



Cross-Monomer Substrate Contacts Reposition the Hsp90 N-Terminal Domain and Prime the Chaperone Activity

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The ubiquitous molecular chaperone Hsp90 plays a critical role in substrate protein folding and maintenance, but the functional mechanism has been difficult to elucidate. In previous work, a model Hsp90 substrate revealed an activation process in which substrate binding accelerates a large open/closed conformational change required for ATP hydrolysis by Hsp90. While this could serve as an elegant mechanism for conserving ATP usage for productive interactions on the substrate, the structural origin of substrate-catalyzed Hsp90 conformational changes is unknown. Here, we find that substrate binding affects an intrinsically unfavorable rotation of the Hsp90 N-terminal domain (NTD) relative to the middle domain (MD) that is required for closure. We identify an MD substrate binding region on the interior cleft of the Hsp90 dimer and show that a secondary set of substrate contacts drives an NTD orientation change on the opposite monomer. These results suggest an Hsp90 activation mechanism in which cross-monomer contacts mediated by a partially structured substrate prime the chaperone for its functional activity.

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Introduction

Molecular chaperones confer stress resistance critical for survival under harsh environmental conditions and maintain protein homeostasis under normal conditions. Beyond their role in protein folding, chaperones affect protein activation

and trafficking, facilitating the degradation of terminally misfolded proteins, and the formation and disassembly of macromolecular complexes. Hsp90 is a highly conserved member of the chaperone family and plays a unique role by its regulatory influence in eukaryotes via the activation of specific classes of substrates (also known as clients), such as nuclear receptors and kinases.¹ This broad regulatory influence is thought to underlie the potent influence of Hsp90 inhibitors on the growth of diverse cancer types.² Despite its fundamental cell biological and clinical importance, the mechanism by which Hsp90 stabilizes and remodels client proteins is not understood.

One confounding problem is that Hsp90 is large, conformationally dynamic, and undergoes dramatic structural changes upon ATP binding and hydrolysis (Fig. 1a).^{3,4,8} Small-angle X-ray scattering (SAXS) and electron microscopy (EM) measurements have

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Abbreviations used: NTD, N-terminal domain; MD, middle domain; CTD, C-terminal domain; SAXS, small-angle X-ray scattering; EM, electron microscopy; FRET, fluorescence resonance energy transfer; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser enhancement; AMPNP, β,γ -Imidoadenosine 5'-triphosphate.

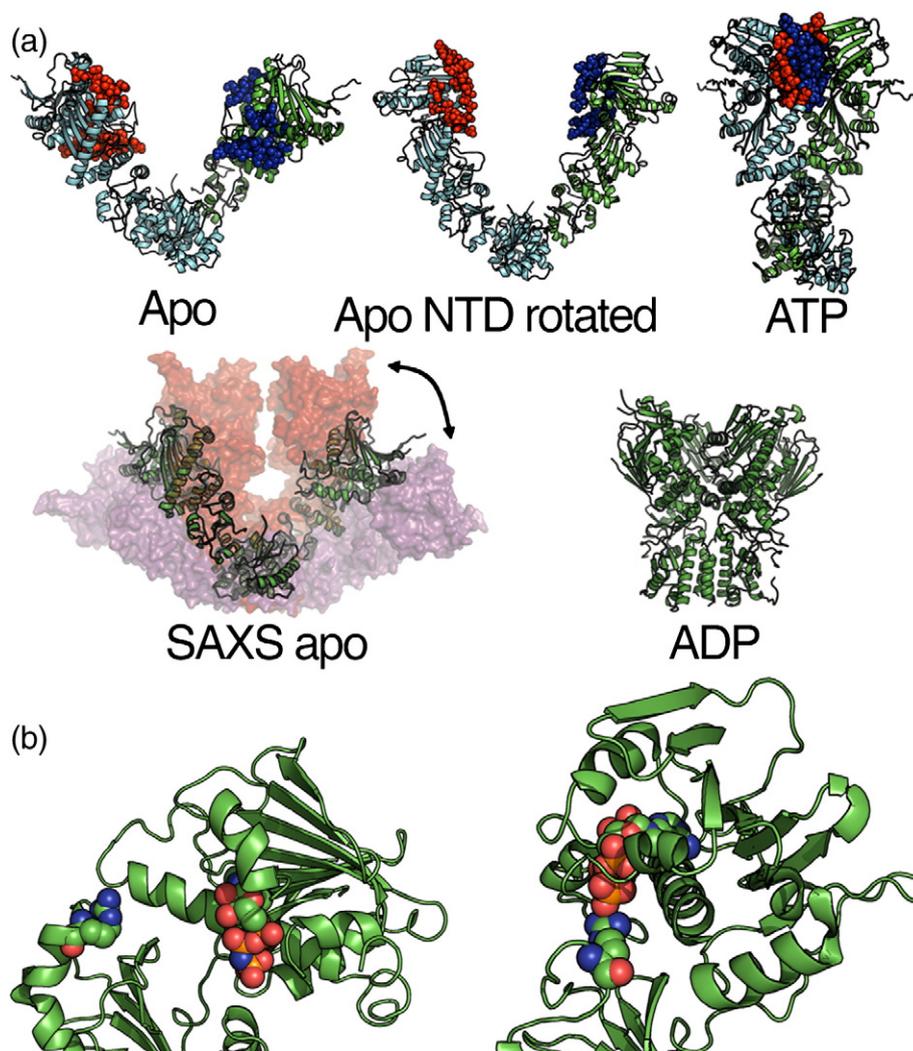


Fig. 1. Hsp90 conformational flexibility. (a) The HtpG apo crystal structure (left³) requires significant structural plasticity to reach the closed ATP state (right⁴). The contacts formed in the closed conformation (red and blue spheres) are significantly out of alignment in the apo structure, prior to NTD rotation (middle). Shown below are the apo solution conformations of HtpG determined by SAXS^{5,6} and the compact ADP state.⁷ (b) The NTD orientation in the Grp94 crystal structure⁸ shows the nucleotide positioned far from the highly conserved arginine (spheres), whereas in the closed conformation, the ATP γ -phosphate makes a direct contact.⁴

revealed an underlying complexity of Hsp90's conformational dynamics.^{5-7,9-11} The Hsp90 monomer is composed of three stable domains [N-terminal domain (NTD), middle domain (MD), and C-terminal domain (CTD)], and conformational flexibility results from their rigid-body-like rearrangement. Under apo conditions, a weak MD/CTD interface allows for a wide range of arm-arm geometries that can be influenced by pH and osmolyte conditions.^{6,12} This striking flexibility has been observed for highly diverse Hsp90 homologs^{7,9} and is postulated to be critically important to Hsp90's ability to recognize a remarkably diverse set of client proteins.

Unlike other molecular chaperones, Hsp90 appears to prefer largely folded but nonnative states. This poses an additional practical challenge, as such

states can be difficult to populate and are prone to aggregation. Previous work introduced a well-behaved model client protein, the partially folded but non-aggregating protein $\Delta 131\Delta$, a fragment of Staphylococcal Nuclease that has been studied extensively by the protein folding community.¹³ Using this model client revealed that, (i) under apo conditions, Hsp90 partially closes around $\Delta 131\Delta$; (ii) Hsp90 binds a highly structured region of $\Delta 131\Delta$; (iii) and $\Delta 131\Delta$ accelerates a nucleotide-driven open/closed transition and stimulates ATP hydrolysis by Hsp90, effectively activating the chaperone by lowering a rate-limiting conformational barrier. Taken in the context that the ligand binding domain of GR enhances the ATPase of the human Hsp90¹⁴ and that the ribosomal subunit L2 enhances the

ATPase of the bacterial Hsp90,¹⁵ this suggests that activation of the rate-limiting Hsp90 conformational transition is a conserved feature of *bone fide* Hsp90 clients, similar to Hsp70 activation by peptide substrates. However, the mechanism by which substrate binding can drive the dramatic Hsp90 open/closed transition is unknown. Indeed, a previous low-resolution SAXS analysis¹³ could not determine whether $\Delta 131\Delta$ makes cross-monomer contacts as has been observed for the activating cochaperone *aha1*¹⁶ or solely intramonomer contacts as observed for an Hsp90-cdc37-cdk4 (chaperone-cochaperone-kinase substrate) EM reconstruction.¹⁷

The Hsp90 ATPase is slow, on the order of 0.1–1 hydrolysis events per minute depending on the homolog and conditions,^{18–20} and mirrors a slow conformational change from the open apo state to the closed ATP conformation.^{13,21} This dramatic transition involves a large change in arm–arm proximity, a domain-level change in the NTD orientation, and local structural changes within the NTD (lid closure over the nucleotide binding pocket, strand exchange between NTDs) and the MD (restructuring of the catalytic loop).^{3,4,19,22} Although the relative importance of these structural changes to the closure rate is not known, the structures of the β,γ -Imidoadenosine 5'-triphosphate (AMPPNP)-bound canine Grp94 (the Hsp90 homolog specific to the ER) and the apo bacterial Hsp90 (HtpG) suggest that the NTD rotational state plays an important role. Both structures exhibit an open resting state in which the NTDs are diametrically opposed, requiring a significant conformational change to come into a closure-competent alignment.^{3,8,23} As illustrated in Fig. 1a, the required movement involves a 90° rotation and a 25-Å translation of the NTD center of mass, rearranging ~2000 Å² at the MD interface. This aligns closed-state contacts (Fig. 1a, red and blue spheres) and also repositions ATP by ~20 Å, allowing the γ -phosphate to contact a highly conserved arginine on the MD (R336 in HtpG) that is essential for both closure and ATP hydrolysis^{4,20,24} (Fig. 1b). Importantly, a full lid closure over the nucleotide pocket, which appears to be necessary for closure,²⁵ cannot occur in the NTD resting state due to a significant steric clash with the MD.⁸ These observations suggest that an NTD rotation may be involved in the timing and order of many critical steps in closure and subsequent ATP hydrolysis.

Here, we use our HtpG-activating substrate to interrogate the open/closed transition and how this process is substrate catalyzed. Key questions include (i) whether substrate contacts are within a single monomer or across monomers, (ii) defining the substrate binding region on HtpG in greater detail than could be achieved from our previous SAXS analysis, (iii) establishing whether a single set or multiple substrate contacts are utilized, and (iv) determining how substrate binding affects HtpG

structural dynamics, particularly at the NTD, and how this is related to the large energetic barrier to closure.

Results

Monitoring NTD movement by fluorescence resonance energy transfer

HtpG can be substrate activated by accelerating the kinetics of a slow open/closed structural transition required for maximal ATP hydrolysis. Previously, closure kinetics were measured by fluorescence resonance energy transfer (FRET), in which opposite monomers were labeled with donor and acceptor pairs.¹³ To monitor NTD/MD rotation, we designed a FRET pair within a single monomer. We identified residues S52/D341 on the NTD/MD that significantly change distance (22–39 Å) in the open/closed conformations yet remain solvent exposed. These sites were mutated to cysteine and labeled with Alexa Fluor 647 and Alexa Fluor 555. Since this pair is on the same monomer, a 20× excess of unlabeled wild-type HtpG was added to ensure only one labeled monomer per dimer.

Figure 2a shows the apo and AMPPNP fluorescence spectra for this FRET pair. There is significant acceptor signal in the apo state, and after a prolonged incubation with AMPPNP, there is a decrease/increase in acceptor/donor signal. Similar to previous studies, these measurements were performed at pH 9 because, for HtpG at this pH, there is a complete conversion between the open/closed state.⁶ Upon addition of AMPPNP, there is a slow time-dependent loss in acceptor signal with single-exponential kinetics (Fig. 2b) and a rate ($k=0.002\text{ s}^{-1}$) that is the same as the arm–arm closure rate measured previously.¹³ As a control, we designed a fixed-point FRET pair within the MD (residues 350 and 362), which does not change distance in the open/closed transition and confirmed that closure did not affect this FRET signal (Supplemental Fig. 1a).

The similar rates for NTD rotation and arm–arm closure suggest simple two-state cooperativity; however, given the large number of structural motions that can occur in Hsp90, this observation does not rule out other intermediates. Cooperativity can only be established by the coincidence of a large number of structural probes. To examine this possibility, we measured closure kinetics by SAXS. Previous work demonstrated that SAXS measurements can determine the conformational equilibrium of HtpG by linear combination fitting of the scattering spectra.^{5,6} Given that closure is slow, it is possible to simultaneously initiate closure on multiple samples and sequentially measure scattering at

different time points. The robotic sample loading system at the SIBYLS beamline at the Advanced Light Source (Berkeley, CA) allows for each measurement to take only ~ 2 min. An advantage of this method is that SAXS reports on all scattering positions, as opposed to the limited sites measured

by FRET. Here, we find that the kinetics of closure by SAXS match well with the arm–arm closure and NTD rotation measured by FRET (Supplemental Fig. 1b). Although these measurements do not indicate whether local sequential conformational changes occur prior to the rate-limiting step, they do indicate that the gross conformational changes associated with HtpG closure obey simple two-state cooperativity.

Given this cooperativity, we wanted to know if NTD rotation was contributing to the high-energy barrier separating the open/closed states. In other words, we wanted to know whether NTD rotation could be significantly populated in isolation on a single monomer or whether this rotation is intrinsically unfavorable and requires stabilization from NTD dimerization contacts with the opposite monomer. To test this question, we made the same NTD/MD FRET pair in the monomeric NM fragment of HtpG (residues 1–495). The acceptor/donor fluorescence spectrum on the NM fragment is similar to the corresponding spectrum on the full-length dimer (data not shown). We find that the acceptor fluorescence on the NM fragment does not undergo any net change upon addition of AMPPNP even in the presence of $\Delta 131\Delta$ (Fig. 2b), demonstrating that the NTD rotation required for closure requires stabilization from the opposite monomer. These results reveal a major energetic mismatch in the local and global energetics associated with Hsp90 closure. NTD rotation creates highly stabilizing dimer contacts in the closed state at the expense of a locally disfavored NTD/MD interface. This suggests that substrate binding could activate HtpG by relieving this rotational penalty.

To investigate this possibility, we tested whether substrate binding is linked to NTD rotation in the HtpG dimer. We first investigated how $\Delta 131\Delta$ affects AMPPNP-driven closure kinetics as monitored by NTD rotation. Previously, we observed that

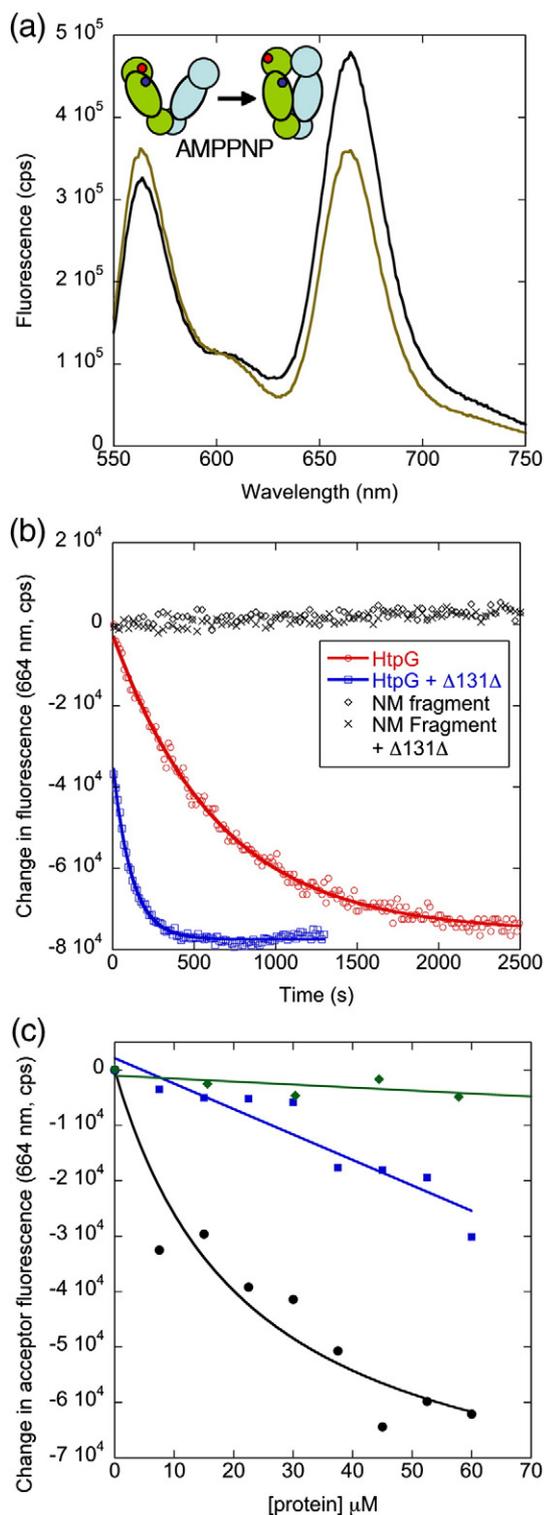


Fig. 2. Substrate binding affects an intrinsically unfavorable NTD rotation required for closure. (a) FRET measurements within a single HtpG monomer track the NTD rotation associated with closure. This rotation is reflected in the apo (black) and AMPPNP (green) fluorescence spectra. (b) Closure kinetics are monitored by the decrease in acceptor fluorescence and are significantly accelerated by $\Delta 131\Delta$; single-exponential fits are shown in continuous lines. The same FRET pair on the NM fragment shows no change in FRET from AMPPNP. (c) Heterodimers consisting of unlabeled HtpG on one arm and the NM FRET pair on the opposite arm allow for NTD rotation to be monitored under apo conditions. $\Delta 131\Delta$ binding changes the NTD orientation, as reflected by a loss in acceptor signal (black circles). The 30-residue peptide corresponding to the dominant binding region on $\Delta 131\Delta$ does not affect the NTD orientation (green diamonds). The W467A heterodimer (one arm NM FRET and second arm W467A, blue squares) shows minimal impact from $\Delta 131\Delta$.

$\Delta 131\Delta$ binding accelerated arm–arm closure kinetics 5-fold,¹³ and here, a similar acceleration of the NTD rotation is observed (Fig. 2b). The acceptor fluorescence for HtpG/ $\Delta 131\Delta$ starts at a value lower than that for HtpG, suggesting that substrate binding alone could affect an NTD rotation. Indeed, under apo conditions, $\Delta 131\Delta$ affects the NTD orientation, indicated by a loss of acceptor fluorescence (black circles, Fig. 2c) directly coupled to an increase in donor fluorescence (data not shown). These results support the idea that substrate binding in the chaperone apo state could prime Hsp90 for ATP-driven closure by affecting an NTD rotation.

Given the large surface that would be rearranged by a substrate-driven NTD rotation, we reasoned that $\Delta 131\Delta$ could affect HtpG hydrogen exchange patterns. In particular, our results suggest that substrate binding may be altering the NTD/MD interface and potentially exposing previously buried surfaces, which should show increased hydrogen exchange. Also, substrate binding itself has the potential to protect regions of HtpG from exchange. To test these predictions, we performed HX-MS measurements on HtpG and HtpG/ $\Delta 131\Delta$. The methodology, described previously for HtpG alone,²⁶ involves rapid dilution into D₂O and allowing exchange for 30 s. H–D exchange is quenched by lowering temperature and pH, and proteolytically digested fragments are then separated and analyzed by a combined HPLC-MS setup.

The effect of $\Delta 131\Delta$ on HtpG H–D exchange shows a striking pattern (Fig. 3a). Regions at the MD/CTD become protected (blue spheres, Fig. 3b), while regions at the NTD/MD interface, as well as a patch at the MD/CTD interface, show increased exchange (red spheres). Two regions at the NTD/MD that become deprotected undergo large rearrangements and become significantly more exposed upon NTD rotation (residues 246–277 and 191–206). These results further support a model in which substrate binding results in an NTD rotation. $\Delta 131\Delta$ -induced HtpG protection (blue spheres, Fig. 3b) is observed in both the CTD and MD, centered at the base of the dimer cleft. This area contains residues 543–565, which are disordered in the apo crystal structure (broken lines, Fig. 3b). In the isolated CTD structure, this region adopts an amphipathic helix, postulated to be involved in substrate interactions.²⁷ Although these results may suggest that $\Delta 131\Delta$ binding extends to the CTD dimer cleft, it is not known whether the dominant source of $\Delta 131\Delta$ -induced protection is from an increase in structure of the amphipathic helices or by a direct interaction.

To distinguish between these scenarios, we measured a ¹⁵N heteronuclear single quantum coherence (HSQC) spectrum of the isolated CTD alone and with $\Delta 131\Delta$ and observed a small number of binding-induced chemical shifts, suggesting the

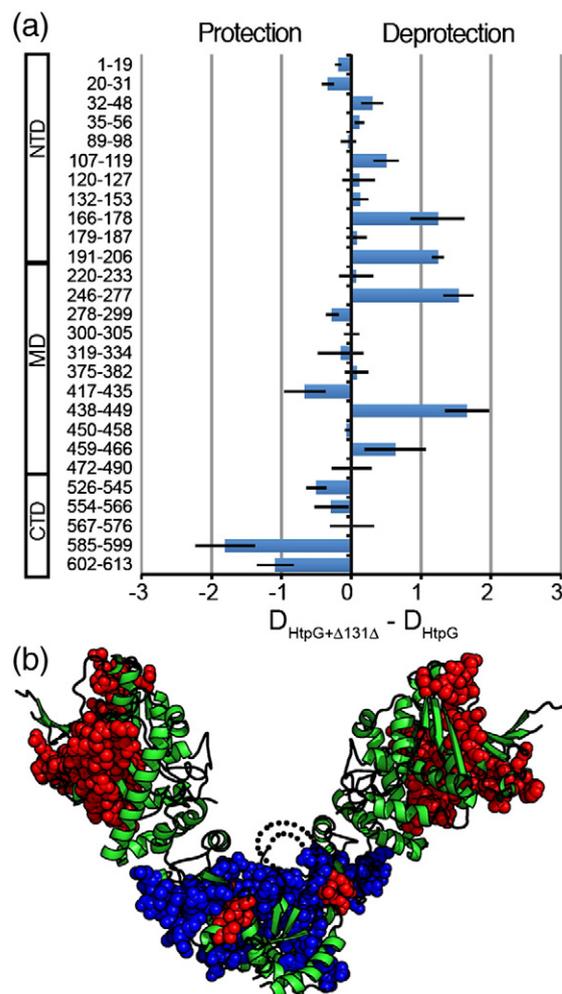


Fig. 3. Substrate binding affects hydrogen exchange patterns across the HtpG structure. (a) Difference values of deuterons incorporated into HtpG after 30 s of exchange in D₂O reflect the structural impact of $\Delta 131\Delta$ binding. Black bars represent the standard error on the mean of three independent measurements. (b) The influence of $\Delta 131\Delta$ on HtpG exchange shows protected regions at the MD/CTD (blue spheres), whereas regions at the MD/NTD interface show increased exchange (red spheres). The $\Delta 131\Delta$ -induced protection at the base of the dimer cleft lies near a region that is disordered in the crystal structure (broken lines).

hydrogen exchange protection has a contribution from a direct interaction (Supplemental Fig. 2). As described below, we also identify a $\Delta 131\Delta$ binding region on the interior of the dimer cleft on the MD, which suggests that $\Delta 131\Delta$ binding may span both the MD and CTD.

Identifying a substrate binding region on the MD

Previous SAXS measurements suggested an MD binding region; however, the measurements were

too low resolution to determine a residue-level surface. To gain this insight, we next used NMR and mutagenesis. The HtpG MD (residues 231–495, 31 kDa) can be purified and ^{15}N labeled for NMR

studies, but the HSQC spectrum is rather crowded (Supplemental Fig. 3a), and the residue assignments are unknown. Given that the structure of the MD is known, we explored an approach utilizing selective

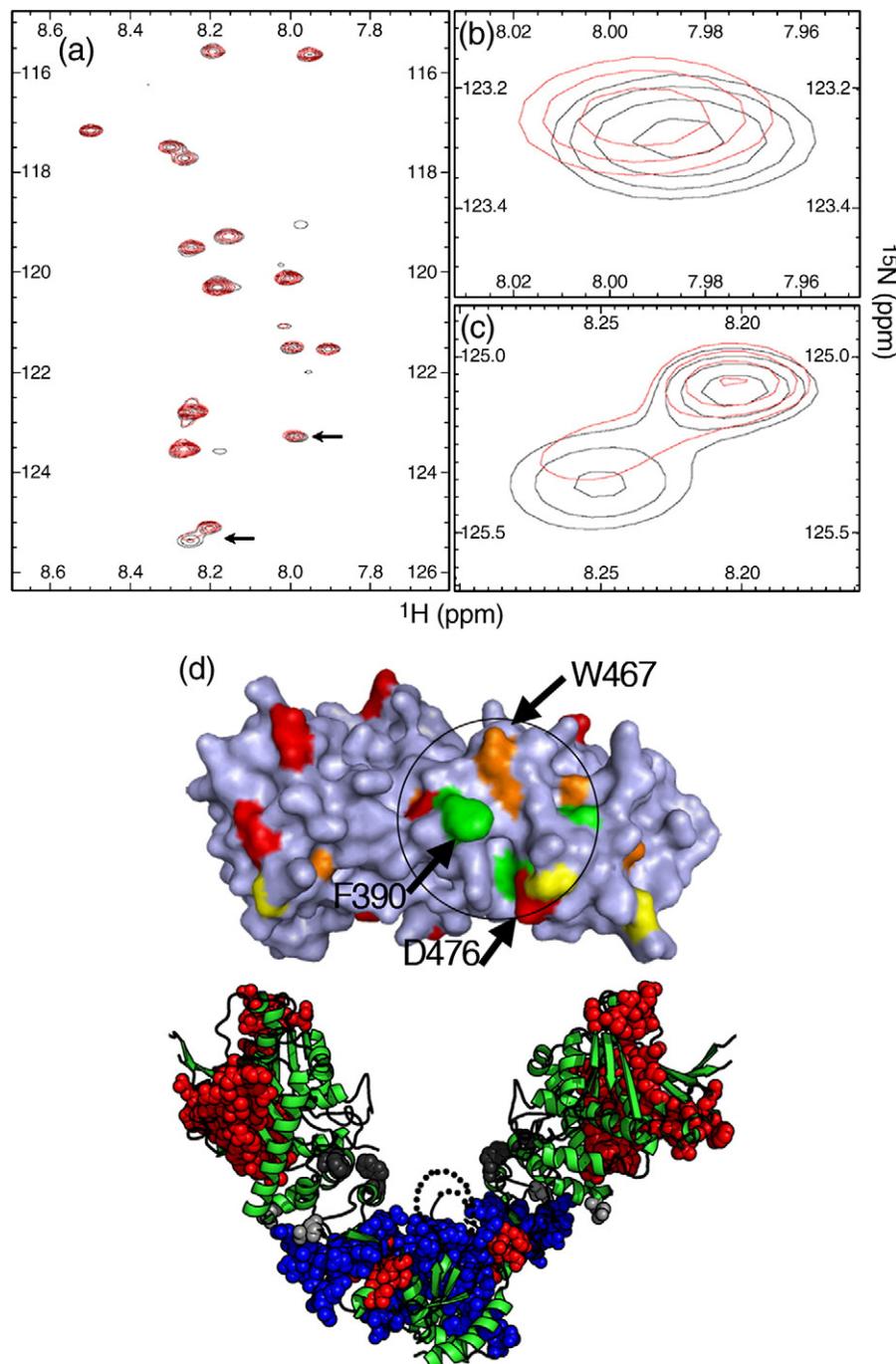


Fig. 4. Mapping a substrate binding location on the HtpG MD. (a) The HtpG MD with ^{15}N -labeled Asp residues shows a dramatic simplification in the HSQC spectrum. (b and c) Both peak intensity and chemical shift changes are observed from $\Delta 131\Delta$. (d) The MD in surface representation with a patch that contains Phe (yellow), Tyr (orange), Asp (red), and Gly (pink). Mutation sites to test this proposed substrate binding region are shown on the apo state HtpG structure (W467, F390, and D476). The mutations that strongly affect substrate binding (dark gray: W467 and F390) lie within the dimer cleft close the regions that show HX protection from $\Delta 131\Delta$ (blue spheres). The charge reversal mutations that make a marginal impact on binding are shown in light gray (D476K and E369K).

amino acid labeling to simplify the process of identifying a $\Delta 131\Delta$ binding location. Specifically, the Volker Doetsch laboratory introduced a method whereby multiple HSQC spectra are collected, each one corresponding to a single type of amino acid being ^{15}N labeled. Through counting of the number of peaks that are affected by the substrate, it is possible to identify one or more patches on the structure that have the correct number of affected residues. Iterating this process with different labeled amino acids can identify a unique region that has correct surface residue composition of the binding site.²⁸ Although this method is advantageous because single amino acid labeling greatly simplifies the HSQC spectra, it only provides a predicted binding region so the results must be independently tested.

We produced four variants of the HtpG MD specifically ^{15}N labeled on Asp, Phe, Tyr, and Gly residues. These residues have an asymmetric distribution over the MD, suggesting the potential to uniquely identify a binding region. $\Delta 131\Delta$ affects both the chemical shifts and intensities for a subset of the labeled residues on the MD; an example with Asp is shown in Fig. 4a–c. We measured chemical shifts and intensity changes, normalized them, and defined their mean and standard deviation. The peaks that were significantly impacted were counted by defining a significance threshold for each amino acid type (see Methods).

This process identified two Phe, three Tyr, two Asp, and one Gly, in the predicted binding region (Supplemental Fig. 3b–i). Inspection of the MD shows a patch facing into the HtpG dimer cleft in the apo state with this surface residue distribution (Fig. 4d), whereas the opposite face shows no such site (Supplemental Fig. 3j). As a test, we mutated three residues within this patch (positive predictions: W467A, F390A, and D476K) and three analogous mutations outside of this patch (negative predictions: W224A, F257A, and E369K). We included charge reversal mutations because the strong salt dependence of $\Delta 131\Delta$ binding suggested an electrostatic contribution.

Using a previously described fluorescence polarization binding assay with IAEDANS-labeled $\Delta 131\Delta$, we measured the binding K_d of these variants (wild-type HtpG has a K_d of 9 μM). The hydrophobic truncations have significantly reduced binding (W467A, 42 μM ; F390A, 38 μM), while the negative predictions are minimally affected (W224A, 11 μM ; F257A, 10 μM), which confirms that $\Delta 131\Delta$ binds to the HtpG interior cleft at the MD. Both the positive and negative prediction charge reversal mutations show an intermediate reduction in binding (D476K, 19 μM ; E369K, 21 μM); one explanation may be that long-range electrostatic interactions between HtpG and $\Delta 131\Delta$ contribute to binding. This would be consistent with the significant

difference in pI between HtpG (5.1) and $\Delta 131\Delta$ (9.5) and also consistent with recent studies of unfolded citrate synthase binding to Hsp90.²⁹ However, if long-range electrostatics are playing a role, then neither mutation (D476K and E369K) is a reliable test for direct binding. Therefore, we used

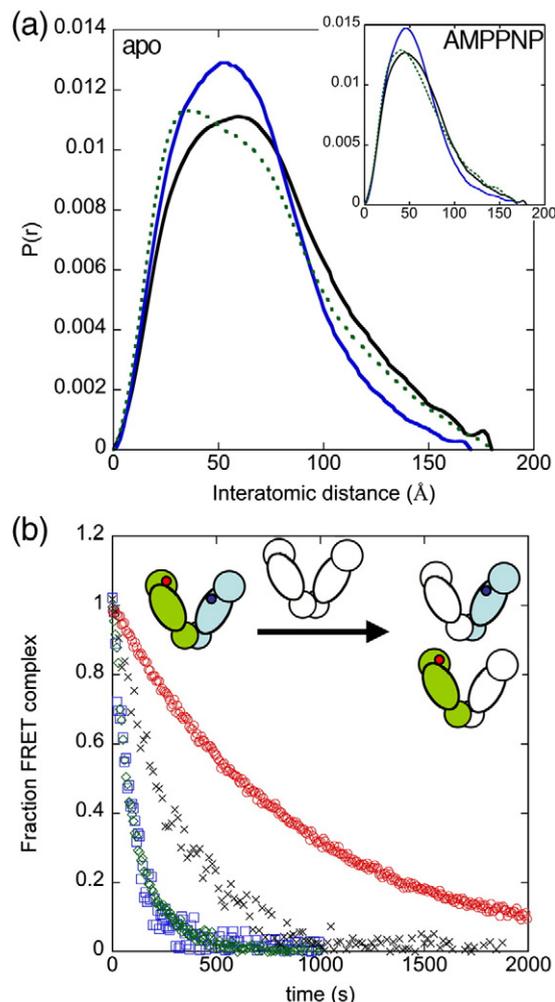


Fig. 5. Cross-monomer substrate contacts are coupled to HtpG conformational changes. (a) The W467A mutation reduces substrate-induced conformational changes in HtpG under apo conditions as measured by SAXS. Here, the wild-type/ $\Delta 131\Delta$ scattering distribution (blue) is significantly more contracted than W467A/ $\Delta 131\Delta$ (black). For reference, the HtpG spectrum in the absence of $\Delta 131\Delta$ is shown in broken lines. Similarly, the W467A mutation reduces substrate-induced conformational changes in HtpG under AMPPNP conditions (inset). Both HtpG and $\Delta 131\Delta$ are at 50 μM . (b) Monomer exchange kinetics were measured by the loss of FRET that results from adding an excess of unlabeled HtpG. $\Delta 131\Delta$ clearly slows exchange (red circles), whereas the peptide (green diamonds) is similar to the HtpG monomer exchange rate in the absence of substrate (blue squares). The $\Delta 131\Delta$ construct that lacks the 30 C-terminal residues has only a modest affect on exchange (black crosses).

the W467A and F390A variants to investigate the relationship between substrate binding and HtpG conformational changes.

First, as a control, we tested the impact of these mutations on $\Delta 131\Delta$ -induced conformational changes in HtpG. For reference, our previous SAXS measurements revealed that substrate binding is coupled to large-scale conformational changes in HtpG under both apo and AMPPNP conditions;¹³ therefore, we expected that by disrupting substrate binding, these large conformational changes should be reduced. The $\Delta 131\Delta$ -induced conformational changes in HtpG are visibly evident in the contracted SAXS $P(r)$ spectrum, which reflects the combined set of scattering distances within the complex. In contrast to wild-type HtpG, the W467A mutant has a significantly reduced conformational change from $\Delta 131\Delta$ under both apo (Fig. 5a) and nucleotide conditions (inset), confirming that the reduction in substrate binding is directly translated in a reduction in the chaperone conformational response. The W467A mutation itself does not affect the HtpG conformation (Supplemental Fig. 4a). Similar results were observed for the F390A mutation, although the mutation itself resulted in a change in the conformational state of HtpG (data not shown).

A secondary set of cross-monomer substrate contacts activates HtpG

A crucial mechanistic distinction concerning $\Delta 131\Delta$ activation of HtpG is whether the substrate activates from within a monomer or across monomers and whether there are single or multiple contacting regions of the substrate. As discussed below, we addressed these questions in three ways: (i) a heterodimer analysis with the W467A variant and NM FRET measurements, (ii) HtpG monomer exchange measurements, and (iii) by studying different fragments of $\Delta 131\Delta$.

Beyond identifying a substrate binding region in greater detail, the W467A mutation provides an opportunity to form heterodimers of HtpG where one arm contains the NM FRET pair, and the opposite arm is either wild-type HtpG or the W467A mutant. This type of heterodimer experiment has been used previously to identify cross-monomer determinants of Hsp90 hydrolysis rate^{20,25} and cochaperone activation.¹⁶ As discussed earlier, under apo conditions, $\Delta 131\Delta$ affects an NTD rotation in the wild-type heterodimer (one arm NM FRET, second arm wild-type HtpG) as seen from a concentration-dependent loss of acceptor fluorescence (black circles, Fig. 2c). In contrast, for the W467A heterodimer (one arm NM FRET, second arm W467A), $\Delta 131\Delta$ only has a modest impact on the NM FRET (blue squares, Fig. 2c). This result shows that substrate binding at the MD of one arm is directly coupled to the NTD rotation on the opposite

arm. Since the heterodimer has a wild-type MD on the FRET-labeled monomer, $\Delta 131\Delta$ should make a modest acceleration of closure by its impact on the opposing NTD. Indeed, in contrast to the 5-fold acceleration of AMPPNP-mediated closure observed for wild-type HtpG, $\Delta 131\Delta$ only accelerates the W467A heterodimer by a factor of 2 (Supplemental Fig. 4b). W467A heterodimers have a similar intrinsic closure rate as the wild-type heterodimers (data not shown).

A second test for cross-monomer contacts is that $\Delta 131\Delta$ should slow the rate of HtpG monomer exchange. Here, we used a FRET-based assay developed in the Buchner laboratory.^{16,21} In this experiment, Hsp90 heterodimers are labeled with donor and acceptor fluorophores on opposite arms, with a resulting FRET signal that can be extinguished by adding an excess of unlabeled wild-type Hsp90 (shown schematically in Fig. 5b). Here, the loss of acceptor fluorescence occurs because monomer exchange randomizes fluorescently labeled monomers with unlabeled monomers. We observe a striking slowdown of monomer exchange kinetics in the presence of $\Delta 131\Delta$ (red circles, Fig. 5b), corroborating that cross-monomer substrate contacts are formed.

There are two models that could explain cross-monomer substrate contacts. The first model is that there is a single dominant substrate binding region that spans the Hsp90 monomers. The second model is that binding is predominantly contained within a monomer with secondary substrate contacts that span the monomers. To discriminate between these models, we investigated a limited construct that only contains the dominant binding region of the substrate. For reference, previous measurements with $\Delta 131\Delta$ suggested that there was a dominant binding region of ~ 25 residues around position 100 in $\Delta 131\Delta$; therefore, we synthesized a 30-residue peptide corresponding to residues 87–116 in $\Delta 131\Delta$. If cross-monomer contacts are due to secondary substrate contacts, then this limited construct will not rotate the NTD or slow monomer exchange. Indeed, although the peptide binds (K_d of 40 μM), it is unable to rotate the NTD (green diamonds, Fig. 2c), does not change the monomer exchange rate (Fig. 5), and has a minimal impact on the closure kinetics (data not shown). SAXS measurements under apo conditions show that HtpG still contracts upon binding the peptide (Supplemental Fig. 4c), indicating an alteration of the MD/CTD interface.

Although these results strongly suggest that multiple regions of the substrate are required to make cross-monomer contacts, rotate the NTD, accelerate closure, and subsequently activate HtpG, a potential confounding factor could be that the peptide either is misfolded or does not have a sufficient level of structure to activate the chaperone. Given that previous studies on $\Delta 131\Delta$ demonstrated that the region around residue 100 has significant

structure,^{30–32} this was a possibility we wanted to explore in detail. Therefore, we performed nuclear Overhauser enhancement spectroscopy (NOESY) measurements (Supplemental Fig. 5a) on the peptide alone. We assigned the peptide using standard methods involving total correlated spectroscopy and NOESY comparisons and a natural abundance ^{13}C - ^1H HSQC (see [Methods](#)). Inspection of the pattern of nonlocal nuclear Overhauser enhancements (NOEs) shows many $i-i+3$ and $i-i+4$ NOEs in the peptide region of the native α -helix in the wild-type structure (residues 98–107, Supplemental Fig. 5b). Weak long-range NOEs suggest modest tertiary organization. Using NOE distance constraints and dihedral constraints based on $\text{C}^\alpha/\text{C}^\beta$ chemical shifts with the DANGLE program,³³ we determined an ensemble of compatible structures with the ARIA program³⁴ (Supplemental Fig. 5c). The peptide structure ensemble reveals a helical region centered on the native α -helix and adjacent N- and C-terminal loops that loosely interact. The central helical region is not present in all members of the ensemble, indicating that the helix is a folding equilibrium, consistent with early $\Delta 131\Delta$ studies.³⁰ This result shows that the dominant substrate region recognized by Hsp90 has a moderate level of structure and that the peptide is not misfolded.

Finally, we wanted to investigate the location of the secondary contacts on the substrate. As shown here, Hsp90 binds to a locally structured region of $\Delta 131\Delta$ around residue 100, and our results show that secondary contacts from this region are required for cross-monomer contacts that affect an NTD rotation, which primes the chaperone for ATP-driven closure. These findings are consistent with our previous NMR measurements that suggested a secondary binding site at the $\Delta 131\Delta$ C-terminus.¹³ To test whether the C-terminal region indeed makes secondary contacts, we investigated a $\Delta 131\Delta$ variant in which the 30 C-terminal residues are removed (residues 111–141). Indeed, this construct only results in a modest slowdown of HtpG monomer exchange (Fig. 5b) and has a minimal impact on the closure kinetics (data not shown). Although these results do not exclude a synergistic contribution from the $\Delta 131\Delta$ N-terminal region, secondary contacts from C-terminal region of $\Delta 131\Delta$ clearly play a central role in Hsp90 activation.

Discussion

The Hsp90 ATPase is required for *in vivo* function^{35,36} and is regulated by numerous

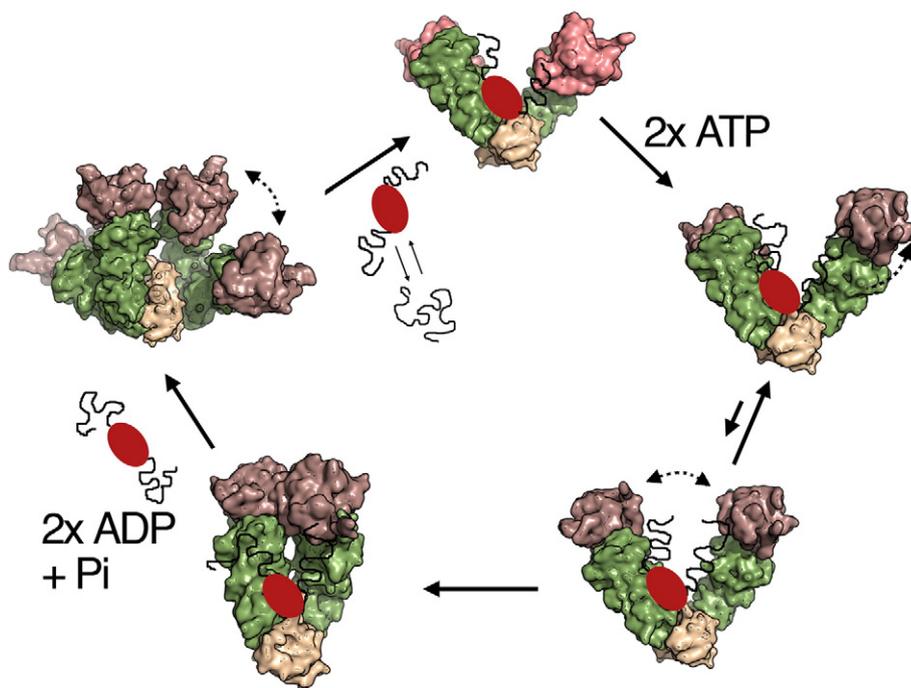


Fig. 6. Model of NTD rotation in substrate activation of Hsp90 ATP hydrolysis cycle. Substrate binding activates Hsp90 by an asymmetric mechanism in which NTD domain rotation makes a significant contribution to the rate-limiting step in Hsp90 closure and ATP hydrolysis. The dominant unit of structure recognized by Hsp90 is a locally structured region (green oval) and is in a local folding equilibrium. However, secondary contacts outside this structured region are required for NTD rotation and acceleration of closure. Although the substrate is shown bound for the entire cycle, given the low affinity of $\Delta 131\Delta$ and the Hsp90 long closure time, many bind and release steps are likely occurring during the course of the cycle.

cochaperones in eukaryotes.³⁷ Previous work with the model client $\Delta 131\Delta$ demonstrated that substrate binding can also regulate the activity of HtpG,¹³ similar to reports on human Hsp90 activation by the ligand binding domain of the glucocorticoid receptor¹⁴ and an *Escherichia coli* ribosomal protein L2 that activates HtpG.¹⁵ Elucidating the Hsp90 functional mechanism requires an understanding of how the chaperone can be activated by substrates. Here, we have focused on NTD structural motions in the HtpG conformational cycle, identifying a client binding region on the MD, and establishing that multiple regions of the substrate make cross-monomer contacts required for HtpG activation (Fig. 6).

This proposed activation of HtpG by $\Delta 131\Delta$ has a parallel with the mechanism of the activating cochaperone Aha1 on the yeast Hsp90.¹⁶ Those authors demonstrate that cross-monomer Aha1 contacts prime Hsp90 for closure and subsequent hydrolysis, while FRET measurements suggested that Aha1 could affect NTD orientation.²¹ There is a second interesting parallel between our HtpG/ $\Delta 131\Delta$ findings and cochaperone-stabilized Hsp90 conformations. Recent EM measurements of human Hsp90 demonstrated that the cochaperone Hop (involved in substrate loading from Hsp70) induces a partial closure of Hsp90 and fully rotates both NTDs into a closure-competent orientation.³⁸ Previous measurements on $\Delta 131\Delta$ showed that substrate binding induces a partial closure of HtpG in the apo state,¹³ similar to the Hop-stabilized conformation. These similarities suggest that Hop stabilizes an Hsp90 conformation that is naturally predisposed for substrate binding, subsequent chaperone closure, and ATP hydrolysis.

Comparison of our results with an EM reconstruction of an Hsp90-cdc37-cdk4 complex¹⁷ reveals differences between these substrates. Modeling suggested the kinase substrate, cdk4, binding both the NTD and MD on a single monomer, in contrast to the cross-monomer contacts we observe. In the Hsp90-cdc37-cdk4 complex, the substrate-bound NTD is in a closure-competent conformation, while the other monomer, bound to cdc37, is hinged away. While the primary $\Delta 131\Delta$ interaction is with the MD, one possibility is that secondary substrate contacts are made to the NTD. This would be consistent with early studies indicating that Hsp90 has two substrate binding sites with different specificities,³⁹ an NTD site that can bind short unstructured peptides and a CTD/MD site that can bind partially folded substrates.⁴⁰ Given that our HX-MS measurements do not show significant $\Delta 131\Delta$ -induced protection at the NTD, if $\Delta 131\Delta$ is contacting the NTD, these contacts are likely transient.

Recent studies have identified that the ribosomal subunit L2 activates HtpG, similar to the effect from $\Delta 131\Delta$; however, L2 is unable to activate the yeast Hsp90 homolog.¹⁵ Interestingly, we find that $\Delta 131\Delta$

does not accelerate closure for the yeast Hsp90 but does accelerate closure for human TRAP1, the mitochondria-specific Hsp90 homolog (T.O.S. and L.A.L., unpublished observations). Although there is very strong evidence that the conformational states of Hsp90 are highly conserved, the degree to which substrates accelerate conformational transitions for Hsp90 homologs appears to be variable.

Numerous studies have established a conserved Hsp90 mechanism in which structural rearrangements resulting in NTD dimerization organizes the catalytic machinery required for ATP hydrolysis.^{4,7,21,25,41} SAXS and EM studies have shown that rigid-body motions between the NTD/MD and the MD/CTD can account for the wide range of Hsp90 conformational states.⁵⁻⁷ The extreme apo state flexibility arises from a weak coupling between the MD and CTD, allowing for a wide range of arm-arm geometries. In contrast, here we find that the NTD/MD rotation required for closure is a high-energy state. The closed conformation, although stable, comes with a cost of adopting an unfavorable NTD/MD interface. However, our measurements do not indicate whether the NM rotation has a large kinetic barrier in addition to an unfavorable equilibrium constant, nor do our measurements assess the degree to which the NM rotation is unfavorable, in terms of kilocalories per mole. Also, it is not known whether $\Delta 131\Delta$ affects a discrete NTD rotation or whether substrate binding weakens the NTD/MD interface, resulting in an ensemble of domain orientations. Further studies are needed to address these questions.

Methods

HtpG, variants of HtpG, and $\Delta 131\Delta$ were purified as described previously.^{5,30} A peptide corresponding to residues 87-116 in $\Delta 131\Delta$ was synthesized (Genemed Synthesis), HPLC purified and confirmed by mass spectrometry. A similar peptide was synthesized with a C-terminal cysteine and labeled with IAEDANS for fluorescence anisotropy measurements. Hydrogen exchange mass spectrometry measurements²⁶ were performed at pH 7.5, 25 mM Tris, 25 mM KCl, and 5 mM MgCl₂. The closure, monomer exchange, and NM FRET measurements were performed at pH 9.0, 25 mM Tris, 50 mM KCl, and 5 mM MgCl₂, 25 °C.

Fluorescence measurements

Fluorescence anisotropy on IAEDANS-labeled $\Delta 131\Delta$ was measured on a Jobin Yvon fluorometer. Excitation and emission monochromator slits were both set to 5 nm, and an integration time of 2 s, and excitation/emission wavelengths of 340/480 nm. The NTD/MD FRET pair (S52C/D341C) was labeled with a 5-fold molar excess of Alexa Fluor 647 and Alexa Fluor 555 (Invitrogen) for 3 h at room temperature and quenched with β -mercaptoethanol. Measurements were performed with the resulting mixture of labeled species with FRET occurring between HtpG

labeled with both fluorophores. HtpG heterodimers with 250 nM labeled monomer and 5 μ M wild-type HtpG were formed by incubation for 30 min at 30 °C. Closure was initiated by 5 mM AMPPNP, either in isolation or with 50 μ M Δ 131 Δ . Excitation and emission monochromator slits were set at 2 and 3 nm, respectively. Monomer exchange measurements were performed with a previously described FRET pair at positions 62 and 341 on HtpG.¹³ Cross-monomer FRET was measured by forming heterodimers (each monomer, 250 nM) by incubation for 30 min at 30 °C. A 20 \times excess of unlabeled HtpG was added, and the loss of acceptor fluorescence was measured at 664 nm. Addition of 25 μ M Δ 131 Δ resulted in slower monomer exchange kinetics.

SAXS measurements

SAXS measurements, as described previously, were performed at the Advanced Light Source in Berkeley.^{5,6,20} The concentrations of HtpG, variants of HtpG, and Δ 131 Δ were 50 μ M. To measure closure kinetics by SAXS, we initiated closure by simultaneously adding 10 mM AMPPNP to multiple samples with a multichannel pipette. SAXS measurements were taken at varying time points on different samples to avoid radiation damage. The linear combination fitting used to determine the population of closed state has been described previously.^{5,6}

NMR measurements

HSQC measurements were performed on a Bruker Avance 800. Fully ¹⁵N-labeled HtpG MD was produced by a 10-mL overnight starter culture, washed in M9 minimal medium, and resuspended in M9 with 1 g/L ¹⁵N ammonium chloride and 0.5 g/L isogrow supplement (Sigma). Selectively labeled MD samples were produced by supplying all ¹⁴N amino acids except the labeled ¹⁵N amino acid. These were added in the following quantities in each liter of minimal media (A:500 mg, R:400 mg, D:400 mg, C:50 mg, Q:400 mg, E:650 mg, G:550 mg, H:100 mg, I:230 mg, L:230 mg, K:420 mg, M:250 mg, F:130 mg, P:100 mg, S:210 mg, T:230 mg, Y:170 mg, V:230 mg, N:300 mg, 500 mg of tryptophan was added after autoclaving the media). NMR buffer conditions were 25 mM 4-morpholineethanesulfonic acid (pH 6.0), 25 mM KCl, and 5 mM MgCl₂.

For each labeled MD sample, chemical shifts and peak intensities were measured in ccpNMR[†]. For changes in the chemical shifts, shifts in the ¹H dimension were normalized in magnitude to the shifts in ¹⁵N dimension by multiplying each shift in ¹H dimension by a ratio of mean shift changes in ¹⁵N over mean shift changes in ¹H. After this, an overall change in chemical shifts was determined in two dimensions, and an overall mean change was found for each spectrum. This mean was subtracted from the chemical shift change for each particular peak, divided by the standard deviation, and plotted to generate Supplemental Fig. 2e–h. For changes in the peak intensities, a ratio of intensities of bound *versus* unbound for each peak was calculated, and a mean and standard deviation

of all ratios for a pair of spectra were found. For generation of Supplemental Fig. 2a–d, the mean ratio was subtracted from the ratio for each particular residue and then divided by the standard deviation. Residues over a 1.5 σ threshold from the mean for either chemical shift or intensity changes were counted. Although the choice of 1.5 σ was an adjustable parameter, values significantly above and below yielded surface residue compositions for the Δ 131 Δ binding site that were incompatible with the MD surface.

NOESY measurements on 800 μ M peptide were performed on a Bruker Avance 800 with a 120-ms mixing time. The spectra were processed with NMRPipe⁴² and analyzed using ccpNMR. Structural ensembles were calculated using ARIA.³⁴

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Supplementary Data

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