The Yeast Heme-responsive Transcriptional Activator Hap1 Is a Preexisting Dimer in the Absence of Heme*

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In the absence of heme, Hap1 is associated with molecular chaperones such as Hsp90 and Ydj1 and forms a higher order complex termed HMC. Heme disrupts this complex and permits Hap1 to bind to DNA with high affinity, thereby activating transcription. Heme regulation of Hap1 activity is analogous to the regulation of steroid receptors by steroids, which involves molecular chaperones. Steroid receptors often exist as monomers when associated with molecular chaperones in the absence of ligand but as dimers when activated by steroids. Furthermore, previous studies indicate that dimerization might be important for heme activation of Hap1. We therefore determined whether Hap1 is a monomer or oligomer in the absence of heme. By coeluting two Hap1 size variants and by comparing DNA binding properties of the HMC and Hap1 dimer, we show that Hap1 is a preexisting dimer in the HMC. Further, increasing overexpression of Hap1 caused progressive increases in Hap1 DNA binding and transcriptional activities. Our data suggest that in the absence of heme, Hap1 exists as a dimer, and the two subunits act cooperatively in DNA binding. Hap1 repression is caused, at least in part, by inhibition of the DNA binding activity of the preexisting dimer.

Dimerization is a common mechanism by which the activity of numerous important biological macromolecules can be regulated. These molecules include receptors for growth hormones (1, 2), steroid hormones (3), other cellular signaling (4, 5), and numerous transcription factors (6, 7). The transcriptional activators of the yeast Gal4 family also require dimerization for DNA binding and transcriptional activation (8, 9). This family includes at least 52 transcription factors that control a wide array of diverse processes ranging from carbon source utilization to oxygen utilization and drug resistance (8, 9). These members all contain a C6 zinc cluster that recognizes a CGG triplet (9–15). Although the DNA binding properties of the C6 zinc cluster proteins are well characterized, the molecular mechanisms by which these members act to control transcription in response to various signals are largely unclear. Interestingly, recent data suggest that, like steroid hormone receptors, certain members of the yeast Gal4 family such as Hap1 and Pdr1 (16, 17) are regulated by Hsp90 and Hsp70 molecular chaperones. In particular, Hap1 is a heme-responsive transcriptional activator, which promotes transcription of genes required for respiration and for controlling oxidative damage in response to oxygen/heme (18–20). In the absence of heme, Hap1 is bound to cellular proteins including Hsp82 (the yeast homologue of Hsp90) and Ydj1, forming a higher order complex termed HMC1 (17, 21, 22). Hap1 DNA binding and transcriptional activities are repressed in this complex. Heme disrupts the HMC and permits Hap1 to bind to DNA as a dimer with high affinity, thereby activating transcription. The formation and disruption of the HMC are the key events in Hap1 repression in the absence of heme and subsequent activation by heme. How does the HMC repress Hap1, and how does the disassembly of the HMC lead to Hap1 activation?

In the case of steroid hormone receptors, a plethora of reports suggested a model for how molecular chaperones control their activities (23–25). In the absence of hormone, steroid hormone receptors form heteromeric complexes containing a receptor monomer, a dimer of Hsp90, and several other proteins including Hsp70 and Ydj1 (23–26). Hormone binding causes the dissociation of Hsp90 and other proteins, permitting steroid hormone receptors to dimerize and bind to DNA with high affinity and activate transcription (23–25). This suggests that dimerization is an important event leading to the activation of steroid hormone receptors. In the case of Hap1, we envisioned two ways by which Hap1 activity could be controlled by the HMC. First, Hap1, like steroid hormone receptors, could exist as a monomer in the HMC and is thus repressed. When the HMC disassembles in the presence of heme, Hap1 is free to dimerize and bind to DNA with high affinity, thereby activating transcription. Second, Hap1 could already be a dimer in the HMC, but its activity is inhibited by molecular chaperones in the HMC. The disruption of the HMC by heme would then relieve this inhibition, thereby leading to Hap1 activation. Previous experiments indicated that the Hap1 dimerization domain plays a role in heme regulation (17); however, it is not clear whether it acts by controlling dimerization or by making molecular interactions critical for Hap1 activity.

In this report we describe a series of experiments aimed at distinguishing whether Hap1 is monomeric or oligomeric in the HMC. First, the two size variants of Hap1 coeluted on affinity columns in the absence of heme. Second, DNA mobility shift assays showed that the HMC recognizes various Hap1-binding sites in a manner similar to Hap1 dimer binding but completely different from monomer binding. Third, increasing overexpression of Hap1 caused graded increases in Hap1 DNA binding and transcriptional activation in the absence of heme, indicating that overexpression of Hap1 can functionally titrate out molecular chaperones. Together, these results strongly suggest that in the absence of heme, Hap1 is a preexisting oligomer in the HMC.

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1 The abbreviations used are: HMC, higher molecular weight complex; PAGE, polyacrylamide gel electrophoresis; DC, dimeric Hap1 complex; GST, glutathione S-transferase; NTA, nitrilotriacetic Acid.
RESULTS
Fusion of the GST or Histag to the Hap1 N terminus Has No Effect on Heme Responsiveness or the Formation of the HMC. A, the activities of wild type Hap1, GST-Hap1, and Histag-Hap1 in heme-deficient (Low) or heme-sufficient (High) cells are shown. Histag-Hap1 oligomerizes in the absence of heme, and GST-Hap1 and Histag-Hap1 from the GAL1–10 promoter were transformed into yeast Δhap1Δhem1 cells (27) bearing the UAS1/CYC1-lacZ reporter. γ-Galactosidase assays were carried out as described under “Experimental Procedures.” Plotted data are averages of values obtained from at least three independent transformants, and standard deviations were within 20%. B, DNA binding complexes formed by Hap1, GST-Hap1, and Histag-Hap1 in the presence or absence of heme are shown. Extracts containing Hap1 (lanes 1 and 2), GST-Hap1 (lanes 3 and 4), and Histag-Hap1 (lanes 5 and 6) were incubated with radiolabeled DNA in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 5 ng/μl heme. The reaction mixtures were analyzed on a 4% polyacrylamide gel. Histag-Hap1 (lane 6) may appear to migrate faster than Hap1 in the presence of heme, likely because the band is much more intense and spread. C, purified proteins by the Ni-NTA column were separated on a 7% SDS-polyacrylamide gel. A Preexisting Hap1 Dimer—To characterize biochemically the HMC and to determine whether it contains a Hap1 monomer or oligomer, we purified the complex. We fused the GST or Histag tag to the Hap1 N terminus and determined how tagged Hap1 fusions respond to heme and how they form the HMC. As shown in Fig. 1A, both Histag-Hap1 and GST-Hap1 respond to heme in the same way.
as Hap1; they showed a low level of activity in the absence of heme, and hence greatly stimulated their activity. Like Hap1, GST-Hap1 and His$_6$-Hap1 formed a high molecular weight complex (HMC) and bound to DNA with low affinity (Fig. 1B). Heme disrupted this complex and permitted Hap1 to bind to DNA as a dimer with high affinity. These results show that fusion of His$_6$ and GST tags to Hap1 has no effect on heme responsiveness of tagged Hap1 fusion proteins and HMC formation. Therefore, the tagged Hap1 fusions can be used to purify the HMC. We initially tested purification by using both GST-Hap1 and His$_6$-Hap1. We prepared extracts from cells expressing a high level of GST-Hap1 and His$_6$-Hap1 from the GAL1–10 promoter. We then loaded the extracts onto glutathione or Ni-NTA columns, respectively. We found that both GST-Hap1 and His$_6$-Hap1 were highly enriched on the columns, but bound GST-Hap1 could not be easily eluted for unknown reasons. However, the bound His$_6$-Hap1 complexes could be successfully eluted by using imidazole. As shown in Fig. 1C, Hap1 was highly enriched in the eluate compared with crude extracts, and several other proteins were coeluted with Hap1 (see Ref. 17). These proteins also cofractionated with His$_6$-Hap1 on a Superose 6 column and were cross-linked to His$_6$-Hap1 (Ref. 17 and data not shown) but were not retained on the Ni-NTA column when extracts containing Hap1 (not His$_6$-Hap1) were used. These results strongly suggest that these proteins were bound to Hap1, not to Ni-NTA beads. The two most abundant proteins, p70 (Hsp70)$^2$ and p60, are in a molar ratio of about 1:1 to His$_6$-Hap1. Further, as expected, the purified HMC bound to DNA with low affinity; heme disrupted the HMC and permitted Hap1 to bind to DNA with high affinity (at least 10-fold higher) (Fig. 1D). This confirms the previous observation that Hap1 DNA binding activity is repressed in the HMC (17, 21, 30).

His$_6$-Hap1ΔKpn and Hap1 Coelute on the Ni-NTA Column in the Absence of Heme—After determining the appropriate conditions for purifying the HMC, we investigated whether Hap1 is a dimer/oligomer in the HMC by coelution. We imagined that if Hap1 exists as an oligomer in the HMC, two different Hap1 size variants should be complexed together in the HMC and thus should coelute on the column that retains one form of the size variants. However, if Hap1 exists as a monomer in the HMC, two Hap1 size variants should not be complexed together and should not coelute on the column. Because all Hap1 domains except for the activation domain are required for forming a stable HMC, we used His$_6$-Hap1ΔKpn (containing Hap1 residues 1–1309 but not residues in the activation domain) and Hap1 as two size variants. Longer Hap1 size variants were not used because of difficulties in cloning and expressing long Hap1 derivatives. We coexpressed His$_6$-Hap1ΔKpn and Hap1 in yeast cells and prepared extracts from these cells under the condition that all Hap1 forms the HMC (22). We then loaded the extracts containing both His$_6$-Hap1ΔKpn and Hap1 (Fig. 2, lane 2) onto a Ni-NTA column. As a control, we loaded extracts containing only Hap1 (Fig. 2, lane 1) onto a Ni-NTA column (for reasons unclear, His$_6$-Hap1ΔKpn appeared to be expressed at a higher level than Hap1; see Fig. 2, lanes 1 and 2). We then extensively washed these columns and eluted them with imidazole. We found that Hap1 coeluted with His$_6$-Hap1ΔKpn, and the ratio of Hap1 to His$_6$-Hap1ΔKpn in the eluate was similar to the ratio in crude extracts (Fig. 2, compare lane 4 with lane 2). Further, no Hap1 was found in the eluate when the extracts did not contain His$_6$-Hap1ΔKpn (Fig. 2, lane 3), suggesting that the retention of Hap1 on the column was not attributable to nonspecific binding to Ni-NTA beads. These results suggest that Hap1 is in a dimeric or possibly higher oligomeric form in the absence of heme in the HMC.

The HMC Binds to Various Hap1-binding and Mutated Sites in a Manner Similar to Dimer Binding but Totally Different from Monomer Binding—The coelution experiment suggests that Hap1 is a preexisting dimer or possibly a higher oligomer in the HMC in the absence of heme. We further ascertained this idea by testing whether Hap1 subunits in the HMC are physically close enough to function together as a dimer. We examined the manner by which the HMC binds to various DNA sites. Hap1 contains a conserved C6 zinc cluster that recognizes a CGG triplet (31). A Hap1 dimer binds cooperatively to asymmetric DNA sites containing a direct repeat of CGG triplets (consensus sequence: CCGnnnTAnCGG) (31, 32). The two C6 zinc clusters are positioned in tandem to recognize the two CGG triplets in a direct repeat (32, 33). Mutating any of the conserved nucleotides leads to reduced Hap1 binding affinity (31). However, when Hap1 is mutated or engineered so that it cannot form a stable dimer, it binds to DNA as a monomer with low affinity (32). These previous studies clearly demonstrated that the affinity of Hap1 monomeric binding is identical whether one or more CGG triplets are present in the DNA sites (32), apparently because a Hap1 monomer can make contacts with only one CGG triplet. The low DNA binding affinity of the HMC may result from monomer binding because the HMC contains a Hap1 monomer or from weakened dimer binding due to the interference by molecular chaperones. If the HMC contains only one Hap1 subunit (a monomer), it should bind identically to various Hap1-binding and mutated sites with one or more CGG triplets. However, if the HMC contains two Hap1 subunits (a dimer) and the C6 zinc clusters are close enough to function together, it should bind to various DNA sites in a manner similar to the Hap1 dimer formed in the presence of heme (32).

Therefore, we determined and compared the ways by which the HMC and Hap1 dimer bind to various Hap1-binding and mutated sites. These sites include UAS1/CYC1 (Fig. 3, lanes 1 and 2); a chimeric site containing a half-site of UAS1/CYC1 and a half-site of UAS/CYC7 (lanes 3 and 4); UAS/CYC7 (lanes 5 and 6); a consensus Hap1 site, H1 (CGGACTTATCGG, lanes 7 and 8); M1, a mutant site with the second CGG of HC1 changed to CCG (CGGACTTATCGG, lanes 9 and 10); M2, a mutant site with the conserved T in the spacer changed to C (CGGACTCATCGG, lanes 11 and 12); and M3, a double mutant site that combines mutations in M1 and M2 (CGGACTCATCGG, lanes 13 and 14) (32). Clearly, Fig. 3 shows that the HMC did not bind identically to these sites, strongly arguing against the idea that the HMC contains a Hap1 monomer. Instead, the HMC and Hap1 dimer bound to these sites in a similar manner. The HMC binding was observed at UAS1/CYC1, UAS/CYC7, the chimera, and the H1 site, where the affinity of Hap1 dimer binding is known to be high (31, 32) (Fig.

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with radiolabeled oligonucleotides containing UAS1/dimer at various sites. Extracts containing Hap1 were incubated
UAS1/CYC1 and a half-site from UAS1/CYC1 and a half-site from UAS1/CYC1 (lanes 3 and 4), UAS/CYC7 (lanes 5 and 6), a consensus site HC1 (CGGACTTATCGG) (lanes 7 and 8), mutant site M1 (CGGACTTATCCG) (lanes 9 and 10), M2 (CGGACTTATCCG) (lanes 11 and 12), and M3 (CGGACTTATCCG) (lanes 13 and 14) in the absence (lanes 1, 3, 5, 7, 9, 11, and 13) or presence (lanes 2, 4, 6, 8, 10, 12, and 14) of 2.5 ng/μl heme. The reaction mixtures were analyzed on a 3.5% polyacrylamide gel (note that this is a gel with a lower percentage of acrylamide than the one in Fig. 1). The positions of the HMC, DC, and free probe are marked.

3, lanes 1–8). At the M2 site, dimer binding was weaker, and HMC binding was barely observable. Binding by both HMC and Hap1 dimer was not observed at the M1 and M3 sites that contain only one CGG triplet. Quantitation showed that the intensity of the HMC band varied to about the same degree as the intensity of the Hap1 dimer band at these sites. The data strongly suggest that the HMC, like the Hap1 dimer formed in the presence of heme, requires a complete site containing a direct repeat of two CGG triplets for binding, suggesting that the HMC recognizes DNA as a Hap1 dimer, not a monomer.

To further verify this result, we carried out competition experiments. We examined the effects of various unlabeled DNA sites on HMC and Hap1 dimer binding to the radiolabeled consensus HC1 site (Fig. 4). We used unlabeled HC1, M1, M2, and M3 oligonucleotides to compete with the binding of HMC and Hap1 dimer to the radiolabeled HC1 site. The cold HC1 oligonucleotides completely out-competed DNA binding by the HMC and Hap1 dimer (compare lanes 3 and 4 with lanes 1 and 2), M2 oligonucleotides competed slightly with the binding by the HMC and Hap1 dimer to labeled HC1 (compare lanes 7 and 8 with lanes 1 and 2), and M1 and M3 did not compete with (perhaps M3 even enhanced) the binding at labeled HC1 (compare lanes 5, 6, 9, and 10 with lanes 1 and 2). Quantitation showed that these oligonucleotides competed with the HMC and dimer binding to the same extent. These results again showed that the HMC and the Hap1 dimer behaved similarly when binding to various DNA sites. These DNA binding experiments (see Figs. 3 and 4) strongly suggested that the HMC, like the Hap1 dimer formed in the presence of heme, cooperatively binds to DNA as a dimer.

Increasing Overexpression of Hap1 Leads to Graded Increases in Hap1 DNA Binding and Transcriptional Activity in the Absence of Heme—The above results suggest that the HMC is a preexisting Hap1 dimer, bound and repressed by molecular chaperones. Thus, we expect that heme should cause the dissociation of molecular chaperones in the HMC, thereby leading to Hap1 activation. To test this idea, we attempted to purify Hap1 in the presence of heme. However, this experiment was not successful because heme binds strongly to the columns (heme is known to be a very sticky molecule, and it stuck to all kinds of beads we tested, including Ni-NTA beads). When extracts were loaded onto the column in the presence of heme, most of the heme stuck to the beads on the top of the column and became ineffective in dissociating the HMC. We also tried to dissociate Hap1 from the HMC by purifying Hap1 from denatured yeast extracts. However, Hap1 is very labile under...
denaturing conditions, and we were not able to recover any full-length Hap1 from the Ni-NTA columns (the longest contained only the DNA-binding domain).

Despite these setbacks, we obtained evidence suggesting that overexpression of Hap1 indeed functionally titrates out the molecular chaperones in the HMC (Fig. 5). As the Hap1 protein level increased, Hap1 DNA binding activity progressively increased (Fig. 5A). First, more and more HMC formed (Fig. 5A, lanes 2, 4, 6, and 8). Second, the amount of the HMC reached its limit, presumably because the molecular chaperones were titrated out, and extra Hap1 formed the lower molecular weight, dimeric Hap1 complex (DC) (Fig. 5A, lane 8). Further, as the Hap1 expression level gradually rose, Hap1 transcriptional activity in vivo simultaneously increased, even in the absence of heme (Fig. 5B). Hap1 activity reached a significant level (about 50-fold higher than the basal transcription level) under the condition that the smaller DC formed (see Fig. 5B, 8-h induction time, and Fig. 5A, lane 8), although this level of activity was still 10-fold less than Hap1 activity in the presence of heme (see Fig. 1A). Together, these results suggest that titration of molecular chaperones in the HMC is functionally linked to Hap1 transcriptional activation.

**DISCUSSION**

In this report, we begin to probe the mechanism by which Hap1 is repressed in the HMC. We present strong evidence suggesting that Hap1 is a preexisting dimer or possibly higher oligomer in the HMC in the absence of heme. First, Hap1 and His6-Hap1ΔKpn coeluted on the Ni-NTA column in the absence of heme (Fig. 2). Second, DNA binding analysis of the HMC showed that the HMC binds to various DNA sites in a manner similar to Hap1 dimer binding but not monomer binding (32) (see Figs. 3 and 4). Third, increasing overexpression of Hap1 leads to increased DNA binding, the formation of the smaller Hap1 dimeric complex, and a significant level of Hap1 transcriptional activity in the absence of heme. These results suggest that Hap1 repression in the absence of heme is due, at least in part, to the interference of DNA binding of the preexisting Hap1 dimer by molecular chaperones. If the HMC was not a preexisting dimer in the absence of heme, and heme were required for Hap1 dimerization, then overexpression of Hap1 would not lead to the formation of the smaller dimeric complex. Further, the correlation between the increase of Hap1 transcriptional activity in vivo (Fig. 5B) and the increase of Hap1 DNA binding activity from both the HMC and DC in vitro (Fig. 5A) suggests that the titration of molecular chaperones is functionally important for Hap1 activation.

Previously, it was postulated that in the absence of heme, Hap1 is prevented from dimerization by other proteins in the HMC; thus, it cannot bind to DNA with high affinity and activate transcription (22, 34). Heme presumably disrupts the HMC, so Hap1 is free to dimerize and thus bind to DNA with high affinity, thereby activating transcription (22, 34). Our data argue against this simple dimerization model. The data show that in the absence of heme, Hap1 preexists as a dimer or possibly higher oligomer. Further, the two subunits of the preexisting dimer act cooperatively in DNA binding; disrupting the interaction of one subunit with one CGG triplet completely abolished HMC binding. Clearly, because of the interference of molecular chaperones, the DNA binding affinity of the HMC is greatly reduced compared with that of the Hap1 dimer formed in the presence of heme (see Figs. 1, 3, 4, and 5). The low DNA binding affinity of the HMC may result from weakened Hap1-Hap1 dimer interactions, weakened Hap1-DNA interactions, or both. In any case, the two Hap1 subunits of the HMC are very likely in direct physical contact with each other because they must be close enough to recognize simultaneously the two CGG triplets, separated by only six base pairs (31, 32).

Taken together, these results suggest a new model for how heme regulates Hap1 activity. In the absence of heme, Hap1 exists as a dimer in the HMC, but its DNA binding and perhaps transcription-activating activities are inhibited by molecular chaperones. When heme binds to Hap1, it may induce certain Hap1 conformational changes, disrupting the inhibitory interactions imposed on the preexisting Hap1 dimer by molecular chaperones in the HMC. Consequently, the inhibition on Hap1 is relieved and allows the Hap1 dimer to bind to DNA with high affinity, thereby leading to Hap1 activation. This model predicts that dissociation of molecular chaperones in the HMC should lead to Hap1 activation. Indeed, data shown in Fig. 5 suggest that molecular chaperones can be functionally titrated out by overexpression of Hap1, and this titration leads to significant increases in Hap1 DNA binding and transcriptional activities. This model provides a new example of how the activity of a dimeric transcription factor can be controlled. Perhaps the activity of many other dimeric transcription factors, such as Mal63 and Pdr1 of the Gal4 family, is also regulated in this manner.

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