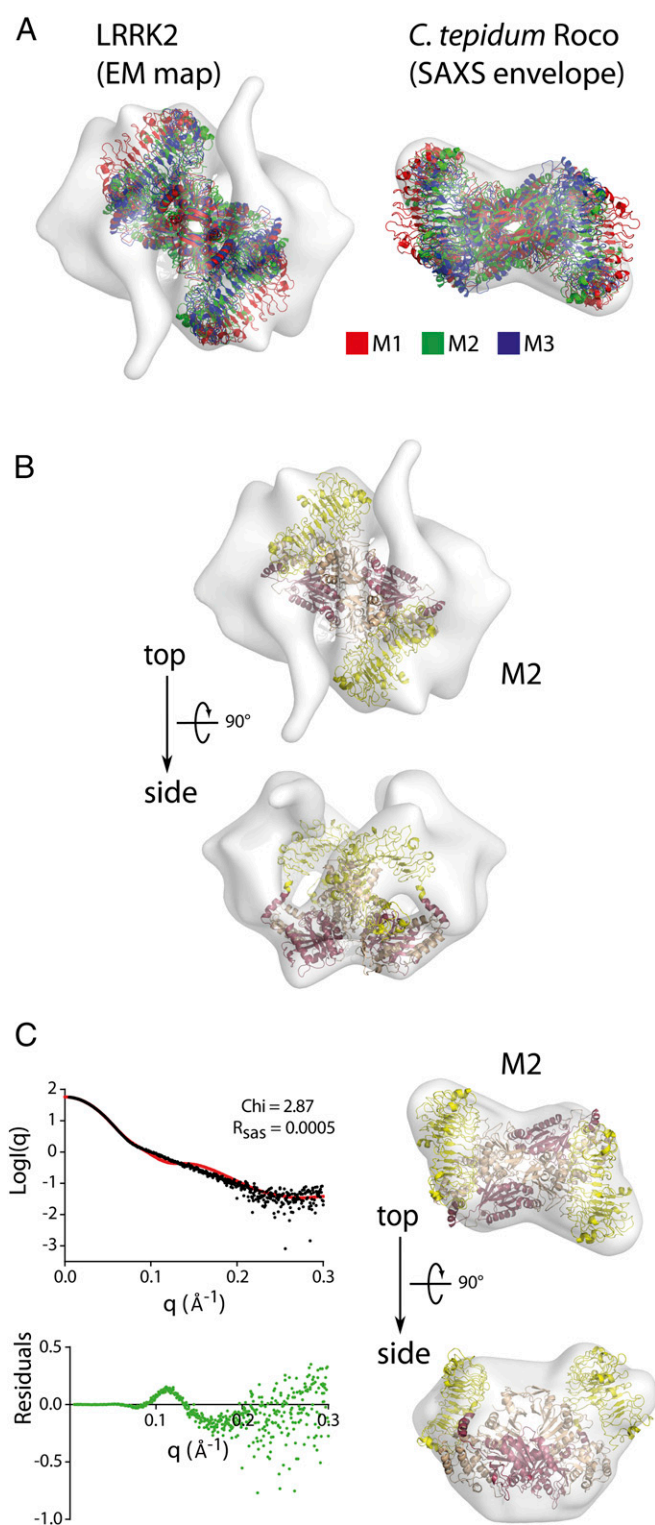


Fig. 5. (A) Superimposed top-scoring LRR-RocCOR conformers M1–M3 fitted to the EM map (Left) as well as to the ab initio averaged bead model envelope calculated from the SAXS data for the *C. tepidum* Roco protein (Right). (B) Docking of the LRR-RocCOR model M2 to the EM map. (C, Upper Left) Fit of the experimental SAXS data of the *C. tepidum* LRR-RocCOR protein (black) on the theoretical scattering curve (red line) corresponding to LRR-RocCOR conformer M2 using the program CRYSOLO. (C, Lower Left) Residuals of the fit are shown below (green). (C, Right) Docking of the LRR-RocCOR model M2 to the ab initio averaged bead model envelope calculated from the SAXS data for the *C. tepidum* Roco protein. The LRR-RocCOR conformer M2 gave the best-scoring final models (Fig. 7). q , scattering vector.

a *C. tepidum* Roco protein and the EM density map of dimeric LRRK2 (Fig. 4).

Hybrid Model for Core of LRRK2 by Docking LRR to RocCOR and Comparison with SAXS Data for the Homologous *C. tepidum* Roco protein. Modeling of LRRK2 quaternary structure was initiated by assembling the LRR domain on the RocCOR domain homology model structures. The recently reported structure of a bacterial Roco protein (*C. tepidum*) was used as a template (36) for the RocCOR dimer. The anchoring of the LRR domain relies mainly on the position of the α -helix (amino acids 1,309–1,328) of the Roc G-domain. The structure of the LRR domain of the *C. tepidum* Roco protein (PDB ID code 5IL7), which served as a template to model the LRRK2 LRR domain, also contains the Roc α -helix (36) at its C terminus, allowing the alignment of the LRR and RocCOR models at this position. Although bacterial Roco proteins lack the kinase domain, they can be used as a model for the LRR-RocCOR region (9). Two different alignments for the LRRK2 and *C. tepidum* Roco LRR domains were tested for threading calculations (*SI Materials and Methods*), leading to two alternative LRR model structures.

The Rosetta Floppy Tail (40) approach was subsequently adapted to sample in an exhaustive manner the possible rigid-body-like orientations of the LRR domain with respect to RocCOR, while keeping them covalently attached through the α -helix. The N-terminally preceding linker (amino acids 1,308–1,315) was sampled with full flexibility to allow local backbone conformational changes to be propagated downstream through the Rosetta internal coordinate system (i.e., foldtree), resulting in rigid-body rototranslations of the RocCOR domain relative to LRR. The best docking solutions (*SI Materials and Methods*) were selected based on cross-linking constraints provided by cross-linking coupled with MS (CL-MS), as well as by fitting to the EM map of dimeric LRRK2. Several good cross-linked fit (XL^{fit}) scoring models were obtained.

To validate the robustness of the top-scoring LRR-RocCOR conformers further, their theoretical scattering curves were compared with the experimental SAXS data of a *C. tepidum* LRR-RocCOR construct (Fig. S10) using CRYSOLO, which resulted in a broad range of χ values ranging from 2.08 to 21. The two LRR-RocCOR conformers that score best based on XL^{fit} and EM map docking, referred to as models M1 and M2, have an χ value of 6.70 and 2.87, respectively. However, both models display an overall conformation that is close to the conformation of the model that shows the best agreement with the experimental SAXS data ($\chi = 2.08$), referred to as model M3 (Fig. S11). An overlay of the resulting LRR-RocCOR models M1–M3 is shown in Fig. S12B. Fig. 5A shows the models docked to the LRRK2 EM map as well as the SAXS envelope of the bacterial Roco protein. All three conformers are compact [experimental radius of gyration (R_g^{exp}) = 45.0 Å, R_g^{M1} = 46.4 Å, R_g^{M2} = 44.8 Å, R_g^{M3} = 44.4 Å], with an LRR domain that folds back toward the C-terminal end/top of the RocCOR module, and show an EM correlation score of >0.84 (Fig. S12A). This general conformation is also in excellent agreement with the ab initio averaged bead model envelope calculated based on the SAXS data (Fig. 5B and C and Fig. S11B). Although models M2 and M3 show good agreement with the SAXS data (especially considering the size of the protein), while clearly fitting the data better than model M1, deviations could also be due to inherent sequence variations between the proteins (human versus *C. tepidum* Roco), where the bacterial protein lacks a kinase, ankyrin, and WD40 domain. All three models, LRR-RocCOR M1 to M3, were then subjected to subsequent docking steps for the remaining domains.

Generation of a Hybrid Model for Dimeric LRRK2 Holoenzyme by Hierarchical Docking. An ensemble-based hierarchical docking approach was used to assemble the ankyrin, kinase, and WD40 domains on the LRR-RocCOR top-scoring conformers. Histograms showing the distributions of either XL^{fit} or EM fit (EM^{fit} , or EM

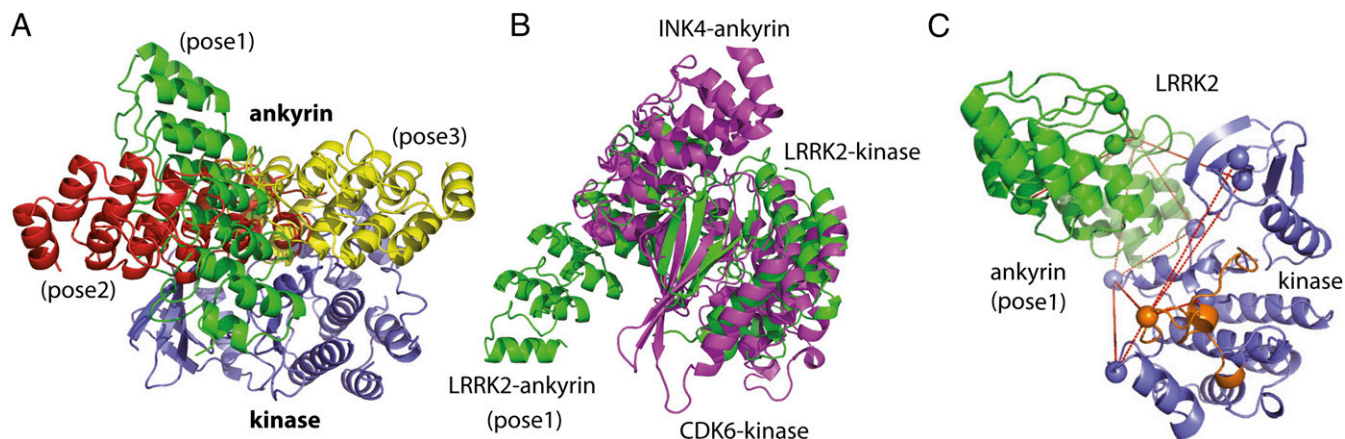


Fig. 6. Ankyrin-domain docked to the kinase domain mode via ZDOCK. (A) Three top poses obtained from the ankyrin domain docking to the kinase domain selected by experimental determined cross-linking constraints. (B) Superposition of one of the most favorable docking solutions to a crystal structure (PDB ID code 1G3N) of the inhibitory complex of the INK4 ankyrin domain with the CDK6 kinase domain (magenta) gives confidence to the model (green). (C) Experimentally determined cross-links (red lines) on the best-scoring ankyrin-kinase docking solution (pose1). The LRRK2 activation loop is highlighted in orange. Cross-linked lysine residues are shown as spheres.

map correlation value) for the three alternative LRR-RocCOR conformers are illustrated in Fig. S12C. The cross-linking data revealed several cross-links of the kinase domain to the ankyrin repeats, suggesting that the ankyrin domain is involved in the regulation of the kinase activity. Therefore, the ankyrin domain was separately docked onto the kinase domain, and the best docking solutions were filtered out through the XL^{fit} scoring approach. The model structure of the kinase in the nucleotide-free (apo) state showed a higher number of satisfied cross-link-related distances (Fig. S13), and was therefore used for the subsequent docking steps. Interestingly, the top-scoring ankyrin-kinase poses (Fig. 6A) are similar to existing crystal structures of a kinase domain of CDK6 with the inhibitory ankyrin domain of INK4 (41), suggesting a common mode of regulation of kinases by these domains (Fig. 6B). The top-scoring model for the LRRK2 ankyrin-kinase module with highest similarity to the latter crystal structure is shown in Fig. 6C, with highlighted cross-links satisfying the defined Euclidean distance limits.

The three best ankyrin-kinase models (Fig. 6A) were subsequently docked to the WD40 model. The nine best representative ankyrin-kinase-WD40 complexes were rigid-body-docked to the top-scoring three LRR-RocCOR conformers by preserving noncrystallographic (point) symmetry (*SI Materials and Methods*) imposed on the LRR-RocCOR model. The best solutions, selected based on cross-link constraints, as well as the 22-Å EM density map, were chosen for subsequent assembling of the remaining armadillo domain. Because no cross-linking constraints satisfying the confidence criteria were determined by the CL-MS method, mainly because of the distribution of lysine residues in this area (Fig. 2D) resulting in rather large peptides, the positioning of the armadillo domain relied primarily on fitting it into the remaining space of the EM density map. The resulting dimeric LRRK2 quaternary structure models were refined on the EM map through 200 cycles of sequential refitting within the University of California, San Francisco (UCSF) Chimera (42) fitmap algorithm. The sequential refitting was carried out by keeping each domain block internally rigid through the following order: LRR-RocCOR (chains A and B), ankyrin-kinase-WD40 (chain A), ankyrin-kinase-WD40 (chain B), armadillo (chain A), and armadillo (chain B).

The resulting top-scoring model for the dimeric LRRK2 holoenzyme shown in Fig. 7 is in good agreement with both the experimental CL-MS data and the EM map. The scoring results for models based on three different LRR-RocCOR conformers (M1–M3) are summarized in *Datasets S3–S5*. For the top pose (model 1)

(*Dataset S4*) based on LRR-RocCOR conformer M2 ($XL^{fit} = 83.4$) the cross-linking distance limits (*Materials and Methods*) were satisfied for 42 of 64 DSS (66%) and 15 of 30 DSG (50%) cross-links, respectively. In addition to the total number of satisfied cross-links, the XL^{fit} metric considers the deviation of the violated cross-links from the distance threshold as well as the distances of consecutive domain terminals. The models showing the best agreement with the CL-MS data had EM^{fit} correlation values greater than 0.90 (M1/M2 = 0.912). The top-scoring model (Fig. 7) had an accumulated terminal distance of 173 Å. An alternative model (model 14) was identified for the conformer M2, satisfying even a higher number of cross-linking constraints (60/ $XL^{fit} = 80.2$) at a similar EM^{fit} (0.9063) but at the expense of a higher accumulated terminal distance of adjacent repeat domains (266 Å). A comparison of both models is shown in Fig. S14A and B.

Interestingly, the final models strongly suggest that there are close contacts of the kinase domain with the N-terminal ankyrin and LRR repeat domains. In the final model, the kinase-ankyrin module localizes in a position close to the LRR domain and the α 0-helix connecting the LRR and the Roc G-domain (Fig. S14C).

Discussion

Understanding the structure of LRRK2 and how mutations alter that structure to disrupt normal function, and ultimately contribute to the pathogenesis of PD, is essential for developing potential therapeutics. Although important progress toward this goal has been made by the determination of the crystal structure of the dimeric RocCOR interface of an orthologous Roco protein from *C. tepidum* (36), as well as the kinase domain of the LRRK2 orthologous *D. discoideum* protein Roco4 (35), the 3D arrangement of the various domains in LRRK2 remains largely unknown. Our results confirm that LRRK2, similar to other Roco protein family members and in contrast to classical small G proteins, forms constitutive dimers (27). To obtain a structural model of the dimeric LRRK2 holoenzyme, we have used an integrative modeling pipeline combining domain homology models with molecular docking and experimental constraints provided by chemical cross-linking, a low-resolution LRRK2 density map generated by EM negative staining, and SAXS data for the homologous *C. tepidum* Roco protein. Both chemical cross-linking of recombinant human LRRK2 and the EM density map from LRRK2 purified from transgenic mice concordantly reveal a compact folding of the dimeric enzyme. Furthermore, the cross-linking approach allowed the identification of various interdomain contacts, including interactions between

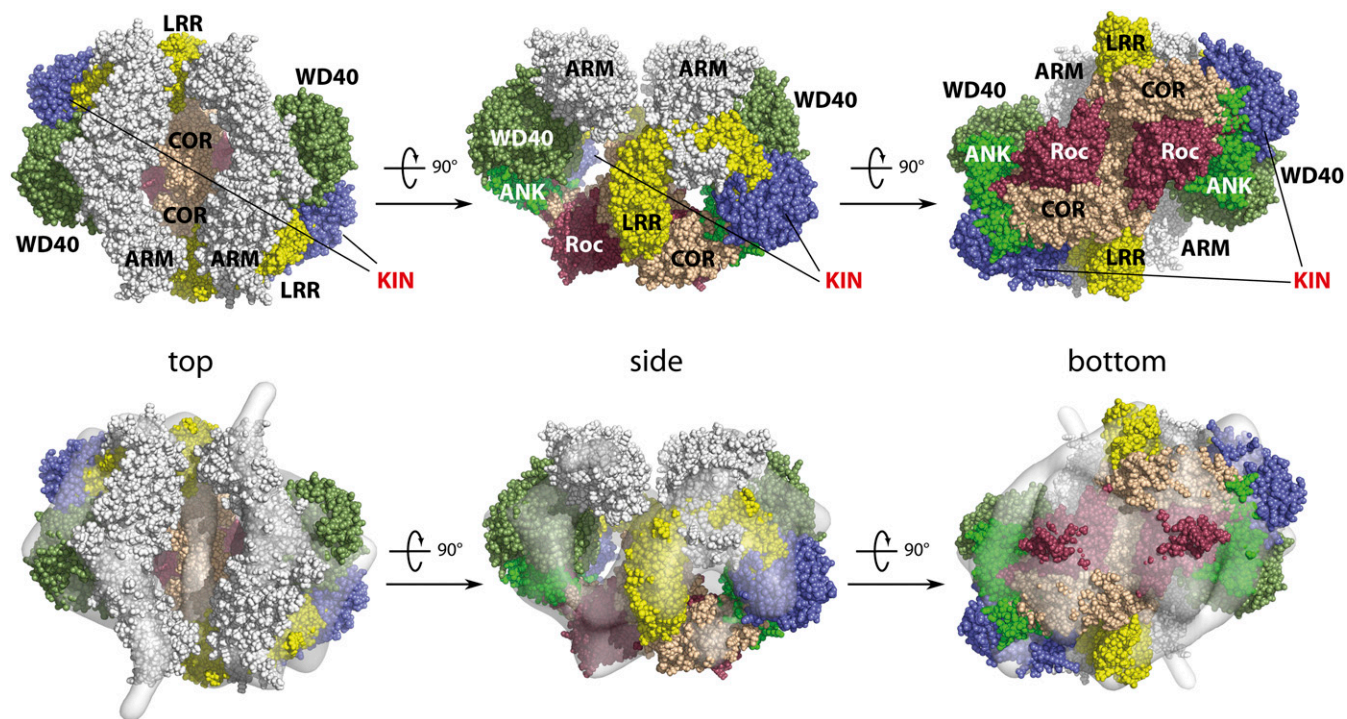


Fig. 7. Final LRRK2 models fitted to the EM map. (*Upper*) Top, side, and bottom views of the resulting top-scoring model (model 1/LRR-RocCOR conformer M2). (*Lower*) Superimposition with the EM negative-stain map. The seven LRRK2 domains are diagrammed: ARM (armadillo repeats), ANK (ankyrin repeats), LRR, Roc (G-domain Roc), COR (COR dimerization domain), KIN (kinase domain), and WD40 (WD40 repeat domain). The scoring results are provided in [Dataset S4](#) and [S5](#).

distant domains in agreement with previous reports (23). Given the presence of a Roc G-domain and kinase domain in one protein and the similarities of these modules to MAP kinase signaling cascades, it has been hypothesized that an intramolecular regulation mechanism involving these domains must exist for LRRK2 (5, 43). On the other hand, by testing direct binding between the G-domain Roc and the kinase domain, previous work demonstrated that these two domains do not bind to each other at high affinity (43). Interestingly, our model reveals spatial proximity of the kinase domain with the ankyrin domain and the LRR domain by chemical cross-linking. Furthermore, the top-scoring docking solutions for the ankyrin domain to the kinase domain resemble the structure determined for a complex from the ankyrin-repeat protein INK4 and the kinase domain of CDK6 (41). INK4 proteins are well-characterized tumor suppressors that inhibit cyclin kinases (44). In addition, based on the LRRK2 primary protein sequence, the LRR domain is directly coupled to both the C terminus of the ankyrin domain and the N terminus of the Roc G-domain. Although mechanistically not well understood, recent studies did show that the N terminus of human LRRK2 and related Roco proteins is essential for function *in vivo* (45). Our data suggest that the LRRK2 ankyrin-LRR domains may play a direct role in the regulation of the LRRK2 kinase activity, guiding future in-depth functional characterization of LRRK2 at a molecular level.

The final structural model, integrating multiple experimental data, supports findings demonstrating that the RocCOR domains form the primary dimerization interface and that the terminal domains are not likely to contribute much to the dimerization of LRRK2. However, although chemical cross-linking provides robust information for physical contacts of different functional domains within a protein, this type of data cannot distinguish intra- versus intermolecular cross-links (i.e., cross-links can be formed *in cis*, within a monomer, or *in trans* between two monomers). Because there is no straightforward analytical strategy to discriminate between *cis* and

trans cross-links, we considered this problem by computational analysis. All *cis* and *trans* distances were considered, with the shortest selected for scoring ([Datasets S6–S8](#)). The resulting models satisfy the highest number of cross-links and show good agreement with the EM density map of dimeric LRRK2. This finding suggests that most of the cross-links occur *in cis*. However, the LRRK2 COR-kinase linker regions (amino acids 1,849–1,878) are in close spatial proximity in the *C. tepidum* RocCOR template structure (36). In consequence, the positions of the C-terminal domains in the final models are also in agreement with an intertwined homodimer as found for other dimeric proteins (46). Therefore, an arrangement where the C-terminal kinase and WD40 domain would interact with the N-terminal ankyrin and LRR domains of the other monomer would still be in agreement with our experimental constraints. For this reason, we did not assign the domains to a specific monomer/chain in the final models.

It also has to be considered that cross-linking data are affected by the presence of multiple conformations. Consistently, we observed heterogeneity in the shape of the EM particles. For a large and dynamic multidomain protein, such as LRRK2, the presence of multiple simultaneous conformations in solution is not unlikely, which could also give a reasonable explanation for the observation that no model was derived that could satisfy all cross-links and EM restraints at the same time.

In conclusion, by combining chemical cross-linking, EM analysis, integrative modeling, and biochemical experiments, we present, for the first time to our knowledge, a compact architecture of the LRRK2 dimer with a domain assembly where distant domains engage in multiple contacts. Our model also suggests that the N-terminal ankyrin and LRR repeats are involved in the intramolecular regulation of the biological activity of LRRK2. The model presented will be useful for future structure-driven functional studies and, furthermore, will support future rational drug design studies (i.e., by

suggesting promising mechanisms for allosteric inhibitors targeting domain–domain contacts).

Materials and Methods

Generation of LRRK2 Expression Constructs. The generation of N-terminal, SF-tagged (NSF), full-length LRRK2 expression constructs has been previously described (19). Similar methods were used to create an N-terminal deletion construct, 1,280-end (Δ 1,279-LRRK2) using the pDEST-NSF-tandem affinity purification (47) Gateway cloning vector.

Cell Culture and Transfection. HEK293T cells (CRL-11268; American Type Culture Collection) were cultured in 14-cm dishes in DMEM (PAA) supplemented with 10% (vol/vol) FCS (PAA) and appropriate antibiotics. Cells were transfected at a confluence between 50% and 70% with 8 μ g of plasmid DNA per dish using polyethyleneimine (Polysciences) solution as described previously (48). After transfection, cells were cultured for 48 h.

Protein Purification. Purification of SF-tagged LRRK2 purified from 4 \times 14-cm dishes (600 cm²) of confluent HEK293T cells was performed as described previously (19, 49). Briefly, after removal of the medium, cells were lysed in 1 mL of lysis buffer and washing buffer [50 mM Hepes (pH 8.0), 100 mM NaCl, 1 mM DTT, 5 mM MgCl₂, 0.5 mM EDTA, 10% (vol/vol) glycerol, 0.01% Triton X-100], supplemented with 0.55% Nonidet P-40 and Complete Protease Inhibitors (Roche), per 14-cm dish. Incubation with lysis buffer was then performed for 40 min at 4 °C on a shaker. Cell debris and nuclei were removed by centrifugation of the lysates at 10,000 \times g for 10 min. Cleared lysates were incubated with 100 μ L of settled Strep-Tactin Superflow resin (IBA) for 1 h at 4 °C. Beads were transferred to microspin columns (GE Healthcare) and washed five times with 500 mL of washing buffer prior to final elution using 400 μ L of elution buffer (washing buffer supplemented with 200 mM D-threitol; IBA).

DLS. DLS was measured using a DynaPro NanoStar instrument with a 50- μ L volume in a disposable cuvette (Eppendorf UVette) using a protein concentration of 0.1 mg/mL. Ten measurements of 10 s each were collected. Data were analyzed using Dynamics v.7.1.9 software.

Cross-Linking Reagents. The 1:1 mixtures of DSS H12/D12 and DSG H4/D4 were purchased from Creative Molecules.

Chemical Cross-Linking. For NHS-ester–based chemical cross-linking, 2 μ L of DSS-H12/D12 solution (12.5 mM in DMSO) or DSG-H6/D6 solution (12.5 mM in DMSO) was added to 100 μ g of purified LRRK2 in a total volume of 0.4 mL of elution buffer. The reactions were carried out under constant shaking for up to 45 min. The reaction was then stopped by adding Tris-HCl (pH 7.5) solution to a final concentration of 100 mM. The mix was incubated for 15 min at room temperature under constant shaking.

Proteolytic Cleavage and SEC Prefractionation. After chemical cross-linking, protein samples were precipitated with chloroform and methanol (49). Proteolysis was performed as described earlier (49), and the resulting peptide mixtures were separated by SEC following the protocols described by Leitner et al. (25). Additional information is provided in *SI Materials and Methods*.

MS Analysis. SpeedVac-dried SEC fractions were redissolved in 0.5% TFA and analyzed by LC-MS/MS using a nanoflow HPLC system (Ultimate 3000 RSLC; Thermo Fisher) coupled to an Orbitrap Velos (Thermo Fisher) tandem mass spectrometer. For identification, cross-linked peptides were separated on the nano-HPLC by 120-min gradients and analyzed by a data-dependent approach acquiring CID MS/MS spectra of the 10 most intense peaks, excluding single- and double-charged ions.

Data Analysis Using xQuest/xProphet. Before analysis via xQuest/xProphet (v2.1.1) (25), MS/MS spectra were extracted from the RAW files using ReAdW (v4.3.1). In the case of Q-Exacte data, the ProteoWizard/msconvert (3.0.6002) suite was used with the parameters described by Leitner et al. (25). The identification of monolinks, looplinks, and cross-links was done based on the identification of DSS H12/D12 or DSG H6/D6 pairs following protocols published by Leitner et al. (25). Briefly, for database search, a database containing 46 proteins was prepared containing common contaminants and interactors of LRRK2. Additionally, a decoy database containing the inverted sequences was generated. For xQuest/xProphet, standard parameters according Leitner et al. (25) were used with carbamidomethyl as a fixed modification and methionine oxidation as a variable modification. For DSS-D12 and DSG-D6, isotope differences of 12.075321 Da and 6.03705 Da, respectively, were used. For CID spectra, the parent ion

tolerance was set to 10 ppm (MS) and the fragment ion tolerance to 0.3 Da (MS/MS). For higher-energy collisional dissociation (HCD) spectra, the fragment ion tolerance was set to 0.02 Da. Only those cross-linked peptides that fulfilled specific minimal criteria (ID score > 28, Δ S < 0.95, FDR < 0.05) were considered for modeling. Additionally, the MS/MS spectra were evaluated by manual inspection to ensure a good representation of the fragment series of both cross-linked peptides.

EM Analysis and 3D Map Reconstruction. A 3- μ L aliquot of LRRK2 [40 ng/ μ L protein, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl] was adsorbed for 45 s onto glow-discharged holey grids (Quantifoil R2/4 on Cu/Rh 300 mesh) coated with thin carbon. The specimen was stained with 2% (wt/vol) uranyl acetate, blotted three times, and finally air-dried. The initial dataset of images used to generate the initial model was selected by imaging the specimens with a TF20 (FEI) field emission gun transmission electron microscope (FEG) at 200 kV under low-dose conditions, using a 4,000 \times 4,000-pixel CCD camera at the equivalent calibrated magnification of 88,249 \times and -1.5 - μ m defocus. A second dataset was taken on a J2100F (JEOL) FEG, using a 2,000 \times 2,000-pixel CCD camera at 63,450 \times and -1.5 - μ m defocus.

CCD images were evaluated for drift and astigmatism in Fourier space, and the contrast transfer function (CTF) was estimated using CTFIND3 (50). An initial dataset of 3,500 dimer particles was selected using EMAN (32) and then normalized and CTF-corrected using Xmipp software (51). The particles were subject to 2DMLF analysis as implemented in Xmipp (52). In this process, particle images with an irregular background, close neighbors, overlapping particles, and aggregates were discarded to yield a dataset of 2,600 particles. The 2DMLF-screened particle images were classified by reference-free alignment into 24 class averages by EMAN. The 24 class averages were then used to reconstruct an initial 3D model by the cross-common lines technique and refined through 16 cycles by EMAN without imposing any symmetry constraint. A second 16-iteration refinement cycle was operated imposing a twofold symmetry to produce a definitive initial model. The second dataset of 65,000 particles was selected, CTF-corrected, and 2DMLF-screened in the same way to yield a total of 51,000 particles. This dataset was used to refine the initial model following the multi-resolution refinement protocol described by Scheres et al. (52) in steps 37–47. The reconstructions of the final volume and the class averages were visualized in EMAN. The Euler angular coverage was calculated by Xmipp (52). The resolution was estimated according to the 0.5 value of the FRC curve, and the maps were low-pass-filtered at this spatial frequency.

SAXS Analysis of the *C. tepidum* LRR-RocCOR Domain Construct. A *C. tepidum* Roco protein fragment corresponding to the LRR-RocCOR domain (amino acids 1–946) was cloned in a pProEX vector and expressed and purified in a nucleotide-free form, as described earlier (36). The SAXS experiment was performed on the BM29 beamline at the European Synchrotron Radiation Facility in Grenoble, France, with an online HPLC set-up. Fifty microliters of an 8-mg/mL sample was injected on a Bio SEC-3 HPLC column (Agilent) equilibrated with 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 5% glycerol, and 1 mM DTT. Individual scatter curves corresponding to the elution peak were buffer-subtracted and averaged. PRIMUS was used for determination of the R_g using Guinier approximation (53) and GNOM for the calculation of the pair distance distribution function P(r) (54). All models of the human LRR-RocCOR module were compared with the experimental data using CRYSOLO (55) and the R_{SAS} metric was calculated with ScÅtter (56). Ab initio envelopes were calculated using DAMMIF (average of 20 runs) (57), and docking into the envelope was performed with Supcomb (58). The Situs pdb2vol module was used to convert the ab initio models into volumetric maps (59). Experimental and modeling parameters for the SAXS analysis are provided in [Dataset S9](#).

Integrative Modeling. A detailed description of the integrative modeling procedure is provided in *SI Materials and Methods* and graphically summarized in Fig. 4 (workflow). Briefly, structures of the single domains were obtained by homology modeling and assembled through an integrative modeling procedure consisting of hierarchical, rigid-body docking calculations carried out by means of the ZDOCK algorithm (60) using established protocols (61–63). Structural models obtained from global docking approaches were scored and filtered by applying cross-link–derived distance filters, using 35-Å and 31-Å thresholds for DSS and DSG, respectively (64). The overall fit of a given docking pose to the cross-link dataset was measured through the XL^{fit} score (*SI Materials and Methods*). Homology models for the ankyrin and kinase domains were used for subsequent docking of the WD40 domain. The best scoring ankyrin–kinase–WD40 ternary complexes were finally used for rigid-body docking on selected LRR-RocCOR conformers; these were obtained by manually placing the LRR domain on the RocCOR structural model by superimposing the common α -helix and by exhaustively sampling the orientation of the covalently attached LRR domain with respect to the RocCOR unit through an adaptation of the Floppy Tail approach (40). The obtained LRR-RocCOR conformers were additionally cross-validated via

comparison with the experimental SAXS data of the *C. tepidum* LRR-RocCOR construct. Final models for dimeric full-length LRRK2 were selected from initial docking solutions combining cross-link constraints and the negative-stain EM map based on XL^{fit} and EM^{fit} scores. See Table S1 for statistics for the crystallographic analysis of the LRR domain of the *C. tepidum* Roco protein.

SDS and BN Gel Electrophoresis. SDS/PAGE was performed using 4–12% NuPage (Life Technologies) gradient gels. BN gel electrophoresis has been performed on 4–12% NativePage gels (Life Technologies) according to the vendor's protocols. The gels have been stained with colloidal Coomassie, as described elsewhere (49).

Kinase Assay. LRRK2 kinase activity was measured by incorporation of ^{32}P into the LRRKtide peptide in 30 °C kinase buffer [consisting of 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF, 5 mM β -glycerolphosphate, 0.02% Triton X-100, 2 mM DTT] in the presence of 500 μ M GDP. To start the reaction, 0.03-mg/mL full-length LRRK2 was mixed with 75 μ M LRRKtide and 25 μ M $ATP\gamma^{32}P$ (2 Ci/mmol) and was stopped by adding 100 mM ice-cold EDTA. Samples were spotted on nitrocellulose filters, washed with 50 mM phosphoric acid, and dried before scintillation counting (PerkinElmer).

GTPase Assay (Charcoal). LRRK2 GTPase activity was measured by adding 10 μ M $ATP\gamma^{32}P$ to 0.1 μ M recombinant LRRK2 at 30 °C. Aliquots of 10 μ L

were taken at certain time points and mixed with 400 μ L of charcoal solution (50 g/L charcoal in 20 mM phosphoric acid) to stop the reaction. The charcoal was pelleted, and the amount of free ^{32}P in the supernatant was determined by scintillation counting.

Statistical Analysis. Linear regression curves were calculated using GraFit v5 (Erithacus Software) weighted by individually determined errors for each data point.

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