

# Tracing an allosteric pathway regulating the activity of the HsIV protease

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Edited by Ann E. McDermott, Columbia University, New York, NY, and approved December 27, 2013 (received for review September 30, 2013)

**The HsIU–HsIV complex functions as a bacterial proteasome, degrading substrate polypeptides to preserve cellular homeostasis.** Here, we use methyl-Transverse Relaxation-Optimized Spectroscopy (TROSY) and highly deuterated, methyl-protonated samples to study the 230 kDa dodecameric HsIV protease component that is structurally homologous to the stacked pair of  $\beta_7$ -rings of the proteasome. Chemical shift assignments for over 95% of the methyl groups are reported. From the pH dependence of methyl chemical shifts, a  $pK_a$  of 7.7 is measured for the amine group of the catalytic residue T1, confirming that it can act as a proton acceptor during the initial step in substrate proteolysis. Analyses involving a series of single site mutants in HsIV, localized to HsIU binding sites or regions undergoing significant changes on HsIU binding, have identified hot spots whose perturbation leads to an allosteric pathway of propagated changes in structure and ultimately, substrate proteolysis efficiency. HsIV plasticity is explored through methyl-TROSY  $^{13}\text{C}$  relaxation dispersion experiments that are sensitive to millisecond timescale dynamics. The data support a dynamic coupling between residues involved in both HsIU and substrate binding and residues localized to the active sites of HsIV that facilitate the allosteric between these distal sites. An important role for dynamics has also been observed in the archaeal proteasome, suggesting a more generally conserved role of motion in the function of these barrel-like protease structures.

protein NMR | protein dynamics | molecular machines

The 20S proteasome core particle (CP) is a molecular machine that plays a critical role in maintaining cell viability through protein degradation, leading to the removal of damaged or misfolded proteins to prevent formation of toxic aggregates. It is also involved in the regulation of the cell cycle and in generating peptides used in the immune response (1, 2). The proteasome barrel-like structure is comprised of a series of four heptameric rings stacked on top of each other and arranged as  $\alpha_7\beta_7\beta_7\alpha_7$ , with active site residues sequestered within the lumen of the proteasome (3, 4). Regulation of the 20S CP function is, in part, accomplished by binding additional large complexes. These partners include the 19S regulatory particle (RP) ATPase that recognizes ubiquitinated protein substrates, 11S RPs that control the sizes and relative distributions of product peptides that are produced, Blm10/PA200 whose binding stimulates the degradation of peptides, and AAA protein complexes, such as Cdc48/p97/VAT (5–7). Although proteasomes are ubiquitous in eukaryotes and archaea and present in some bacteria, most bacteria have what is considered to be a primitive proteasome system that degrades target proteins in an ATP-dependent manner (8). The protease component, HsIV, is a double hexameric ring structure (Fig. 1A), with each of the concentrically arranged rings analogous to  $\beta_7$  in the proteasome. The proteolytic activity of HsIV can be enhanced several orders of magnitude (8) through the binding of the hexameric ATPase HsIU that unfolds protein substrates and translocates them through a central channel to HsIV for degradation (9–11).

The 20S CP and the HsIV protease share many features. For example, both degrade proteins through active sites localized to

the lumen of a barrel structure, with function controlled through binding of additional regulatory particles (4, 9). Structurally, the  $\beta$ -subunits of the 20S CP and each of 12 subunits of HsIV are highly homologous, and it is believed that both the 20S CP and HsIV cleave substrate using a similar catalytic mechanism involving an amino terminal threonine residue (9, 12). However, the HsIV protease is simpler, because it is comprised of only a single polypeptide chain, whereas the 20S CP is made up of a pair of proteins,  $\alpha$  and  $\beta$ , in the case of the archaeal version or seven different  $\alpha/\beta$ -subunits for the eukaryotic complex (1, 2, 7). Some similarities also exist between the 19S and the HsIU RPs that interact with the 20S CP and HsIV, respectively. For example, the hexameric HsIU RP consists of six identical AAA ATPases that are positioned on the top and bottom of the HsIV donut structure (9). Although the 19S RP is much more complex, with 19 canonical subunits, it too includes a ring of six AAA ATPases, architecturally similar to HsIU, that binds to the 20S CP  $\alpha_7$ -rings (1, 2, 7).

The inherent simplicity of the HsIU–HsIV system makes it attractive as a model for understanding the structure–dynamics–function paradigm in proteasome-like structures using solution NMR, a technique that has, until recently, been limited to much smaller protein systems. Furthermore, HsIV from the malaria-causing parasite *Plasmodium falciparum* may be an attractive antimicrobial drug target (13), especially because the HsIV protease is not present in humans. In this regard, a detailed understanding of the mechanism of substrate proteolysis and the general role of dynamics in enzyme function could be important for the design of effective pharmaceuticals. Here, we focus on the *Haemophilus influenzae* HsIV protease because high-resolution X-ray structures are available (9, 11, 12) as well as a significant body of biophysical and biochemical data (14–16). This protease has an aggregate

## Significance

**Molecular machines, such as the proteasome in eukaryotes and archaea as well as the HsIU–HsIV complex in bacteria, play critical roles in maintaining cellular homeostasis. Here, we have used methyl-Transverse Relaxation-Optimized Spectroscopy to study the 230 kDa HsIV dodecamer that cleaves substrate polypeptides. The initial catalytic step is investigated by measuring the  $pK_a$  of the terminal amine of catalytic residue T1. Furthermore, we show that single site mutations in key regions, which contact HsIU or change on HsIU binding, lead to propagated changes in structure along an allosteric pathway, affecting proteolysis rates. NMR relaxation experiments are consistent with a coupling of millisecond timescale dynamics throughout regions of HsIV that link HsIU and substrate binding with catalysis.**

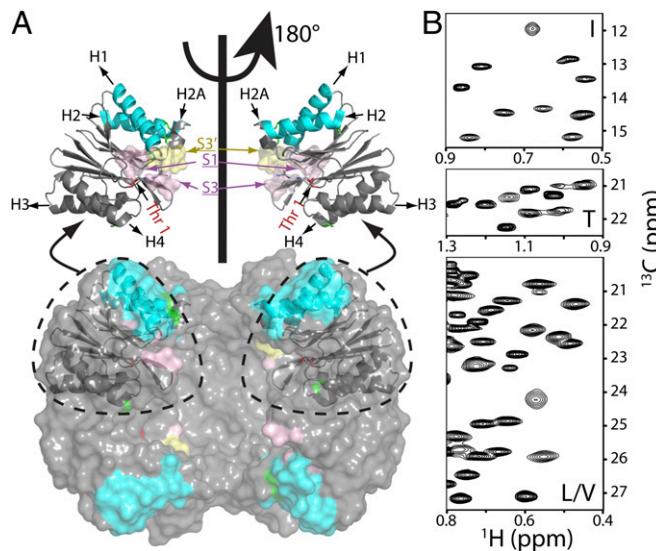
Author contributions: L.S. and L.E.K. designed research, performed research, analyzed data, and wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318476111/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318476111/-DCSupplemental).



**Fig. 1.** Near-complete I, L, M, T, and V methyl chemical shift assignments for HsIV. (A) Surface model showing the dodecameric structure of HsIV, comprising a pair of hexameric rings that are aligned coaxially (9) (Protein Data Bank ID code 1G3K) with the front four subunits removed to show the proteolytic chamber. All monomers of the hexamer are equivalent. Shown in *Upper* are ribbon diagrams for a pair of monomers, corresponding to the two front-facing subunits of the surface model, related to each other by a 180° rotation about an axis of sixfold symmetry. Helices are labeled H1, H2 (highlighted in cyan), H2A, H3, and H4, with catalytic residue T1 in red. Parts of the substrate binding pocket (S1, S3, and S3' from an adjacent molecule) are also indicated. (B) Regions of  $^{13}\text{C}$ ,  $^1\text{H}$  HMQC spectra of [ $^2\text{H}$ ;  $\text{Ile}^{\delta 1-13}\text{CH}_3$ ;  $\text{Leu}, \text{Val}^{\delta 1-13}\text{CH}_3/^{12}\text{CD}_3$ ;  $\text{Met}^{\delta 1-13}\text{CH}_3$ ]- or [ $^2\text{H}$ ;  $\text{Ile}^{\delta 1-13}\text{CH}_3$ ;  $\text{Met}^{\delta 1-13}\text{CH}_3$ ;  $\text{Thr}^{\delta 1-13}\text{CH}_3$ -labeled HsIV (18.8T and 40 °C).

molecular mass of 230 kDa, with each equivalent monomer of ~19 kDa.

The development of isotope labeling strategies, in which highly deuterated, methyl-protonated proteins are readily produced (17), along with NMR experiments that exploit the labeling to minimize the decay of NMR signals (18) have significantly increased the use of solution NMR for quantitative studies of supramolecular complexes (17, 19). Below, we use these NMR methods in a study of the HsIV protease, with the results compared with data obtained on the 20S CP previously. Central to the motivation of the present work is the recent demonstration of an allosteric pathway of communication in the 20S CP that extends from the  $\alpha$ -rings, where RPs bind, to the  $\beta$ -ring active sites that are 75 Å removed (20). The high degree of homology between the structures of the  $\beta$ -subunits from the 20S CP and the subunits of the HsIV protease led us to wonder whether a pathway might be found in HsIV as well and if present, whether it could regulate proteolytic activity. Here, we show that conservative single point mutations in the HsIV binding interface of HsIV lead to significant chemical shift changes of methyl probes extending to the active site/substrate binding regions of HsIV, with concomitant changes in proteolysis rates, identifying an allosteric pathway. By contrast, mutations that do not result in shift changes have little effect on hydrolysis. Relaxation dispersion experiments establish that the regions most affected exchange between conformations on a millisecond timescale, suggesting that inherent plasticity is a key component in mediating the observed allosteric.

## Results

**Methyl Group Assignments and Characterization of the 230 kDa HsIV Complex.** Central to any NMR study is the assignment of key probes to specific sites in the primary amino acid sequence.

Here, we have chosen I, L, V, M, and T methyl groups as spies of molecular structure and dynamics using [ $^2\text{H}$ ;  $\text{Ile}^{\delta 1-13}\text{CH}_3$ ;  $\text{Leu}, \text{Val}^{\delta 1-13}\text{CH}_3/^{12}\text{CD}_3$ ;  $\text{Met}^{\delta 1-13}\text{CH}_3$ ]- or [ $^2\text{H}$ ;  $\text{Ile}^{\delta 1-13}\text{CH}_3$ ;  $\text{Leu}, \text{Val}^{\delta 1-13}\text{CH}_3/^{12}\text{CD}_3$ ;  $\text{Met}^{\delta 1-13}\text{CH}_3$ ;  $\text{Thr}^{\delta 1-13}\text{CH}_3$ ]-labeled protein samples, which has been done in previous studies of the proteasome by our group (20–23). Near-complete assignments (91 of 95 methyl groups) were obtained through a combined approach involving mutagenesis (*SI Appendix*) and analysis of NOE datasets that exploited the availability of a high-resolution X-ray structure of *H. influenzae* HsIV (9). Fig. 1B shows regions of 2D  $^{13}\text{C}$ ,  $^1\text{H}$  HMQC methyl correlation maps of HsIV (40 °C and 18.8T) with assignments provided in *SI Appendix*, Fig. S1. Stereospecific assignments were achieved using a labeling strategy introduced by Gans et al. (24), where the proR(S) methyl of Leu and Val side chains is  $^{13}\text{CH}_3/^{12}\text{CD}_3$  (*SI Appendix*, Fig. S1).

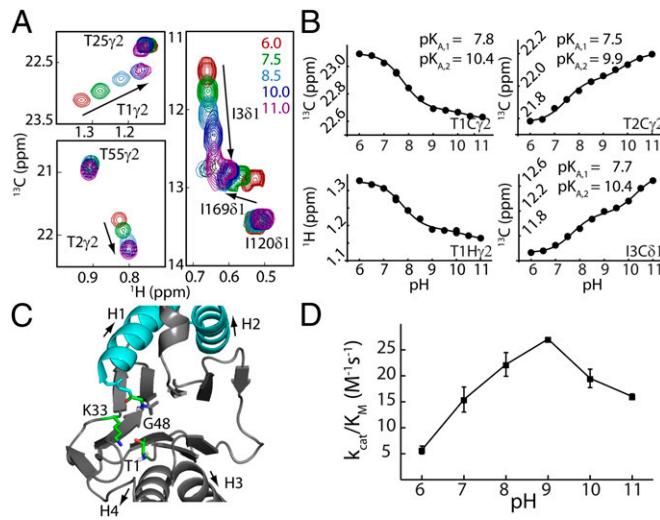
We have also validated that the proteins in our NMR samples were of the size expected by recording pulsed-field gradient diffusion experiments (*SI Appendix*, Fig. S2) on a number of protein samples, including HsIV (230 kDa), a single ring of the 20S CP ( $\alpha_7$ ; 180 kDa), the half proteasome ( $\alpha_7\alpha_7$ ; 360 kDa), and the 8.9 kDa protein ubiquitin (25 °C). The calculated diffusion rates are inversely proportional to the cube root of the molecular mass, assuming that each of the diffusing particles is spherical. The values for WT HsIV and a series of point mutants (see below) are very similar ( $3.8 \pm 0.4 \times 10^{-7} \text{ cm}^2/\text{s}$ ) and between those of  $\alpha_7$  ( $4.5 \pm 0.1 \times 10^{-7} \text{ cm}^2/\text{s}$ ) and  $\alpha_7\alpha_7$  ( $2.6 \pm 0.1 \times 10^{-7} \text{ cm}^2/\text{s}$ ) as expected, whereas ubiquitin diffuses much more rapidly ( $13.5 \pm 0.1 \times 10^{-7} \text{ cm}^2/\text{s}$ ). The diffusion data, thus, provide strong evidence that the recombinantly produced samples of HsIV, both WT and mutants (see below), have the expected molecular architecture of the intact complex.

**NMR Titration Data Support Similar Proteolysis Mechanisms for HsIV and the 20S CP.** As described in detail elsewhere, methyl groups can potentially provide detailed structural and dynamics information, even in studies involving very high-molecular mass protein complexes (17, 19). Most applications have focused on I, L, and V probes and sometimes, M (22, 25) and A (26) methyl groups. Although these residues remain important for much of the work described here, extending  $^{13}\text{CH}_3$  labeling to threonine is critical, because the catalytic residue in HsIV is T1. Therefore, we have used the assignments of I, L, V, and M methyl groups (80 of 84 methyl groups) that were obtained in the initial phases of our work along with NOE data from  $^{13}\text{C}$ -edited 3D spectra (*SI Appendix*, Fig. S3) to obtain complete assignments for all 11 T methyl groups in highly deuterated samples of HsIV prepared with  $^{13}\text{CH}_3$  label at I, L, V, M, and T sites. Key to the success of these experiments is the availability of  $[\alpha, \beta^2\text{H}; \gamma^{13}\text{C}]$ -T, which has been produced using a biosynthetic scheme described previously (27). We have shown that deuteration at the T $\alpha$ - and T $\beta$ -positions is critical for recording high-quality datasets when applications to large complexes, such as HsIV, are considered.

The availability of assignments for methyl group probes throughout the HsIV complex, including for the catalytic residue T1, allowed us to address key mechanistic questions at an early stage in the analysis. For example, in analogy with the proteasome, the catalytic mechanism for peptide bond hydrolysis is thought to involve a nucleophilic attack of the substrate peptide bond by T1 O $\gamma$ 1 after first removing the hydroxyl proton (*SI Appendix*, Fig. S4) (3, 28). In its neutral form, the T1 amino group can serve as a base in this first step of the catalytic cycle by accepting the proton that is released from the hydroxyl group (9). However, the ionization state of this site, and hence its ability to function as a base, remains to be confirmed. As we describe below, the titration of methyl group chemical shifts from T1, T2, and I3 as a function of pH facilitates the measurement of the T1 amino group pK<sub>a</sub> value and

hence, the determination of the ionization state of the T1 amino moiety.

Fig. 2A shows a superposition of specific regions of  $^{13}\text{C}$ - $^1\text{H}$  correlation spectra focusing on T1, T2, and I3 recorded over a pH range extending from 6.0 to 11.0, where HsIV is stable. Not surprisingly, the methyl groups of these three residues titrate over a much larger chemical shift range than other I and T methyls, providing confidence in the assignments. Fig. 2B plots chemical shift vs. pH profiles for methyl groups of these residues. Notably, none of the profiles could be well-fitted to the Henderson–Hasselbalch equation with only a single  $\text{pK}_a$ . However, they were well-fit assuming a model involving a pair of protonation events each of which is thermodynamically independent but where the chemical shifts of the reporter nuclei depend on the ionization state of both titratable groups (29). Profiles from each of T1, T2, and I3 were fit independently, and the pairs of  $\text{pK}_a$  values obtained ( $\text{pK}_{a1} = 7.7 \pm 0.2$  and  $\text{pK}_{a2} = 10.2 \pm 0.3$ ) are in excellent agreement with each other. These values are consistent with those for an unperturbed protein amino terminus (8.0) and a Lys amino group (10.5), respectively (30). X-ray structures of both HsIV (9) and the  $\beta$ -subunit of the 20S CP (3) show that K33 is in close proximity to T1 (within 2.9 Å) (Fig. 2C) and functional assays involving mutations of K33 to A, H, or R in the archaeal 20S CP from *Thermoplasma acidophilum* (31) or to H or R in HsIV reduce protease activity to <0.1% of the WT enzyme. Therefore, it is likely that the  $\text{pK}_a$  values measured derive from the titration of the amino groups of T1 and K33. Notably, the activity vs. pH profile of HsIV is shifted into a range where the amino terminus of T1 is neutral, and therefore, it can serve as a base, accepting T1  $\text{H}_\gamma 1$  (Fig. 2D). The results from this analysis are in agreement with those from a previous study of the 20S CP, where it was shown that T1 of each  $\beta$ -subunit of

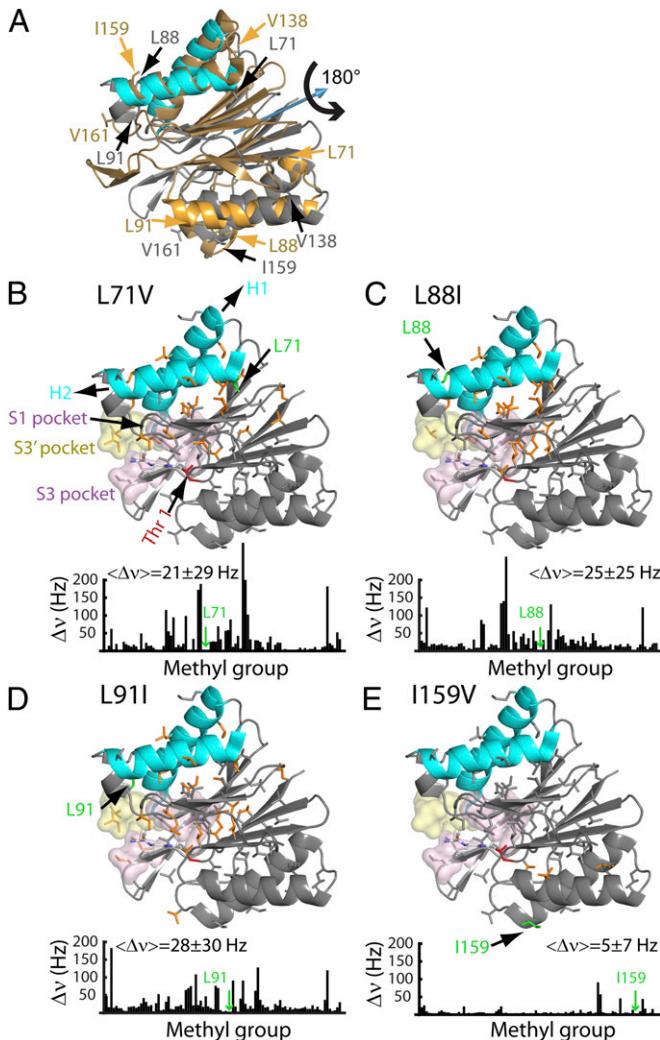


**Fig. 2.** Measurement of active site T1 amine  $\text{pK}_a$  establishes the first step of the catalytic mechanism. (A) Superposition of selected regions of HMQC spectra as a function of pH, focusing on residues T1, T2, and I3. (B) Chemical shift titration data were fit on a per-residue basis to a model that involved ionization of two groups, which is described in the text. The  $^{13}\text{C}$  and  $^1\text{H}$  shifts of a given methyl group were fit simultaneously (e.g., T1 $\gamma 2$ ). Duplicate measurements at pH 7.5 and pH 10 were used to estimate errors in measured chemical shifts; the error bar is within the size of each dot. (C) Ribbon diagram of a region of a single HsIV monomer highlighting the active site (Protein Data Bank ID code 1G3K). Residues that are critical for the proposed peptidase activity are shown in ball-and-stick representation (SI Appendix). (D) pH profile of HsIV peptidase efficiency based on hydrolysis rates of the substrate Z-GGL-AMC (*N*-carbobenzoxy-Gly-Gly-Leu-amido-4-methylcoumarin; 40 °C).

the proteasome is also neutral at a pH that is optimal for activity (23).

**Allosteric Pathway.** In addition to providing information about the catalytic mechanism, methyl group probes can also be used to obtain insight into the relation between dynamics and function (17, 19). In a previous study of the 20S CP, we observed that mutations of key amino acids in regions forming the binding sites for RPs ( $\alpha$ -subunits) resulted in chemical shift changes (hence, structural perturbations) that were propagated over a distance of 75 Å from the  $\alpha$ -rings to the substrate binding pockets in the  $\beta$ -rings (20). The relation between allosteric and dynamics was clarified by additional solution NMR studies showing that the 20S CP interconverts rapidly between a series of distinct conformers, whose relative populations are changed on introduction of point mutants at key sites in the enzyme. Given the structural similarities between the  $\beta$ -subunits of the proteasome and the subunits of HsIV, we wondered whether an allosteric pathway might be present in HsIV as well and what the role of dynamics would be in this case. As a first step, we constructed a series of HsIV mutants that were localized to regions that either interact directly with HsIU or change on HsIU binding, with some of the sites of mutation indicated in Fig. 3A. For example, L71 and V76 (mutated to V and A, respectively) are part of helix H2 that, along with H1, binds the carboxyl-terminal tail of each of the subunits of HsIU. Other sites include L88 and L91, which are part of a small four-residue helical structure, H2A, immediately after helix H2. A comparison of the X-ray structures of HsIV, free and bound to HsIU, shows that this region undergoes the largest conformational change on binding, with H2A becoming unstructured (9). In addition to mutations in the RP binding region, we also considered several mutations in H4 at the opposite end of the molecule that is effectively the symmetry-related counterpart of H2. The structure of each HsIV monomer is approximately symmetric about a pseudotwofold axis that runs through the center of the protein (Fig. 3A). Shown in gray (cyan for H1/H2) is the structure of HsIV using the same orientation as in Fig. 1A, whereas the structure obtained by applying a 180° rotation about the symmetry axis (blue arrow) is indicated in gold in Fig. 3A. Many of the sites of mutation considered in this analysis are labeled in Fig. 3A. Importantly, all mutants have the same oligomeric structure as WT, which was established by pulsed-field gradient diffusion measurements (SI Appendix, Fig. S2) and gel filtration chromatography protein elution profiles (SI Appendix, Fig. S5).

Fig. 3 and SI Appendix, Fig. S6 show ribbon diagrams of one of the subunits from the dodecameric HsIV complex, highlighting all I, L, V, M, and T methyl groups that serve as probes. Methyl groups with changes in chemical shifts  $\Delta\nu = \sqrt{\Delta\nu_H^2 + \Delta\nu_C^2} > 25$  Hz on mutation of the WT protein, where  $\Delta\nu_j, j \in \{H, C\}$  is the difference in shift in the  $j$  dimension of the  $^{13}\text{C}$ - $^1\text{H}$  HMQC spectrum, are color-coded orange on each structure. Immediately underneath each structure is a plot of  $\Delta\nu$  as a function of residue. Relatively large mean shift changes are observed for L71V ( $21 \pm 29$  Hz) (Fig. 3B), L88I ( $25 \pm 25$  Hz) (Fig. 3C), and L91I ( $28 \pm 30$  Hz) (Fig. 3D), excluding those methyl groups within 8 Å of the residue mutated. By contrast, very little shift difference is observed for I159V ( $5 \pm 7$  Hz) (Fig. 3E), where I159 (H4) is symmetry-related to L88 (H2) through a 180° rotation about the axis shown in Fig. 3A. This scenario, in which large changes in shifts are observed for residues at the HsIU binding site (H1/H2) but not at symmetric positions (H3/H4), has also been noted for the L91I (H2)-V161I (H4) pair (Fig. 3 and SI Appendix, Fig. S6). Similar observations have also been made for other residues that are not at symmetric positions but nevertheless, brought into proximity on 180° rotation about the symmetry axis, such as L71V (H2)-V138I (H3) or V76A (H2)-V149A (H4).



**Fig. 3.** An allosteric pathway regulating the function of HslV. (A) The structure of each HslV monomer is approximately symmetric about a pseudodotwofold axis that runs through the center of the protein, which is indicated by the blue arrow. Shown in gray (cyan for H1/H2) is the structure of an HslV monomer using the same orientation as in Fig. 1, right subunit, whereas the structure obtained by applying a 180° rotation about the symmetry axis (blue arrow) is indicated in gold. The positions of some of the mutations used are indicated. (B–E) Chemical shift changes relative to the WT protein are indicated in bar chart form and color-coded on the structure. Each methyl-containing residue is depicted in stick representation, and those methyls that undergo changes in shift  $\geq 25$  Hz are highlighted in orange. The mean chemical shift change ( $\pm 1$  SD) is indicated, excluding methyl groups  $\leq 8$  Å from the site of mutation. Note that the L88I mutation (H2) shows large changes in chemical shifts, whereas the mutation involving the symmetry-related position I159V shows little change. Where changes are observed, they extend from helices H1/H2 to regions surrounding the active site and/or substrate binding sites (S1, S3, and S3') as indicated.

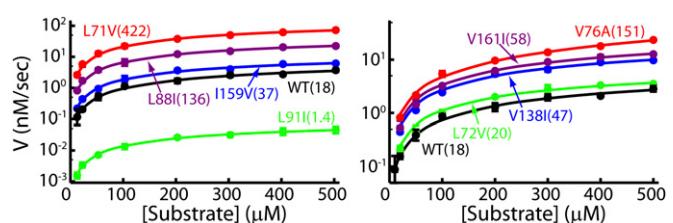
Proteolytic activities of the mutants considered in this study were measured by monitoring changes in fluorescence resulting from hydrolysis of a large excess of the fluorogenic substrate peptide *N*-carbobenzoxy-Gly-Gly-Leu-amido-4-methylcoumarin (8). Fig. 4 plots the reaction velocity as a function of the concentration of substrate and lists  $k_{cat}/K_M$  values. All mutated residues are distal to the active site or substrate binding regions, with distances  $\geq 13$  Å for all sites, with the exception of I159 (10 Å) and V161 (7 Å). Nevertheless, some mutations result in changes in  $k_{cat}/K_M$  ratios by over an order of magnitude from the

WT enzyme. Notably, mutations that lead to significant changes in chemical shifts are those that change proteolysis rates, such as L71V, L88I, and L91I (Fig. 3) as well as V76A (SI Appendix, Fig. S6). For the remaining mutants (I159V, V161I, V138I, V149A, and L72V), there is little change in either chemical shifts or  $k_{cat}/K_M$ .

**Spin Relaxation Experiments Confirm the Plasticity of HslV.** The structural plasticity of HslV, suggested by the mutational study described above, has been further examined by recording NMR spin relaxation experiments that are sensitive to motion on the millisecond timescale (32, 33). Conformational exchange in this time regime gives rise to stochastic changes of chemical shifts, leading to an increase in effective transverse relaxation rates of NMR spins,  $R_{2,eff}$ . This effect can be modulated by the application of a series of chemical shift refocusing pulses, each separated by a delay- $\delta$  [ $\nu_{CPMG} = 1/(2\delta)$ ], to produce dispersion profiles ( $R_{2,eff}$  vs.  $\nu_{CPMG}$ ) that are then fit to extract rates of exchange between conformers and the fractional populations of each of the interchanging states. We have recorded  $^{13}\text{C}$ - $^1\text{H}$  multiple quantum (33) and  $^1\text{H}$  single quantum (34) relaxation dispersion profiles on samples of WT HslV (Materials and Methods). Fig. 5 shows a number of these curves (recorded at 18.8T and 40 °C). All  $^{13}\text{C}$  (18.8T and 14.0T) and  $^1\text{H}$  data (18.8T), with the exception of I3C81, could be fit together reasonably well assuming a model of two-site exchange (Fig. 5, solid lines) ( $E \xrightleftharpoons[k_{GE}]{k_{EG}} G$ ), with  $k_{ex} = k_{EG} + k_{GE} = 620 \pm 30 \text{ s}^{-1}$  and a value for the fractional population of the excited state ( $E$ ) of  $p_E = 2.4 \pm 0.1\%$ . Residues for which methyl groups have dispersion profiles with  $R_{ex} = R_{2,eff}(\nu_{min}) - R_{2,eff}(1,000 \text{ Hz}) > 5 \text{ s}^{-1}$  ( $\nu_{min} = 50$  and 100 Hz for  $^{13}\text{C}$  and  $^1\text{H}$  dispersions, respectively) are plotted in orange on the ribbon structure of an HslV monomer in Fig. 5. Dynamic residues are found in all regions of the protein but particularly, helices H1/H2 and the  $\beta$ -sheet interface connecting them with H3/H4. Interestingly, residues that play an important role in catalysis (T1), are proximal to the active site (I3), are involved in substrate binding (L22 and I109), or form the binding sites for the carboxyl-terminal tails of HslU (L56, L72, V76, L104, and V112) are all dynamic on the millisecond timescale.

## Discussion

Of all the NMR parameters that can be measured, chemical shifts are obtained most accurately, and they can be exquisitely sensitive to changes in conformation. In this study, we have assigned over 95% of the I, L, V, M, and T methyl groups in the 230 kDa HslV complex (Fig. 1), and therefore, they can be used as probes of function and dynamics. Methyl groups are uniquely



**Fig. 4.** Peptidase assay for the HslV mutants considered. A fluorogenic peptide Z-GGL-AMC was used as a substrate. The  $V$ -substrate curves were fitted to a simple Michaelis-Menten equation (solid lines) as indicated. Note that mutations showing large chemical shift perturbations (Fig. 3 and SI Appendix, Fig. S6) also give rise to an order of magnitude change in activity (either increase or decrease), whereas mutants (for example, I159V) producing small chemical shift changes have little effect on activity. Values of  $k_{cat}/K_M$  ( $\text{M}^{-1}\text{s}^{-1}$ ) are indicated. The values, including errors, are WT =  $18 \pm 2$ ; L71V =  $422 \pm 8$ ; L72V =  $20 \pm 4$ ; V76A =  $151 \pm 10$ ; L88I =  $136 \pm 5$ ; L91I =  $1.40 \pm 0.03$ ; V138I =  $47 \pm 5$ ; I159V =  $37 \pm 8$ ; and V161I =  $58 \pm 2$ .

suit for studies of high-molecular mass complexes, because they can be readily  $^{13}\text{C}$ ,  $^1\text{H}$ -labeled in an otherwise highly deuterated background, they are localized to molecular interfaces and in the present case, to active sites, and they give rise to NMR spectra that can be quantified at a level of detail that is often reserved for studies of small- to medium-sized proteins using traditional labeling schemes (17, 19). Here, we have used the assigned methyl groups to test aspects of the reported mechanism of proteolysis involving catalytic residue T1 and to address the plasticity of the HsIV protease.

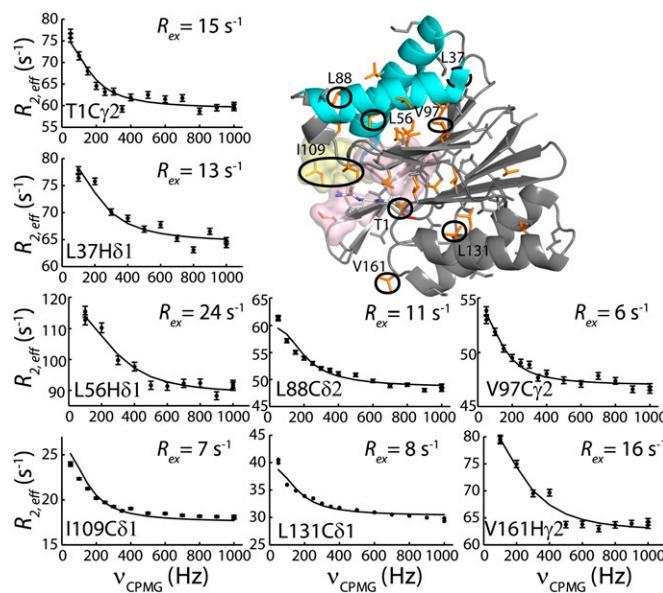
We have used the methyl groups of T1, T2, and I3 as reporters of the ionization state of the amine group of T1, providing evidence that it functions as a base by abstracting the T1 hydroxyl proton in the first step of catalysis. If proton abstraction is to happen efficiently, then the amine must be neutral over the pH optimum activity range of HsIV. Chemical shift titrations vs. pH have been fit with a  $\text{pK}_a$  value  $\sim 7.5$ , consistent with a neutral form for the terminal amine at pH 8–9, where HsIV is optimally active (Fig. 2). Interestingly, from a similar analysis on the 20S CP, a  $\text{pK}_a$  value of 6.3 was obtained for the amino terminal amine of T1 of the  $\beta$ -subunits (23).

Elegant X-ray studies of HsIV and the HsIU–HsIV complex establish that HsIV is an allosteric enzyme (9). On binding HsIU, the apical helices of HsIV (Fig. 1, cyan) shift substantially, leading to conformational changes that are propagated to residues proximal to the active site to increase proteolysis rates by two orders of magnitude (8). Moreover, it has been shown that, in an asymmetric complex in which only a single HsIU was attached to the HsIV double ring, the HsIV ring bound with HsIU is active in the sense that it could react with inhibitor, whereas the second is not (35). Although X-ray studies have not been able to explain the allosteric properties of the 20S CP that have been observed in biochemical assays (36), a recent methyl-Transverse Relaxation-Optimized Spectroscopy (TROSY) NMR study of the 20S CP from *T. acidophilum* shows an allosteric pathway extending from the sites of RP binding on the  $\alpha$ -rings of the proteasome to residues involved in substrate binding localized to the  $\beta$ -rings. This pathway could be manipulated through mutations of

residues that are critical for RP binding, leading to changes in the distribution of proteolysis products (20).

The similarities in structure between the  $\beta$ -rings of the 20S CP proteasome and the HsIV protease as well as the crystallographic data described above suggest that HsIV is likely to be inherently plastic, as observed for the proteasome in NMR studies (20), with this plasticity important for function. To explore this issue further, we have produced a series of single site mutants in HsIV that are localized to HsIU binding sites (apical helices H1/H2) or regions undergoing significant changes on HsIU binding and quantified methyl chemical shift changes relative to the WT enzyme. In all cases where substantial chemical shift changes were observed, there were order of magnitude changes in  $k_{\text{cat}}/K_M$  (Figs. 3 and 4 and *SI Appendix*, Fig. S6), and where only very minor shifts were recorded, few changes to enzyme activity were noted. Moreover, mutations of residues that are related to amino acids on the apical helices by a 180° rotation of the structure about its pseudosymmetry axis (Fig. 3A) result in only small changes in chemical shifts and correspondingly, little effect on  $k_{\text{cat}}/K_M$ . Thus, the effects of the mutations on chemical shifts and hence, structure are unidirectional, extending from H1/H2 to the interior of the protein, ultimately leading to changes in activity. Despite the inherent symmetry of the molecule, it is not possible to effect the same changes in structure/function by mutations in the corresponding positions on H3/H4. Hot spots on H1/H2 are primed to sense perturbations through either HsIU binding or mutation, which was noted for the 20S CP (20). By contrast, these hot spots are not found on H3/H4, because these helices carry out a function distinct from the role of H1/H2. Helices H3/H4 stabilize the HsIV complex through packing interactions involving neighboring subunits, and therefore, there is no need for them to sense and respond to HsIU binding. Furthermore, it may well be the case that the packing interactions involving adjacent stacked rings dampen the effects of mutations to H3/H4, providing a structural rational for the observed directionality of mutational effects from H1/H2 to the active site region of the protease, despite the inherent symmetry within a given HsIV subunit.

To explore HsIV plasticity in more detail, we have performed methyl-TROSY  $^{13}\text{C}$  and  $^1\text{H}$  relaxation dispersion experiments that are sensitive to millisecond timescale dynamics. Large dispersion profiles are found at many sites (Fig. 5), including residues L88 and L91, where mutation leads to a 10-fold increase (L88) or decrease (L91) in  $k_{\text{cat}}/K_M$ . Previous biochemical studies have shown that the proteolytic activity of HsIV can be enhanced several orders of magnitude through the binding of HsIU (8), suggesting that the major state of the isolated protease in solution corresponds to a proteolytically repressed conformation. We were, thus, interested in testing whether changes in activity observed for the various HsIV mutants could be explained in terms of a shift of relative populations of conformers interconverting between repressed and derepressed states. To this end, we compared relaxation dispersion profiles from experiments recorded on WT, L71V (20-fold more active), and L91I (12-fold less active) HsIV. As with the WT protein, a large number of dispersion profiles was also observed for both mutants, involving, for the most part, the same residues in all three proteins, and these profiles were fit to a two-site exchange model. Values of  $p_E = 2.6 \pm 0.3\%$  and  $1.6 \pm 0.3\%$  were obtained for L71V and L91I mutants, respectively, and were similar to  $2.4 \pm 0.1\%$  for WT HsIV. The dispersion data argue that the millisecond dynamics do not derive from exchange between inactive and active conformers, because very significant differences in  $p_E$  would then be obtained for each of the HsIV systems examined. More likely, the dispersion profiles are reporting a dynamic coupling between residues involved in HsIU and substrate binding and amino acids localized to the active sites.



**Fig. 5.** Representative single quantum  $^1\text{H}$  or  $^{13}\text{C}$ - $^1\text{H}$  multiple quantum CPMG relaxation dispersion profiles ( $R_{2,\text{eff}}$  vs.  $\nu_{\text{CPMG}}$ ) for WT HsIV (18.8T and 40 °C). Methyl-containing side chains are highlighted on the ribbon diagram and color-coded in orange in the case where  $R_{\text{ex}} > 5 \text{ s}^{-1}$ .

We have further extended the relaxation analysis to include a comparison of picosecond–nanosecond timescale motion of WT, L71V (20 times more active), L91I (12 times less active), and V138I (approximately the same as WT activity) HsIV as described in *SI Appendix*. For each residue, an order parameter squared,  $S_{axis}^2$ , that is related to the amplitude of motion of the methyl group symmetry axis was obtained (37). Values of  $S_{axis}^2 = 1$  or 0 correspond to a methyl axis that is completely rigid or disordered, respectively, whereas values  $0 < S_{axis}^2 < 1$  reflect intermediate levels of ordering. Values of  $\Delta S_{axis}^2 = S_{axis}^2(\text{mutant}) - S_{axis}^2(\text{WT})$  (*SI Appendix*, Fig. S7) are small (<0.2), indicating only small differences in methyl axis ordering between WT and mutant HsIVs. Taken together, the relaxation data indicate that both picosecond–nanosecond and millisecond timescale dynamics are similar in each of the examined mutants of HsIV; hence, the changes in activity do not derive from large global changes in motion but from structural rearrangements that propagate to active/substrate binding sites through the coupled dynamic network. This picture is consistent with and adds to the results from comparative X-ray studies (9), in which a shift in the position of G48 on binding HsIU provides stabilization of the active form of HsIV (*SI Appendix*, Fig. S4).

In summary, we have used methyl-TROSY NMR spectroscopy to show that the HsIV protease is an inherently plastic molecule that is poised to undergo a conformational change on HsIU binding, leading to activation. Key residues at the binding interface serve as hot spots in the sense that their mutation leads to changes in structure as reported by chemical shift perturbations and concomitantly, changes in proteolysis rates. Many of these residues participate in a concerted millisecond timescale dynamic process involving regions of structure linking HsIU binding

with substrate binding and proteolysis. Dynamics, thus, facilitate an allosteric pathway that communicates HsIU binding to sites of proteolysis in HsIV, leading to increased activity. The important role of plasticity that is established here and by earlier X-ray studies of HsIV (9) is also seen with the 20S CP proteasome (20), suggesting that both structure and dynamics are critical components for the function of these barrel-like protease systems.

## Materials and Methods

Proteins were expressed as described in *SI Appendix*. All NMR experiments were performed at 40 °C using 18.8T or 14.0T Varian Inova spectrometers equipped with room temperature (18.8T) or cryogenically cooled (14.0T) pulsed-field gradient triple resonance probes. Sample concentrations varied from 300 μM to 2 mM in HsIV (subunit) concentration, depending on the application. HsIV protein was dissolved in a buffer of 75 mM KCl, 1 mM EGTA, 5 mM MgCl<sub>2</sub>, 20 mM Hepes (pH 7.6), and 0.05% azide in 99.9% D<sub>2</sub>O (0.1% H<sub>2</sub>O, vol/vol) for all experiments except the pH titration, where a mixture of buffering reagents was used, including 25 mM monosodium citrate, 25 mM dipotassium phosphate, 25 mM Tris, 25 mM boric acid, 1 mM EGTA, 5 mM MgCl<sub>2</sub>, and 0.05% azide. Additional experimental details are provided in *SI Appendix*.

**ACKNOWLEDGMENTS.** We thank Professor J. Forman-Kay for valuable discussions, A. Velyvis for the production of methyl-labeled threonine, R. Muhandiram for NMR support, and Professor R. Melnyk for use of the microplate reader. L.S. is a recipient of postdoctoral fellowships from the Province of Ontario and the Canadian Institutes of Health Research. L.E.K. holds a Canada Research Chair in Biochemistry. This work was funded by research grants from the Canadian Institutes of Health Research and Natural Sciences and Engineering Research Council (to L.E.K.).

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