ATPase Activity and ATP-dependent Conformational Change in the Co-chaperone HSP70/HSP90-organizing Protein (HOP)*

Received for publication, January 24, 2014 Published, JBC Papers in Press, February 17, 2014, DOI 10.1074/jbc.M114.553255

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Co-chaperones help to maintain cellular homeostasis by modulating the activities of molecular chaperones involved in protein quality control. The HSP70/HSP90-organizing protein (HOP) is a co-chaperone that cooperates with HSP70 and HSP90 in catalysis of protein folding and maturation in the cytosol. We show here that HOP has ATP-binding activity comparable to that of HSP70/HSP90, and that HOP slowly hydrolyzes ATP. Analysis of deletion mutants revealed that the ATPase domain of HOP is in the N-terminal TPR1-DP1-TPR2A segment. In addition, HOP changes its conformation in the presence of ATP. These results indicate that HOP is a unique co-chaperone that undergoes an ATP-dependent conformational change.

The 70 kDa and 90 kDa heat shock proteins (HSP70 and HSP90)† are major molecular chaperones in the eukaryotic cytosol. These chaperones play essential roles in protein quality control by preventing protein aggregation, catalyzing the folding of newly synthesized proteins, and promoting degradation of denatured proteins (1–3). HSP70 recognizes hydrophobic surfaces of unfolded and partially folded proteins, and it releases these substrate proteins after undergoing conformational changes over the course of its ATPase cycle (4). HSP70 consists of a highly conserved N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD) (5, 6). HSP90 consists of three functional domains, the N-terminal, middle, and C-terminal dimerization domains. The middle domain is involved in ATP hydrolysis, client protein binding, and co-chaperone binding, whereas the C-terminal domain is required for HSP90 dimerization (7, 8). During the ATPase cycle, open and closed structures of HSP90 are produced, and these conformations are important for stabilization of the client proteins (9, 10).

HSP70 and HSP90 cooperate with co-chaperones during the process of protein folding, including maturation of steroid hormone receptors, kinases, and p53 (11, 12). The HSP70/HSP90 organizing protein (HOP, also known as stress-inducible protein 1 or STI1) is a co-chaperone that cooperates with HSP70 and HSP90 in protein folding. HOP acts as a scaffold for HSP70/HSP90 and modulates the functions of these chaperones (12–16). HOP is a monomeric protein (9, 17) composed of three tetratricopeptide repeat domains (TPR1, TPR2A, and TPR2B) and two aspartic acid-proline domains (DP1 and DP2) (16). The TPR domains are protein-protein interaction modules containing helix-turn-helix structures (18). HOP binds HSP70 and HSP90 through the TPR1 domain or the TPR2B domain and the TPR2A domain via ionic interactions (19–21). The solution structure of the yeast HOP/STI1 DP domains has been determined by nuclear magnetic resonance (NMR) analysis, which revealed that the DP1 and DP2 domains consist of six and five helices, respectively (21). The DP2 domain provides important support for the chaperone activity of HSP90 (21, 22). However, the detailed mechanisms of HOP-assisted, chaperone-dependent protein folding are largely unknown. Here, we report that HOP has ATPase activity and changes its conformation in the presence of ATP. We discuss the possible roles of ATP-dependent conformational changes in regulating HOP function.

* This work was supported by a Grant-in-Aid for Young Scientists (B), a Sasakawa Scientific Research Grant from the Japan Science Society (to S. Y.), and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to H. K. and H. I.).

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‡ The abbreviations used are: HSP, heat shock protein; HOP, HSP70/HSP90-organizing protein; NBD, nucleotide-binding domain; SBD, substrate-binding domain; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

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EXPERIMENTAL PROCEDURES

Expression Vectors and Purification—Expression vector encoding human full-length HOP-(1–543) was constructed as previously described (10). DNAs encoding domain-deletion mutants (containing amino acids 106–543, 352–543, 1–359, 1–224, 106–224, 225–309, and 106–309) were generated by polymerase-chain reaction (PCR) using specific primers, and then subcloned into the pCold I hexahistidine-tagged (His6) protein expression vector (Takara, Tokyo, Japan). The resultant vector was transformed into Escherichia coli BL21, and the bacteria were grown in L-broth at 37 °C. Expression of His6-tagged full-length HOP and its deletion mutants were induced in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 24 h at 15 °C. Bacteria were harvested, resuspended in 10 mM Tris-HCl buffer, pH 7.4, and lysed by sonication on ice. Supernatant was recovered after centrifugation at 20,000 rpm for 5 min at 4 °C and then applied onto a Ni-NTA column (GE Healthcare, Amersham Biosciences) equilibrated with buffer A (50 mM NaCl in 25 mM HEPES-KOH, pH 7.4) for 5 min at 4 °C and then applied onto a Ni-NTA column (GE Healthcare, Amersham Biosciences) equilibrated with buffer A supplemented with 200 mM NaCl. After incubation, partially digested HOP fragments were separated by SDS-PAGE on 11% gels and blotted onto polyvinylidene difluoride filters. After blotting, the filter was stained with 0.025% Coomassie Brilliant Blue R250 in 40% ethanol for 30 min at room temperature, de-stained by washing in 50% ethanol, and washed in 100% ethanol. Protein bands excised from filters were N-terminally sequenced using a LC 491 protein sequencer (Applied Biosystems).

NMR Spectrometry—NMR experiments were recorded using a 600 MHz spectrometer (DRX-600, Bruker Biospin) equipped with a 5 mm triple-resonance inverse (TXI) probe or with a 5 mm BBO (Broadband observe) probe. For monitoring of protein signals, proteins (0.3 mM) were dissolved in 20 mM potassium phosphate buffer (pH 6.5), 50 mM KCl, and 5 mM MgCl2 containing 10% (v/v) D2O, and then titrated ATP at a 10-fold molar excess. Proton temperature was set at 25 or 5 °C, and the water signal was suppressed using the WATERGATE pulse sequence.

For determination of dissociation constants, all the samples were prepared in 25 mM MES and 5 mM MgCl2, pH 6.5. For the titration experiment, HOP was used at a concentration 135 μM. Various amounts of ATP stock solutions were added to HOP solutions, and 1H and 31P NMR spectra of ATP were recorded after each titration. To determine the dissociation constant (Kd) of HOP with ATP, the chemical shift changes of H2 (8.25 ppm) and H8 (7.88 ppm) protons of ATP were taken, because these proton signals exhibited chemical shift changes as the ATP concentration was gradually increased. NMR data were processed using XWIN-NMR ver. 3.5 (Bruker Biospin).

Kd values were determined by a non-linear fitting method using Equation 1.

\[
D_{\text{obs}} = D_{\text{max}} \left( \frac{(K_d + L_o + P_o) - \sqrt{(K_d + L_o + P_o)^2 - 4L_oP_o}}{2P_o} \right)
\]

Here, \(L_o\) and \(P_o\) indicate the molar concentrations of HOP and ATP, respectively; \(D_{\text{obs}}\) is the observed chemical shift change; and \(D_{\text{max}}\) is the change in chemical shift at saturation.

RESULTS AND DISCUSSION

HOP Binds ATP with an Affinity Comparable to That of HSP70 and HSP90 and Slowly Hydrolyzes ATP—Using purified HOP expressed in Escherichia coli, we analyzed the ATPase activity of the purified HOP and determined the kinetic parameters using the Michaelis-Menten equation (Fig. 1A). The catalytic constant (kcat), which represents the number of ATP molecules hydrolyzed by HOP, was estimated to be 3.8 ± 0.3 × 10⁻³ mol ATP/mmol HOP/min. The ATP hydrolysis rate of HOP was lower than those of porcine brain HSP70 (kcat = 85.3 ± 15.0 × 10⁻⁵) and HSP90 (kcat = 27.9 ± 3.4 × 10⁻³). To determine the dissociation constant (Kd) of HOP for ATP, we performed NMR titration experiments. We first examined the 31P chemical shift changes of the ATP phosphate group; no significant changes were observed (data not shown). Therefore, we analyzed the chemical shift changes on adenine H2 and H8 protons of ATP. We observed chemical shift changes in both the H2 and H8 proton signals at each titration point (Fig. 1C). These chemical shift changes were used for the determination
of $K_d$ values using non-linear fitting methods, and $K_d$ was estimated to be 350 $\mu M$ (Fig. 1D). These results indicated that HOP binds to ATP with considerable affinity and then slowly hydrolyzes ATP.

To determine the domains responsible for the ATPase activity, we constructed a series of deletion mutants and purified the protein segments from bacteria (Fig. 2A). These segments were incubated with ATP at 37 °C for up to 120 min, and then ATP hydrolysis activities were determined (Fig. 2, B and C). The ATPase activity of the HOP-(1–359) fragment (TPR1-TPR2A) was very similar to that of full-length HOP-(1–543), defined here as 100%. By contrast, the 106–543 ($\alpha$1) and 352–543 (TPR2B-DP2) fragments exhibited significantly reduced ATPase activities (57.2 ± 4.3% and 6.1 ± 8.5%, respectively). These results indicated that the 1–359 region plays an important role in ATPase activity. We further analyzed the 1–359 region by analyzing smaller sub-fragments. The ATPase activity of the 106–359 fragment (DP1-TPR2A) was 36.0 ± 3.1%, and the ATPase activities of the 1–224 (TPR1-DP1), 106–224 (DP1 domain), and 225–359 (TPR2A domain) fragments were almost negligible (9.6 ± 2.7%, 5.7 ± 2.5%, and 1.4 ± 1.8%, respectively). These results indicated that the 106–359 region is essential for the ATPase activity of HOP, where the 1–105 region makes a significant, but smaller contribution to ATPase activity.

We next used NMR spectroscopy to determine whether ATP directly interacts with the ATPase domain of HOP (Fig. 3). Specifically, we obtained the $^1$H NMR spectra of full-length HOP and the 1–359 region containing the ATPase domain (TPR1-TPR2A). $^1$H chemical shift changes were observed for full-length HOP and the 1–359 region containing the ATPase domain (TPR1-TPR2A). $^1$H chemical shift changes were observed for full-length HOP and the 1–359 region containing the ATPase domain (TPR1-TPR2A).
To identify the ATP-binding site of human HOP, we searched the amino acid sequence of HOP for known ATP-binding motifs, i.e. the Walker A motif (G/A XXXXGK(X)/S/T, where X indicates any amino acid), also know as the P-loop and phosphate-binding motif (25) and the Walker B motif (R/K-X\_2..10-O-X-O-D/E, where O indicates a hydrophobic amino acid) (26). The Walker B motif binds a divalent ion and the adenosine residue of ATP, and this motif is present in HSP70, HSP90, and other chaperones (27). HOP contains a Walker B motif in the TPR1, DP1, and TPR2B domains (Fig. 4A), but no Walker A motif. We focused on the Walker B motif of the DP1 domain because this region was essential for ATP hydrolysis (Fig. 3, B and C). To disrupt the Walker B motif, we constructed the D186A mutant in DP1. However, this mutation caused no significant change in ATPase activity (Fig. 4B). These results indicate that the Asp-186 residue is not essential for HOP ATPase activity, and suggest that HOP binds ATP using motifs other than Walker A and B. The NBD of the HSP70 and HSP90 structures are similar to those of actin and DNA-gyrase B, respectively (5, 28), and the ATP hydrolysis mechanisms of these non-Walker ATPases were recently elucidated (4, 8, 12, 21). Another non-Walker ATPase is the chaperonin GroEL; this chaperone has a unique ATP-binding motif (DGTTT) and phosphate-recognition region, i.e. a P-loop arrangement located between two $\alpha$-helices (25, 29). Thus, HOP may contain non-Walker ATP-binding motifs, although these motifs remain to be determined.

HOP Changes Its Conformation in the Presence of ATP—The\textsuperscript{1}H chemical shift change of full-length HOP and the 1–359 region containing the ATPase domain suggested that HOP might undergo conformational changes upon ATP binding (Fig. 3). In addition, most molecular chaperones change their conformations during ATP binding and hydrolysis (8). To determine whether HOP exhibits ATP-dependent conformational changes, we first performed protease digestion assays (30). HOP was incubated with trypsin in the presence and absence of various nucleotides (Fig. 5). HOP was more effectively protected from trypsin digestion in the presence of ATP (Fig. 5A, lane 3) than in the presence of ADP and AMP-PNP, a
non-hydrolyzable analog of ATP; the protection efficiency was 54.3 ± 7.5% for ATP, 31.0 ± 8.9% for ADP, and 28.6 ± 10.1% for AMP-PNP, respectively (Fig. 5B). Furthermore, the proteinase K digestion experiment suggests that the conformation of HOP in the presence of ATP differs from that in the presence of ADP or AMP-PNP, because the proteinase K-digested HOP fragments produced in the presence of ATP were partly different from those produced in the presence of ADP or AMP-PNP (Fig. 5C). Although HSP70 and HSP90 change their conformations upon nucleotide binding and hydrolysis (8, 31), the chaperonin CCT/TRiC changes its conformation only during ATP hydrolysis but not upon nucleotide binding (30). These observations suggest that the role of HOP in the ATP-dependent conformational changes may be similar to that of CCT/TRiC, but not to those of HSP70 and HSP90.

We next investigated the effect of ATP on the secondary structures of HOP by analysis of circular dichroism (CD) spectra. However, no significant change was detected (data not shown). Taken together with the results of the NMR and protease digestion experiments (Figs. 3A and 5), these results suggest that in the presence of ATP, HOP changes the relative orientations, but not the secondary structures, of its domain.

To further analyze the regions affected by the ATP-dependent conformational changes, we determined the amino acid sequences of trypsin-digested HOP fragments (Fig. 6, A and B). This sequencing analysis revealed that the region between TPR1 and DP1 was sensitive to protease digestion. Similarly, the hinge region between DP1 and TPR2A was susceptible to enzymatic cleavage. Because, the relative proportions of the fragments produced by digestion at these cleavage sites (#3, #4, and #5) changed in the presence of ATP, these finding indicated...
that the structure of HOP is altered, at least somewhat, at the N-terminal region near the DP1 domain. However, the level of the large #2 fragment rose in the presence of ATP, suggesting that the conformation of the C-terminal region is also affected by ATP. Thus, the conformational change at the N-terminal ATPase domain may trigger structural alteration of the C-terminal domain, although this possibility remains to be conclusively demonstrated. Nevertheless, these observations support the notion that the ATP-dependent conformational change of HOP occurs throughout the entire HOP molecule, rather than in a small restricted region.

HOP is a multi-domain protein containing three TPR and two DP domains. The TPR domains are protein-interaction modules that act as a scaffold for the HSP complex. TPR1 and TPR2B bind to HSP70, whereas TPR2A binds to HSP90 (19, 21). Recently, the TPR1-DP1 segment was suggested to serve as an HSP70-client delivery system for the TPR2A-TPR2B-DP2 segment, because HSP70 changes the HOP binding domain from TPR1 to TPR2B during the maturation of substrate proteins (21). The role of the DP1 domain is not well understood (32, 33). By contrast, the DP2 domain is known to be essential for HSP90-assisted protein maturation, because mutants lacking the DP2 domain are unable to assist in glucocorticoid receptor activation (21, 22, 34). In this study, we demonstrated that the ATP-dependent conformational change of HOP may contribute to HSP70/HSP90-assisted protein folding and maturation by rearranging the orientations of the five domains of HOP (35).

HOP, which is ubiquitously expressed in all cell type, and localizes in the cytoplasm, nucleus, and endoplasmic reticulum, as well as on the cell surface (33, 36–41). Transportation of HOP from the cytoplasm to the nucleus is regulated by phosphorylation (41). HOP affects maturation of the cystic fibrosis transmembrane conductance regulator protein through S-nitrosylation (37). In addition, HOP functions in neuroprotection and neuritogenesis by interacting with the prion protein on the cell surface (40, 42). These studies suggest that HOP has a variety of functions in vivo, in addition to its co-chaperone function for HSP70 and HSP90.

Based on our finding, we conclude that, cooperation between the N-terminal ATPase domain and the C-terminal chaperone interacting domain may be required for co-chaperone activity of HOP or other HOP-dependent cellular functions.

Acknowledgments—We thank members of the Systems Glycobiology Research Group, RIKEN, for their suggestions and valuable discussion.

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