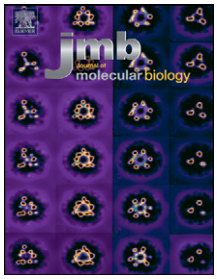

Elucidating the Mechanism of Substrate Recognition by the Bacterial Hsp90 Molecular Chaperone

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http://dx.doi.org/10.1016/j.jmb.2014.04.001
Edited by J. Buchner

Abstract

Hsp90 is a conformationally dynamic molecular chaperone known to promote the folding and activation of a broad array of protein substrates ("clients"). Hsp90 is believed to preferentially interact with partially folded substrates, and it has been hypothesized that the chaperone can significantly alter substrate structure as a mechanism to alter the substrate functional state. However, critically testing the mechanism of substrate recognition and remodeling by Hsp90 has been challenging. Using a partially folded protein as a model system, we find that the bacterial Hsp90 adapts its conformation to the substrate, forming a binding site that spans the middle and C-terminal domains of the chaperone. Cross-linking and NMR measurements indicate that Hsp90 binds to a large partially folded region of the substrate and significantly alters both its local and long-range structure. These findings implicate Hsp90's conformational dynamics in its ability to bind and remodel partially folded proteins. Moreover, native-state hydrogen exchange indicates that Hsp90 can also interact with partially folded states only transiently populated from within a thermodynamically stable, native-state ensemble. These results suggest a general mechanism by which Hsp90 can recognize and remodel native proteins by binding and remodeling partially folded states that are transiently sampled from within the native ensemble.

Introduction

A large network of highly conserved molecular chaperones protects cells against protein misfolding and aggregation. Given the diversity of the proteome, different molecular chaperones have evolved to stabilize initial or late events in protein folding and to help recover from thermal or other proteotoxic stresses. For example, some chaperones (e.g., Hsp60, Hsp70, Hsp104) use nucleotide binding and hydrolysis to drive conformational changes that modulate chaperone affinity for nascent polypeptides or largely unfolded protein substrates. Other chaperones work independently of nucleotide but can populate different assembly states (e.g., small heat shock proteins [1]) or undergo conformational changes from differing conditions, such as the redox environment (e.g., Hsp33 [2]) or low pH (e.g., HdeA [3]). Despite their abundance, the detailed mechanisms of many of these chaperones are only poorly understood.

Hsp90 is a ubiquitous nucleotide-dependent molecular chaperone. The Hsp90 dimer is composed of three stable domains [N-terminal domain (NTD), middle domain (MD), and C-terminal domain (CTD)]. Nucleotide binding occurs at the NTD and dimerization occurs at the CTD. The apo state of Hsp90 is highly flexible, where a weak interface between the MD/CTD (Fig. 1) creates a wide range of open extended states [7,8]. Addition of non-hydrolyzable ATP results in a closed state wherein the two arms

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make contact at the NTD (Fig. 1 [6]), organizing the catalytic machinery for ATP hydrolysis. In addition, a transiently populated, highly compact state has been observed by electron microscopy under ADP conditions [9]. Hsp90 has a slow rate of ATP hydrolysis, indicating that a large energetic barrier governs the chaperone activity. Although both ATPase and a common set of conformational states have been observed for Hsp90 homologs ranging from bacteria (HtpG) to human [5,10–12], the absolute rates and conformational equilibria are species specific [9]. Despite these significant advances, little is known about the relationship of Hsp90's conformational dynamics to its function as a chaperone.

Among its many functions in the cell, Hsp90 can facilitate kinase activation and nuclear receptor ligand binding by a currently unknown mechanism [13]. This modulation of native activity has suggested that Hsp90 can interact with non-native states of substrates that are late in their folding pathway. Furthermore, the ability of Hsp90 to facilitate protein–protein or protein–ligand interactions suggests that the chaperone can actively influence the conformation of bound substrates, which could alter the substrate folding process and subsequent functional outcome. Unfortunately, because partially folded states are typically sparingly populated and aggregation prone, exploring the Hsp90 mechanism in biochemical and biophysical detail has proven very challenging. A second complication is that eukaryotic cytosolic Hsp90s typically work in collaboration with a host of co-chaperones that serve to modulate the Hsp90 ATPase cycle and aid substrate binding and release. As a result, little is known about how Hsp90 recognizes specific structural states of its substrates or how the chaperone affects substrate structure and folding.

To alleviate many of the practical limitations of Hsp90/substrate studies, we have focused on a minimal system using the *Escherichia coli* Hsp90 homolog, $Hsp90_{Ec}$ (HtpG), which lacks the numerous co-chaperones and post-translational modifications found in eukaryotic Hsp90s, and have combined this with a well-studied, NMR-tractable partially folded model substrate, Δ131Δ. This truncation variant of staphylococcal nuclease (SN) is non-aggregating and compact [14] and retains some local native-like structure [15,16]. NMR order parameters [17] suggest a locally formed hydrophobic core within residues ~70–120. The presence of residual structure within Δ131Δ suggested that it could be a suitable model system to determine how Hsp90 can recognize structural features beyond short unstructured regions of polypeptide.

Previous work demonstrated that Δ131Δ binds to $Hsp90_{Ec}$, shifts the conformation of apo from an extended conformation to a V shape, and activates its ATPase. By contrast, Δ131Δ that is refolded via a tight binding inhibitor fails to interact [18]. Moreover, $Hsp90_{Ec}$ binds to the most structured region of Δ131Δ, around residues 80–115, indicating a very different strategy from Hsp70, which recognizes very short and fully unfolded protein regions. Although these findings established a basic phenomenology of an Hsp90/substrate interaction, because binding was mapped by the localized loss of heteronuclear single quantum coherence (HSQC) peaks, the underlying mechanism and structural consequences of Δ131Δ recognition remained obscure. Furthermore, these findings did not indicate whether Hsp90 could interact with thermodynamically stable proteins that have already undergone nascent folding and thus only rarely sample non-native states.

A combination of small-angle X-ray scattering (SAXS) and NMR was used to identify a Δ131Δ binding region on the MD toward the base of the $Hsp90_{Ec}$ dimer cleft [19]. More recently, the same region was identified in an *E. coli* genetic screen for $Hsp90_{Ec}$-defective mutations and then extended to the adjacent region of the CTD [20] Altogether, eight $Hsp90_{Ec}$ mutations at the MD/CTD significantly disrupt Δ131Δ binding (Fig. 1). These mutation sites cluster in the apo-state crystal structure, whereas on SAXS models of the dominant fully open conformation [7], the sites are distended and disorganized (Fig. 1). These results suggest a mode of molecular recognition in which the chaperone utilizes its conformational flexibility to position hydrophobic regions on the MD/CTD into a continuous surface. Importantly, the same region was shown to be functionally relevant for client activation by yeast Hsp90 [20], attesting to conserved underlying mechanisms for Hsp90s from prokaryotes to eukaryotes.

Here we build on this previous work to elucidate the mechanism of Hsp90/substrate recognition and determine how the chaperone affects the structure and folding of its target protein. We find that Hsp90's flexibility at the MD/CTD is critical for binding Δ131Δ and that a minimal construct of the MD/CTD is sufficient to bind a large partially folded region of Δ131Δ. Using chemical cross-linking and mass spectrometry (MS) along with computational modeling of the data, we show that the interactions between Hsp90 and Δ131Δ result in significant local and long-range structural changes within the substrate. Finally, using native-state hydrogen exchange (HX), we discover that Hsp90 can interact with partially folded states of SN that are only transiently populated under folding conditions. Together these findings provide the basis of a structural mechanism by which Hsp90 can alter the functional outcome of substrates by binding and remodeling partially folded states that are transiently sampled from the native ensemble.

Results

The positioning of Δ131Δ binding sites on Hsp90 (Fig. 1) immediately suggests a recognition mechanism in which Hsp90's flexibility around the MD/CTD

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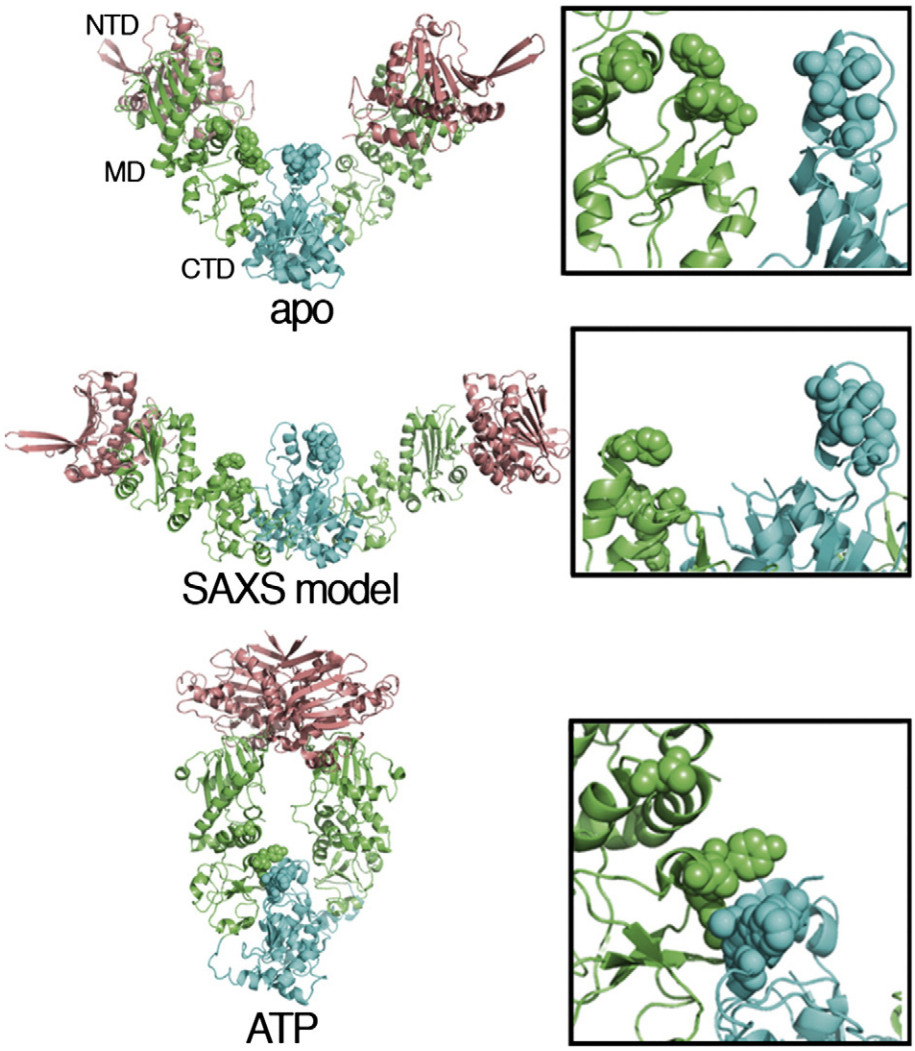

Fig. 1. (a) Hsp90 conformational heterogeneity is highlighted by dramatic conformational changes from nucleotide binding. Residues that significantly reduce Δ131Δ binding are shown in spheres at the MD/CTD interface. Positioning of these residues is significantly altered in the apo $Hsp90_{Ec}$ crystal structure [4], the SAXS model of the fully open state [5], and the closed state crystal structure of nucleotide-bound yeast Hsp90 [6].

is critical for binding. To test this idea, we investigated whether disrupting the MD/CTD interface affects substrate binding. Previous work identified a pH-dependent conformational change in $Hsp90_{Ec}$ controlled by histidine 446 buried between the MD/CTD [8]. At neutral pH, the wild-type $Hsp90_{Ec}$ adopts an equilibrium between a fully extended and a partially closed state (similar to the Grp94 crystal structure [21]). At higher pH, the fully open conformation is favored, and at lower pH, the Grp94 conformation is favored [8]. The H446E mutation pushes the equilibrium to the fully open state, which can still progress to the closed ATP state. If MD/CTD reconfiguration is required for substrate recognition, then the H446E mutation should reduce substrate binding and any associated chaperone conformational changes.

The effect of H446E on Δ131Δ binding was assessed by fluorescence polarization anisotropy [18]. Although residue 446 is not located at the known Δ131Δ binding site (Fig. 2a, see red spheres in inset), the H446E mutation effectively abrogates binding (Fig. 2a, blue squares). In addition, we used SAXS to assess whether Δ131Δ could alter the conformation of H446E as it does with wild-type $Hsp90_{Ec}$, shown schematically in the Fig. 2b inset [18]. The $P(r)$ curve, which summarizes the inter-atomic scattering distances within $Hsp90_{Ec}$, shows how Δ131Δ binding to wild-type $Hsp90_{Ec}$ (Fig. 2b, broken lines) reduces the scattering probability at long distances (>100 Å) and

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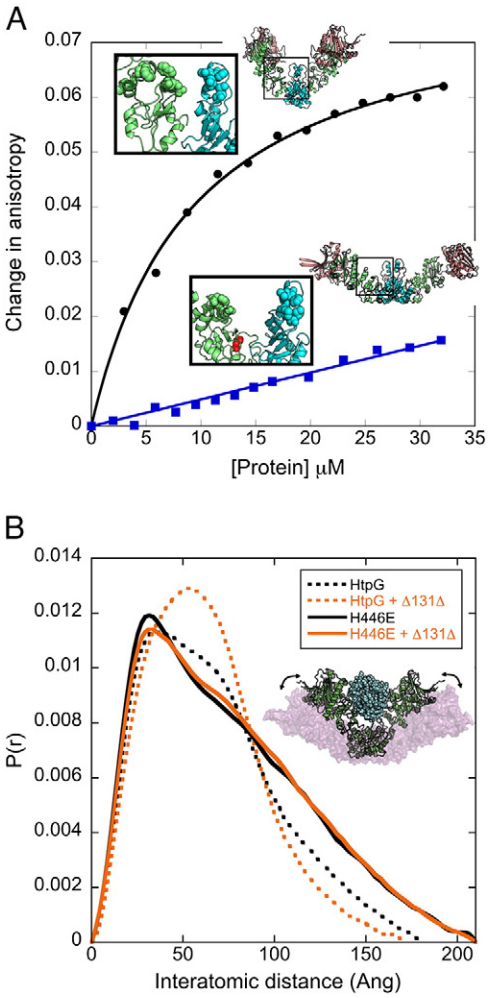

Fig. 2. (a) Binding measurements of $Hsp90_{Ec}$ (black circles) and the H446E variant (blue squares) show a dramatic difference in affinity for Δ131Δ. The insets show that the Δ131Δ binding region forms a continuous surface in the wild-type interface, but in H446E, this surface is broken. Position 446 is shown in red spheres. (b) SAXS measurements show that the conformation of $Hsp90_{Ec}$ is significantly altered by Δ131Δ [18], while the H446E variant is not significantly affected. Conditions: 25 mM Tris (pH 7.5), 25 mM KCl, and 10 mM $MgCl_2$.

increases the probability of intermediate distances. By contrast, the H446E mutation adopts a fully extended conformation as described previously [8], and, importantly, its conformation is unaffected by Δ131Δ (Fig. 2b, continuous lines). These measurements support a recognition mechanism in which structural reconfiguration at the MD/CTD is necessary for binding substrate. Next we sought to investigate whether the MD/CTD region is sufficient for binding Δ131Δ.

Previous NMR measurements indicated that $Hsp90_{Ec}$ interacts with a highly structured region of Δ131Δ (residues ~80–115 [18]). However, the large size of the $Hsp90_{Ec}$ dimer (140 kDa) and potential conformational changes within this region resulted in complete disappearance of HSQC peaks around the binding region on Δ131Δ, which precluded a more detailed description of the binding interaction. Given that a continuous MD/CTD surface is required for optimal Δ131Δ binding, we explored whether a monomeric MD/CTD fragment lacking the CTD dimerization helix (residues 383–604; referred to as MC) would allow detailed interactions to be addressed by NMR via chemical shift mapping.

In contrast to the peak losses observed previously, it is now evident that addition of MC causes chemical shifts in ^{15}N-labeled Δ131Δ (Fig. 3a). The binding interaction is in intermediate exchange with increasing MC concentrations resulting in an initially small peak movement with broadening and a subsequent appearance of a new peak (a detailed example is shown in Supplemental Fig. 1a). Some residues, such as V104, show multiple chemical shifts, suggesting modest local conformational heterogeneity. To ensure that these chemical shifts were the result of specific interactions, we measured the Δ131Δ HSQC in the presence of BSA (*b*ovine *s*erum *a*lbumin) and indeed did not observe any significant changes (Supplemental Fig. 1b). These results indicate that the MC region on $Hsp90_{Ec}$ is sufficient to bind a large partially folded region of Δ131Δ.

The change in chemical shifts in the ^{15}N and ^{1}H dimensions are plotted across the Δ131Δ sequence in Fig. 3b. The affected residues span the region (residues 80–115 on Δ131Δ) that disappeared upon binding to full-length $Hsp90_{Ec}$. In native SN, this binding region consists of the final strand of the β-barrel, β6, followed by a central helix, α2, followed by the loop connecting the C-terminal α3 helix (inset, Fig. 3b). The residues showing large chemical shift changes (e.g., $\Delta\delta^{15}N > 0.2$) (D83, Y85, G86, Y91, I92, L103, V104, G107, V114) are primarily hydrophobic. A comparison of the direction of these chemical shifts to average values for random coil, helix, and strand (Supplemental Fig. 2) suggests that the helical core and associated strands are preserved.

$Hsp90_{Ec}$ significantly alters the structure of Δ131Δ

While the abovementioned measurements indicate which residues on Δ131Δ are involved in Hsp90 binding, they do not provide a clear picture of what kinds of conformational changes take place within Δ131Δ. Resolving this by NMR represents a major challenge due to Δ131Δ conformational heterogeneity. However, we reasoned that the ability of chemical cross-linkers to trap transient states combined with tandem MS to identify the cross-linked sites could provide these structural insights. That is, we will look for changes in intramolecular cross-links that occur upon Δ131Δ binding to $Hsp90_{Ec}$ (see Methods).

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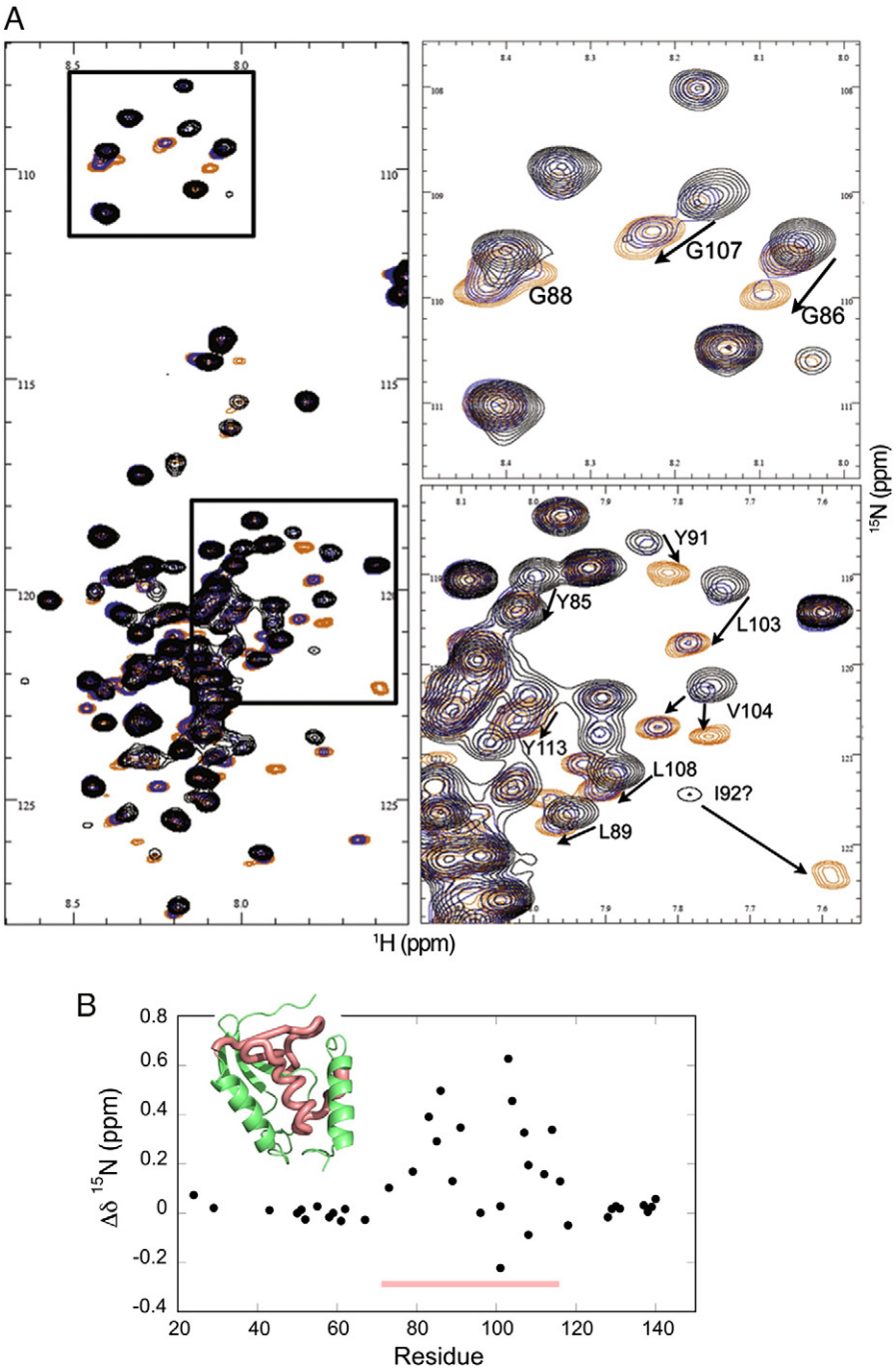

Fig. 3. (a) A two-dimensional ^{15}N HSQC NMR spectrum of Δ131Δ alone (black contours) and with increasing concentrations of MC (blue and orange contours) shows a subset of residues affected by the interaction. (b) The chemical shift changes are plotted across the sequence of Δ131Δ. Conditions: 25 mM 4-morpholineethanesulfonic acid (pH 6.0), 75 mM KCl, 10 mM $MgCl_2$ (25 °C), 150 μM Δ131Δ (black), 100 μM MC (blue), and 300 μM MC (orange).

Analyzing the complex set of cross-links is empowered by recent developments in bioinformatic tools for automatic analysis of liquid chromatography (LC)-MS/MS data to identify cross-linked peptides [22–25]. Cross-links identified within $Hsp90_{Ec}$ alone confirm that the C^{α} of linked lysine residues are within the expected maximal distance of 24 Å (DSS length, 12 Å; lysine side-chain length, 6 Å). Of the 11 cross-

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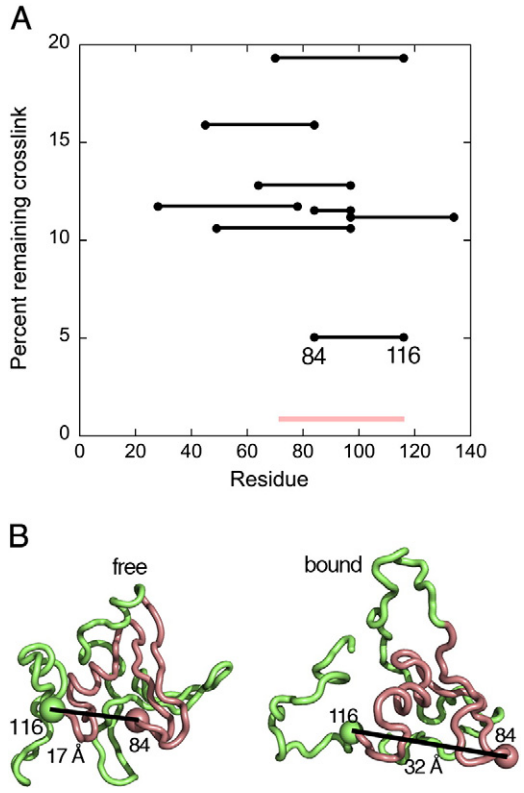

Fig. 4. (a) The changes in intramolecular Δ131Δ cross-linking due to $Hsp90_{Ec}$ are most evident in binding site identified by NMR. The cross-link most affected by $Hsp90_{Ec}$ spans residues 84:116 in Δ131Δ. (b) Modeling of the cross-linking data resulted in collections of bound and free structures. Structures from the top scoring model show a general expansion upon binding $Hsp90_{Ec}$ and associated exposure of the chaperone binding sites.

linked lysines (Supplemental Table 1), all had C^{α}/C^{α} distances less than 20 Å (average: 14.1 ± 4.3), indicating structural integrity of the complex under our cross-linking conditions.

Many intramolecular cross-links were observed within Δ131Δ alone (31 total), consistent with its large number of lysine residues and its conformational flexibility. The relative abundance of each cross-link was quantified by ion intensity, with repeated measurements showing similar results (Supplemental Table 1). The Δ131Δ sample incubated with $Hsp90_{Ec}$ contained many of the same cross-linked lysine pairs, except a subset were significantly reduced (to <20% of peak intensity in the absence of $Hsp90_{Ec}$). As shown in Fig. 4a, the yield of 84:116 cross-link was the most reduced (95% reduction). Notably, this position spans the dominant binding region on Δ131Δ, suggesting that $Hsp90_{Ec}$ specifically rearranges Δ131Δ structure. All 10 of the most significantly reduced cross-links involve at least one lysine residue within the $Hsp90_{Ec}$ binding region. The long-range nature of some of these destabilized contacts suggests that $Hsp90_{Ec}$ also alters the Δ131Δ conformation distant from the immediate binding site. However, an alternative possibility could be that the change in cross-links reflects a change in the global flexibility of Δ131Δ in the bound configuration.

In an effort to quantitatively interpret the cross-linking data, we used structural modeling. Briefly, about 9×10^6 coarse-grained C^{α}-only conformations of Δ131Δ were generated with varying levels of distance restraints to allow sampling of states ranging from fully unfolded to highly native-like. Since Δ131Δ exists in an ensemble of configurations, a combination of up to four structures was used to fit the cross-linking data. Including more than four states (for both bound and free conformations) provided little improvement of the fit to the data. The modeled structures were scored by how they recapitulate the quantitative changes in cross-linking for all 31 lysine–lysine pairs (Supplemental Table 1). The result is a collection of structures that correspond to Δ131Δ alone and Δ131Δ upon interacting with $Hsp90_{Ec}$. The scores shown in Supplemental Fig. 4a show how well different bound/free structure pairs describe the cross-linking data. For the top three scoring models, an accounting of how well they describe the cross-linking data is shown in Supplemental Fig. 4b–d. Detailed structural examples of the top three scoring models are shown in Supplemental Fig. 5.

The modeling approach appears to identify $Hsp90_{Ec}$-induced structural changes. For example, inspection of the top scoring structures immediately reveals that Δ131Δ undergoes an expansion upon binding to $Hsp90_{Ec}$ (Supplemental Fig. 5). This structural rearrangement is evident in the top scoring pair of bound and free states (Fig. 4b), with the increase in distance between residues 84 and 116 reflecting the observed loss of cross-linking in the bound state. A second observation from the modeling is that $Hsp90_{Ec}$ opens the local conformation around the binding site on Δ131Δ. This opening is clear from a surface area analysis of the top three scoring models, shown in Supplemental Fig. 6, which shows a significant increase in solvent-accessible surface area at the interaction sites identified by chemical shift mapping (D83, Y85, G86, Y91, I92, L103, V104, G107, V114). The opening is most likely a direct consequence of these residues binding to $Hsp90_{Ec}$. While still qualitative, the modeling suggests that the chaperone competes for a specific set of hydrophobic contacts on the structured part of Δ131Δ; by interacting with these sites, the chaperone opens and remodels the surrounding substrate structure. Unfortunately, the lysine cross-linking information is too sparse, and the conformational variability is too large, to make additional claims about identity of specific contacts between $Hsp90_{Ec}$ and Δ131Δ.

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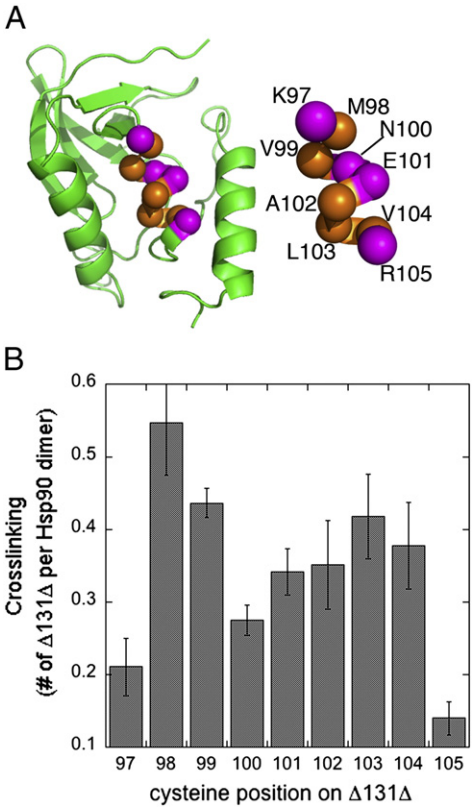

Fig. 5. (a) Residues tested in cysteine cross-linking are on the amphipathic helix in the center of the region of SN that binds to $Hsp90_{Ec}$. The helical pattern of hydrophobic residues is highlighted. (b) The degree of cross-linking across this portion of the Δ131Δ sequence shows a clear pattern in which contact with W467C is mediated by the hydrophobic face on this amphipathic helix.

As an alternative, we used scanning cysteine cross-linking across both the hydrophobic and the polar residues of the central amphipathic helix (Fig. 5a) known to be critical for Δ131Δ binding to assess interactions with $Hsp90_{Ec}$. In the native state, the hydrophobic residues (M98, V99, A102, L103, V104) are relatively buried compared to the polar residues (K97, N100, E101, R105). Therefore, if $Hsp90_{Ec}$ has no effect of the structure of this region, it would be expected that cross-linking would preferentially occur at the exposed polar sites. In contrast, if $Hsp90_{Ec}$ interacts with the hydrophobic face, and thus disrupting the local structure, then cross-linking would preferentially occur on the hydrophobic residues with a characteristic helical pattern.

As previous work had identified W467 on the Hsp90 MD as part of the Δ131Δ binding interface [19], we quantified cysteine cross-linking between a W467C mutant and each of the nine positions from 97 to 105 on Δ131Δ by integrating bands on a non-reducing gel. This clearly revealed a periodic pattern of cross-linking between $Hsp90_{Ec}$ and the hydrophobic face of the amphipathic helix (Fig. 5b). The cross-linking saturated at a ~1:1 molecular stoichiometry (dimer $Hsp90_{Ec}$:Δ131Δ). The cross-linking periodicity (two cycles over 9 residues) is close to that expected for two turns of an α-helix (7.2 residues). A modest level of mobility within the bound state is suggested by the roughly equivalent cross-linking across that the hydrophobic face on Δ131Δ. Together the abovementioned findings show how hydrophobic contacts at Hsp90's flexible MD/CTD interface can bind to and reconfigure a partially folded substrate protein.

$Hsp90_{Ec}$ interacts with transiently populated unfolded states of native full-length SN

While Hsp90 is known to facilitate folding via handoff from Hsp70, many functions of Hsp90 are believed to result from its reconfiguration of native substrates. In principle, Hsp90's recognition mechanism described above should work equally well in the folding and unfolding direction. While conventional binding studies failed to reveal interactions with full-length native SN [18], it might be possible to detect interactions between $Hsp90_{Ec}$ and transient non-native states that exist within the full-length native SN ensemble. Although SN folding is cooperative, high-energy partially folded states are sampled prior to complete unfolding [26–28]. Since HX can provide a very sensitive measure of the populations of such partially unfolded states, we used this approach to probe the ability of $Hsp90_{Ec}$ to alter the SN folding landscape.

A native SN HSQC shows that peak heights were uniformly decreased in response to increasing concentrations of $Hsp90_{Ec}$ (peaks offset for clarity, Supplemental Fig. 7), similar to that observed with the p53 client [29]. Possible explanations include a weak native SN interaction with $Hsp90_{Ec}$ resulting in peak disappearance from slow tumbling and potential conformational changes or interactions with a transiently populated SN state leading to higher populations of partially unfolded states and/or changes in folding dynamics. These possibilities can be separated by HX measurements with sub-stoichiometric quantities of $Hsp90_{Ec}$. Specifically, if $Hsp90_{Ec}$ interacts with the native state of SN, then the HX rate should decrease and stoichiometric quantities of $Hsp90_{Ec}$ will be required to observe a significant effect. In contrast, if $Hsp90_{Ec}$ interacts with unfolded or partially folded states of SN, then only a limited chaperone concentration would be required for their stabilization, which would lead to increased HX that can be measured on the free SN population.

We first measured HX on SN in the absence of $Hsp90_{Ec}$. For full-length SN, many amide protons exchange upon dilution into D_2O within the deadtime

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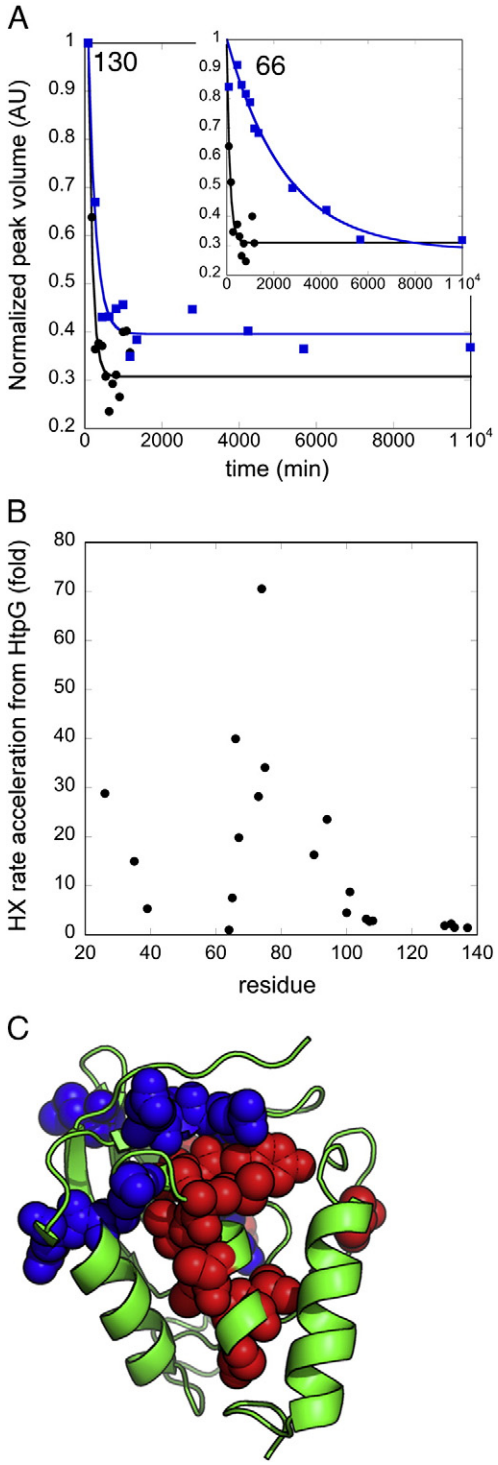
of the first HSQC reflecting fast excursions around the native ensemble. The remaining amide protons show varying levels of exchange, as has been measured previously [26–28]. For example, residues 66 and 130 show significantly different exchange rates (Fig. 6a, blue squares) consistent with subglobal unfolding events exposing residue 130 before residue 66.



HX measurements in the presence of $Hsp90_{Ec}$ show a striking change in SN exchange rates. For example, position 66 has dramatically accelerated exchange whereas residue 130 is not significantly altered (black circles, Fig. 6a). Only a sub-stoichiometric amount of $Hsp90_{Ec}$ (30 μM for 150 μM SN) is needed to observe this acceleration, indicating that the $Hsp90_{Ec}$ interaction is with a transiently populated partially folded state of SN. We used 30 μM $Hsp90_{Ec}$ as this is significantly above the K_d for binding Δ131Δ (~10 μM); thus, any unfolded SN states can be readily bound by $Hsp90_{Ec}$.

We quantified the HX acceleration due to $Hsp90_{Ec}$ (Fig. 6b) and found significant acceleration starting at residue ~20, becoming maximal between residues 60 and 80, and extending out to residue 100. There were no significant changes at the C-terminus of SN. Residues with HX acceleration greater than 10× are shown on the native SN structure in blue and residues with large chemical shifts or residues with significant cysteine cross-linking are shown in red (Fig. 6c). These findings indicate that $Hsp90_{Ec}$ can dramatically enhance the probability of alternate conformations accessible within the native ensemble. However, since HX changes can occur both from stabilization of a non-native state and from a structural changes within a state, these measurements alone cannot demonstrate how $Hsp90_{Ec}$ affects folding intermediates.

Our HX measurements were performed at pH* 7.5, where SN is in the EX2 limit [26], which means that HX rates are governed by the equilibrium population of partially folded states that expose amide positions to bulk solvent. This means that many folding/unfolding events occur before HX happens, both in the presence and in the absence of $Hsp90_{Ec}$. The EX1/EX2 regime can be simply confirmed by measuring HX at a different pH, with EX2 behavior predicting uniformly faster exchange at higher pH, due to increased catalysis of HX by hydroxyl ions. Indeed, when we repeated the HX experiments at pH* 8.5 we found a similar fold acceleration of SN HX from $Hsp90_{Ec}$ with all the rates uniformly faster (data not shown). Given the EX2 regime, these measurements enable the free energy by which $Hsp90_{Ec}$ stabilizes non-native SN to be

Fig. 6. (a) HX kinetics of SN at positions 66 and 130 are shown in blue squares, highlighting an order of partial unfolding events in SN. Addition of a sub-stoichiometric concentration of $Hsp90_{Ec}$ leads to accelerated HX (black circles), although position 66 is significantly more accelerated than 130. (b) The HX acceleration from $Hsp90_{Ec}$ across SN shows that positions between 20 and 100 are significantly affected. (c) All sites with HX acceleration greater than 10× are colored blue on the SN structure, while all contacting sites identified from chemical shifts and cysteine cross-linking are shown in red.

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determined. The slowest exchanging residues in SN correspond to those exposed only during global unfolding [30]. Therefore, $Hsp90_{Ec}$-driven change in folding free energy can be calculated for a residue as $\Delta\Delta G_{Hsp90} = -RT \ln(k_{SN}^{res}/k_{SN:Hsp90}^{res})$, which averages between 2 and 2.5 kcal/mol, depending on the reporting residue.

Discussion

Despite extensive research, it is not yet known how Hsp90 recognizes non-native states of its substrates and how the chaperone affects substrate structure and folding. Equally mysterious is how Hsp90 can facilitate protein–protein and protein–ligand interactions, behaviors that are associated with native folded proteins. Here we have used $Hsp90_{Ec}$ and a model substrate to investigate how Hsp90 recognizes and alters the structure of a partially folded target protein. We then demonstrate that Hsp90 can interact with non-native states of this substrate even in the context of a stable and fully native construct that only rarely samples non-native conformations. These findings provide the basis of a structural mechanism by which Hsp90 can alter the functional outcome of its substrates by binding and remodeling partially folded states that are transiently sampled from within the native ensemble.

The recent observation that the Δ131Δ binding site on $Hsp90_{Ec}$ extends from the MD to the CTD [20] (Fig. 1) immediately suggests that rearrangement of these domains allows the chaperone to adapt its structure to match potential binding sites on the substrate. Indeed, we showed here that, by blocking Hsp90 conformational changes at the MD/CTD interface (via the H446E mutation), Δ131Δ binding was dramatically reduced and the chaperone no longer undergoes substrate-driven conformational changes (Fig. 2). Furthermore, the MD/CTD region alone is sufficient to recognize a large partially folded region of Δ131Δ (Fig. 3). It is interesting to point out that a recent crystal structure of the closed state of the zebrafish mitochondria-specific Hsp90 (TRAP1) shows two distinct MD/CTD conformations [31]. One arm has a continuous hydrophobic surface, similar to the yeast crystal structure [6], whereas the other arm has an MD/CTD interface that is splayed out with the hydrophobic Δ131Δ interaction sites in a highly discontinuous surface. Consistent with our findings here, Δ131Δ binds to and activates the ATPase of human and zebrafish TRAP1 and binds better to the arm with the intact MD/CTD interface [31].

The dramatic defect in Δ131Δ binding from the H446E mutation provides a potential mechanistic explanation for functional defects observed in early Hsp90 genetic screens. Of four mutations in yeast Hsp90 that severely impaired nuclear receptor function [32], two were at the MD/CTD interface (T525I, A576T/R579K). One of the other mutations, E431K, is adjacent to a residue implicated in Δ131Δ binding (F390 in $Hsp90_{Ec}$, T433 in yeast Hsp90). Similarly, the CTD region of $Hsp90_{Ec}$ implicated in Δ131Δ binding has now also been implicated in the interaction of the ER Hsp90 (Grp94) with Toll-like receptors and integrins [33]. These observations are part of mounting evidence indicating that core features of the Hsp90 mechanism are universal among its diverse homologs. For example, the structural states of Hsp90 are conserved among homologs from divergent species but the population of these states are highly species dependent [9]. Similarly, we propose that there are conserved features of substrate binding by Hsp90 while specific substrates may have additional modes of interaction tailored for specific needs.

The abovementioned observations suggest a mechanism of substrate recognition in which conformational changes at the MD/CTD allow the chaperone to recognize and adapt to specific, locally structured regions of the client that would normally be buried in the native state. A critical functional question is whether Hsp90 also significantly affects Δ131Δ structure and folding. Answering this has been a major experimental challenge; however, important insights have come from a novel MS cross-linking approach that focuses on changes in the pattern and abundance of Δ131Δ intramolecular cross-links resulting from interaction with Hsp90 (Fig. 4). Computational modeling of the cross-linking data suggests that $Hsp90_{Ec}$ remodels a previously buried hydrophobic cluster within Δ131Δ. Indeed, cysteine cross-linking confirmed that specific hydrophobic contacts are readily formed between Hsp90 and an amphipathic helix in Δ131Δ (Fig. 5). These observations show that Hsp90 is capable of making significant structural alterations to specific regions in folding intermediates. Thus, Hsp90 has the potential to reroute folding rather than just passively bind and release non-specific segments of protein chain.

A critical mechanistic distinction is whether Hsp90 only interacts with partially folded proteins when they are populated constitutively, as with Δ131Δ and during unfolding conditions, or whether Hsp90 can bind partially folded states that are only transiently sampled from a thermodynamically stable native state. The latter ability is directly supported by our observations on wild-type SN, for which no significant bulk binding is observed. First, we found that $Hsp90_{Ec}$ reduces SN HSQC peak intensity, similar to observations made with Hsp90 and p53 [29]. As with studies on p53, HSQC measurements alone were insufficient to determine if intensity loss was a consequence of a native interaction or an interaction with one or more partially folded states. However, we found that only a sub-stoichiometric concentration of $Hsp90_{Ec}$ was required for significant HX acceleration, demonstrating that $Hsp90_{Ec}$ stabilizes a low population partially folded state (Fig. 6).

Please cite this article as: Street Timothy O., et al, Elucidating the Mechanism of Substrate Recognition by the Bacterial Hsp90 Molecular Chaperone, J Mol Biol (2014), http://dx.doi.org/10.1016/j.jmb.2014.04.001

Given their modest stability, most proteins will fold and unfold many times during the lifetime of the protein, with many more partial unfolding events. Thus, the findings outlined above indicate that Hsp90 has the potential to alter the folding and activation fate of a client every time it partially unfolds. This, in turn, could facilitate a client's ability to reach conformations relevant for interactions with ligands or other partners. This suggests that clients, such as kinases, could be recruited to the chaperone in the native state by co-chaperones, such as Cdc37, and that holding the client in proximity to Hsp90 would allow sufficient time for the client to sample a partially unfolded state, which then can be recognized and reconfigured by Hsp90. Further work with different clients is needed to test this proposed model.

Methods

Proteins were purified and isolated as descried previously [18,19]. Binding measurements were performed on a Jobin Horiba fluorometer with IAEDANS-labeled Δ131Δ [18] and varying concentrations of $Hsp90_{Ec}$ or the H446E variant. The excitation/emission wavelengths were 340/480 and both slit widths were set to 5 nm, with an integration time of 1 s. SAXS was measured at the SIBYLS beamline (12.3.1) at the Advanced Light Source in Berkeley. Data were collected with 0.5, 2, and 5 s integration times and buffer subtracted. The scattering data were analyzed with the GNOM program [34] and transformed to a $P(r)$ representation.

NMR measurements were performed on a Bruker Avance 800. Isotopically labeled protein was expressed in M9 minimal media supplemented with 1 g/L ^{15}N ammonium chloride and 0.5 g/L Isogro supplement (Sigma). Chemical shifts were measured with the ccpNMR software[†]. Random coil, helical, and strand chemical shifts were taken from the Biological Magnetic Resonance Bank[‡]. For HX experiments, native ^{15}N-labeled SN was concentrated to 1.5 mM and diluted 10-fold into D_2O, 25 mM Tris (pH* 7.5), 50 mM KCl, and 10 mM $MgCl_2$, at 25 °C, and successive HSQC measurements were recorded.

Scanning cysteine cross-linking was performed by incubating 50 μM of a Δ131Δ variant with 5 μM W467C for 3 h at room temperature with air oxidation, 25 mM Tris (pH 7.5), 50 mM KCl, and 10 mM $MgCl_2$. Gels were imaged on a Kodak GelLogic 100, and band quantification was performed with ImageJ[§]. The fractional degree of cross-linking was determined by integrating the area of the W467C monomer band (W) and the W467C:Δ131Δ band (W:D) and then calculating W:D/(W + W:D).

For global lysine cross-linking, proteins were cross-linked with 5-fold molar excess of DSS [in 25 mM Hepes, 25 mM KCl, and 5 mM $MgCl_2$ (pH 8.5)] at room temperature for 30 min. To minimize the impact of the cross-linker on Hsp90 conformation, we preclosed the chaperone with AMPPNP. Previous work demonstrated that Δ131Δ can bind $Hsp90_{Ec}$ in the closed state [18]. Protein samples were separated by SDS-PAGE gel, in-gel digested and analyzed by LC-MS and LC-MS/MS as described previously [35]. The concentration of protein and cross-linker was optimized so that only a small number of cross-links per molecule would be formed and only specific cross-links within the Hsp90/Δ131Δ complex could be observed (Supplemental Fig. 3). Briefly, an aliquot of the digestion mixture was injected into an Dionex Ultimate 3000 RSLCnano UHPLC system with an autosampler (Dionex Corporation, Sunnyvale, CA) and was separated by a 75/25 cm PepMap RSLC column (100 Å, 2 μm) at a flow rate of 330 nL/min. The eluant was connected directly to a nanoelectrospray ionization source of an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA). LC-MS data were acquired in an information-dependent acquisition mode, and cycling between a MS scan (m/z 315–2000) was acquired in the Orbitrap, followed by low-energy CID analysis on three most intense multiply charged precursors acquired in the linear ion trap. Activation time was 30 ms; automatic gain control targets were set at 200,000 for MS scans and 30,000 for MS/MS scans. Data were analyzed using an integrated bioinformatic workflow on the in-house version of the University of California San Francisco Protein Prospector (version 5.9.2), as described previously [22]. A precursor mass tolerance of 15 ppm and a fragment mass tolerance of 0.5 Da were used for protein database search. Cross-linked peptides were further inspected manually to assure correct identification.

To determine structural models corresponding to the lysine cross-linking data, we first defined a Bayesian scoring function, which is obtained from the posterior probability of having a model satisfying the cross-linking data and additional structural constraints. A full description of the theory is outlined in Supplementary Information. The ensemble of bound and unbound Δ131Δ conformations that optimize the scoring function are organized into a parsimonious set of structures (a multi-state model with up to four states) that simultaneously explains the ratio of ion intensities cross-linked peptides (from Supplemental Table 1), native contacts (derived from HX data collected on SN, see below), basic secondary structure propensity (from SN crystal structure), and stereochemistry. This modeling assumes that Δ131Δ lysine residues remain solvent accessible in the bound state.

To create models with a range of structure, we used the foldon classification from native-state HX of SN [26]. Foldons are defined as local substructures that hierarchically lose their native content along with the folding and unfolding reaction. The SN foldons are named by colors, and in order of unfolding, they are as follows: *r*ed, *y*ellow, *g*reen, *b*lue. Therefore structures with all foldons used as constraints (*bgyr*) are more native-like and structures with only a subset of foldons (such as *bg*) have more structural freedom. We organized the modeling into a two-step protocol: First, we generated, by sampling the posterior distribution, the most probable multi-state models given the binary cross-linking data. Second, we used the pool of generated models to best fit the quantitative cross-linking ratio data by enumerating all possible combination of models for the bound and unbound states.

Acknowledgements

Funding for this project was provided by the Howard Hughes Medical Institute. The Chu laboratory was supported by the National Institute of Food and Agriculture/U.S. Department of Agriculture Grant

H00567. We thank members of the Agard laboratory and Christopher Miller for many helpful suggestions and critical feedback. We thank Dr. Y. Spill and Prof. M. Nilges for the free energy calculations of the DSS cross-linker. R.P. was supported by grants from the Swiss National Science Foundation (PA00P3-139727 and PBZHP3-133388).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2014.04.001.

Received 10 January 2014;
Received in revised form 1 April 2014;
Accepted 2 April 2014
Available online xxxx

Keywords:
Hsp90;
chaperone;
protein folding;
HtpG

†http://www.ccpn.ac.uk
‡http://www.bmrb.wisc.edu
§http://rsbweb.nih.gov/ij/

Abbreviations used:
NTD, N-terminal domain; MD, middle domain; CTD, C-terminal domain; SN, staphylococcal nuclease; HSQC, heteronuclear single quantum coherence; SAXS, small-angle X-ray scattering; MS, mass spectrometry; LC, liquid chromatography; HX, hydrogen exchange.

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