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Review

Molecular mechanism of heme signaling in yeast: the transcriptional activator Hap1 serves as the key mediator

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Abstract. Heme is a key molecule in mediating the effects of oxygen on various molecular and cellular processes in many living organisms. In the yeast *Saccharomyces cerevisiae*, heme serves as a secondary signal for oxygen; intracellular heme synthesis directly correlates with oxygen tension in the environment. In yeast, oxygen sensing and heme signaling are primarily mediated by the heme activator protein Hap1, which, in response to heme, activates the transcription of genes required for respiration and for controlling oxidative damage. Heme regulation of many genes required for anaerobic growth is mediated by the aerobic repressor Rox1, whose expression is controlled by heme. In this review, we summarize recent knowledge about (i) how heme synthesis may be controlled by oxygen tension, (ii) how heme precisely and stringently controls Hap1 activity and (iii) whether other transcriptional activators can also mediate heme action.

Key words. Heme signaling; Hap1; transcriptional regulation; oxygen sensing; yeast Rox1.

Introduction

Heme is central to oxygen sensing and utilization in many living organisms. Heme plays key roles in numerous molecular and cellular processes for systems that sense or use oxygen. The functions of heme can be classified into the following major categories. First, heme plays an indispensable role in oxygen sensing in many living organisms. In the bacteria *Rhizobium meliloti*, the heme moiety in the oxygen sensor, Fix L, permits oxygen binding and initiates a cascade of events in response to changes of oxygen tension [1–3]. In the yeast *Saccharomyces cerevisiae*, heme directly serves as a secondary signal for oxygen (see below) [4–7]. In mammals, evidence suggests that oxygen sensors are hemoproteins [8, 9]. Second, heme permits oxygen binding in oxygen carriers, such as hemoglobin, and in enzymes, such as cytochrome c. Third, heme serves as a redox reactive center and functions in electron transport in enzymes such as cytochromes. Fourth, heme is essential for inactivation and activation of H_2O_2 carried out by catalase and peroxidases. Finally, heme directly regulates a wide array of biological processes for systems that sense or use oxygen [10–12]. For example, heme stimulates the differentiation of erythroid, hepatic and nerve cells [10–12]. Heme promotes the transcription of globin chains and cytochomes, enhances protein synthesis in reticulocytes, and stimulates the assembly of hemoprotein complexes [11, 13–16].

All these heme functions are directly linked to two characteristics of the heme iron. First, the heme iron (ferrous or ferric), attached to the porphyrin ring, can coordinate two ligands at the axial positions (fig. 1). The heme iron will readily coordinate amino acid residues in hemoproteins, or small molecules such as oxygen, NO and CO [17–19]. Those amino acid

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residues bound to the heme iron include histidine, cysteine and methionine [19]. Therefore, in proteins and enzymes, heme can be attached to proteins at one of the axial ligand positions by coordinating amino acid residues, while a small molecule such as oxygen can still bind at the other position. In some enzymes, such as cytochrome c, heme is covalently attached to its apoprotein through the side chains of the porphyrin ring. Second, the heme iron can oscillate between ferrous and ferric forms, so it can serve as a redox reactive center and permit electron transport. Heme regulation of numerous molecular and cellular processes likely involves direct heme binding to numerous regulatory proteins, but whether or not redox reactions play a role in heme regulation remains unknown. This review focuses on the regulatory functions of heme in *S. cerevisiae*. We first discuss briefly how heme is synthesized and how heme synthesis is controlled in yeast. We then review extensively how heme controls the activity of the heme activator protein 1 (Hap1) [20, 21] and exerts its effect on the transcription of numerous genes required for respiration and for controlling oxidative damage [6, 7]. Other regulators that may play a role in heme regulation are also considered.

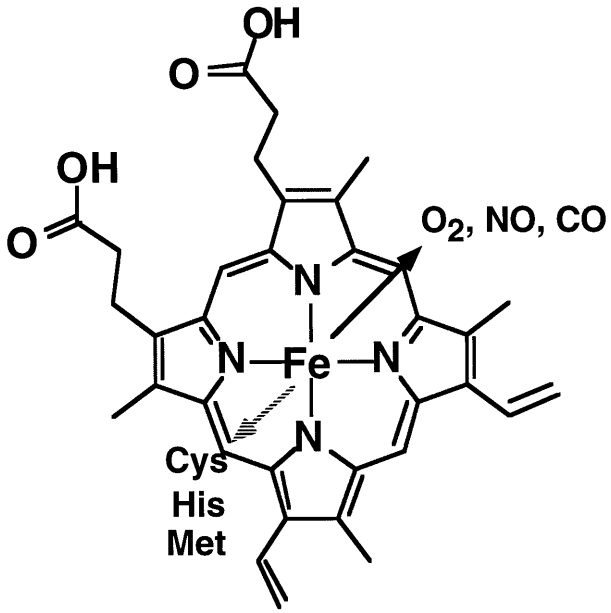

Figure 1. Heme structure. Also shown are the two axial ligand positions of the heme iron. At one axial ligand position, the heme iron may coordinate amino acid residues Cys, His or Met in proteins or peptides; at the other ligand position, it may coordinate oxygen, NO or CO, and so on.

Heme synthesis and its role in oxygen sensing and utilization in yeast

In *S. cerevisiae*, many lines of evidence suggest that the intracellular heme concentration correlates with oxygen tension [4, 5, 7]. In yeast, as well as in mammals, heme is synthesized from glycine and succinyl coenzyme A (CoA) [5]. To synthesize one molecule of heme, eight molecules of glycine and succinyl CoA and one ferrous ion are required. Heme synthesis requires eight enzymes [5]: 5-Aminolevulinate synthase, porphobilinogen synthase, porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen decarboxylase, coproporphyrinogen III oxidase, protoporphyrinogen oxidase, and ferrochelatase, encoded by the *HEM1* [22], *HEM2* [23], *HEM3* [24], *HEM4* [25], *HEM12* [26], *HEM13* [27], *HEM14* [28] and *HEM15* [29] genes, respectively (fig. 2). 5-Aminolevulinate synthase, protoporphyrinogen oxidase and ferrochelatase are located in mitochondria, while the other five enzymes are located in the cytosol [5]. The characteristics of these enzymes have been extensively reviewed previously [5]. This review, therefore, does not focus on these enzymes, but only summarizes several key characteristics relevant to the control of heme synthesis in yeast. First, heme synthesis in yeast correlates with oxygen tension; heme concentration is high under aerobic growth conditions, but low under anaerobic growth conditions [4, 5]. Second, the expression of *HEM1*, *HEM2*, *HEM3* and *HEM12* genes is largely constitutive and is unaffected by oxygen or carbon sources [22–24, 30]. Third, *HEM13* is the only gene whose expression is repressed by oxygen/heme [31]. Fourth, the synthesis of porphobilinogen is likely the rate-limiting step under aerobic conditions, whereas the synthesis of protoporphyrinogen IX is likely the rate-limiting step under anaerobic conditions, [29]. The mechanism by which oxygen tension controls heme synthesis is unclear. It has been suggested that oxygen tension dictates heme synthesis by controlling the rate of coproporphyrinogen III oxidase and protoporphyrinogen oxidase, both of which use oxygen as a substrate [29]. However, this view was questioned because the K_m of these enzymes for oxygen is low [32]. Under high oxygen tension, evidence [29] suggested that the rate of porphobilinogen synthase is limited and determines the level of heme synthesis. Perhaps an oxygen sensor senses oxygen tension and modulates the activity of porphobilinogen synthase.

Once synthesized in mitochondria, heme is delivered to other cellular compartments for diverse purposes (fig. 3). In nuclei, heme controls gene transcription through the transcriptional activator Hap1. Heme activates Hap1, which promotes the transcription of many genes encoding functions required for respira-

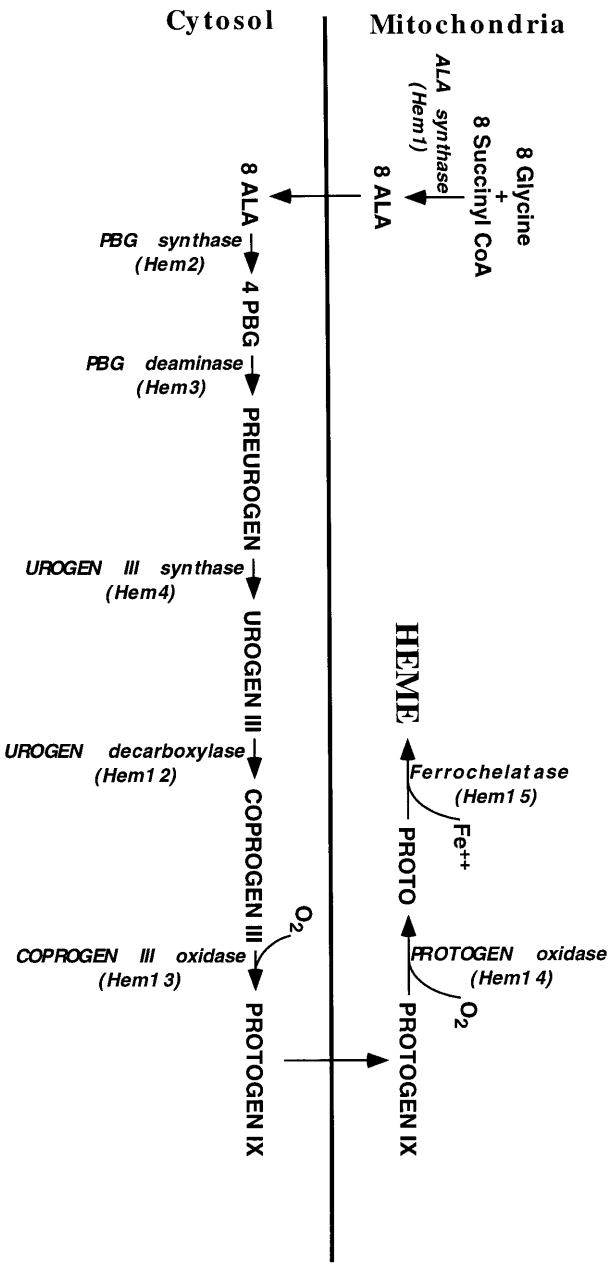

Figure 2. The heme synthesis pathway in the yeast *S. cerevisiae*. ALA, 5-aminolevulinic acid; PBG, porphobilinogen; preurogen, hydroxymethylbilane; urogen, uroporphyrinogen; coprogen, coproporphyrinogen; protogen, protoporphyrinogen; proto, protoporphyrin.

tion and for controlling oxidative damage, including *CYC1* (iso-1-cytochrome c) [33], *CYC7* (iso-2-cytochrome c) [34, 35], *CYT1* (cytochrome c1) [36, 37] and *CYB2* (cytochrome b2) [38] (see [7, 32] for a complete review of heme-responsive promoters). Hap1 also activates the transcription of the *ROX1* gene, encoding the aerobic repressor Rox1, which represses the transcription of genes encoding anaerobic specific functions, such as *ANB1* [6, 7, 34, 39, 40]. In other cellular compartments, including mitochondria, cytosol and peroxisomes, heme is incorporated as a prosthetic group in various proteins and enzymes, such as flavohemoglobin, catalase, peroxidases and cytochromes [36, 37, 41–44]. Heme not only serves as a prosthetic group in these protein complexes but also controls the expression of these proteins, including transcription and possibly translocation and assembly of these enzymes [6, 7]. In this way, heme efficiently coordinates cellular responses to changes of oxygen tension. The genesis of functional complexes required for oxygen utilization is activated when oxygen tension is high and repressed when oxygen tension is low. Likewise, the genesis of functional complexes required for anaerobic growth is repressed when oxygen tension is high and activated when oxygen tension is low through the action of Hap1 and Rox1 (fig. 3) [6, 7].

Importantly, despite the lack of understanding of the mechanism controlling heme synthesis, the correlation between heme concentration and oxygen tension is overwhelmingly supported by the existing experimental evidence [6, 7]. The expression patterns of various oxygen/heme-responsive genes, such as *CYC1*, *ANB1* and *ROX1*, under various oxygen tension or heme concentrations are all consistent with the idea that heme serves as a secondary signal for oxygen. In strains in which heme synthesis and oxygen tension are unlinked, the intracellular heme concentration, not oxygen tension, controls the expression of these genes. Aerobic genes, such as *CYC1* and *ROX1*, are activated by high heme concentrations and repressed by low heme concentrations, regardless of the level of oxygen tension [43, 45]. Conversely, anaerobic genes, such as *ANB1*, are repressed by high heme concentrations and activated by low heme concentrations, regardless of the level of oxygen tension [34, 46].

The Hap1-heme signaling pathway

Hap1 activity is precisely and stringently controlled by heme

The *HAP1* gene, encoding 1483 amino acid residues, was independently cloned in the laboratories of Guarente and Slonimski [20, 21]. Hap1 is a DNA binding transcriptional activator. Three Hap1 domains were initially identified [20, 21]: the DNA binding domain, containing a zinc finger-like structure, the activation domain, containing acidic residues, and the heme domain, containing six repeats, which were later shown to be able to bind directly to heme and named as heme-responsive motifs (HRMs) [47] (fig. 3). In the absence of heme, Hap1 is unable to activate transcription, but it represses the expression of *ROX1*, *SOD1* and *ERG11*

genes [40, 45, 48–50], and is required for anaerobic or heme-deficient growth of *S. cerevisiae* [51]. As the heme concentration rises, Hap1 activity increases, reaching its maximum activity at micromolar heme concentrations. Table 1 shows the effect of increasing heme concentration on Hap1 activity, in a strain with the *HEM1* gene deleted, permitting experimental control of heme concentration by addition of the heme precursor, 5-aminolevulinic acid. At the highest heme concentration tested, Hap1 activity is stimulated more than 100-fold. These and previous data [43, 52] show that Hap1 activity is highly responsive to heme concentration; increasing heme concentration increases Hap1 transcriptional activity, whereas decreasing heme concentration decreases Hap1 transcriptional activity.

Interestingly, it was suggested that heme might act as a redox-sensitive group that in turn controls Hap1 activity [32]. If this idea were correct, then reactive oxygen species should enhance Hap1 activity, whereas reducing agents should diminish Hap1 activity. We measured Hap1 activity in the presence of several reactive oxygen species, including hydrogen peroxide, menadione and paraquat, and in the presence of the reducing agent glutathione at low, intermediate and high heme concentrations. At all heme concentrations, these reactive oxygen species did not enhance Hap1 activity, and neither did glutathione diminish Hap1 activity (data not shown). These results argue against the idea that Hap1 is redox-sensitive, but support the idea that heme serves as a direct effector of Hap1 activity.

Because Hap1 must bind to DNA to activate transcription, Hap1 DNA binding plays a central role in the control of Hap1 transcriptional activity. Therefore, a complete understanding of the mode of Hap1 DNA binding is a prerequisite for understanding heme regulation of Hap1 activity. Remarkably, recent studies have elucidated how Hap1 binds to DNA [53–56], leading to a better understanding of how heme may control Hap1 activity. These studies are summarized below.

Hap1 is a member of the Gal4 transcriptional activator family and binds to DNA asymmetrically

The yeast Gal4 family includes at least 52 transcription factors that control a wide array of diverse processes, ranging from carbon source utilization to oxygen uti-

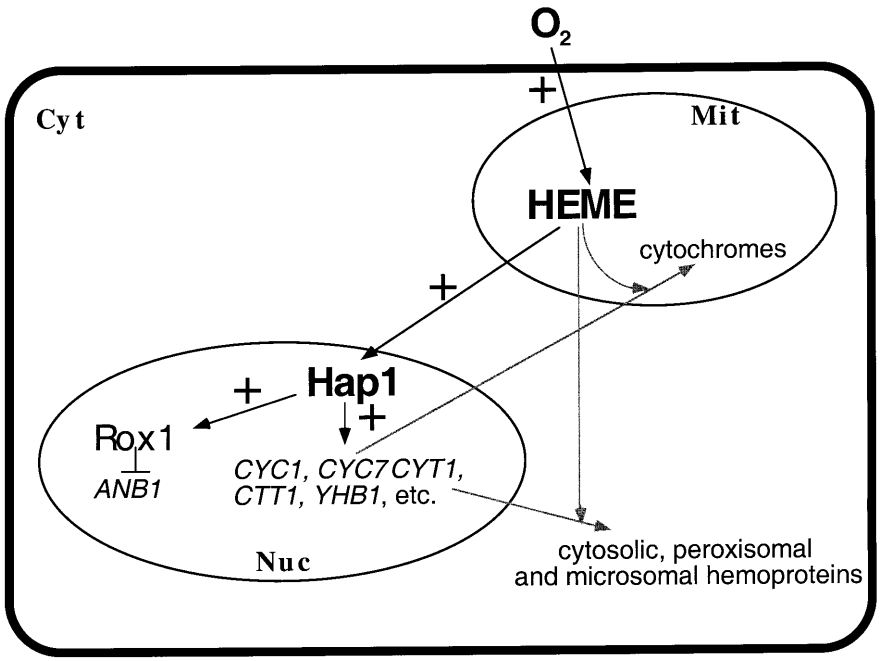

Figure 3. The Hap1-heme signaling pathway in yeast. When cells are grown aerobically, heme is synthesized in mitochondria (Mit). Then, heme activates Hap1 in the nucleus (Nuc), permitting Hap1 to bind upstream activation sequences and to promote transcription of many genes required for oxygen utilization and for controlling oxidative damage, such as those encoding cytochrome c-iso-1 (*CYC1*) [33], iso-2 (*CYC7*) [34, 35], cytochrome c1 (*CYT1*) [36, 37], catalase (*CTT1*) [37, 38] and flavohemoglobin (*YHB1*) [41, 42]. Hap1 also activates the expression of the *ROX1* gene, encoding the aerobic repressor Rox1, which represses the expression of genes encoding functions specifically required for anaerobic growth, such as *ANB1* [34, 46]. Once synthesized, these enzymes and proteins may bind to heme and are then transported into various cellular compartments, including mitochondria, cytosol and peroxisome, to carry out their functions.

Table 1. The effect of heme on Hap1 activity*.

[ALA] μg/ml	2	6	17	38	75	150	300
Hap1 activity	<1	<1	3.2 ± 0.4	16 ± 3	39 ± 4	67 ± 9	124 ± 22

*Yeast *Δhem1* cells bearing the Hap1-driven UAS1/*CYC1* reporter were grown in media containing various amounts of the heme precursor 5-aminolevulinate, and β-galactosidase activities were detected.

lization and to drug resistance [57, 58]. These members all contain a conserved C6 zinc cluster that recognizes a CGG triplet [55, 58–65]. Many of these factors, such as Gal4 and Ppr1 [59, 60], were shown to dimerize and bind to symmetrical DNA sites containing an inverted repeat of two CGG triplets. Hap1 was also shown to bind to DNA as a dimer, and the minimal Hap1 dimerization element was mapped between residues 115 and 148 [66]. Like those of Gal4 and Ppr1, the Hap1 dimerization element contains a 4–3 hydrophobic repeat, coiled-coil dimerization sequence [66].

However, unlike Gal4 and Ppr1, Hap1 binds to asymmetric DNA sites containing a direct repeat of two CGG triplets [56, 67]. Selection of high-affinity Hap1 binding sites in vitro revealed that Hap1 binds selectively to the optimal sequence CGGnnnTAnCGG [67]. All naturally occurring Hap1 binding sites are related and are imperfect direct repeats compared with this optimal sequence [33–38, 41, 42, 45, 48–50, 67]. Footprint analysis suggests that two zinc clusters of a Hap1 dimer are positioned asymmetrically in a directly repeated orientation to make the same contacts with the two CGG triplets [67]. An extensive biochemical analysis of various Hap1-Ppr1 chimeric proteins suggested that the two C6 zinc clusters in a Hap1 dimer are positioned on DNA asymmetrically in tandem, by an interaction between the residues within the zinc cluster of one Hap1 subunit and the residues immediately N-terminal to the C6 zinc cluster of the other Hap1 subunit [53]. This asymmetric interaction not only positions the two zinc clusters in a directly repeated orientation, but it also enhances Hap1 dimerization when Hap1 binds to DNA. Disruption of this asymmetric interaction by substitution of the Hap1 zinc cluster with that of Ppr1 abolishes cooperative dimeric binding and allows the Hap1-Ppr1 chimera to bind DNA predominantly as a monomer, with lower affinity [53].

These biochemical analyses are now confirmed by the X-ray crystal structure of the Hap1-DNA complex [54]. Indeed, Hap1 binds to DNA in a dramatically asymmetric fashion. The asymmetry is largely a result of extensive hydrophobic interactions between the zinc cluster of the right protein subunit with the N-terminal arm, linker and the zinc cluster domain of the left subunit. These interactions contribute to a large part of the dimer interface, which also include the previously identified coiled-coil dimerization element [66]. Interestingly, the N-terminal arm of the left zinc cluster also makes extensive contacts with nucleotides in the spacer in the minor groove [54, 55]. These studies provide a clear understanding of how a Hap1 homodimer can bind to an asymmetric DNA site.

Hap1 forms a high molecular weight complex (HMC) in the absence of heme

Because Hap1 is a transcriptional activator, its activity could be regulated at several possible levels, including nuclear localization, DNA binding and transcriptional activation. By indirect immunofluorescent staining, it was shown that in the absence of heme, Hap1 is localized in the nucleus [68]; thus, Hap1 activity does not appear to be regulated at the level of nuclear entry. Further analysis of whole cell Hap1 extracts showed that, in the absence of heme, Hap1 is bound by certain cellular proteins and forms an HMC [48, 68]. As the heme concentration increases, the Hap1 in the HMC gradually changes to a dimeric Hap1 complex with high DNA binding affinity, thereby leading to Hap1 activation. At a low expression level, all the Hap1 is in the HMC form, as detected by DNA mobility shift assays [48, 68]. As the level of Hap1 gradually increases, the amount of HMC formed reaches a limit; extra Hap1 forms a dimeric complex similar to the one formed in the presence of heme [48, 68]. This suggests that non-Hap1 components of the HMC are titrated out when the Hap1 concentration is high.

The HMC and the dimeric complex exhibit very different sedimentation properties in a 10–45% linear sucrose gradient [69]. In the absence of heme, Hap1 exists predominantly as the HMC and sediments at a fast rate. Addition of heme liberates Hap1 from the HMC; all Hap1 is found in the smaller dimeric complex, which sediments at a much slower rate. In the absence of heme, the HMC exhibits a molecular mass of nearly 1000 kDa. Strikingly, HMC formation by several Hap1 mutants is directly correlated with Hap1 heme responsiveness [48, 69]. When the Hap1 heme domain is deleted, no HMC is observed by DNA mobility shift assays or by sucrose gradient centrifugation [69]. Rather, the mutant Hap1 forms the dimeric complex in the absence of heme and activates transcription consti-

tutively. Similarly, a Cys to Tyr mutation at amino acid position 1048 [70] largely abolishes HMC formation and is highly active in the absence of heme [69]. It is not clear what caused the discrepancy between this [69] and the earlier study [70], which shows that HMC formation is similar for the wild-type and mutant forms of Hap1, as detected by DNA mobility shift assays. Interestingly, a Hap1-Ppr1 chimeric protein with the dimerization domain of Hap1 replaced by that of Ppr1 forms an intermediate complex smaller than the HMC but larger than the dimeric Hap1 complex [69]. This chimeric protein is also transcriptionally active in the absence of heme. Similarly, a Hap1 mutant containing the Gly to Asp mutation at amino acid 235 in the dimerization domain exhibits heme-independent expression of the *CYC1* and *ROX1* genes and binds to DNA as the smaller dimeric complex in the absence of heme [46]. These data directly link HMC formation to Hap1 heme responsiveness [48, 69].

Molecular chaperones including Hsp82 and Ydj1 are associated with Hap1

Using the Hap1 protein tagged with His_6, our lab has recently purified the HMC [69]. We found that in the absence of heme, at least four proteins cofractionate with His_6-Hap1 on Ni-NTA and Superose 6 columns, suggesting that these proteins are components of the HMC. By immunodetection, we showed that Hsp82 and Ydj1 are associated with Hap1 and present in the purified complex. In addition, the two most abundant proteins associated with Hap1 are a 70-kDa protein (p70) and a 60-kDa protein (p60). The transcriptional repressor Ssn6 is not detected in the purified complex, although Ssn6 affects heme regulation of Hap1 by an unknown mechanism [52].

Genetic analysis supports a functional role of Hsp82 in Hap1 activation [69]. In a strain that expresses 5% of the wild-type level of Hsp82, Hap1 activity is greatly reduced, even in the presence of heme, while the activity of other transcriptional activators, including Hap2/3/4/5, Gcn4 and Bas1/2, is largely unaffected [69]. The effect of a reduced level of Hsp82 on Hap1 activity is similar to its effect on the activity of steroid receptors [71]. Perhaps the regulation of Hap1 activity by heme is analogous to the regulation of steroid hormone receptors by steroids. Hap1, like steroid receptors, is associated with molecular chaperones and is repressed in the absence of ligand. Heme, a functional equivalent of steroids, binds to Hap1, disrupts the interaction of Hap1 with molecular chaperones, and activates Hap1 (the receptor for heme). The involvement of molecular chaperones in heme regulation of Hap1 is also supported by our recent finding that p70 is Hsp70. Hsp70 and Ydj1 function together as molecular chaperones in protein folding. The Hsp70-Ydj1 molecular chaperones have been shown to be involved in the regulation of a range of transcription factors, including HSF1, WT1 and steroid hormone receptors [72–74]. In yeast, genetic evidence suggests that Hsp70 is also important for the function of the transcription factor Pdr1, a member of the Gal4 activator family [75]. Perhaps both Hsp90 and Hsp70 molecular chaperones are involved in the regulation of certain members of the yeast Gal4 family, including Hap1 and Pdr1, as they are in the regulation of mammalian steroid hormone receptors. However, whether these molecular chaperones affect Hap1 and steroid receptors by a similar mechanism remains to be investigated.

Multiple Hap1 elements mediate heme regulation of Hap1 activity

Initial inspection of Hap1 sequence identified the region encompassing residues 244–444 as the heme domain (fig. 4) [20, 21]. The heme domain contains a short

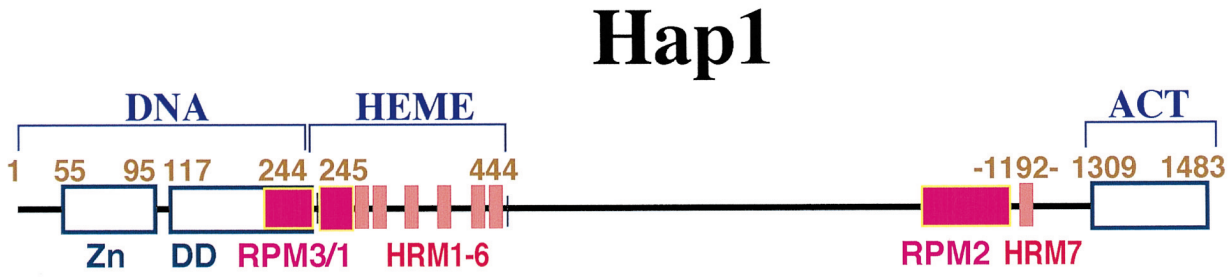

Figure 4. Hap1 domain structure. Shown here are the zinc cluster (Zn), the dimerization domain (DD), the repression modules RPM1-3 (RPM3/1 and RPM2) [76], the HRM1-6 motifs, the HRM7 motif and the activation domain (ACT). The boundaries of the originally designated DNA binding domain and the heme domain are indicated [20, 21]. The three repression modules are responsible for Hap1 repression in the absence of heme, whereas HRMs, in particular HRM7, mediate heme activation of Hap1 [76].

sequence motif, namely, the HRM, Lys/Arg, Cys, Pro, Val/Ile, Asp, His, which is repeated six times across 200 amino acids [20, 21]. A synthetic peptide containing one HRM of Hap1 can directly bind to heme in vitro, as shown by spectrophotometric methods and gel filtration chromatography [47]. The peptide drastically changes the heme absorption spectrum; it shifts the strongest heme absorption peak (Soret band) [19] from 388 nm to 362 nm, or from 412 nm to 362 nm in the presence of imidazole [47]. The Cys residue is required for heme binding, while the Pro residue aids the interaction between the peptide and heme. Presumably, the SH group in Cys serves as an electron donor to chelate the heme iron as an axial ligand, thereby providing an energy source for heme-HRM interactions. This shift of the Soret band as a result of HRM binding is unique among the known interactions between heme and proteins or peptides. All previously known interactions between heme and proteins, such as cytochromes and globins, or peptides, result in a shift of the Soret band to a longer wavelength [17–19]. Whether this feature is relevant to the fact that the Hap1-heme interaction is regulatory and highly reversible is not yet clear.

Recent experiments conclusively showed that the Hap1 mutant with the heme domain deleted is constitutively active in heme-deficient cells, supporting the idea that the heme domain is critical for heme regulation of Hap1 [48, 69]. However, it is increasingly clear that other Hap1 domains or elements are also involved in heme regulation. First, a seventh HRM is located in a distal part of Hap1 near the activation domain [47]. This motif alone is able to mediate a low level of heme regulation of a lexA-Hap1 chimeric protein. Second, mutation of Cys to Tyr at the amino acid position 1048 renders Hap1 active in the absence of heme [70]. These results strongly suggest that amino acid residues distal to the previously designated heme domain are also critical for heme regulation. Third, replacement of the Hap1 dimerization domain with that of Ppr1 abolishes heme regulation of Hap1 [69]. The Hap1-Ppr1 chimera is derepressed and gains a high level of activity in the absence of heme; heme further stimulates its activity only two- to three-fold [69]. Taken together, these results suggest that elements other than HRM1–6 of the heme domain are critical for heme regulation of Hap1.

On the basis of these experiments, our lab recently carried out a systematic analysis to determine Hap1 elements critical for heme regulation [76]. We divided the dimerization domain, the heme domain and the domain containing the HRM7 motif into small regions encompassing 30–40 amino acid residues. We deleted each individual region and determined the activity of deletion mutants in heme-deficient and heme-sufficient cells. We found that a new class of modules, namely, three repression modules (RPM1 in the N-terminus of the heme domain, RPM2 immediately N-terminal to the HRM7 motif and RPM3 in the C-terminus of the dimerization domain, fig. 4), is critical for Hap1 repression in the absence of heme. Deletion of any one of these modules causes Hap1 derepression, permitting Hap1 to gain a high level of activity in the absence of heme. Interestingly, a previously characterized mutation of Gly to Asp at amino acid 235 [46] is located within RPM3 and causes Hap1 derepression in the absence of heme. Strikingly, deletion of all HRM1–6 in the heme domain did not affect Hap1 repression or heme activation. However, mutation of the critical Cys residue in HRM7 causes Hap1 to be hyperrepressed and diminishes Hap1 activity in the presence of heme. Simultaneous deletion of HRM1–6 and mutation of HRM7 causes Hap1 to be even more repressed and further diminishes Hap1 activity in the presence of heme, while Hap1 repression in the absence of heme is largely unaffected. These results suggest that heme regulation of Hap1 involves at least two classes of elements: the repression modules (RPMs), which are responsible for Hap1 repression in the absence of heme, and the HRMs, which permit heme binding and mediate heme activation (fig. 4).

A tentative model for how heme controls Hap1 activity

The existing evidence described above provides a plausible, but still sketchy model for heme regulation of Hap1 activity. Evidently, in the absence of heme, Hap1 is bound by multiple proteins, including Hsp82, Ydj1, p70 and p60, and forms a high molecular weight complex (HMC, fig. 5). The HMC binds to DNA with low affinity and therefore fails to activate transcription of target genes. The low DNA binding affinity of the HMC does not appear to be caused by Hap1 monomer binding. Recent experiments in our laboratory [76a] suggest that DNA binding by the HMC still requires a complete Hap1 binding site containing a direct repeat of two CGG triplets. The HMC, like the Hap1 dimer, does not bind DNA sites containing one CGG triplet, which, however, is sufficient to allow Hap1 monomer binding [53]. Therefore, DNA binding by the HMC is likely weakened by inhibitory interactions imposed on the Hap1 DNA binding domain by molecular chaperones and heme regulatory domains. The HMC appears to be sensitive to high temperature but largely resistant to high salt, suggesting that the interaction between Hap1 and molecular chaperones is hydrophobic and involves a transition from a disordered structure to a highly organized one. RPMs are very likely involved, directly or indirectly, in the interactions between Hap1 and molecular chaperones. RPMs may form the interaction surfaces or provide a backbone for forming the interaction surfaces with molecular chaperones, thereby medi-

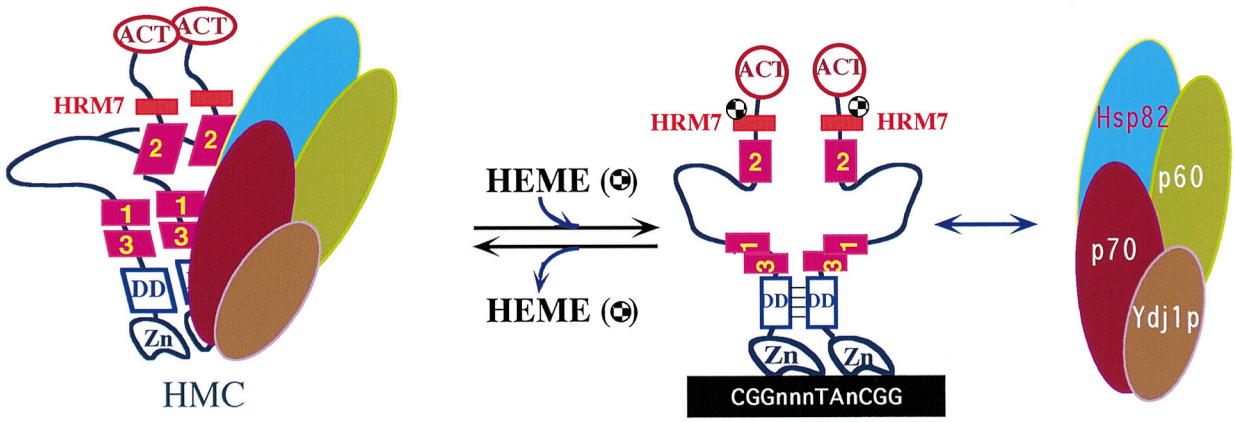

Figure 5. A model of heme regulation of Hap1 activity. Shown are the Hap1 functional domains: the Zn cluster (Zn), the dimerization domain (DD), the three repression modules RPM1-3 (1, 2 and 3), the HRM7 motif (HRM7) and the activation domain (ACT). In the absence of heme, four cellular proteins, including Hsp82, p70, p60 and Ydj1 (shown by four shaded ovals), interact with Hap1, forming a higher-order complex termed HMC [69]. Hap1 is repressed in this complex. The three RPM modules very likely mediate Hap1 repression by permitting HMC formation [76]. When heme concentration increases, HRM7 presumably binds to heme and permits the disassembly of the HMC, thereby leading to Hap1 activation. Activated Hap1 then binds to DNA with high affinity and promotes transcription. The transition between the HMC and Hap1 dimer is very likely dynamic and highly responsive to heme concentrations.

ating Hap1 repression in the absence of heme. Hap1 repression may be achieved by repressing the DNA binding domain or both the DNA binding domain and the activation domain.

When the heme concentration increases, heme presumably binds to Hap1 through the HRMs, particularly HRM7 [76], leading to a conformational change in Hap1. Consequently, all or some of the proteins in the HMC dissociate from Hap1, relieving the repression on the DNA binding domain and perhaps also on the activation domain. As a result, Hap1 is able to bind DNA with high affinity, thereby activating transcription. Heme binding to HRMs is highly reversible, which may enable Hap1 to sense heme concentrations. Very likely, in vivo, a dynamic equilibrium exists between the repressed HMC and the active dimeric complex. High heme concentrations would shift the equilibrium towards the dimeric complex, while low heme concentrations would shift the equilibrium towards the HMC. In this manner, Hap1 activity can be responsive to changes of heme concentration (fig. 5). Molecular chaperones, such as Hsp82, may not only contribute to Hap1 repression in the absence of heme but may also be involved in heme activation of Hap1, as is proposed in the regulation of steroid receptors [71, 77, 78]. Indeed, recent evidence suggests that molecular chaperones may play roles in both Hap1 repression in the absence of heme and Hap1 activation by heme [69].

The exact molecular interactions involved in the heme-mediated transition between the HMC and dimeric Hap1 complex need further investigation. For example, HRMs can bind to heme in vitro and are important for heme activation in vivo [47], but it is possible that other Hap1 elements, and even other factors in the HMC, can bind heme and mediate heme action. Interestingly, Ydj1 contains a putative cytochrome c family heme-binding site in its conserved zinc finger-like domain [72, 79–81]. It is tempting to speculate that this site may be important for heme regulation of Hap1. Another important question is whether all non-Hap1 components of the HMC dissociate upon heme binding. Given the drastic difference between the mobility of the HMC and the dimeric Hap1 complex, some, if not all, proteins in the HMC very likely dissociate upon heme binding. However, some of these proteins may remain bound to Hap1 and function together with Hap1 in transcriptional activation. Further experiments are needed to address these issues.

The aerobic repressor Rox1

The Rox1 repressor is responsible for the repression of anaerobic genes, such as *ANB1*, under hypoxic or heme-deficient conditions [6, 7, 31, 34, 39, 40]. Rox1 consists of 368 amino acid residues and contains two known functional domains, the HMG DNA binding domain encompassing residues 1–100 and the repression domain encompassing residues 123–368 [6, 39]. Rox1 binds as a monomer to DNA sites containing the sequence PyPyPyATTGTTCTC [6, 39]. The HMG domain of Rox1 appears to adopt a tertiary structure

similar to that of the HMG domain of the human transcriptional activator protein SRY [6]. The Rox1 repression domain presumably binds to the general repressor Tup1/Ssn6 complex and mediates Rox1 repression [40]. The Tup1/Ssn6 repressor is required for mediating repression by $\alpha 2$ and Mig1 repressors [82–87]. Interestingly, Tup1/Ssn6 are also involved in heme regulation of Hap1, probably by an indirect mechanism [52].

The repression function of the Rox1 protein is heme-independent [40, 45, 48–50]. However, heme-dependent repression by Rox1 is mediated by activation of *ROX1* gene transcription. In response to heme, Hap1 is activated and promotes the transcription of the *ROX1* gene, thereby increasing Rox1 synthesis and causing the repression of anaerobic genes [6, 39, 40]. Intriguingly, previous evidence suggested that Hap1 also represses the expression of *ROX1*, *ERG11* and *SOD2* genes under heme-deficient conditions [40, 45, 48–51]. We confirmed the repression of *ROX1* expression by Hap1. In the presence of heme (high [ALA]), the *ROX1* promoter activity was stimulated about twofold by Hap1 (compare the reporter activity in the wild-type *HAP1* strain with the *Δhap1* strain, table 2), as expected [40]. In the absence of heme (low [ALA]), Hap1 repressed the *ROX1* promoter about fivefold (compare the reporter activity in the wild-type *HAP1* strain with the *Δhap1* strain, table 2). When the *HAP1* gene was deleted, heme concentrations had very little effect on the *ROX1* promoter activity, suggesting that Hap1 is responsible for mediating the effect of heme on *ROX1* expression. Hap1 activation of *ROX1* expression in the presence of heme and Hap1 repression of *ROX1* expression in the absence of heme collectively allowed approximately 10-fold stimulation of Rox1 synthesis by heme. Strikingly, this level of regulation of Rox1 synthesis resulted in a significantly much higher level of regulation of *ANB1* expression, which was repressed 100-fold by heme. Hap1 activation of *ROX1* expression is likely mediated by Hap1 binding directly to the *ROX1* promoter, which contains a Hap1-binding site, T*GG*AAC*TA*C*CGG*, at position –420. Perhaps under heme-deficient conditions, Hap1 can interact with some of its target sequences and thereby repress *ROX1* expression, as suggested by previous studies [40, 45, 48–51].

Other heme-responsive transcriptional activators in yeast

Under aerobic growth conditions, oxygen, by oxidative phosphorylation, provides the majority of energy source for growth in yeast. Therefore, it is conceivable that oxygen and its secondary signal, heme, could affect a wide spectrum of processes involved in cell growth, such as carbon source utilization and amino acid synthesis. Indeed, the activity of the Hap2/3/4/5 transcriptional activator is stimulated about 10-fold by heme [88–90]. The Hap2/3/4/5 complex binds to the CCAAT box and activates genes required for the utilization of nonfermentable carbon sources, such as lactate. The Hap2/3/4/5 complex activates a wide array of genes, including those activated by Hap1 and those not activated by Hap1, such as *CYC1* [33], *SOD2* [49] and *CYT1* [37]. Because the activity of the Hap2/3/4/5 complex is substantial only in the presence of nonfermentable carbon sources and because cells will not grow in nonfermentable carbon sources without oxygen (that is, heme, in this case), it is difficult to investigate how heme activates the activity of the Hap2/3/4/5 complex. Interestingly, a human homologue of the yeast Hap2/3/4/5 complex, the NFYA/B/C complex, was also shown to be heme-responsive [91, 92]. Heme enhances the DNA-binding activity of the NFY complex in human erythroid K562 cells, which may be caused indirectly by an increased level of the NFYA subunit [92]. Perhaps heme can also enhance the DNA binding activity of the Hap2/3/4/5 complex in yeast. Interestingly, there is evidence from our laboratory suggesting that the activity of the Gln3 activator is controlled by heme (J. Lee and L. Zhang, unpublished data). Gln3 activates the transcription of a number of genes whose products permit yeast cells to use a variety of compounds as the source of nitrogen when preferred nitrogen sources are not

Table 2. The effect of Hap1 on the expression of *ROX1* and *ANB1* genes*.

	HAP1		*Δhap1*	
	low [ALA]	high [ALA]	low [ALA]	high [ALA]
UAS1/*CYC1*	<1	68 ± 5	<1	<1
ROX1	4.6 ± 0.6	41 ± 2	21 ± 3	19 ± 3
ANB1	617 ± 120	6 ± 1.5	7.2 ± 1.5	21 ± 2

*Yeast wild-type cells (*HAP1*) or cells with the *HAP1* gene deleted (*Δhap1*) carrying the UAS1/*CYC1*, *ROX1* or *ANB1* reporter were grown in media containing 2.5 μg/ml (low [ALA]) or 250 μg/ml(high [ALA]) of the heme precursor 5-aminolevulinate, and β-galactosidase activities were detected.

available [93–95]. Genes activated by Gln3 include *GLN1* (encoding glutamine synthetase) [95], *GDH1* (encoding NADP-linked glutamate dehydrogenase) [96], *GAP1* (encoding a general amino acid permease) [97] and genes in the urea, allantoin and arginine catabolic pathways [98]. Interestingly, Gln3 and the Hap2/3/4/5 complex act together to control the expression of the *GDH1* gene [96]. Possibly, heme may serve as a global regulator of numerous genes involved in the utilization of carbon sources, nitrogen sources and oxygen.

Perspective

Recent studies discussed above helped elucidate how heme mediates its regulatory effects on gene expression. Nevertheless, many questions about the heme signaling pathway in yeast still remain unanswered. First, what is the exact mechanism by which heme synthesis is controlled? When the oxygen concentration is much higher than the K_m of coproporphyrinogen III oxidase and protoporphyrinogen oxidase for oxygen, heme synthesis appears to be controlled by the rate of PBG synthase [5]. How the activity of PBG synthase is controlled by oxygen tension and whether an oxygen sensor is involved in this process are entirely unknown. Second, once heme is synthesized, how is heme transported and delivered to other cellular compartments? Because heme is a rather insoluble and toxic compound, intracellular heme carriers and transporters are probably required to store and transport heme, but heme transporters have not yet been identified in yeast. Third, although much has been learned about how heme activates Hap1 activity, many molecular interactions responsible for Hap1 repression and heme activation remain uncharacterized. For example, how do molecular chaperones, such as Hsp90 and Hsp70, interact with Hap1 and control Hap1 activity? Do these molecular chaperones affect Hap1 activity in the same manner as they affect the activity of steroid hormone receptors? Finally, how does heme affect the activity of other transcriptional activators, such as the Hap2/3/4/5 complex, Gln3, and perhaps even still other activators? These activators may not play a dominant role in oxygen sensing and heme signaling, but may be important for mediating the effects of heme on other cellular processes that are not directly related to oxygen utilization.

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