pH-Dependent Conformational Changes in Bacterial Hsp90 Reveal a Grp94-Like Conformation at pH 6 That Is Highly Active in Suppression of Citrate Synthase Aggregation

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The molecular chaperone Hsp90 depends upon large conformational rearrangements for its function. One driving force for these rearrangements is the intrinsic ATPase activity of Hsp90, as seen with other chaperones. However, unlike other chaperones, structural and kinetic studies have shown that the ATPase cycle of Hsp90 is not conformationally deterministic. That is, rather than dictating the conformational state, ATP binding and hydrolysis shift the equilibrium between a preexisting set of conformational states in an organism-dependent manner. While many conformations of Hsp90 have been described, little is known about how they relate to chaperone function. In this study, we show that the conformational equilibrium of the bacterial Hsp90, HtpG, can be shifted with pH. Using small-angle X-ray scattering, we identify a two-state pH-dependent conformational equilibrium for apo HtpG. Our structural modeling reveals that this equilibrium is observed between the previously observed extended state and a second state that is strikingly similar to the recently solved Grp94 crystal structure. In the presence of nonhydrolyzable 5′-adenylyl-β,γ-imidodiphosphate, a third state, which is identical with the solved AMPPNP-bound structure from yeast Hsp90, is populated. Electron microscopy confirmed the observed conformational equilibria. We also identify key histidine residues that control this pH-dependent equilibrium; using mutagenesis, we successfully modulate the conformational equilibrium at neutral pH. Using these mutations, we show that the Grp94-like state provides stronger aggregation protection compared to the extended apo conformation in the context of a citrate synthase aggregation assay. These studies provide a more detailed view of HtpG’s conformational dynamics and provide the first linkage between a specific conformation and chaperone function.

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Introduction

Hsp90 is a member of a diverse class of proteins known as molecular chaperones. Molecular chaperones are required in order to maintain the correctly folded state of other proteins in the cell. This is generally accomplished by recognition of hydrophobic surfaces on the client protein and then repeated rounds of ATP hydrolysis-dependent conformational changes by the chaperone, facilitating substrate folding.1 Two well-studied members of this class, Hsp70 (DnaK) and Hsp60 (GroEL), inter-
act with nascent polypeptide chains and promote their folding.\textsuperscript{22} In addition to the stress response role in preventing aggregation,\textsuperscript{4} Hsp90 under unstressed conditions appears to act at later stages of the protein folding pathway and interacts with client proteins in their near-native states. Hsp90 induces subtle conformational changes that promote the binding and release of ligands or interaction with partner proteins.\textsuperscript{3–11} Hsp90’s client proteins include serine/threonine and tyrosine kinases, steroid hormone nuclear receptors, transcription factors, and tumor-suppressor proteins.\textsuperscript{9,12–14} Many of Hsp90’s client proteins, including Cdk4, c-src, and v-src, are oncogenic or otherwise required for cell proliferation, making Hsp90 an attractive target for anticancer therapeutics.\textsuperscript{15} Inhibition of Hsp90 with small molecules such as geldanamycin and its derivatives has been shown to be antitumorigenic, and several of these compounds are currently in clinical trials.\textsuperscript{16–19}

While the molecular mechanisms of Hsp70 and Hsp60 are well characterized, the conformational changes and determinants of substrate recognition associated with Hsp90 function remain unclear. For Hsp70 and Hsp60, client protein binding stabilizes unstructured states; only upon release (either into solution or into the lumen of the Hsp60 cavity) can folding occur. Thus, in the bound state, the client protein adapts its conformation to the binding surfaces for these chaperones.\textsuperscript{20} By contrast, Hsp90 substrates have already achieved a partially folded or almost fully folded conformation before they interact with Hsp90,\textsuperscript{7} suggesting that Hsp90 must adapt its conformation to match each substrate or that different conformations recognize different substrates. This issue is particularly pronounced for the bacterial Hsp90, HtpG, for which there are no known cochaperones to improve the efficiency of client protein recruitment.

Hsp90 exists as an obligate dimer, and the crystal structure of the nucleotide-free (apo) state reveals exposed hydrophobic surfaces on each of the three domains within a protomer [N-terminal domain (NTD), middle domain (MD), and C-terminal domain (CTD)] that likely serve for client protein binding.\textsuperscript{21} Other crystal structures show that binding of nucleotide to the NTD induces remarkably distinct conformational states that decrease [the nonhydrolyzable ATP analog 5′-adenylyl-3′,5′-imidodiphosphate (AMPPNP)]\textsuperscript{22} or completely bury (ADP)\textsuperscript{21} exposed hydrophobic patches. These conformational changes represent unexpectedly large changes where the NTD moves by approximately 50 Å between each state (Fig. 1). These rearrangements presumably act to alter client protein structure and to modulate client protein binding. Although localized changes in the active site lid\textsuperscript{25,26} (residues 100–126) and the src loop\textsuperscript{27} (residues 281–296) occur upon nucleotide binding, these vastly different conformational states are well described by rigid-body rearrangements of the domains about flexible linkers. A crystal structure of the full-length endoplasmic reticulum homolog Grp94 has recently been solved,\textsuperscript{24} revealing a state that appears to be inter-

**Fig. 1.** Previously solved structures demonstrating the conformational flexibility of Hsp90. Apo and ADP forms were crystalized using HtpG,\textsuperscript{23} ATP was crystallized in yeast,\textsuperscript{27} Grp94 was the crystal structure of the canine mitochondrial homolog,\textsuperscript{28} and the SAXS model was obtained from solution studies of apo HtpG.\textsuperscript{24} NTD is shown in blue, MD is shown in green, and CTD is shown in gold. Hydrophobic patches on the surface are highlighted in purple.
equilibrium is heavily biased towards the extended state even in the presence of ATP,\textsuperscript{31} whereas in yeast, the equilibrium is shifted predominantly towards the closed state with the addition of nucleotide.\textsuperscript{31,32} Notably, the prokaryotic Hsp90 seems poised to equally sample the extended and closed states in the presence of ATP.\textsuperscript{24,31}

As described here, HtpG displays an even broader range of conformational states than previously anticipated. By varying the pH and by examining the solution conformation with SAXS, we show that at low pH a previously unknown HtpG conformation, closely resembling the reported crystal structure of Grp94, dominates in solution. In the absence of nucleotide, a two-state equilibrium with a pH midpoint around pH 7.2 exists between the extended state and the Grp94-like conformation described here. Through mutagenesis of a single histidine residue, we can bias the conformation of HtpG independent of pH, providing powerful new tools for examining the functional properties of each conformation. Importantly, we also demonstrate a functional difference between these two apo conformations, where the Grp94-like conformation is the active state in a citrate synthase (CS) chaperoning assay. These results provide the first direct evidence for distinct functional behaviors of different apo conformations.

Results

pH causes the solution structure of HtpG to shift from an extended state to a more compact state

Our previous SAXS study of HtpG revealed a structure in solution that is more open and extended than that observed in the crystal.\textsuperscript{24} This model was determined at pH 9 given that both SAXS data and

![Fig. 2. Scattering data for apo HtpG show a more compact conformation at low pH. (a) Averaged and scaled solvent-subtracted scattering curves (I(Q)) at varied pH values (pH 6, purple; pH 6.5, gold; pH 7, green; pH 7.5, red; pH 8, blue; pH 9, gray). (b) Interatomic distance distribution functions (P(r)) calculated from the scattering data shown in (a). The pH conditions are colored as in (a). The P(r) curves are normalized to have equivalent areas under the curve, and a continuous vertical line marks an interatomic distance of 54 Å. (c) Two-state pH titration curve-fitting values for P(r) at 54 Å. Data were fitted with a standard two-state binding model y = Δ(y)/(1 + 10^{-pK_a-pH}) + minimum(y), giving a pK_a of 7.2.](image-url)
EM studies had suggested that HtpG was more homogenous at pH 9. However, at physiological pH, both SAXS and EM studies indicate that HtpG is conformationally heterogeneous. These results suggest that varying the pH could be an effective and important tool for investigating the conformational dynamics of HtpG in solution.

Indeed, SAXS measurements show dramatic changes between pH 6 and pH 9. The scattering intensity $I(Q)$ was measured for scattering angles $(Q=4\pi\sin(\theta)/\lambda)$ from 0.01 to 0.3 Å$^{-1}$ (Fig. 2a). The scattering curves for each pH represent the average of multiple curves (see Materials and Methods), and the differences seen between the pH conditions are very robust especially for low $Q$, where the largest differences are seen between the different pH conditions (average error of 2% for $Q=0.06–0.12$). The $I(Q)$ data were then converted into the interatomic distance distribution function $P(r)$ by Fourier transform (Fig. 2b). The $P(r)$ function provides a distribution of interatomic distances found within the molecule, giving information on shape and maximum end-to-end distance, and provides a particularly sensitive indicator of conformational changes. Both the $I(Q)$ and the $P(r)$ functions for HtpG at different pH values demonstrate a clear transition to a more compact state as the pH of the solution is lowered. HtpG at pH 6 has a radius of gyration, $R_g$, of 46.3 Å as compared to an $R_g$ of 55.7 Å for HtpG at pH 9. For a clear analysis of the pH dependence of the conformational transition, $P(r)$ at pH 5 can be plotted versus pH (Fig. 2c). The transition is well fit by a two-state pH titration curve, resulting in a $pK_a$ of 7.2. The resulting titration curve is the same when you use the $P(r)$ values at 50 or 126 Å (data not shown). That the data are best fit with a two-state model indicates that two distinct conformations are present in solution.

A $pK_a$ of 7.2 immediately suggests the involvement of histidine in the conformational change. Fitting the titration data with a multiple binding site model shows that 1–2 histidines are required for the transition between the two populations (Supplemental Fig. S1). There are 14 histidines present in one monomer of HtpG. To narrow down the list of candidates, we focused on domain interfaces. As was previously noted, the differences in the various conformations of Hsp90 result from large domain rearrangements, where the origins of these motions lie at the NM interface (between the NTD and the MD) or at the MC interface (between the MD and the CTD). There are 8 histidines clustered at the NM interface and 3 histidines clustered at the MC interface. To isolate which interface is relevant to the pH-dependent conformational changes, we analyzed the effect of pH on a monomeric truncation mutant containing only the NTD and the MD using SAXS. Scattering data were collected for the truncation mutant (NMHtpG; residues 1–495) for $Q=0.009–0.3$ at pH 6, pH 7, pH 8, and pH 9. No significant differences were seen for the scattering data at different pH values (Supplemental Fig. S2). The slight differences seen in the pH 6 curve (purple) are due to aggregation in the sample and do not result from actual conformational differences in the monomer. These results indicate that the pH-dependent conformational changes are localized to the MC domain interface.

**Modeling of scattering data reveals the presence of a second Grp94-like population**

From the $P(r)$ data collected in this study and the pH titration analysis, it appears that a two-state conformational equilibrium exists, with one state (at high pH) represented by the previously described extended conformation (Fig. 3a). If this hypothesis is true, the SAXS data collected at the other pH values would be fit by a linear combination of the previous model and a new model that represents the low-pH state. To maximally use the information from all of the SAXS data, we decided to simultaneously fit all of the pH data as a linear combination of two conformations. To do so, we modified a previously developed rigid-body modeling program PRFIT that was used to fit the SAXS data of HtpG at high pH. In the modified version, rigid-body refinement was performed on the low-pH conformation while the extended state (the previously determined pH 9 state) was held fixed.

In order to model the low-pH state, we treated the NM domains as rigid bodies that pivot around the MC interfaces given that the NM domain does not exhibit pH-dependent conformational changes. We also maintained a 2-fold dimer symmetry. Residue 500 on the flexible linker between the MD and the CTD was defined as the hinge point, and conformational space was systematically searched at defined angles about a general rotation axis, as described in our previous work. We found that −70° to 70° sufficiently covered the conformational space accessible to the HtpG dimer. At each refinement step, the best-scoring linear combination for each pH condition was calculated, and the combination with the best overall R-factor for the six pH conditions was chosen as the best model (Fig. 3a and b).

The $P(r)$ data for each of the six pH values are very well fit by a linear combination of the extended high-pH state and the more closed compact low-pH state (Table 1; Supplemental Fig. S3). The low-pH model was also confirmed using negative-stain EM (Fig. 3c and d). EM images were collected for HtpG at pH 5.5 and pH 7.5. From an examination of the two-dimensional (2D) images, a significantly larger population of more compact particles is present at low pH compared to neutral pH. High pH (pH 9) was also tested, and extended particles predominated (data not shown). In order to better compare our compact low-pH SAXS model to the EM data, we collected ~1000 single particles and generated reference-free class averages. Class averages were not generated for the neutral pH particle given the heterogeneity of the particles. Reference-free alignments of the more compact particles were then compared to 2D projections of the low-pH SAXS model. Remarkably, when the
averages are aligned to 2D projections of our SAXS model, the conformations appear nearly identical, confirming the structure of this compact state and establishing the accuracy of our SAXS modeling methods (Fig. 3d).

The low-pH SAXS model is surprisingly reminiscent of the Grp94 crystal structure (Fig. 4). While the Grp94 crystal structure and the low-pH model (Grp94-like state) have similar open angles, the Grp94-like state has a more pronounced U shape, while the Grp94 crystal structure more closely resembles a V shape. This difference is the result of rotations of both the MD and the NTD of the Grp94-like state, as depicted in Fig. 4b. While the two structures are similar, the combinations with the low-pH model fit the data best (Fig. 4c). Linear combinations of the Grp94 crystal structure and the extended state give higher R-factors overall (pH 6, 6.14; pH 6.5, 4.74; pH 7, 2.62; pH 7.5, 2.34; pH 8, 4.16) than mixtures with the low-pH model (pH 6, 5.52; pH 6.5, 2.57; pH 7, 3.17; pH 7.5, 2.07; pH 8, 2.8).

The AMPPNP-bound conformation of HtpG is dependent on pH

From previous SAXS and EM studies, it is clear that nucleotide binding also significantly influences the conformational equilibrium of HtpG. The addition of AMPPNP at pH 8 shifts the conformation of HtpG from the apo state to an approximately 40:60 equilibrium between the apo state and the closed AMPPNP-bound state identified crystallographically.24,31 Given the importance of pH in influencing the conformational equilibrium for apo HtpG, we sought to assess the effect of pH on the conformational equilibrium of the AMPPNP-bound state.

Table 1. Populations of extended state and low-pH state (Grp94-like) for HtpG at varied pH values

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<thead>
<tr>
<th>pH</th>
<th>% Extended state</th>
<th>% Low-pH (Grp94-like) state</th>
<th>R-factor (%)</th>
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<tr>
<td>6</td>
<td>41</td>
<td>59</td>
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<tr>
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<td>100</td>
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<td>3.12</td>
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Scattering data were collected from pH 6 to pH 9 for HtpG in the presence of saturating levels of AMPPNP. As can be seen in Fig. 5, there also exists a pH-dependent conformational shift in the presence of nucleotide. Contrasting with the apo data, we were unable to fit the AMPPNP data with a simple two-state titration model, suggesting that a third conformation may exist. To better define the source of the conformational equilibrium in the presence of AMPNP, we examined the structure of the NM domain. SAXS data for NMHtpG in the presence of AMPPNP support the presence of the AMPPNP-bound crystal state as an additional conformation in solution. At low pH, NM domain scattering matches the single NTD–MD conformation found in the low-pH and high-pH apo states, while at high pH, the NMHtpG data indicate that the structure of the AMPPNP-bound NM domain matches that seen in the full-length yeast crystal structure. The best fit was achieved with a mixture of monomeric and dimeric forms of the AMPPNP-bound NM domain (R-factor of 3.5% with 74% dimer and 26% monomer; Supplemental Fig. S4). A DAMMIN reconstruction also indicated the presence of the AMPPNP dimer at high pH (data not shown). This is the first observation of an NM dimer in solution and may help explain the residual ATPase activity for the isolated NM domain.

Fig. 4. The low-pH Grp94-like HtpG state closely resembles the Grp94 crystal structure. (a) Top-down and side views of the Grp94 crystal structure and the Grp94-like HtpG model. (b) Cartoon representation of the Grp94-like model depicting the rotations necessary to transform this state into the Grp94 crystal state. Rotations were calculated with the program LSQMAN. (c) Representative fits to the SAXS data at pH 8 (black) using a linear combination including the low-pH Grp94-like model (blue) or the Grp94 crystal structure (brown). The fit is better, especially at large distances, when using the low-pH Grp94-like model.
domain. To test for the yeast crystal state in full-length HtpG, we fitted each pH state with a linear combination of three conformations: the two apo states (extended and Grp94-like states) described above and the ATP state (closed state), as seen in the yeast crystal structure (Table 2; Supplemental Fig. 6a). The three conformations were sufficient to provide good fits to the AMPPNP data, suggesting that at physiologic pH in the presence of AMPPNP, HtpG is in equilibrium between these three states.

In the presence of AMPPNP, the amount of each conformation varies significantly with pH, as seen in Fig. 5b. The dominant states are represented by the Grp94-like state and the closed state. Interestingly, the Grp94-like state is greatly stabilized relative to the extended state by the presence of AMPPNP especially around pH 7.5 (Fig. 5b and c). The lack of a closed state at low pH is not, however, indicative of differential AMPPNP binding. Using a standard tryptophan fluorescence assay, we confirmed that the binding of AMPPNP is independent of pH (data not shown). This leads to the question of what the roles of each of these conformations are in the functional cycle of HtpG.

**Table 2.** Populations of extended state, Grp94-like state, and closed state for HtpG/AMPPNP at varied pH values

| pH | % Extended state | % Grp94-like state | % Closed state | R-factor (%)
<table>
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<td>40</td>
<td>60</td>
<td>2.26</td>
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<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>3.15</td>
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In the presence of AMPPNP, the amount of each conformation varies significantly with pH, as seen in Fig. 5b. The dominant states are represented by the Grp94-like state and the closed state. Interestingly, the Grp94-like state is greatly stabilized relative to the extended state by the presence of AMPPNP especially around pH 7.5 (Fig. 5b and c). The lack of a closed state at low pH is not, however, indicative of differential AMPPNP binding. Using a standard tryptophan fluorescence assay, we confirmed that the binding of AMPPNP is independent of pH (data not shown). This leads to the question of what the roles of each of these conformations are in the functional cycle of HtpG.

**Mutations at H446 shift the conformation of HtpG independently of pH**

Given that the titration analysis of the apo conformational change suggests the involvement of one to two histidine residues, it is reasonable to assume that a histidine could be mutated to shift the conformation of apo HtpG regardless of pH. As previously
discussed, histidines (H446, H530, and H574) located at the MC interface (Fig. 6a) are the most likely candidates for the responsible titratable group. We systematically mutated these groups to lysines to mimic the protonated state of the histidine expected at low pH. To ensure that the mutations did not disrupt the dimerization interface, we determined the molecular weight of each mutant using multiangle light scattering. Both the H446K and the H530K mutants formed dimers, whereas the H574K mutant was a monomer in solution (data not shown). Residue H574 was therefore not further pursued. The mutants were also examined by CD spectroscopy, and no differences were seen between H446K, H530K, and wild-type (WT) HtpG (data not shown), indicating that the overall secondary structure is unchanged.

The SAXS profiles for the H446K and H530K mutations were initially collected at pH 6 and pH 9, and the resulting $P(r)$ values were compared to the $P(r)$ values for the WT protein. The H446K mutation significantly shifts the conformational equilibrium towards the low-pH state at both pH 6 and pH 9 (Fig. 6b). This shift is consistent with the pH dependence seen in the WT protein. The H530K mutation, on the other hand, shifts the conformational equilibrium towards the high-pH state (Supplemental Fig. 6a). We also examined H530K in the context of a nontitratable, yet polar, mutation of H446 (H530K/H446Q). This showed a complete shift to the high-pH extended state (Supplemental Fig. 6c).

Because the H446K mutation is consistent with the dominant pH effect seen for the WT protein while H530K behaves in an opposite manner, we focused on residue 446 for further analysis. Modeling of the H446K scattering data shows that the solution state is a combination of ~80% of the low-pH state.

Fig. 6. H446 is primarily responsible for the observed pH-dependent conformational equilibrium. (a) Cartoon depicting the location of histidine residues at the MC interface. The histidines are represented as red spheres, with the NTD in blue, the MD in green, and the CTD in gold. (b and c) SAXS profiles for (b) H446K and (c) H446E (pH 6, gold; pH 9, purple) compared to WT (pH 6, black; pH 9, blue). (d) ATPase activity of WT, H446K, and H446E HtpG. ATPase activity was measured using radioactively labeled ATP, as described previously.
and ~20% of the high-pH state, regardless of pH (Fig. 7b). Residue 446 was then mutated to glutamic acid (H446E), and the conformation shifted completely to the extended state, as predicted (Figs. 6c and 7b). In the presence of AMPPNP, both H446K and H446E are still able to populate the closed ATP state (data not shown), suggesting that both the extended state and the Grp94-like state are able to convert into the closed state. Both mutations are also able to turn over ATP in an ATPase assay; however, their overall activity is ~1/3 that of WT HtpG (Fig. 6d).

H446Q was made as a nontitratable mutation that should be able to act either as a hydrogen bond donor or as a hydrogen acceptor. As such, we expected it to remove any pH dependence resulting from residue 446. Surprisingly, this mutant appears to be almost identical with the WT protein in terms of its pH dependence (Supplemental Fig. 6b), suggesting that another residue must be involved (Fig. 7b). Since, as discussed above, mutation of the only other relevant histidine residue (H530) produced an effect opposite to that observed with the WT protein, this result appears paradoxical. Examination of the relevant crystal structures shows that H530 resides in a highly polar/charged environment in both the extended state and the Grp94-like state; hence, the H530K mutation could result in interactions with neighboring groups that are quite distinct from those interacting with the WT histidine. Thus, while H530 undoubtedly contributes to the overall pH effects seen in the WT protein, the complexity of the local environment makes it difficult to probe its contributions in a straightforward manner.

By contrast, our SAXS data indicate that H446E and H446K shift the conformation in a way that is consistent with WT protein behavior. Given the potential importance of these mutations for testing the functional role of different HtpG conformations without having to shift the pH from neutrality, we sought a confirmation of the SAXS results by negative-stain EM. As expected, the H446K mutant HtpG particles incubated at pH 7.5 were predominately in a more compact state identical with WT HtpG at low pH. Conversely, H446E particles at pH 7.5 were more extended and similar to WT HtpG at high pH. Reference-free alignments of the H446K particles match 2D projections of the Grp94-like model remarkably well, confirming that the compact structure seen for H446K is the same structure seen in WT HtpG at low pH (Fig. 7a).

Different conformations have distinct functional roles in suppressing the aggregation of CS

Since it has been postulated that client proteins first bind to the apo state, of key importance is determining whether or not the two conformations are functionally distinct. To test this, we used the well-established CS aggregation assay. It has been shown that at 43 °C, CS aggregates in solution, and that this aggregation can be suppressed by adding Hsp90. This suppression of aggregation does not depend on nucleotide.

The CS assay emphasizes the importance of mutations that alter the conformation of Hsp90 while maintaining a constant pH. Changes in the pH of the system cause the aggregation behavior of CS to vary greatly (data not shown). The H446K and H446E mutations allow us to assess the role of Hsp90 conformation in the aggregation assay in the absence of any extraneous pH effect. As expected, the addition of HtpG at pH 7.5 greatly reduces CS aggregation (Fig. 8). We tested both the H446K mutant (Grp94-like conformation) and the H446E mutant (extended conformation) at pH 7.5 for their ability to prevent CS aggregation. As shown in Fig. 8, H446K, the Grp94-like conformation, is considerably more effective than the WT protein in preventing the

![Fig. 7.](image-url) H446K shifts the conformation to the Grp94-like state at neutral pH, whereas H446E shifts the population to the extended conformation. (a) Single-particle negative-stain EM of H446K at pH 7.5 shows more compact particles that are identical with the 2D projections of the Grp94-like model. The number of single-particle images included in each average is listed. The scale bar represents 250 Å, and single-particle boxes are 250 Å across. (b) Relative populations of the Grp94-like state (black) and the extended state (blue) for H446K (continuous) and H446E (broken).
aggregation of CS, whereas H446E, the extended conformation, has little effect on the aggregation of CS. Moreover, WT activity corresponds to the fraction of the low-pH state normally present at pH 7.5. WT HtpG shows a slight aggregation over 18 min at 43 °C, but this aggregation is negligible compared to that of CS. Both mutants displayed no aggregation after 18 min at 43 °C (Supplemental Fig. 7). These data indicate that there are significant functional differences between the two conformations of HtpG.

Discussion

It is becoming more and more apparent that structural dynamics plays an important role in the function of Hsp90, and understanding the conformational ensemble is imperative for better defining its molecular mechanism. Large conformational changes occur throughout the ATPase hydrolysis cycle, and previous structural and kinetic studies have shown that the conformational state is not rigorously determined by the bound nucleotide, but that nucleotide binding results in a conformational equilibrium between the apo state and the nucleotide state.24,29,31 Conformational equilibria have been shown as functionally important in other systems as well. For example, NMR studies have revealed that the protooncogene Ras exists in a conformational equilibrium that can be shifted with point mutations. The equilibrium shift then promotes interactions with effector proteins, leading to an increase in oncogenic potential.40 In another example, single-molecule FRET studies revealed that even in the absence of substrate, adenylate kinase exists in both the open state and the closed state. Notably, the closed state, which has been previously thought to exist only in the presence of substrate, is favored.31 These examples also support the realization that the functional pathway of Hsp90 must be considered as a stochastic process rather than as a deterministic ATP machine, as has been observed for other chaperones.

Here, both SAXS and single-particle EM have revealed an unanticipated solution conformation of apo HtpG that exists in a pH-dependent two-state conformational equilibrium with the previously described extended apo state. Interestingly, the apparent pK_a for the conformational transition between the extended state and the Grp94-like state is 7.2, suggesting that under physiological conditions, the system is optimized for maximum levels of both conformations. By using a linear combination of two structures together with rigid-body modeling, we were able to dissect out the two populations of HtpG and to discover that the low-pH conformation of HtpG is remarkably similar to the recent Grp94 crystal structure. While the exact rotations of the MD and the NTD differ between our low-pH structure and the Grp94 crystal structure, the overall architecture is surprisingly similar. Although our SAXS data is fit very well with a two-state model, it is likely that a third state, the closed AMPPNP-bound crystal state, is present as well. Recent single-particle EM studies have directly observed the presence of the closed state under apo conditions.31 The closed state has also been identified under apo conditions for yeast by single-molecule FRET, and analysis of energy barriers suggests that all states observed in the presence of nucleotide are accessible in the absence of nucleotide.52 If the closed state represents less than 5–10% of the total population, its effect on SAXS modeling would be negligible. Therefore, the apo state of HtpG is most likely represented by the extended state, the Grp94-like state, and a small amount of the closed state. The conformational equilibrium of HtpG persists in the presence of saturating AMPPNP, with the closed state now being significantly populated. The AMPPNP-bound equilibrium can also be shifted with pH, with the Grp94-like conformation being most populated at low pH and with the closed state being most populated at high pH.

Given that the Grp94-like state has both conformational features of the extended state (NTD–MD angle) and the closed state (MD–CTD angle), it is possible that the low-pH Grp94-like state (G) represents an intermediate step along the pathway proceeding from the extended state (E) to the closed state (C) (pathway (1)). Alternatively, the Grp94-like state could be off-pathway (pathway (2)), or the Grp94-like and extended states could be combined in a three-way pathway with the closed state (pathway (3)):

\[ E \rightarrow G \rightarrow C \]  
(1)

\[ G \rightarrow E \rightarrow C \]  
(2)
Typically, kinetics measurements are used to determine the connectivity of a particular pathway. However, it seemed that similar information should be extractable from the pH equilibrium data. We know from the mutant HtpG SAXS data in the presence of AMPPNP that both the extended state and the Grp94-like state can interconvert into the closed state, suggesting that pathway (3) is the relevant pathway. Given that the only structural changes required for the transformation from the extended state to the Grp94-like state occur at the MC interface, we assumed that this pH-dependent equilibrium is unaffected by the addition of AMPPNP. With this assumption, we modeled the pathways using standard kinetic equations and confirmed that only a model allowing all three states to interconvert, pathway (3), can recapitulate the steady-state levels observed in the presence of AMPPNP. The modeling indicates that the conversion from the extended state into the Grp94-like state and the conversion from the extended to the closed state both have similar pK_a values, whereas the conversion from the Grp94-like state to the closed state is independent of pH. This suggests that the main role of pH is to shift the equilibrium for the MC domain conformation. Because the Grp94-like state is stabilized in the presence of AMPPNP, the dominant pathway from the extended state to the ATP state most likely goes through the Grp94-like state, despite the fact that all three species can interconvert. A recent hydrogen exchange study of HtpG further supports this idea with the demonstration of an intermediate state even in the presence of CS. The inability of CS to shift the conformational equilibrium of HtpG also suggests that the interaction is weak and transitory. It is also possible that the CS/HtpG interaction is mediated by local changes in HtpG that occur between the two apo states. For these and other possible modes of interaction to be distinguished, more detailed structural studies of the interaction of HtpG with CS will be required.

In order to better understand the molecular mechanism for the conformational switch, we examined the residues contacting H446 in both the extended high-pH model and the Grp94-like low-pH model. Unfortunately, no obvious contacts that would account for the pH dependence of the conformational change were being made. Comparison of the analogous residue to H446 in the Grp94 structure (F554) provides some insight into the molecular basis of the conformational switch. In Grp94, F554 is nicely packed in a hydrophobic pocket comprising residues that are conserved in HtpG as hydrophobic. The one nonconserved residue contacting F554 is a serine (S725) in Grp94 and a glutamate (E604) in HtpG. This residue could explain the preference of HtpG for the Grp94-like state only when the histidine is either protonated at low pH or replaced with a lysine. Both of these would be able to form a favorable electrostatic interaction with E604, whereas the H446E mutation would be expected to clash with E604, shifting the population towards the extended state as is observed.

The use of histidines to modulate the conformation of Hsp90 appears to be a uniquely bacterial trait. The histidines involved in HtpG are not conserved in higher organisms. The similarity of the functionally active apo HtpG state to the Grp94 structure, however, suggests that Grp94 may utilize the same structural pathway as other Hsp90 homologs, and that the different structural states are conserved from bacteria to higher eukaryotes. Given the evidence that the extended, ATP, and ADP states are structurally conserved from bacteria to higher
eukaryotes, it is probable that the intermediate Grp94-like conformation found here is also important in higher eukaryotes. Recent FRET studies have shown that yeast Hsp90 has at least two intermediate states between the already described open apo state and the closed ATP state.\textsuperscript{32,33} One possibility is that one of the intermediate states in yeast is related to the Grp94-like state described here, but this hypothesis will require further characterization of the yeast solution structure.

Our previous work}\textsuperscript{24,31} and FRET studies demonstrate that nucleotide shifts the conformational equilibrium as opposed to explicitly determining the conformational state of Hsp90. An important difference between eukaryotes and prokaryotes may be the other factors that determine the conformational equilibrium. One hypothesis is that the equilibrium in eukaryotes has evolved to be controlled by client/cochaperone binding instead of pH. Bacteria may rely more heavily on the finely tuned conformational equilibrium to optimize the levels of both conformations because of the lack of cochaperones. It is also possible that the HtpG pH equilibrium acts as a sensor to tune chaperone response in times of metabolic stress. Identification of HtpG client proteins will assist in the development of \emph{in vivo} HtpG functional assays and provide information as to the types of client proteins that interact with each conformational state.

The functional differences of the different apo states observed here may also be relevant to other homologs. The question as to the generalizability of the preferential binding of the Grp94-like structure to client proteins remains. One possibility is that different substrates are recognized by different conformations of Hsp90, and this structural diversity is therefore one mechanism by which Hsp90 recognizes such a broad set of substrate proteins. All of these questions will require further studies of yeast and human proteins and their interactions with client proteins. The results discussed here with HtpG provide critical insights into the mechanism of substrate activation and the differences in regulation between different species, and these results are an important step in better understanding the chaperone cycle of Hsp90.

**Materials and Methods**

**Protein expression and purification**

Full-length HtpG (residues 1–624) was cloned into pET29b (Invitrogen), and the HtpG truncation mutant NMHtpG (residues 1–495) was cloned into pET151 using the TOPO cloning system (Invitrogen). Point mutations were made using PCR and 30–35 nucleotide primers encoding for the mutated amino acid. Parental DNA was digested with DpnI after the completed reaction, and the resulting plasmid was transformed into TOP10 (Invitrogen) cells. All mutations were confirmed by sequencing of the appropriate regions. All constructs were expressed in \emph{Escherichia coli} using BL21(DE3) cells (Invitrogen). The full-length WT protein was purified by successive passage over DE52 (Whatman), monQ, and gel-filtration (Amerham Biosciences) columns with a final buffer consisting of 20 mM Tris (pH 7.5) and 100 mM NaCl. NMHtpG and the point mutations were first passed over Ni-NTA agarose, and the collected peak was cleaved with tobacco etch virus overnight. The cleaved protein was then purified by gel filtration (Amerham Biosciences) into a final buffer consisting of 20 mM Tris (pH 7.5) and 100 mM NaCl. All proteins ran as a single monodisperse peak on gel filtration. CD spectroscopy of the proteins at 20 μM in 50 mM Tris (pH 8), 50 mM KCl, and 10 mM MgCl\textsubscript{2} showed no difference in 2º structure between the different constructs. The purified proteins were collected and concentrated using 10-kDa molecular-mass-cutoff Amicon Ultra spin concentrators (Millipore). With the use of a desalting column (GE Healthcare), the samples were exchanged into 50 mM buffer [4-morpholineethanesulfonic acid (pH 6), bis-Tris (pH 6.5), or Tris (pH 7, pH 7.5, pH 8, or pH 9)]. 50 mM KCl, 10 mM MgCl\textsubscript{2}, and 1 mM DTT. With the use of Millipore Ultrafree\textsuperscript{\textregistered}–0.5 Centrifugal Filter Units, samples were concentrated to between 10 and 30 mg/ml, as estimated by absorbance at 280 nm. The $A_{280}$ nm/$A_{280}$ nm ratio was measured as <0.6, giving confidence that no nucleotide was present in the purified sample. Samples were split into two aliquots, with 10 mM AMPPNP added to one aliquot. Samples were then flash frozen in liquid nitrogen and stored at ~80 °C.

**SAXS data collection**

Initial SAXS measurements were collected at beamline 4-2 of the Stanford Synchrotron Radiation Laboratory (SSRL) and at the BioCAT beamline (18-ID) of the Advanced Photon Source. Data reported here were collected at SSRL beamline 4-2 and at SIBYLS beamline (12.3.1) of the Advanced Light Source (ALS). To minimize aggregation, we spin samples in a table-top microcentrifuge for 5 min immediately before data collection. Data were then collected at 25 °C at 2.5, 5, and 10 mg/ml. At the ALS, samples were exposed for 70 and 70 s at a detector distance of 1.6 m. At SSRL, samples were exposed for five 30-s exposures at a detector distance of 2.5 m. The data were collected on a Mar165 CCD detector, and detector channels were converted into Q = 4πsinθ/λ, where θ is the scattering angle and λ is the wavelength, using a silver behenate sample as calibration standard. The data were circularly averaged over the detector and normalized by incident beam intensity.

**SAXS data analysis**

The raw scattering data I(Q) were scaled, and the buffers were subtracted. Variations were seen in the amplitude of the high-Q data for data sets that should be identical. To correct for this variation, we linearly extrapolated data from multiple concentrations to the buffer background signal. The experimental buffers were then scaled to match the calculated buffer background signal. This correction was only necessary for a precise comparison to molecular models and did not affect the overall shape of either the scattering curves or the interatomic distance profiles (P(r)). Individual scattering curves for a given nucleotide condition collected at different concentrations and over different scattering angle ranges were scaled and merged to yield a single low-noise average scattering curve. The radii of gyration ($R_g$) were initially calculated from the Guinier
plot as implemented in the program PRIMUS.\textsuperscript{42} The interatomic distance distribution functions (P(r)) were then calculated using the program GNOM.\textsuperscript{43} D\textsubscript{max} was determined by constraining r\textsubscript{max} to zero and then varying r\textsubscript{max} between 150 and 200 Å. D\textsubscript{max} was chosen as the r\textsubscript{max} where the P(r) smoothly approached zero based upon perceptual criteria. The overall shape of the P(r) function was very robust for D\textsubscript{max} = 10 Å. The molecular modeling of the scattering data was then performed using the in-house software PRFIT, as previously described.\textsuperscript{24}

**Electron microscopy**

HtpG protein was negatively stained with uranyl format on thin-carbon layered (40–50 Å thick) 400-mesh copper grids (Pelco), as described previously.\textsuperscript{13,44} Prior to staining, WT and H446K HtpG (200 nM) were incubated at 37 °C for 10 min in either 20 mM Tris (pH 7.5) or 20 mM 4-morpholineethanesulfonic acid (pH 5.5) and 50 mM KCl. Samples were imaged using a Tecnai G2 Spirit TEM (FEI) operated at 120 keV. Micrograph images were recorded using a 4000 × 4000 charge-coupled device camera (Gatan) at a magnification of 68,000× with a 2.21-Å pixel size.

Two-dimensional single-particle analysis was carried out using the EMAN software package.\textsuperscript{45} Reference-free class averages were determined using *stars* classes from a set of 811 and 1109 single particles (collected from 9 and 20 micrographs, respectively) for WT (pH 5.5; Fig. 3d) and H446K (pH 7.5; Fig. 7a), respectively. This method involves a k-means clustering analysis where the particles are separated into a given number of classes, and no other information, such as information on the SAXS models, was used for alignment or as a reference to generate the image. This was critical for generating unbiased averages that were not influenced by known structures. Between 1% and 5% of the total particles collected were aligned in each of the average images. This was expected given the apparent random orientation of the particles on the grid. The class averages (27 and 22, respectively) were then lowpass filtered (25 Å), aligned, and rotated (using *classesbymerge* and *align2d*) to 2D projections of the low-pH HtpG SAXS model for qualitative comparison.

**CS assay**

CS aggregation was monitored with a Jobin Yvon FluoroMax-3 fluorescence spectrophotometer with a temperature-controlled jacket and automatic stirring. The excitation and emission wavelengths were both set to 500 nm with slit widths of 2 nm. CS was purchased from Sigma and prepared for thermal aggregation, as described previously.\textsuperscript{38} HtpG and variants at 300 nM dimer were incubated for 5 min at 43 °C and 40 mM Hepes (pH 7.5) before rapidly diluting CS 1:100 to a final concentration of 75 nM dimer and monitoring the resulting light scattering.

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.04.080

**References**


