The charged linker of the molecular chaperone Hsp90 modulates domain contacts and biological function

Markus Jahn^{a,1}, Alexandra Rehn^{b,1}, Benjamin Pelz^a, Björn Hellenkamp^a, Klaus Richter^{b,c}, Matthias Rief^{a,c,2}, Johannes Buchner^{b,c,2}, and Thorsten Hugel^{a,2,3}

^aPhysik-Department E22 and ^bDepartment Chemie, Technische Universität München, 85748 Munich, Germany; and ^cMunich Center for Integrated Protein Science, 85748 Garching, Germany

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The heat shock protein 90 (Hsp90) is a dimeric molecular chaperone essential in numerous cellular processes. Its three domains (N, M, and C) are connected via linkers that allow the rearrangement of domains during Hsp90's chaperone cycle. A unique linker, called charged linker (CL), connects the N- and M-domain of Hsp90. We used an integrated approach, combining single-molecule techniques and biochemical and in vivo methods, to study the unresolved structure and function of this region. Here we show that the CL facilitates intramolecular rearrangements on the milliseconds timescale between a state in which the N-domain is docked to the M-domain and a state in which the N-domain is more flexible. The docked conformation is stabilized by 1.1 k_BT (2.7 kJ/mol) through binding of the CL to the N-domain of Hsp90. Docking and undocking of the CL affects the much slower intermolecular domain movement and Hsp90's chaperone cycle governing client activation, cell viability, and stress tolerance.

single molecule | optical tweezers | Hsp90 conformation | FRET | asymmetric state

The molecular chaperone Hsp90 (heat shock protein 90) is essential for the folding, maturation, and activation of approximately 10% of the yeast proteome. The set of substrate proteins is structurally and functionally diverse and ranges from telomerase to kinases and transcription factors (1–3). Processing of these substrates requires ATP turnover and large conformational rearrangements within Hsp90 (4–6). Interestingly, these conformational states of yeast Hsp90 are not strictly coupled to the binding of nucleotides (7) but are recognized and regulated by the interaction with cochaperones (8) and substrate proteins (9).

Hsp90 is a dimer with each monomer consisting of three domains (N, M, and C). The N-terminal domain comprises the nucleotide binding site, whereas the M-domain is important for the binding of many substrates. The C-terminal domain is mainly responsible for the dimerization of the protein. A long charged linker (CL) region, amino acids 211–272 in yeast, connects the N- and M-domain in eukaryotes (Fig. 1A). The crystal structure of yeast Hsp90 was obtained by partly deleting the CL region and shows a closed, compact conformation in the presence of AMP-PNP [Adenosine 5'-(β,γ -imido)triphosphate] and Sba1/p23 (10). The fact that the CL region is difficult to map structurally led to the assumption that this region is disordered and flexible, thereby enabling the conformational rearrangements of Hsp90 (11, 12). Besides the structural indetermination of the CL, its ultimate function remains elusive as well (13). Early studies show that parts of the CL region (amino acids 211–259) are dispensable in yeast (14), whereas more extended deletions affect cell viability, substrate maturation, and regulation by cochaperones (11). These deficiencies can be partially rescued by a short linker consisting of an artificial sequence (11), but its specific amino acid sequence is associated with a gain of function, probably an additional Hsp90 regulatory mechanism (15).

Single-molecule experiments have recently provided detailed insight into subtle biochemical processes. Single-molecule force spectroscopy using optical tweezers allows defined manipulation of biomolecules with exquisite force (sub-pN) and time resolution (sub-ms). These experiments revealed the complex dynamics and

energetics in protein folding of small proteins and DNA structures (16–18). Complementary single-molecule FRET (smFRET) experiments permit observation of biomolecules without external perturbation, with high spatial resolution on the nanometer scale. Confocal smFRET experiments are ideal to accurately obtain state populations (19), whereas smFRET experiments in TIR (total internal reflection) geometry reveal dynamics in real time (20).

Here we use single-molecule force spectroscopy on Hsp90 to drive and observe docking/undocking transitions of the CL in real time and in addition single-molecule fluorescence methods to investigate how the CL and its substitutions by GGS repeats impact the dynamics of the N-domains. Together with biochemical methods and in vivo experiments, we delineate the conformations, kinetics, and the biological function of this unresolved CL region.

Results

Unfolding of Hsp90 Reveals Its Domains and a Transition at Low Forces. To investigate the stability of the various domains and the CL region, we performed single-molecule mechanics experiments with Hsp90 monomers using an optical trap (Fig. 1A). A single molecule of Hsp90 was suspended between two 1- μ m glass beads using two 185-nm-long DNA handles and specific coupling of the protein to the beads via cysteines (details are provided in *SI Appendix, Methods*). By moving the glass beads

Significance

Many proteins consist of domains that are connected by flexible linkers. Because of their flexibility they are usually unresolved in crystal structures. Using single-molecule force spectroscopy we were able to measure free energy changes around the thermal energy and obtained for the first time, to our knowledge, structural information on the linker connecting the N-domain and M-domain in Hsp90. We show a sequence-dependent rapid equilibrium between the docked and undocked state of these two domains. This is facilitated by transient binding of the linker to the N-domain. Single-molecule FRET, analytical ultracentrifugation, and cell viability assays show that the equilibrium between these two states affects intermolecular domain movement, client activation, cell viability, and stress tolerance.

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¹M.J. and A.R. contributed equally to this work.

²To whom correspondence may be addressed. Email: thorsten.hugel@ph.tum.de, mrief@ph.tum.de, or johannes.buchner@tum.de.

³Present address: Institute of Physical Chemistry, University of Freiburg, 79104 Freiburg, Germany.

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Fig. 1. Unfolding of Hsp90 by optical tweezers shows a structured CL. (A) Schematics of the experimental setup (not to scale). Monomeric Hsp90 was clamped between two glass beads (spheres), which were trapped by focused laser beams to apply and measure forces. DNA (chain) and ubiquitins (circles) serve as handles to manipulate Hsp90 in the center of the optical tweezers setup. (B) A typical force–extension trace at constant velocity (here, 10 nm/s) shows the successive unfolding of Hsp90’s three domains as large peaks. The contour length increase obtained from WLC (worm-like chain) fits (dashed lines) for each domain matches the number of amino acids within the domain (average values are given between WLC fits). The first peak (violet to green) accounts for the unfolding of the C-domain, the second peak (green to blue) for the N-domain, and the last (blue to orange) for the M-domain. The colored trace shows filtered data; gray is the full-resolution data at 20 kHz. (Inset, violet) Magnified view of the region before any of the domains unfold (arrow). Rapid fluctuations between two states were observed, which are caused by docking and undocking of the CL.

relative to each other, force was applied to the molecule, and force–extension curves were recorded. A typical force–extension trace from the fully folded to the fully unfolded state is shown in Fig. 1B. As expected for Hsp90, we observed three peaks that mark the consecutive unfolding of the three domains (when the attachment points are at the N and C terminus). Comparison of the number of amino acids folded in each domain with the measured contour length increase after each unfolding peak allowed us to assign unfolding events to single domains. Until an extension of approximately 350 nm, all domains remain fully folded (purple part). The first unfolding event (transition from purple to green) with an associated length gain of 42.9 ± 2.3 nm ($n = 62$) matches well with the resolved part of the C-terminal domain in the crystal structure (10) (amino acids 538–671). At higher forces the N-domain unfolded (green to blue), followed by the M-domain (blue to orange). The contour length increases for the N- and M-domain (68.4 ± 2.1 nm and 85.4 ± 1.0 nm, $n = 62$) match the number of amino acids folded. More sample traces, as well as statistics of forces, lengths, and domain association are provided in *SI Appendix*, Fig. S1 and Tables S1, S2 and S3. In addition to the unfolding of the domains, a transition at low forces in the purple part, before unfolding sets in, was also observed (Fig. 1B, arrow and *Inset*). Here a change in the contour length of approximately 28 nm was measured, which fits approximately to the length of the CL (62 aa; details in *SI Appendix*, *Comment S1*).

The CL Forms a Structural Element in Contact with the N-Domain. To investigate whether the low-force fluctuations in the constant velocity optical tweezers experiments (Fig. 1 and Fig. 2A) are due to unfolding of structural elements in the CL, we substituted large parts of the CL by glycine-glycine-serine repeats (GGS repeats). The crystal structure suggests not only direct contacts between the N- and M-domain but also a stabilization of amino acids 264–272 of the CL by the N-domain (10). To test whether these nine amino acids are sufficient to stabilize the N–M-domain interface, we compared two GGS substitution mutants that differed in these nine amino acids. The first substitution is from amino acids 211–263 (Sub211-263) and the second from amino acids 211–272 (Sub211-272), hence spanning the complete CL region. Interestingly, both substitution mutants did not show any mechanical stability of the N–M-domain interface; that is, under force, the N- and M-domains could move apart freely tethered by the unstructured GGS linkers (Fig. 2B). In other words, we did

not find a state where the N-domain is docked onto the M-domain, only the state where the N-domain is detached from the M-domain. Therefore, amino acids 264–272 alone cannot be responsible for the stabilization of the N–M-domain interface. In fact, the sequence of the CL between amino acids 211 and 263 seemed crucial for the observed stabilization (docked state). This is surprising, because amino acids 211–263 have often been considered as unstructured (12). To scrutinize whether this stabilization is caused by a structure within the CL or by contacts of the CL with other Hsp90 domains, we constructed deletion mutations of Hsp90 comprising only the CL with the M- and C-domains (Fig. 2C, *Upper*), as well as the CL with the N-domain (Fig. 2C, *Lower*). We did not see any stabilization with the construct lacking the N-domain. On the other hand, a similar stabilization

Fig. 2. The CL interacts with the N-domain in a sequence-dependent manner. (A) Close-up view of the force-extension trace of the WT protein showing the two-state behavior of the CL (flipping between the two dashed WLC fits). (B) Substitution of amino acids 211–263 (*Upper*) and amino acids 211–272 (*Lower*) by GGS repeats. No docked state is observed, indicating that the N–M interface is not stabilized anymore. (C) N-domain deletion prevents the docked state (*Upper*), whereas M- and C-domain deletion shows a two-state behavior similar to the WT protein. All Hsp90 mutants show similar length increases and domain stabilities like WT (*SI Appendix, Tables S1 and S2*).

compared with WT Hsp90 was observed for the construct with the N-domain alone (Fig. 2C, *Lower*). Therefore, the CL region needs to be in contact with the N-domain to form a stable secondary structure, by itself or including parts of the N-domain. The CL on its own or in contact with the M- or C-domain does not form a stable secondary structure. Full-length traces of the regions depicted in Fig. 2, as well as scatter plots and constant distance traces, are shown in *SI Appendix, Figs. S2, S3, and S4*. Taken together these results imply that the CL binds to the N-domain and that the sequence between amino acids 211 and 263 is crucial for this interaction.

CL Kinetics Are Fast and the Stabilizing Energy Is Low. To assess the force-dependent docking and undocking kinetics as well as the free energy involved, we used an experimental assay in which the forces do not change continuously as in the force-extension cycles shown in Figs. 1 and 2. Therefore, we held the two traps at constant positions, hence imposing a constant average force on the fluctuating protein (passive mode or constant distance) (21). By changing the trap distance we can change the average applied force and can thus affect the equilibrium between the docked and the undocked state of the CL. The force-dependent shift of the equilibrium populations then allowed us to extract the free energy of docking of the CL with an energetic model for the complete system comprising beads, DNA, and protein. Likewise, the dwell times of the docked and undocked states directly yield the force-dependent docking/undocking rate constants (details in ref. 16 and *SI Appendix, Methods*).

Fig. 3A depicts a small part of a typical force vs. time trace at two different average forces. For clarity only short time traces are displayed, even though we can routinely observe individual molecules at a bandwidth of 20 kHz for more than 100 s (an example is shown in *SI Appendix, Fig. S5*). The assignment of the docked state (blue) and undocked state (red) (Fig. 3B, *Inset*) was done using Hidden Markov analysis (22). From the force-dependent probabilities of docking and undocking of the CL (example experiment in Fig. 3B), we obtained a stabilizing free energy of 1.1 ± 0.4 k_BT, meaning the probability of the docked state is $75\% \pm 8\%$ (average value and SD of 34 individual molecules, altogether 4,315 s of constant distance experiments). The rates were then extracted (*SI Appendix, Fig. S6*) from the time intervals that are spent in each of the two states and corrected for missed events (22). The force-dependent docking/undocking rate constants for a single molecule are plotted in Fig. 3C. As expected intuitively, the undocking rate constants (blue) increased with force, whereas the docking rate constants (red) decreased. Using an energetic model, extrapolation of those rate constants to zero force (fits in Fig. 3C) yielded a docking rate of 173 ± 102 s⁻¹ and an undocking rate of 75 ± 41 s⁻¹ (average value and SD of 34 individual molecules; more details in *SI Appendix*). In addition, the distances to the transition state can be extracted. We obtain 20.8 nm for the distance from the undocked state and 8.5 nm for the distance from the docked state. The energy landscape is visualized in *SI Appendix, Fig. S7*. Consequently, even in the absence of force, the CL is a highly dynamic structural element in Hsp90.

CL Substitutions Modulate the N-Terminal Dimerization of Hsp90. Large N-terminal conformational changes between an open and a closed state are essential for the function of the Hsp90 dimer. To investigate whether the CL affects these, we exchanged one WT monomer with a CL substitution (either amino acids 211–263 or amino acids 211–272 replaced with GGS repeats), labeled the dimers with donor and acceptor dyes, and performed smFRET experiments. To prevent dimer dissociation at the concentrations needed for single-molecule experiments, Hsp90 dimers were C-terminally zipped with a recombinant coiled-coil motif (7). In the closed state, small distances between the fluorophores yield a high FRET signal, whereas the open state results in a low FRET signal. Details are provided in *SI Appendix, Methods and Fig. S8*.

Fig. 3. Undocking-docking kinetics and energetics of the CL. (A) Equilibrium transitions between the docked (high force, blue) and the undocked (low force, red) state of the CL at two different average forces (pretensions). As expected the pretension changes the kinetics and the population of the two states. In optical tweezers experiments the CL was elongated (stretched) in the undocked state by up to 4 nm (deflection). (B) The force-dependent probability to be in the docked state (blue) or undocked state (red). A global fit (*SI Appendix, Methods*) allows extrapolation to zero force. Averaging all measured molecules ($n = 34$) resulted in a 75% probability of being in the docked and a 25% probability of being in the undocked state at zero force. (C) The force-dependent rate constants were fitted (*SI Appendix, Methods*) and yielded an undocking rate constant of 75 s⁻¹ and a docking rate constant of 173 s⁻¹. All data shown in this panel represent an example experiment from the exact same molecule.

In confocal experiments we obtain snapshots of Hsp90s conformation of thousands of molecules. Fig. 4A shows histograms of the FRET efficiencies for the WT and the CL mutants in the absence (apo, *Left*) and presence of nucleotides (ATP, *Right*). As previously described, WT Hsp90 can partially be found in a closed conformation in the apo state but is even more shifted toward the closed conformation in the presence of ATP (7, 23). Here the ATP-induced shift to the closed state is approximately 5% for WT and substitution mutants (although absolute numbers show some variation for different preparations). More interesting and pronounced is the shift between CL substitutions heterodimers and WT homodimers. Replacing one WT monomer with Sub211-272 shifted the equilibrium toward the N-terminal closed state (approximately 13%) in apo and ATP conditions. Similarly the Sub211-263 variant shows a shift of approximately 9%.

Additionally, smTIR-FRET experiments were performed. Here, individual immobilized Hsp90 dimers can be monitored over time. The WT and the mutants show closing and opening kinetics on the seconds timescale, and histograms of multiple traces (34 for WT, 41 for Sub211-263, and 51 for Sub211-272) confirm the shift to the closed state for the CL mutants (*SI Appendix, Fig. S9*).

The unnatural nucleotides ATP γ S and AMP-PNP strongly shift Hsp90 to a closed state. Confocal FRET experiments in the presence of ATP γ S show the same FRET efficiencies for all constructs (*SI Appendix, Fig. S8D*), indicating a similar conformation in this nucleotide state. To monitor the transition from the apo to the closed ATP γ S or AMP-PNP state, we carried out fluorescence bulk experiments (*SI Appendix, Fig. S10*). The WT and the mutants show closing kinetics upon the addition of ATP γ S or AMP-PNP, except the homodimers of the substitution

Fig. 4. N-terminal dimerization is modulated by the CL. (A) Histograms of FRET efficiencies in the absence of nucleotide (Left, apo) and in the presence of ATP (Right) for WT (black), GGS substitutions from amino acids 211–263 (blue), and amino acids 211–272 (orange). Each histogram contains a minimum of 923 (up to 5,565) FRET events. (B) Cross-linking experiments using the cysteine-specific cross-linker BM(PEG)₃ with homodimers of WT, Sub211-272, and Sub211-263. First lane is without cross-linking agent yielding monomer bands. Other lanes are with cross-linking agent (X-Linker) in apo (second lane), AMP-PNP (third lane), and ATPγS (fourth lane) condition, respectively, yielding monomer and dimer bands. (C) ATPase activity of the mutants at 30 °C, error bars represent SD. (D) Different concentrations of the cochaperone Aha1 were added to the mutants, and the ATPase activity was determined. The figure shows the stimulation of the Hsp90 ATPase activity compared with the value obtained in the absence of Aha1. (E) The binding of the different Hsp90 constructs to Aha1 was analyzed by analytical ultracentrifugation. dc/dt plots are shown for Aha1-FAM (magenta) and the mixtures of Aha1-FAM with the indicated Hsp90 constructs in the absence of nucleotide. (F) Analytical ultracentrifugation experiments for the interaction between Atto488-labeled p23/Sba1 and the Hsp90 mutants in the presence of AMP-PNP.

mutant Sub211-272, which did not show any closing kinetics with AMP-PNP.

Fig. 2 already showed that the interaction of the CL with the N-domain is demolished in the substitution mutants. Additionally, our fluorescence experiments indicate that these mutants are shifted toward a more closed conformation. To further understand this increased flexibility of the N-domains in the closed state, cross-linking experiments with the short, cysteine-specific cross-linker BM(PEG)₃ were performed. To this end, C-terminally zipped homodimers of WT, Sub211-272, and Sub211-263 were allowed to form intermonomer cross-links between its cysteines at position 61 in the presence of the cross-linker (Fig. 4B). The crystal structure (in the presence of AMP-PNP and p23/Sba1) of the closed conformation of Hsp90 suggests that amino acids at positions 61 are pointing away from each other (10) and would therefore not cross-link at all. Interestingly, we were able to observe cross-linking events in the WT in the absence and presence of nucleotides, implying that the CL is not only able to dock and undock in the open conformation but also in the N-terminally closed state, even though the cross-linking events were less prominent with ATPγS or AMP-PNP. The substitution mutants, on the other hand, can only occupy the undocked state and show a drastic increase in cross-linking probability compared with WT. These results imply that the undocking of the CL from the N-domain results in an increase of rotational flexibility of the N-domain itself.

CL Substitutions Affect Cochaperone Interaction with Hsp90. Because the single-molecule experiments clearly suggested a structural role for the CL, we next set out to clarify whether these structural rearrangements affect Hsp90 in its *in vitro* and *in vivo* functions. First, Hsp90's intrinsic ATPase activity and its modulation by cochaperones were analyzed. Compared with the WT protein, Sub211-263 showed a mild increase in its ATPase activity, whereas

substitution of amino acids 211–272 surprisingly led to a decrease in ATP turnover (Fig. 4C).

Because the cochaperones Aha1 and p23/Sba1 recognize different conformations of Hsp90 (23, 24), we used those to explore the ability of the substitution mutants to adopt different conformations during the ATPase cycle.

The cochaperone Aha1 is an activator of the Hsp90 ATPase (25–27) and facilitates closing of Hsp90 (28), thereby accelerating its ATPase. Although Hsp90 WT was stimulated as previously described (27), both substitution mutants showed a decreased activation (Fig. 4D). To exclude the possibility that the substitution of the CL by GGS repeats abrogated Aha1 binding, we performed analytical ultracentrifugation runs with labeled Aha1 (Fig. 4E). Complexes were found for all Hsp90 mutants, indicating that the decrease in ATPase stimulation was not due to an impaired binding to Aha1.

p23/Sba1 is a conformation-specific cochaperone that only binds to Hsp90 in the fully closed AMP-PNP state and thereby inhibits its ATPase activity (29, 30). Analytical ultracentrifugation runs with labeled p23/Sba1 in the presence of the non-hydrolyzable ATP analog AMP-PNP revealed that all Hsp90 variants except Sub211-272 were able to form a complex with p23/Sba1, indicating that Sub211-272 does not reach the conformation necessary for p23/Sba1 binding (Fig. 4F), which is in agreement with the lack of closing kinetics in the fluorescence experiments (SI Appendix, Fig. S10).

CL Substitutions Affect the Biological Function of Hsp90. Because the CL has been shown to play a crucial role for the function of Hsp90 *in vivo* (11, 15), we set out to analyze the CL substitution mutants in *Saccharomyces cerevisiae*. Additionally, the variants were tested in a Δ cpr7 strain, because Cpr7 becomes essential when the CL is deleted (13). Cells carrying Sub211-263 as the sole source of Hsp90 were viable in the absence and presence of Cpr7, whereas Sub211-272 only conferred viability in the

Fig. 5. CL conformations in Hsp90's chaperone cycle. The Hsp90 cycle is driven by large and slow conformational changes, leading from the open to the closed state. In addition, the CL can rapidly adopt a docked or an undocked state. The docked conformation fixes the N-domain to the M-domain (e.g., Open 1a and Closed 1a), whereas the undocked conformation provides some flexibility, but still close proximity of the domains (e.g., Open 1b and Closed 1b). In principle, both CLs can undock at the same time, but we have omitted this unlikely case (6%) for clarity. Undocking of the CL is not restricted to the open conformation (Open 1b), thus leading to a previously unknown state of Hsp90, namely Closed 1b. In the Closed 2 state (Protein Data Bank ID 2CG9), the CL may adopt an additional conformation, which has not been measured directly (dashed box). Here a segment of the CL (amino acids 263–272) might be important for the integrity of the Closed 2 state, which is essential for proper biological function.

presence of Cpr7 (*SI Appendix, Fig. S11A*). Additionally, we tested these strains toward stressors like heat or UV light. (*SI Appendix, Fig. S11 B and C*). Yeast carrying Sub211–272 already exhibited an impaired growth at 30 °C and did not survive at temperatures above 37 °C. Additionally, these cells reacted very sensitively to UV light. In contrast, Sub211–263 yeast showed a comparable growth behavior in the WT background at all temperatures tested, which is in agreement with previous results (15). In the Δ cpr7 background, however, Sub211–263 displayed growth defects and reacted also more sensitively to the presence of UV light (*SI Appendix, Fig. S11C*). Comparison of the two substitution mutants in regard to their effects toward the client proteins glucocorticoid receptor (GR) and v-src revealed that the Sub211–263 mutant activated both clients comparable to WT, whereas substitution of amino acids 211–272 affected the chaperoning activities (*SI Appendix, Fig. S11 D and E*). All in vivo experiments show that substitution of amino acids 211–272 dramatically impaired Hsp90's function in yeast, whereas Sub211–263 is only restricted under Δ cpr7 conditions.

Discussion

Our integrated approach comprising multiple single-molecule methods as well as biochemical and in vivo experiments allowed us to dissect the dynamics and structural and functional aspects of Hsp90's CL.

Our results indicate that the CL is not a mere loop connecting the N- and M-domains but is crucial for the structural flexibility between these two domains. It is able to mediate the formation of a docked state in which the N-terminal domain is stabilized relative to the M-domain, as well as an undocked conformation in which the N domain can reorient (Figs. 2 and 4B). The docked state is stabilized by an energy of 1.1 k_BT (2.7 kJ/mol) through the binding of the CL in a sequence-dependent manner to the N-domain. It is occupied for approximately 75% in the absence of force (Fig. 3). In the remaining 25%, the CL is not significantly interacting with the N-domain, and the N- and M-domains are undocked. These docking and undocking kinetics of the CL

occur with a frequency of hundreds per second and are much faster than all previously measured kinetics of Hsp90.

However, what are the impacts of the docking/undocking of the CL on Hsp90's conformations? Our smFRET results show that the docking/undocking of the CL also modulates larger conformational changes in Hsp90 like its N-terminal dimerization. Heterodimers with one monomer lacking the docked state still show N-terminal dimerization dynamics but exhibit a higher probability for an N-terminal closed state (Fig. 4A and *SI Appendix, Fig. S9B*). The adoption of a more closed conformation of the CL substitution mutants is also consistent with the slight shift to higher $s_{20,w}$ values in the sedimentation profiles of the analytical ultracentrifugation experiments (Fig. 4E and F). A clear difference between the docked and undocked state is an easier reorientation of the N-domains, which might in turn enable the formation of additional intermolecular N–N contacts, resulting in an N-terminally more closed conformation. This suggests that the CL acts as a return spring, preventing some N–N contacts. The most significant reorientation might be a rotation, which has already been shown to be a requirement for closure of the N-domains in the bacterial Hsp90 ortholog HtpG (31). Moreover, a gain in rotational freedom in the undocked state is a plausible explanation for the increased cross-linking events of the substitution mutants compared with WT (Fig. 4B).

Are the docking/undocking events restricted to the open conformation, or are they possible in the closed state as well? In the literature at least two closed states have been reported. The so-called Closed 1 state (28) can already be populated in the absence of nucleotides (7) and is stabilized by Aha1 (27) (see below). The Closed 2 state, on the other hand, reflects the twisted Hsp90 conformation obtained from the crystal structure in the presence of AMP-PNP and p23/Sba1 (10). In both states the positions 61 of the two monomers point away from each other. Surprisingly, Hsp90 WT dimers can be cross-linked via cysteines at positions 61 (Fig. 4B). Therefore, undocking events that (temporarily) enable the rotational freedom of the N-domain so that positions 61 point toward each other have to occur in the closed state. This necessitates postulation of an additional closed state. We term this heretofore undescribed state “Closed 1b” and rename Closed 1 as “Closed 1a.”

Because the substitution variants do not show docking, they mainly occupy the more flexible Closed 1b state. This is consistent with hydrogen exchange experiments, in which substitution mutants show decreased protection of the N-domain and increased protection of the M-domain compared with WT (15).

Do the undocking/docking events of the linker affect Hsp90's cochaperone-mediated conformational cycle? To answer this question, the actions of the conformation-specific cochaperones Aha1 and p23/Sba1 were investigated. Aha1 shifts Hsp90 from the N-terminal open conformation to one of the Closed 1 states, which is already more occupied in both substitution mutants and is in good agreement with a decreased Aha1 stimulation (27). The reason for the even stronger effect on the Sub211–272 variant could be an additional impairment of this mutant in reaching the Closed 2 state (see below).

The cochaperone p23/Sba1 recognizes and binds to the Closed 2 state of Hsp90, which is preferentially populated upon the addition of AMP-PNP. This state is supposed to mimic the hydrolysis-competent state of Hsp90 and was crystallized (10). The substitution mutant 211–272 is unable to bind p23/Sba1 in the presence of AMP-PNP (Fig. 4F) and shows no indication for this (closed) state (*SI Appendix, Fig. S10*). Therefore, we propose that the CL mutant cannot reach Closed 2, and binding to p23/Sba1 cannot occur. This could be explained by amino acids 264–272 being essential to efficiently reach the Closed 2 state. Nevertheless, the Sub211–272 mutant is able to undergo N-terminal dimerization and hence efficiently reaches the Closed 1 state (Fig. 4A). The problems in reaching or populating the Closed 2 state are also reflected in the strongly decreased Aha1 stimulation and the in vivo defects of this mutant (*SI Appendix, Fig. S11*).

What are the effects of the docking/undocking of the CL on the biological function of Hsp90? As previously described, deletion and/or substitution of the CL results in various growth defects in yeast (11, 15). Here we show that the ability to switch between the docked/undocked states is not the cause of immediate growth effects, because Sub211-263 (undocked) and WT (docked) do not differ in that respect at any temperature tested. Moreover, Sub211-263 did not show any limitations in client protein activation (*SI Appendix, Fig. S11*). However, additional restraints like the loss of Cpr7 or the additional substitution of amino acids 264–272 (conformational restraint) cannot be tolerated without any effects, thereby implying a function for the CL docking/undocking *in vivo*.

Altogether we have shown that Hsp90's CL is not permanently disordered but can adopt a sequence-dependent secondary structure. The formation of this structure is mediated by the N-domain of Hsp90 and it stabilizes the N/M-domain contacts leading to a docked state. This docked state is stabilized with an energy of 1.1 k_BT. Therefore, both the docked (75%) and undocked (25%) state are significantly populated in Hsp90 monomers. Assuming independent docking and undocking in the Hsp90 dimer we obtain 56% of dimers with both CLs docked, 38% of dimers with one CL docked, and 6% of dimers with both CLs undocked. Docking and undocking of the CL is possible in the N-terminally open and closed conformation (Fig. 5). The CL therefore significantly impacts Hsp90's conformational cycle. In addition, the CL offers a further opportunity for cochaperones and clients to interfere with Hsp90. Even cochaperones with low affinities could modulate the kinetics and the equilibrium between the docked and undocked state facilitating new global conformations. Such modulations and the general

weak coupling in Hsp90 might be the key to understand how few isoforms of Hsp90 can handle diverse of functions in the cell.

Methods

For optical trapping we used a custom-built, high-resolution dual trap optical tweezers with back-focal plane detection. Genetically engineered Hsp90 mutants were coupled via cysteine-maleimide chemistry to two 185-nm double-stranded DNA handles. DNA handles in turn carried modifications at their ends that can bind to functionalized 1-μm beads, which were trapped in optical tweezers. In this dumbbell geometry, force was exerted by moving the beads away from each other with constant speed, forcing the molecule to unfold (constant velocity) or by fixing the trap positions measuring the protein response in equilibrium (constant distance). Constant-distance experiments allow us to extract energetic and kinetic information depending on force. Energies and rates at zero force can be calculated using an energetic model that accounts for beads, DNA handles, and protein.

N-terminal opening and closing of Hsp90 was quantified using confocal single-pair FRET. Fluorescent dyes were covalently attached to C-terminally zipped Hsp90 mutants by maleimide chemistry. Each CL variant and WT carried a donor dye (Atto 550) at amino acids position 61. They were mixed with WT carrying an acceptor dye (Atto 647N) at amino acids position 385, yielding heterodimers with substituted CL in one of both monomers. Then FRET efficiencies of freely diffusing Hsp90 dimers were measured in a home-built confocal microscope using two pulsed lasers.

All measurements were carried out in 40 mM Hepes, 150 mM KCl, and 10 mM MgCl₂ pH 7.4 (if not stated otherwise). A detailed description of methods and material used is given in *SI Appendix, Methods*.

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