Structural basis of protein substrate processing by human mitochondrial high-temperature requirement A2 protease

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The human high-temperature requirement A2 (HtrA2) protein is a trimeric protease that cleaves misfolded proteins to protect cells from stresses caused by toxic, proteinaceous aggregates, and the aberrant function of HtrA2 is closely related to the onset of neurodegenerative disorders. Our methyl-transverse relaxation optimized spectroscopy (TROSY)–based NMR studies using small-peptide ligands have previously revealed a stepwise activation mechanism involving multiple distinct conformational states. However, very little is known about how HtrA2 binds to protein substrates and if the distinct conformational states observed in previous peptide studies might be involved in the processing of protein clients. Herein, we use solution-based NMR spectroscopy to investigate the interaction between the N-terminal Src homology 3 domain from downstream of receptor kinase (drk) with an added C-terminal HtrA2-binding motif (drkN SH3-PDZbm) that exhibits marginal folding stability and serves as a mimic of a physiological protein substrate. We show that drkN SH3-PDZbm binds to HtrA2 via a two-pronged interaction, involving both its C-terminal PDZ-domain binding motif and a central hydrophobic region, with binding occurring preferentially via an unfolded ensemble of substrate molecules. Multivalent interactions between several clients and a single HtrA2 trimer significantly stimulate the catalytic activity of HtrA2, suggesting that binding avidity plays an important role in regulating substrate processing. Our results provide a thermodynamic, kinetic, and structural description of the interaction of HtrA2 with protein substrates and highlight the importance of a trimeric architecture for function as a stress-protective protease that mitigates aggregation.

Significance

Protein aggregates are often toxic, leading to impaired cellular activities and disease. The human HtrA2 trimeric enzyme cleaves such aggregates, and mutations in HtrA2 are causative for various neurodegenerative disorders, such as Parkinson’s disease and essential tremor. The mechanism by which cleavage occurs has been studied using small peptides, but little information is available as to how HtrA2 protects cells from the pathologic effects of aggregation involving protein molecules that can form well-folded structures. Using solution NMR spectroscopy, we investigated the structural dynamics of the interaction between HtrA2 and a model protein substrate, demonstrating that HtrA2 preferentially binds to an unfolded substrate ensemble and providing insights into how HtrA2 function is regulated.

Many proteins must fold into a well-defined three-dimensional (3D) structure in order to properly function. When this process fails, these molecules can form toxic aggregates via exposed hydrophobic regions that are typically sheltered in the properly folded structure. Increasing evidence suggests that the accumulation of toxic aggregates of misfolded proteins is causative for various neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease (1–3). Cells are equipped with quality-control machinery to counteract these stresses, which either assist protein refolding or target proteins for degradation. High-temperature requirement A (HtrA) proteases are an important class of stress-protective enzymes, widely conserved from bacteria to mammals, whose main function is to cleave toxic proteins in an adenosine 5’-triphosphate (ATP)-independent manner (4–6). Their activities are not limited to the degradation of misfolded proteins, however, but also include roles as protein chaperones and proteases of regulatory proteins involved in various cell-signaling pathways, such as cellular proliferation, mobility, and apoptosis (4).

Human HtrA2, also known as the Omi protease, is an HtrA protease that is primarily localized to the mitochondrial intermembrane space (7–9). There is increasing evidence that HtrA2 plays a prominent neuroprotective role by degrading toxic aggregates in mitochondria (9–14) and that its aberrant function results in deregulation of mitochondrial and, ultimately, in the onset of neurodegenerative disorders (10, 12–16). This notion is further supported by the finding that missense mutations in the HTRA2 gene are present in patients with Parkinson’s disease or essential tremor (17, 18). The crystal structure of HtrA2 has been solved, revealing a pyramid-shaped homotrimer, in which each HtrA2 monomer is composed of one copy each of serine protease and PDZ (PSD-95, DLG, and ZO-1) domains, with intertrimer interactions mediated by protease–protease contacts (Fig. 1 A, Upper Left and Right) (19). In the crystal structure, the catalytic center in the protease domain is formed by H198, D228, and S306, which is occluded by the PDZ domain, precluding substrate binding. Thus, the crystal structure is thought to represent a closed-inactive conformation that cannot bind and, thus, cleave client molecules (6, 19).

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As described in the introduction, studies of HtrA proteins have largely focused on peptide mimics of protein substrates. In a previous set of studies, using solution-state NMR spectroscopy focusing on side-chain methyl groups (31, 32), we elucidated the activation mechanism of HtrA2 by using two different short peptides (30), one that contains the PDZ-binding motif (DDGQYYFV; in what follows, referred to as PDZ-peptide) and a second sequence with an efficiently cleaved site (IRRVSYSF; hereafter referred to as substrate peptide), as illustrated in Fig. 1A. We demonstrated that, in the absence of these peptides, HtrA2 adopts a closed conformation, which is consistent with the known crystal structure (referred to in what follows as the C state). The binding of PDZ-peptide to trimeric HtrA2 occurs in a stepwise manner with positive cooperativity, leading to the formation of an open conformation, whereby the PDZ and protease domains are dissociated from each other (Fig. 1A). Our NMR analyses and peptidase assays indicated that this PDZ-peptide–bound open conformation is not catalytically competent (open inactive [OI] state). Subsequent binding of the substrate peptide to the exposed catalytic center of the OI state leads to a catalytically active conformation, i.e., the open active (OA) state, which involves cooperative structural transitions of the protease domains, revealing an extensive interprotomer network that regulates HtrA2 function (32).

These studies, and others preceding them (30, 33–35), focused on small peptides. As many known clients of HtrA2 are intact proteins (9, 21–26), it is of considerable interest to examine a protein substrate and to establish whether the same HtrA2 conformations identified using small peptide substrates are at play when processing protein molecules that can assume folded conformations. Further, what are the structures of protein clients when bound to HtrA2, and how does HtrA2 discriminate between substrate and nonsubstrate proteins? Solution NMR spectroscopy is well-suited to these types of investigations, as it offers the ability to analyze dynamic enzyme–substrate interactions at the atomic level. In contrast, the potentially disordered nature of substrate clients and the structural heterogeneity of the resulting complexes challenges similar studies by many other techniques.

Here, we present an NMR-based, thermodynamic, kinetic, and structural analysis of the interaction between HtrA2 and a metastable model protein substrate, the N-terminal Src homology 3 domain from downstream receptor kinase (drkN SH3), to which a PDZ-binding motif has been added to its C terminus, as is present in physiological substrates, to promote docking to HtrA2. We show that HtrA2 binds to this substrate protein via a two-pronged interaction involving 1) the C-terminal PDZ-binding motif of drkN SH3 and the PDZ domain of HtrA2; and 2) a hydrophobic region in the middle of the drkN SH3 molecule and the catalytic center in the protease domain. Based on proteolytic assays of HtrA2 using an oligomeric drkN SH3 substrate, we demonstrate that HtrA2 cleaves oligomeric clients more efficiently than their monomeric counterparts, suggesting that binding avidity plays a significant role in regulating substrate cleavage. Our NMR study establishes that HtrA2 preferentially binds to a transiently sampled unfolded state of drkN SH3, rather than the folded conformation, via a conformational selection mechanism. More generally, it provides insight into how HtrA2 recognizes and processes protein clients and adds to our understanding of the mechanism by which HtrA2 functions as a stress-protective protease.

### Results

**HtrA2 Cleaves drkN SH3 Possessing a C-Terminal PDZ-Binding Motif.** As described in the introduction, studies of HtrA proteins have largely focused on peptide mimics of protein substrates. In a previous set of studies, using solution-state NMR spectroscopy focusing on side-chain methyl groups (31, 32), we elucidated the activation mechanism of HtrA2 by using two different short peptides (30), one that contains the PDZ-binding motif (DDGQYYFV; in what follows, referred to as PDZ-peptide) and a second sequence with an efficiently cleaved site (IRRVSYSF; hereafter referred to as substrate peptide), as illustrated in Fig. 1A. We demonstrated that, in the absence of these peptides, HtrA2 adopts a closed conformation, which is consistent with the known crystal structure (referred to in what follows as the C state). The binding of PDZ-peptide to trimeric HtrA2 occurs in a stepwise manner with positive cooperativity, leading to the formation of an open conformation, whereby the PDZ and protease domains are dissociated from each other (Fig. 1A). Our NMR analyses and peptidase assays indicated that this PDZ-peptide–bound open conformation is not catalytically competent (open inactive [OI] state). Subsequent binding of the substrate peptide to the exposed catalytic center of the OI state leads to a catalytically active conformation, i.e., the open active (OA) state, which involves cooperative structural transitions of the protease domains, revealing an extensive interprotomer network that regulates HtrA2 function (32).

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substrates. In order to provide insight into interactions involving proteins that can assume fully folded structures, we have used drkN SH3 (residues 1 to 59) as a potential substrate of HtrA2. Unlike many other SH3 domains that form stably folded β-barrel structures, drkN SH3 exists in an equilibrium between folded and unfolded states at near-neutral pH and ambient temperature (folded:unfolded ratio of ~2:1 at pH 6 and 20°C) (Fig. 1B) (36, 37). Because of this metastability, drkN SH3 has been utilized as a model system to study aspects of protein folding or to investigate chaperone-client interactions (38–41).

We first performed a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)-based proteolysis assay monitoring the cleavage of drkN SH3 by wild-type (WT) HtrA2. To compare the proteolytic activity between the closed and open forms of HtrA2, we measured the activity in the presence and absence of 1 mM PDZ-peptide (DDGQYYFV) that binds to the PDZ domain of HtrA2, inducing an open conformation that is required for activity (19, 31). In addition, we prepared a fusion protein in which the PDZ-binding motif (bm), GQYYEV, is attached to the C terminus of drkN SH3 (referred to as drkN SH3-PDZbm) (Fig. 1C). Since it has been proposed that HtrA2 recognizes the C-terminal tails of substrates through their PDZ domains, drkN SH3-PDZbm is expected to serve as a mimic of a physiological substrate. Cleavage was not observed, neither in the presence nor absence of PDZ-peptide, upon incubating WT HtrA2 (10 µM as a monomer) with 5 equivalents of drkN SH3 at 37°C. In contrast, efficient cleavage of drkN SH3-PDZbm was observed, forming ~4-kDa fragments (Fig. 1D). Liquid chromatography–mass spectrometry (LC-MS) analyses of the cleaved products were performed, identifying cleavage sites at I27–L28, L28–N29, and M30–E31, consistent with cleaved fragments of ~4 kDa, as observed via SDS-PAGE (SI Appendix, Table S1).

These results establish that drkN SH3 requires a C-terminal PDZ-binding motif for efficient cleavage by HtrA2, suggesting a synergistic binding process involving contacts between 1) the PDZ-binding motif and the PDZ domain; and 2) the substrate cleavage site and the active site on the enzyme. To test whether PDZ-binding motifs are required for the efficient processing of other protein substrates, we performed the same proteolysis assays using the DNA binding domain of the α-helical telomeric repeat binding factor 1 (hTRF1) and a triple mutant of the β-sheet containing Src homology 3 domain from Gallus gallus Fyn tyrosine kinase (A39/V/N53/P/V55L Fyn SH3), both of which showed marginal folding stability and have been widely used to analyze chaperone-client interactions (42–49) (SI Appendix, Fig. S1). Similarly, as observed for drkN SH3, these proteins were efficiently cleaved only when the PDZ-binding motif was attached to the C terminus.

**Stepwise Binding of drkN SH3-PDZbm Induces the Formation of the Catalytically Active Conformation of HtrA2.** Having established that drkN SH3-PDZbm serves as a model substrate for HtrA2, we next used NMR to analyze the structural changes of HtrA2 that accompany formation of a drkN SH3-PDZbm–protease complex. As the molecular mass of trimeric HtrA2 is 105 kDa, methyl transverse relaxation optimized spectroscopy (methyl–TROSY) was employed, which facilitates the recording of high-resolution and high-sensitivity NMR spectra of protein complexes with molecular masses of hundreds of kilodaltons (50, 51). In the approach used here, samples were prepared with 13C3 labeling at Ile61, Leu61 (proR), Val61 (proR), and Met positions in an otherwise deuterated background (referred to as U-2H, proR ILVM-13CH3 labeling in what follows) (52–54) and 13C-1H heteronuclear multiple quantum coherence (HMQC) spectra, which preserve slowly relaxing NMR signals, were recorded. In the following NMR analyses, two background mutations were introduced into HtrA2, including S306A, in which the catalytic Ser residue was replaced with Ala to suppress self-cleavage at high protein concentrations, and 1441V, which suppresses the formation of a ligand-binding-incompetent hexamer (31).

In our previous NMR analyses, we identified several well-resolved methyl probes that are sensitive to different conformational states of HtrA2 (31, 32). Fig. 2A shows spectra with cross-peaks derived from C, OI, and OA states that were obtained with samples containing only HtrA2 (Fig. 2 A, Left), HtrA2 with PDZ-peptide (Fig. 2 A, Center), and HtrA2 with both PDZ and substrate peptides (Fig. 2 A, Right), respectively. The methyl-probes from the PDZ domain (I362, L377, and M420) inform on the binding of PDZ-peptide to generate the OI state, while I150 at the interprotomer interface of each of the three equivalent protease domains is sensitive to the formation of the OA state. Some of the probes from the protease domain (I164 and I274) are sensitive to the presence of both OI and OA states. The assignment of these signals to specific states was confirmed by magnetization-exchange experiments correlating chemical shifts from different conformers that exchange slowly on the NMR chemical-shift timescale (SI Appendix, Fig. S2 B and C). It should be noted that distinct chemical shifts were often observed for methyl probes in each state, consistent with their slow interconversion that is accompanied by significant structural changes. We next prepared a U-2H, proR ILVM-13CH3-labeled HtrA2 sample and recorded 13C-1H HMQC spectra in the presence of various concentrations of U-2H,15N drkN SH3-PDZbm (Fig. 2B and SI Appendix, Fig. S2A). Upon addition of drkN SH3-PDZbm, we observed the successive transition from C to OI states up to an approximate 1:1 drkN SH3-PDZbm:HtrA2 monomer concentration, as well as a transition from OI to OA states when an excess of drkN SH3-PDZbm was added (Fig. 2B). These results establish that binding of drkN SH3-PDZbm to HtrA2 proceeds via a two-pronged mechanism, as previously characterized using short peptides (31, 32), with the first prong involving the C-terminal PDZ-binding motif–PDZ-domain interaction, forming a tethered substrate and responsible for the C to OI transition at the level of an individual HtrA2 protomer, and the second prong resulting from binding of drkN SH3-PDZbm to the protease active site, inducing the OI to OA conformational switch.

Prior to a quantitative analysis of the titration profile (Fig. 2 B and C) by model fitting, a number of important points can be made through a simple inspection of the data. First, the population of the C state decreases rapidly with increasing ligand and is largely depleted when an equimolar amount of ligand is added, while the OA state increases relatively slowly (I150 and I274 of Fig. 2C). This suggests that the OA state is only substantially formed after the PDZ domains are almost fully occupied, signals from both OI and OA states were observed for I150 and I164. Thus, despite the high local concentration of substrate available, not all of the active sites on HtrA2 are bound (second prong of the interaction, triggering formation...
of OA), consistent with a relatively weak contact involving the substrate and protease domains. Finally, the signal intensities reporting on the OA state continue to increase, even after the addition of more than one equivalent of drkN SH3-PDZbm, suggesting that additional substrate molecules can access HtrA2’s active sites without first binding to the PDZ domain.

We have fit a thermodynamic model to the NMR titration analyses as described above (Fig. 2 A, C, and D). The model, motivated by previous peptide studies (31, 32), includes free (P₃), partially ligated (P₃L) and fully bound (P₃L₃) trimeric states describing the stepwise binding of the C-terminal PDZ-binding motif of drkN SH3-PDZbm to the PDZ domains of HtrA2. As highlighted in Fig. 2D, the promoters in these states range from all closed (P₃) to all open (P₃L₃), but the HtrA2 molecules are inactive. In addition, two distinct catalytically active states, P₃L₃ and P₃L₄ (asterisk denotes the OA state), are included, with substrates bound at the protease active sites. These substrates can either be tethered to HtrA2 via their PDZ domains (P₃L₃) or one of the four untethered (three of the four ligands in P₃L₄ are tethered), accounting for the fact that the population of the OA state increases at substrate concentrations beyond where the PDZ domains of the enzyme are saturated. Following our previous analyses of the binding of PDZ-peptides to HtrA2 (31), we assume that the unbound and bound promoters in each P₃L₃ (j ∈ 0–3) conformer contribute to signals from the C and OI states, respectively.

Fig. 2. Binding model of drkN SH3-PDZbm to HtrA2. (A, Left) Close-up view of HtrA2 monomer structure showing methyl probes used in the titration analyses as purple spheres (PDB ID code 1LCY). (A, Right) The ¹³C-¹H HMQC correlation maps of the methyl signals that show distinct chemical shift differences between the C (blue), OI (purple), and OA (red) states. The cartoon representations of each state are shown on top of the spectra. The spectra of OI and OA states were recorded in the presence of 1 mM PDZ-peptide (OI) or both 1 mM PDZ-peptide and 2 mM substrate peptide (OA) (40 °C and 23.5 Tesla). (B) The ¹³C-¹H HMQC correlation maps of 150 μM (monomer concentration) U-²H, proR ILVM-¹³CH₃ S₃₀₆A/I₄₄₁V HtrA2 with varying concentrations of U-²H,¹⁵N drkN SH3-PDZbm (40 °C and 18.8 Tesla). (C) Plots of the intensities of methyl signals as a function of the concentration of drkN SH3-PDZbm. The solid lines are the fitted curves, and the 95% CI of each fitted curve is contained within the thick line estimated from Monte Carlo error analyses (84). The fractional populations of the C, OI, and OA states calculated from the fitted parameters are shown in C, Right Bottom. In each plot, the concentration of one equivalent added ligand is indicated as a dotted vertical line. (D) Thermodynamic binding model along with cartoon representations of each state used in fits of the titration data. The best-fit values and the estimated errors of the parameters are shown below the model (see Stepwise Binding of drkN SH3-PDZbm Induces the Formation of the Catalytically Active Conformation of HtrA2 for details). Equations for fractional populations of the C, OI, and OA states and the total protein concentration are also listed. Eq., equivalent; N term, N terminus; C term, C terminus.
The thermodynamic model described in the previous section assumes that formation of the OA state occurs only after all PDZ domains in a trimer are bound with substrate, consistent with results from our previously described protomer-mixing experiments (32), where preventing substrate binding to a single protomer was sufficient to eliminate activity. In this case, it might be predicted, therefore, that substrates able to form a tripartite interaction involving tethering to all three PDZ domains at once would be more efficiently cleaved by HtrA2. In order to test this, we performed a proteolytic activity assay against an engineered oligomeric substrate that is expected to exhibit strong binding avidity.

We designed a trimeric version of drkN SH3-PDZbm and compared proteolysis rates relative to a drkN SH3-PDZbm monomer (Fig. 3 A and B). To stabilize the trimeric structure, a trimerization domain from the T4 bacteriophage fibritin protein was fused to the N terminus of drkN SH3-PDZbm (55–58), with a flexible linker (seven repeats of GGGGS) inserted between the trimerization domain and drkN SH3-PDZbm to ensure that all three drkN SH3-PDZbm copies had enough flexibility to simultaneously interact with trimeric HtrA2. The formation of the client trimer was confirmed by using multieangle light scattering coupled with size-exclusion chromatography (SEC-MALS), where the molecular mass of the fusion protein (41.7 ± 0.4 kDa) was about threfold larger.

Synergistic Binding of Substrates Enhances the Proteolytic Activity of HtrA2. The thermodynamic model described in the previous section assumes that formation of the OA state occurs only after all PDZ domains in a trimer are bound with substrate, consistent with results from our previously described protomer-mixing experiments (32), where preventing substrate binding to a single protomer was sufficient to eliminate activity.
drkN SH3-PDZbm Is Unfolded When Bound to HtrA2 via Both Prongs. To further explore the binding mechanism of drkN SH3-PDZbm to HtrA2, we sought to characterize the structural dynamics of the substrate in the HtrA2-bound state. To this end, we prepared a U-2H, ILVM-[13]CH3 drkN SH3-PDZbm [only one of the pair of isopropyl methyls of Leu and Val was 13CH3-labeled (52, 53)] and recorded [13]C-1H HMQC spectra with and without U-2H, S306A/I441V HtrA2. As drkN SH3-PDZbm is a metastable protein, we observed two separate sets of signals in the absence of HtrA2, one for each of the folded (F) and unfolded (U) conformations. The relative populations of these two states were estimated to be ~35% and ~65%, respectively, at 40 °C. In the presence of HtrA2, we observed a new set of signals whose chemical shifts were similar, but distinct, particularly in the 1H dimension, from the unfolded state, which showed large chemical-shift changes in the presence of HtrA2, consistent with a stimulation of HtrA2 proteolytic enzyme interaction. Although most of the B- and U-state signals were monitored from the methyl signals of V65, a residue at the C-terminus of the PDZ-binding motif of drkN SH3-PDZbm, that showed large chemical-shift changes in the presence of HtrA2, presumably reflecting the direct association of substrate with enzyme. Although most of the B- and U-state signals were severely overlapped, assignments could be established through 13CH[t1]-max[1H[t2]] ZZ-exchange experiments (59), with the methyl chemical shifts of the B state unambiguously read out in many cases from exchange cross-peaks connecting B signals with
the well-resolved F-state signals (SI Appendix, Fig. S5 A and B). The obtained B-state chemical shifts are consistent with random coil values (60), so that the B-state structure of drkN SH3-PDZbm (two binding prongs are engaged) is largely unfolded (SI Appendix, Fig. S5 C).

Our titration results (Stepwise Binding of drkN SH3-PDZbm Induces the Formation of the Catalytically Active Conformation of HtrA2) suggested a two-pronged client–enzyme interaction, with contacts involving protease domains formed less frequently than those associated with PDZ moieties. We therefore wondered whether an intermediate PDZ-tethered, protease-domain unbound complex (i.e., only one prong formed) could be observed directly, providing support for our binding model. To test for this state, we recorded a pair of experiments that are sensitive reporters of methyl-group dynamics and focused on the F-state signals of drkN SH3-PDZbm (61, 62), with and without one equivalent of HtrA2 (Fig. 4 B and C and SI Appendix, Fig. S6). The experiments included 1) measurement of apparent transverse relaxation rates of methyl \(^{13}\text{C}\) nuclei, with potential exchange contributions suppressed by applying a train of closely spaced chemical-shift refocusing pulses (2-kHz Carr–Purcell–Meiboom–Gill experiment) (61, 63), and 2) quantifying \(S^2_{\text{axi}}\tau_c\) values, where \(S^2_{\text{axi}}\) and \(\tau_c\) are the order parameter squared of the methyl threefold axis and the effective rotational correlation time of the methyl, respectively (62). Notably, the apparent \(^{13}\text{C}\) transverse relaxation rates at 40°C (Fig. 4B) and \(S^2_{\text{axi}}\tau_c\) values at 10°C (Fig. 4C) were larger in the presence of HtrA2, indicating that the overall tumbling of the F state of drkN SH3-PDZbm was significantly reduced with added enzyme. This reduction was similarly observed at low temperature (10°C; SI Appendix, Fig. S6A), where the exchange between each conformer is expected to be slow, so that the increased relaxation rates do not arise from rapid relaxation in the B state coupled with chemical exchange (i.e., B to F). Also, the effect is not due to increased viscosity associated with the addition of HtrA2, as the dynamics of drkN SH3 did not change when the native drkN SH3 domain without the PDZ-binding motif, which does not form a stable complex, was mixed with HtrA2 (SI Appendix, Fig. S6 B and C). These results paint a picture of a binding intermediate, whereby the folded conformation of drkN SH3-PDZbm binds to HtrA2 through its PDZ domain (tethered), but does not unfold, so that one of the two prongs of the binding interaction is formed. In this model, drkN SH3-PDZbm exchanges between F, U, and B structural ensembles, where F and U are not solely unbound states, but also include contributions from C-terminal–tethered conformers that are bound to PDZ domains. Thus, there is a distinction in terms of the SH3 domain client structure between a bound (and unfolded) conformation, where the core of drkN SH3 interacts with the HtrA2 protease domain, and a tethered state, in which only PDZ contacts are made.

drkN SH3-PDZbm Binds to HtrA2 via a Conformational Selection Mechanism. Although the B state of drkN SH3-PDZbm is unfolded, it remains an open question as to whether HtrA2 binds first to the F state of drkN SH3-PDZbm to unfold the structure or preferentially to an already-existing U state. In the context of ligand–protein interactions, these two limiting cases are referred to as induced fit (IF) and conformational selection (CS), respectively (64, 65). Discrimination between these two possibilities requires measurement of fluxes between states, as described (41, 66). With this in mind, the interconversion between the F, U, and B states of drkN SH3-PDZbm, which occurs in the slow-exchange regime on the NMR chemical-shift timescale, was quantified by using ZZ-exchange experiments.

In order to minimize signal overlap, we made use of residue-specific \(^{13}\text{C}\)-methylene sulfonate (MMTS) labeling of an engineered Cys side chain in drkN SH3-PDZbm (67). As drkN SH3-PDZbm does not have intrinsic Cys residues, Met30 was mutated to Cys, followed by labeling with \(^{13}\text{C}\)-MMTS to form an S-methyliothiocystine (MTC) group that serves as a site-specific NMR probe (Fig. 4 D, Top). The advantage of using a single MTC probe is that cross-peaks fall in an “empty” region of the \(^{13}\text{C}\)-\(^{1}\text{H}\) spectrum, so that overlap with signals from other residues is not an issue. Three distinct peaks from the MTC group, corresponding to F, U, and B states of U-\(^{2}\text{H}\), ILV-\(^{13}\text{CH}_3\), and M30C \(^{13}\text{C}\)-MMTS–labeled drkN SH3-PDZbm, were observed in the presence of 0.5 equivalent of U-\(^{2}\text{H}\) S306A/I441V HtrA2, which could be used to probe the thermodynamics and kinetics of the interconversion between these three states (Fig. 4D).

The \(^{13}\text{C}\)-edited ZZ-exchange (\(^{1}\text{H}\)[\(t_1\)]–\(^{1}\text{H}\)[\(t_2\)]) experiments (68) were recorded as a function of mixing time (Fig. 4 D and E). In the spectrum recorded with \(t_{\text{mix}} = 50\ ms\), the exchange cross-peaks between the F and B states were much weaker than those between U and B, unequivocally demonstrating that the U state of drkN SH3-PDZbm predominantly interacts with HtrA2 to form the B state. The ZZ-exchange profiles were fit to a triangular kinetic scheme in which F, U, and B states interconvert with each other (Fig. 4B). The obtained six rate constants, as well as the populations of each state, are indicated in Fig. 4F, where the U and B states are depicted to include both unbound and C-terminally tethered states, as described in drkN SH3-PDZbm Is Unfolded when Bound to HtrA2 via Both Prongs. In order to determine which of the IF and CS pathways is dominant, it is important to compare the fluxes between the two pathways (41, 66). With this in mind, fluxes were calculated (\(F_{\text{F\rightarrow B}} = k_{\text{F\rightarrow B}}[\text{drkN SH3-PDZbm}]\) for the IF pathway and \(F_{\text{U\rightarrow B}} = k_{\text{U\rightarrow B}}[\text{drkN SH3-PDZbm}]\) for the CS pathway, where \(F_k\) and \(U_k\) are the equilibrium populations of the F and U states) with \(F_{\text{U\rightarrow B}}\sim 17\)-fold larger than \(F_{\text{F\rightarrow B}}\). We also calculated the flux for the three-state pathway \(\{F_{\text{F\rightarrow B}} = (F_{\text{F\rightarrow U}} + F_{\text{U\rightarrow B}})^{-1}\}\), where \(F_{\text{F\rightarrow U}} = k_{\text{F\rightarrow U}}[\text{drkN SH3-PDZbm}]\), which was also larger than \(F_{\text{B\rightarrow F}}\) (Fig. 4G). These results establish a dominant CS pathway for the binding of drkN SH3-PDZbm to HtrA2.

The Hydrophobic Region of drkN SH3-PDZbm Interacts with the Catalytic Center of HtrA2. Our studies of the drkN SH3-PDZbm/HtrA2 binding mechanism (Fig. 2D) and the structural dynamics of the enzyme–substrate complex (Fig. 4) made use of side-chain methyl probes, as the size of the complex prohibited recording high-quality backbone amide data. However, by working with drkN SH3 without the C-terminal PDZ-binding motif, which has a much lower affinity for HtrA2 than drkN SH3-PDZbm, and exploiting its rapid on/off binding kinetics, it is possible to “read out” structural properties of the SH3 bound state in high-quality spectra of the free form of the molecule and to identify interaction sites by inspecting residue-specific differential broadening or intensity reductions that inform on the transient binding to HtrA2 (69). Fig. 5A illustrates schematically the exchanging system that was used, in which the OH HtrA2 state was created through the addition of saturating amounts of PDZ-peptide.

Amide \(^{15}\text{N}\)-\(^{1}\text{H}\) spectra of drkN SH3 were recorded in the presence and absence of PDZ-peptide–bound HtrA2 (10°C to minimize hydrogen exchange) (70). Although separate bound-state signals were not observed, owing to the fast–intermediate exchange regime, individual correlations for residues in the
drkN SH3 F and U states, which interconvert slowly, were present. This hampers the residue-specific analysis of two-dimensional spectra due to cross-peak overlap. We, therefore, recorded 3D HNCO spectra of U-13C,15N drkN SH3, to which varying amounts (0, 0.2, or 0.33 equivalents) of unlabeled S306A/I441V HtrA2 in complex with PDZ-peptide was added. Fig. 5 B, Left shows site-specific intensity reductions in many of the signals relative to the isolated SH3 domain, and, notably, these reductions were more prominently observed in the U state (Fig. 5 B, Left), consistent with the preferential binding of HtrA2 to the U state as discussed in drkN SH3-PDZbm Binds to HtrA2 via a Conformational Selection Mechanism. Significant intensity reductions in the U state were observed primarily in the β4–β5 region, and to correlations from residues in β6–β7 to a lesser extent, where we annotate the U state according to secondary structure formed in the folded conformation (Fig. 5 B, Right Top and Middle). Not surprisingly, one of these regions includes the cleavage sites (residues I27–L28, L28–N29, and M30–E31 of strand β4), identified in our LC-MS analysis, so that the interactions observed in the HNCO spectra very likely correspond to those involving the protease domain that are required for proteolysis. The cleaved region is predicted to lie in a hydrophobic stretch based on the Kyte–Doolittle scale (71), suggesting that HtrA2 may recognize hydrophobic regions of unfolded substrates for cleavage (Fig. 5 B, Right Bottom).

To further validate that the intensity reductions in spectra of drkN SH3 reflect direct binding to HtrA2, we carried out a transferred cross-saturation (TCS) experiment (72–74). In the TCS experiment, the intensities of amide correlations from a uniformly deuterated drkN SH3 sample are quantified in data-sets where the aliphatic protons of HtrA2 are either saturated by an applied radio-frequency field or unperturbed. As saturation transfer from HtrA2 to drkN SH3 occurs efficiently for amide protons on drkN SH3 that are proximal to the binding interface, a binding site can be ascertained by monitoring site-specific intensity reductions of the drkN SH3 amide signals in the unbound state (Fig. 5 C, Left Upper). The 3D TROSY-HNCO spectra of U-13C,15N drkN SH3 with (orange) or paramagnetic (orange) label on HtrA2 (drkN SH3:HtrA2 monomer concentrations in a ratio of 2:1) (D, Right) Plots of PRE rates of the folded and unfolded states of drkN SH3. Average + SD values are indicated as orange horizontal lines. Residues with PRE rates larger than average + SD are colored orange. All of the measurements were performed at 10°C and 14.0 Tesla. Asterisks indicate the Pro residue (P49) or residues that were not analyzed due to signal overlap or broadening, Eq., equivalent.

Fig. 5. Mapping of HtrA2 interaction sites on drkN SH3. (A) Schematic cartoon describing the non tethered interaction between drkN SH3 without the C-terminal PDZ-binding motif and the open, inactive state of HtrA2 bound to PDZ-peptide. (B, Left) The 15N-1H planes from 3D HNCO spectra of U-13C,15N drkN SH3 with (pink) or without (navy) unlabeled S306A/I441V HtrA2 bound to PDZ-peptide. F and U denote the folded and unfolded states of drkN SH3. (B, Right Top and Middle) Plots of signal height ratios of drkN SH3 correlations in the presence of 0.2 (triangle, purple) or 0.33 (pink circle) equivalents of HtrA2. The signal heights were normalized against those in the absence of HtrA2. The secondary structure elements in the folded state are shown above the plots. The average – SD values are indicated as dotted horizontal lines (average over all residues, both from F and U states). Residues with ratios below the average – SD are shown as filled symbols. (B, Right Bottom) Plot of hydrophobicity calculated based on the Kyte–Doolittle scale using an averaging window size of 7 (71). (C, Left Upper) Schematic representation of the TCS experiment. (C, Left Lower) The 15N-1H planes from 3D TROSY-HNCO spectra of U-13C,15N drkN SH3 with (orange) or without (navy) 1H radio-frequency (R.F.) irradiation of 306A/I441V HtrA2 bound to PDZ-peptide. A ratio of drkN SH3:HtrA2/PDZ-peptide of 2:1 (monomer) was used. (C, Right) Plots of the cross-saturation effects for the folded and unfolded states of drkN SH3. The average – SD values are indicated by orange horizontal lines. Residues with a ratio above average – SD are colored orange. (D, Left Upper) A close-up view of the structure of the catalytic center of HtrA2 (PDB ID code 1LCY). The catalytic residues (residues 198, 228, and 306) are shown as yellow sticks, and the beta carbon of V226 are indicated by orange horizontal lines. Residues with a ratio below average – SD are colored orange. All of the measurements were performed at 10°C and 14.0 Tesla. Asterisks indicate the Pro residue (P49) or residues that were not analyzed due to signal overlap or broadening, Eq., equivalent.
and β6–β7 in the unfolded state (Fig. 5 C, Right), consistent with the regions identified from intensity reductions (Fig. 5B) and providing further evidence that these are the interaction sites on drkN SH3. We repeated the experiment on a sample containing only drkN SH3 and confirmed that the observed intensity reductions are not from intramolecular spin diffusion caused by direct irradiation of residual protons of the U–H, 13C, 15N drkN SH3 sample (SI Appendix, Fig. S7).

As a final validation of the interaction site on drkN SH3, we measured intermolecular paramagnetic relaxation enhancements (PRE) (75), with a spin label attached to HtrA2 V226C that is located close to the catalytic center residue (D228) (Fig. 5 D, Left Upper). Intermolecular PREs were quantified from increases in 2H linewidths in HNCO spectra of U–13C, 15N drkN SH3, to which V226C 2, 2, 6, 6-tetramethylpiperidine 1-oxyl (TEMPO)-labeled S306A/I441V HtrA2 was added (drkN SH3:HtrA2 = 2:1, monomer to monomer; Fig. 5 D, Left Lower and Right). Significant PREs were again observed in β4–β5 and β7 of drkN SH3 in the unfolded state. Taken together, our data demonstrate that the hydrophobic stretch of residues, mainly comprising β4–β5, as well as β6–β7, are the interaction sites on drkN SH3 and that the catalytic centers of the HtrA2 protease domains are involved in drkN SH3 binding (second prong).

Discussion

HtrA2 is a key stress-protective protease that cleaves misfolded proteins in an ATP-independent manner, with aberrant functions of the enzyme closely linked to pathogenic neurological conditions. Most biochemical and structural studies have involved short peptide fragments, such as 1) PDZ-peptides that bind to the PDZ domains of HtrA2 to form an open, albeit inactive, structure; or 2) substrate mimics that bind to the active sites of HtrA2 when PDZ-peptides have first been added. These studies have elucidated important roles of oligomeric assembly and interprotomer allostery (19, 30–32), but are limited because HtrA2 substrates are proteins, not short peptides, and many are capable of forming folded structures. Physiological substrates, thus, include PDZ-binding regions, localized to their C termini (5, 30), as well as an additional segment that is bound and cleaved by the protease domains of the enzyme. Understanding the structural dynamics of binding when there are two prongs in the same molecule, as well as the binding mechanism in this case, is therefore of considerable interest.

In this study, we used the metastable protein substrate drkN SH3-PDZbm and studied its interaction with HtrA2 by using a combination of solution-based NMR methods and proteolytic activity assays. We show that a similar activation scheme is in play for the SH3 domain as that proposed on the basis of our previous NMR studies involving PDZ-peptides and substrate peptides (31, 32). The two-pronged drkN SH3-PDZbm/HtrA2 interaction, occurring via conformational selection (Fig. 4), involves three distinct conformational states of HtrA2. The C-terminal PDZ-binding motifs of drkN SH3-PDZbm clients successively bind to the PDZ domains of HtrA2 trimers to induce the structural transition from state C to state OI, followed by the binding of a hydrophobic segment of drkN SH3 containing the sites of hydrolysis to the catalytic center of the enzyme, leading to the OI to OA transition and catalytic activity (Fig. 2). Notably, the second prong of the interaction, involving the hydrophobic region of drkN SH3, is weak. Proteolysis therefore likely only occurs when substrates are tethered to the enzyme via PDZ domains and their local concentration is high, so that all PDZ-binding sites are occupied. In this context, it may well be that anchoring of substrates close to the enzyme’s protease domains allows the active sites to rapidly “search” for cleavable sequences within the tethered substrate. This is consistent with the results of the proteolytic assay of Fig. 1, where HtrA2 efficiently cleaved drkN SH3 only when the PDZ-binding motif was attached to its C terminus. The requirement for linking the two interacting sites on the same substrate molecule is similarly observed for the bacterial HtrA protease DegP (76), suggesting that the mechanism proposed here may be applicable to the whole HtrA family.

Our study highlights the importance of carrying out experiments on each component of a complex, multistep binding reaction to build a reliable model of association (Fig. 2D). For example, in the drkN SH3-PDZbm–HtrA2 titration, only HtrA2 was NMR-active (Fig. 2), providing information about the evolution of the HtrA2 conformational states, from C to OI to OA as a function of added substrate. No insight into the status of the substrate—for example, at what stage it goes from tethered (single-prong interaction involving only the PDZ domain) to bound (double-prong association with PDZ and protease domains)—could be obtained. Our previous studies, involving the use of separate peptides as activators and substrates, allows separation of the individual enzyme conformations (C, OI, and OA) since the OA state can only form when substrate is added (after addition of PDZ-peptide), indicating that hydrolysis does not occur until all PDZ domains on the enzyme are saturated (31, 32). However, it is unclear whether binding of a single drkN SH3 domain to the protease domain (second prong) is sufficient for triggering formation of OA or whether additional substrates must be bound in a similar way to HtrA2 prior to activation. Correspondingly, our relaxation studies focusing on drkN SH3 in the presence of NMR invisible HtrA2 allow the separation of unfolded, folded, and bound substrate conformations (Fig. 4), showing that an intermediate exists in which substrates are tethered to PDZ domains, but not interacting with protease domains, but they do not inform on the conformational status of HtrA2. However, by combining results from these two sets of experiments, it is possible to generate a more nuanced model of substrate binding, as we show in Fig. 2D. In particular, it becomes possible to estimate the number of protease domain–substrate interactions that are formed when the enzyme is saturated with substrate (i.e., HtrA2 in the P*L3, P*L4, and P*L4 states). Consider monomer concentrations of 150 μM for drkN SH3-PDZbm and 75 μM for HtrA2, as used in the experiments of Fig. 4 D and E. The molar concentrations of each species depicted in Fig. 2D can be calculated from the parameters extracted from the drkN SH3-PDZbm–HtrA2 titration, along with the concentration of free drkN SH3-PDZbm, to give: [P*] = 0 μM, [P*L3] = 0.2 μM, [P*L3] = 2.0 μM, [P*L4] = 13.6 μM, [P*L4] = 3.9 μM, [P*L4] = 5.4 μM, and [L] = 72.1 μM. Assuming that only one molecule of drkN SH3-PDZbm interacts with the protease domain of HtrA2 in each of the P*L3 and P*L4 states to give rise to the B-state signal of Fig. 4 D (i.e., only a single protease subunit must be occupied to form OA), the fractional population of the B state is expected to be ([P*L3] + [P*L4])/ [L] = 0.062 (L = 150 μM, the total concentration of drkN SH3-PDZbm), which is much smaller than the experimentally observed fractional population of the B state derived from magnetization exchange (pB = 0.324; Fig. 4B). When three molecules of drkN SH3-PDZbm are required to interact with the protease domain to form P*L3 and P*L4 (i.e., the active state), the fractional population of the B state is (3[P*L3] + 3[P*L4])/ [L] = 0.184, more similar to the experimental value.
Thus, the trimeric architecture of HtrA2 functions both as a scaffold to select toxic aggregates, while at the same time suppressing uncontrolled cleavage of nonsubstrate proteins. The trimeric HtrA2 structure also supports an extensive interprotomer network that regulates catalytic activity, while also functioning as the basic unit to form an auto-inhibited hexameric state (31, 32). Therefore, the oligomeric assembly of HtrA2 plays a variety of nuanced roles in selecting substrates and in regulating its catalytic activity. Other families of HtrA proteases are also integral for the removal of toxic aggregates. These include HtrA1, involved in the degradation of toxic tau fibrils (77, 78), which shares a similar domain architecture with HtrA2, suggesting a similar mechanism of function potentially for many HtrA enzymes and explaining the evolutionary conservation of oligomeric structures across these families.

Our spin-relaxation studies of drkN SH3-PDZbm side-chain methyl groups demonstrate a highly dynamic substrate–enzyme complex. When the C terminus of drkN SH3-PDZbm is tethered to the HtrA2 PDZ domain, the core region of drkN SH3 is in equilibrium between folded, unfolded, and unfolded protease-bound states, reflecting the weak and transient second prong of the binding interaction. This explains why structural studies of HtrA-substrate complexes have focused on small peptides, with models of HtrA in complex with protein substrates limited to oligomeric cages where the bound substrate is poorly resolved (76, 79–82). In this regard, solution NMR spectroscopy offers a unique window into studies of protein client–HtrA interactions, demonstrating how the oligomeric architecture of the HtrA family plays a critical role in regulating enzyme function.

Materials and Methods

HtrA2 and drkN SH3 proteins were expressed in Escherichia coli and purified by Ni-affinity chromatography, hydrophobic interaction chromatography (for HtrA2), and SEC. All NMR measurements were performed at 23.5 Tesla (1-GHz1H frequency), 18.8 Tesla (800-MHz1H frequency), or 14.0 Tesla (600-MHz1H frequency). Structures of proteins for visualization were generated by using UCSF ChimeraX (83). Details of protein expression, purification, NMR experiments, and data analysis are provided in SI Appendix.

Data Availability. All relevant data are included in the paper and in SI Appendix. Backbone NMR chemical shifts of drkN SH3 have been deposited in the Biological Magnetic Resonance Bank, https://bmbio.iucr.org/ (entry 51327).

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