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Heme binds to a short sequence that serves a regulatory function in diverse proteins

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Heme is a prosthetic group for numerous enzymes, cytochromes and globins, and it binds tightly, sometimes covalently, to these proteins. Interestingly, heme also potentiates binding of the yeast transcriptional activator HAP1 to DNA and inhibits mitochondrial import of the mammalian δ-aminolevulinate synthase (ALAS) and the catalytic activity of the reticulocyte kinase, HRI. All three of these proteins contain a short sequence, the heme regulatory motif (HRM), that occurs six times adjacent to the HAP1 DNA binding domain, twice in the leader targeting sequence of ALAS and twice near the catalytic domain of the HRI kinase. Here we show that a 10 amino acid peptide containing the HRM consensus binds to heme in the micromolar range, and shifts the heme absorption spectrum to a longer wavelength, a direction opposite to the change caused by cytochromes or globins. Further, we show that a single HRM regulates the acidic activation domains of HAP1 and GAL4 independently of regulation of DNA binding of the transcription factors. These findings thus establish a novel heme binding sequence which is structurally distinct from sequences in globins or cytochromes and which has a regulatory function.

Key words: activation domain/HAP1/heme binding module/heme regulation/transcriptional activator

Introduction

Heme serves as a redox-active cofactor in cytochromes and as an oxygen carrier in globins (Bock *et al.*, 1978). Recently, heme has been shown to function as an effector molecule that can regulate many biological processes, including transcription, translation, protein translocation and erythroid differentiation (for a review see Padmanaban *et al.*, 1989). Heme levels in yeast cells are proportional to oxygen concentrations in the media (Matton *et al.*, 1979). The yeast transcriptional activator HAP1 activates transcription of genes encoding cytochromes in response to oxygen/heme (Pfeifer *et al.*, 1987; Creusot *et al.*, 1988). HAP1 contains a DNA binding domain (residues 1–244), an acidic activation domain (residues 1309–1483) and the heme domain (residues 245–444) that is adjacent to its DNA binding domain and mediates induction by heme (Pfeifer *et al.*, 1989). This region represses the activity of the protein *in vivo* and DNA binding *in vitro* in the absence of heme. Previously, Fytlovich *et al.* (1993) and we (Zhang and Guarente, 1994) have shown that HAP1 is complexed with a cellular repressive factor via the heme domain and forms a high mol. wt complex in the absence of heme. This HAP1 complex binds to DNA with low affinity in the cold and is transcriptionally inactive. Addition of heme allows the repressive cellular factor to dissociate from HAP1, thereby promoting HAP1 DNA-binding and transcriptional activation.

The repression of HAP1 activity by a cellular factor is similar to the repression of glucocorticoid receptor (GR) by HSP90 (Picard *et al.*, 1988, 1990). In the absence of steroid hormone, HSP90 binds to GR and represses its activity. Binding of glucocorticoids to GR changes the receptor conformation and releases HSP90. Subsequently, GR enters the nucleus and activates transcription from its cognate DNA site. It is not clear whether heme mediates the action of HAP1 directly, by binding to the molecule via the heme domain or indirectly, for example, by triggering some post-translational modification, such as phosphorylation of the repressor IκB in response to mitogens which then releases NFκB to enter the nucleus and activate transcription (Henkel *et al.*, 1992).

Strikingly, the HAP1 heme domain contains a short sequence motif, Lys/Arg-Cys-Pro-Val/Ile-Asp-His (Figure 1B), that is repeated six times across 200 amino acids (Figure 1A; Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). A seventh repeat located in a distal part of the protein is discussed below. In a second example, the import of the mammalian mitochondrial δ-aminolevulinate synthase (ALAS) into mitochondria is repressed by the presence of heme (Lathrop and Timko, 1993). The leader targeting sequence of ALAS contains two copies of a sequence similar to the HAP1 repeat, termed heme regulatory motifs (HRMs), which are required for the repression by heme (Figure 1B). In a third case, heme stimulates translation in reticulocytes by inactivating the HRI kinase, thus preventing phosphorylation and consequent inactivation of the translational initiation factor eIF2α (Chen *et al.*, 1991). The catalytic domain of the HRI kinase also contains two copies of an HRM-related sequence (Figure 1B).

The HRM is different from sequences found in cytochromes and globins, which are commonly a histidine/methionine pair or bis-histidine and bind heme very tightly, sometimes covalently (Bock *et al.*, 1978). Thus, it is an important question whether the HRM binds heme directly to mediate heme regulation of HAP1 activity or whether the heme signal is transduced via a less direct pathway. Using spectrophotometric and chromatographic techniques, we demonstrate that the HRM binds heme directly with an affinity at micromolar levels. Furthermore, we show that one HRM can mediate an effect on HAP1 transcriptional activity independent of DNA binding. We also speculate on how the heme responsive motifs in

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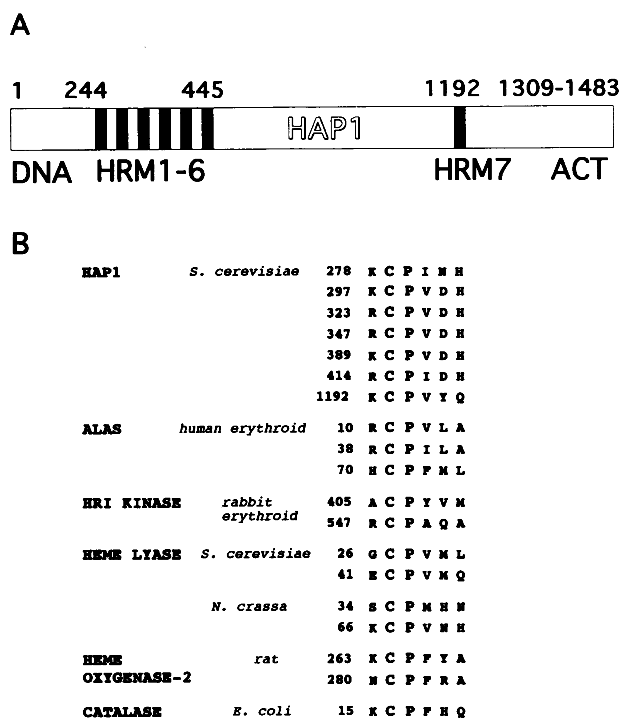

Fig. 1. (**A**) Domains in the yeast transcriptional activator, HAP1. The DNA binding (DNA) and transcriptional activation (ACT) domains lie at the amino- and carboxyl-termini of the protein. The HRMs (solid bars) lie in two regions, the first is a cluster of six HRMs between residues 244 and 445 (the previously identified heme domain; Pfeifer *et al.*, 1989), and the second is a single HRM beginning at residue 1192. (**B**) Sequences of HRMs from HAP1 and other proteins. The indicated HRMs are from HAP1 (Pfeifer *et al.*, 1989), the human erythroid ALAS (Lathrop and Timko, 1993), the rabbit erythroid HRI kinase (Chen *et al.*, 1991), heme lyase from *S.cerevisiae* or *Neurospora crassa* (Dumont *et al.*, 1987; Drygas *et al.*, 1989), the rat heme oxygenase-2 (Rotenberg and Maines, 1991) and the *Escherichia coli* catalase (Triggs-Raine *et al.*, 1988). The sequences in HAP1 and ALAS have been shown to confer regulation by heme, as described in the text. The other sequences were identified by a EMBL database search. The cysteine–proline dipeptide is absolutely conserved and there is a tendency for a basic residue in the first position and a hydrophobic residue in the fourth position.

HAP1 and other proteins mediate the regulation of their activity by heme.

Results

Heme binds to the HRM

Since the HRM contains a cysteine, creating the possibility of bonding between the sulfur of the cysteine and the Fe of a heme molecule, we investigated the possibility that the HRM binds heme directly. We synthesized a peptide corresponding to the consensus of HRMs adjacent to the DNA binding domain of HAP1 (Ala-Lys-Arg-Cys-Pro-Val-Asp-His-Thr-Met) and used spectrophotometry to analyze whether this peptide bound heme (Braterman *et al.*, 1964; Smith and Williams, 1970). Figure 2A and B shows the effect of peptide addition on the absorption spectrum of hemin (20 μM, line 1). As the concentration of the peptide increased from 5 to 20 μM, the peak of the strongest heme absorption band, the Soret band (Smith and Williams, 1970), shifted toward a shorter wavelength (lines 2–4). The maximum of hemin absorption was shifted by ~26 nm (from 388 to 362 nm) upon addition of 20 μM peptide. Addition of 40 μM (line 5) or more (up to 1 mM, not shown) of the peptide did not change the absorption profile considerably. Therefore 20 μM peptide saturated 20 μM hemin to form hemin–peptide complexes with different absorption properties. The peptide itself absorbed minimally across these wavelengths (line 6). The Soret band of heme results from the Π–Π* transition of the porphyrin ring (Braterman *et al.*, 1964; Smith and Williams, 1970). The binding of the peptide to heme may distort the porphyrin ring and increase the energy gap of the Π–Π* transition.

There were three isosbestic points (see arrows in Figure 2B) when the ratio of hemin and hemin–peptide complexes was changed by varying the concentration of peptide, suggesting the existence of a two-state chemical equilibrium between heme and a hemin–peptide complex (Marshall, 1978). Further, since equal molar concentrations of peptide and heme caused a total shift in the Soret band, we conclude that the stoichiometry of the hemin–peptide complex is 1:1, and that the peptide binds heme with a dissociation constant in the micromolar range or lower. Previous experiments indicated that 1–10 μM hemin induced DNA binding by HAP1 in yeast extracts (Pfeifer *et al.*, 1987).

We next tested whether this shift in the hemin absorption spectrum was specific. First, we determined that addition of cysteine alone did not alter the heme absorption spectrum (not shown). Next we tested peptides that differed from the HRM peptide by single amino acid changes. Mutation of the cysteine residue to alanine abolished the ability of the peptide to shift the hemin absorption spectrum at concentrations of peptide up to 200 μM (Figure 2C). The cysteine in the HRM is therefore absolutely essential for binding to heme. Since the adjacent proline is also completely conserved in HRMs, we tested a mutant peptide in which the proline was changed to alanine (Figure 2D). Although this mutant peptide shifted the hemin spectrum, a higher concentration was required to do so. This finding suggests that the proline increases the affinity of interaction between hemin and the HRM.

A similar experiment was carried out in the presence of 10 mM imidazole, which shifts the heme maximum to a longer wavelength because it chelates the Fe^{3+} of hemin (Braterman *et al.*, 1964; Smith and Williams, 1970). Figure 2E is a composite showing the heme peak in the presence of imidazole [now shifted from 388 nm (line 1) to 412 nm (line 2)], and the effect of adding the peptides. The wild type peptide shifted the absorption maximum to 362 nm (line 3), although more peptide was required (400 μM) than above because imidazole competed for heme binding. Nonetheless, the concentration of peptide was lower than the concentration of imidazole by 25-fold, indicating that the peptide bound to heme much more strongly than imidazole bound to heme. The mutant peptides are shown in Figure 2F. The cysteine to alanine mutant peptide (line 3) showed no detectable binding and a spectrum identical to hemin–imidazole alone (line 1). The proline to alanine mutant peptide partially shifted the absorption peak (line 4) compared with the wild type (line 2), indicating that a portion of the heme was bound to the peptide. These findings indicate that the wild type peptide binds to heme,

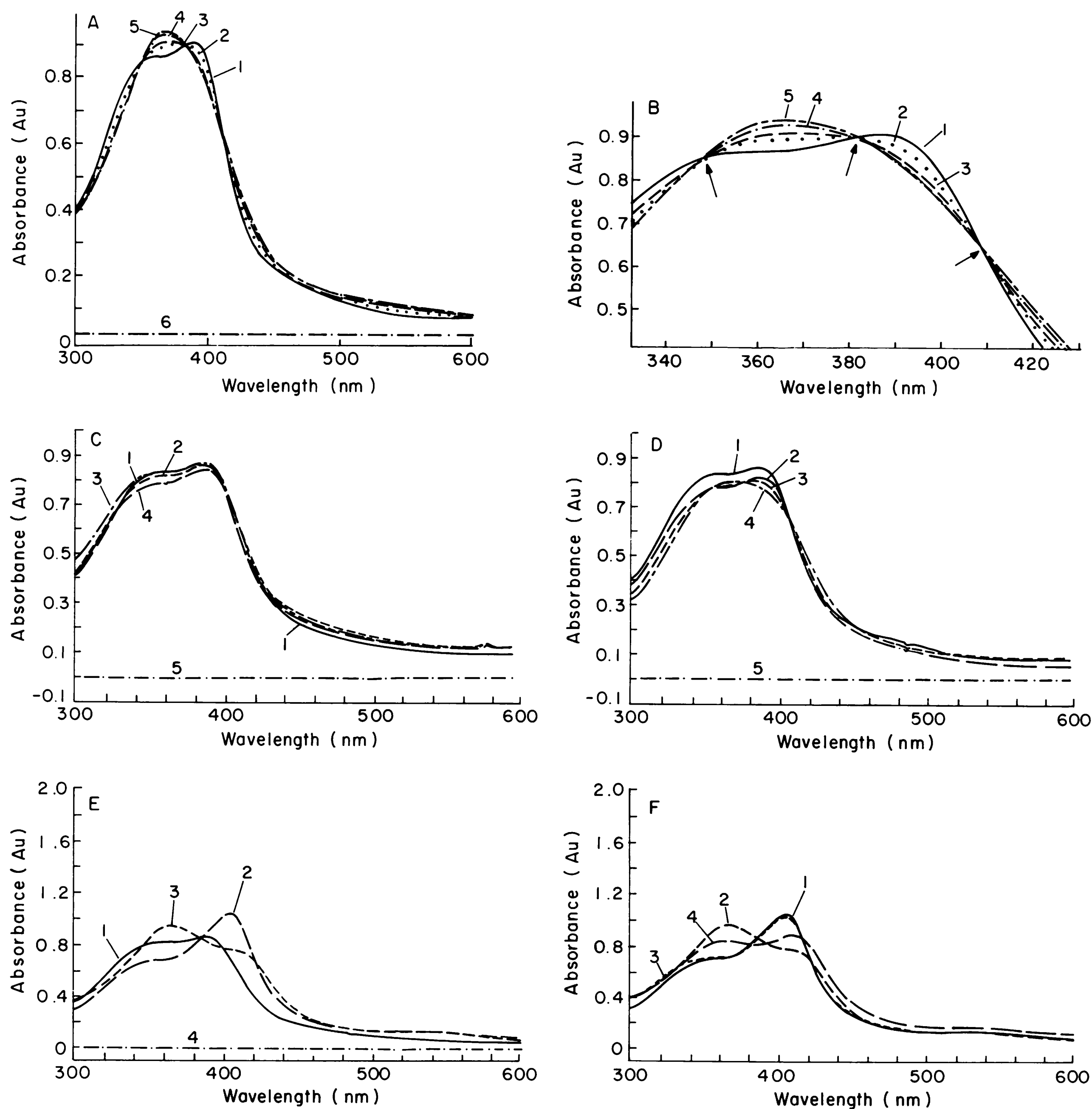

Fig. 2. Absorption spectrum of heme in the absence and presence of the HRM peptide. (**A** and **B**) The effect of wild type HRM peptide on the heme absorption spectrum (B is an enlarged version of the Soret band of the spectra in A). Shown are the spectrum of hemin (20 μM for all the spectra shown in this figure) with no peptide (line 1), hemin + 5 μM peptide (line 2), hemin + 10 μM peptide (line 3), hemin + 20 μM peptide (line 4), hemin + 40 μM peptide (line 5) and peptide alone at 1 mM (line 6). The three isosbestic points are shown by three arrows in B. (**C**) The effect of Cys to Ala mutant peptide on the heme absorption spectrum. Shown are the spectrum of hemin alone (line 1), hemin + 20 μM peptide (line 2), hemin + 100 μM peptide (line 3), hemin + 200 μM peptide (line 4) and peptide alone (line 5). (**D**) The effect of Pro to Ala mutant peptide on the heme absorption spectrum. Shown are the spectrum of hemin alone (line 1), hemin + 10 μM peptide (line 2), hemin + 20 μM peptide (line 3), hemin + 40 μM peptide (line 4) and peptide alone (line 5). (**E**) The effect of wild type peptide on the heme absorption spectrum in the presence of imidazole. Shown are the spectrum of hemin alone (line 1), hemin + 10 mM imidazole (line 2), hemin + 10 mM imidazole + 400 μM wild type peptide (line 3) and imidazole alone (line 4). (**F**) Comparison of the effect of mutant peptides on the heme–imidazole absorption spectrum with that of wild type peptide. Shown are the spectrum of hemin + 10 mM imidazole (line 1), hemin + 10 mM imidazole + 400 μM wild type peptide (line 2), hemin + 10 mM imidazole + 400 μM Cys to Ala peptide (line 3) and hemin + 10 mM imidazole + 400 μM Pro to Ala peptide (line 4).

that the cysteine is critical for binding and that the proline aids the affinity of binding.

To confirm the above findings we also demonstrated the binding of heme to the peptide by column chromatography. We hypothesized that if the peptide binds to heme, the heme–peptide complex will be larger than heme alone. As a result, the heme–peptide complex will be able to pass through certain gel filtration columns while heme itself will not. Indeed, we found that heme did not pass through a Sephadex-G50–80 column (Sigma) while heme passed readily through the column when mixed with the wild type peptide (in columns packed with Sephadex with smaller pore size such as G25, heme itself eluted as quickly as in the presence of peptide). Figure 3A shows

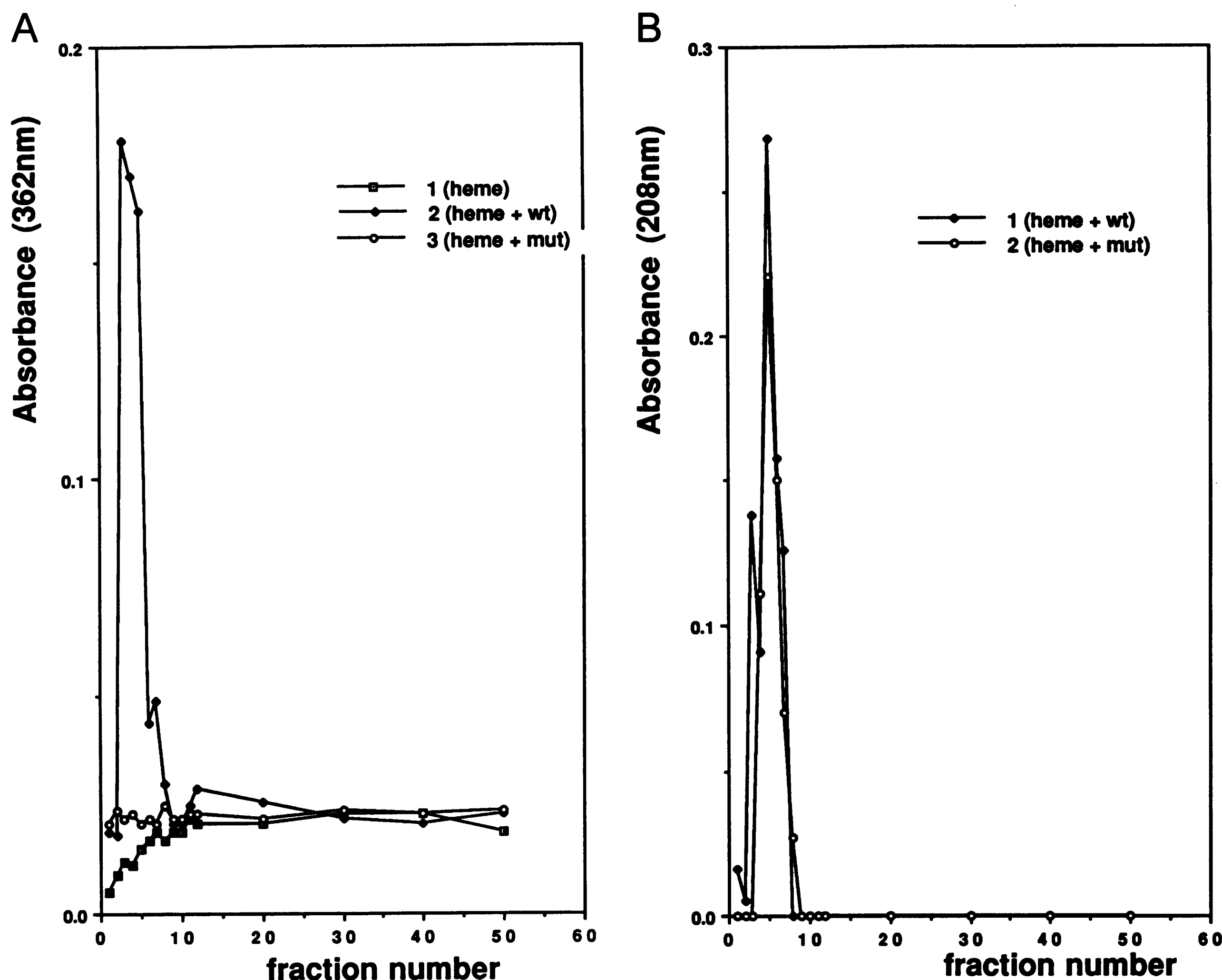

Fig. 3. Elution profiles of heme and heme–peptide mixtures in Sephadex-G50–80 columns. (**A**) Elution profiles of the heme–peptide complex monitored by absorption of hemin at 362 nm. Shown are the profiles of hemin alone (line 1), hemin + the wild type peptide (line 2), and hemin + the Cys to Ala mutant peptide (line 3). (**B**) Elution profiles of the peptides as by absorption at 208 nm. Shown are the profiles of the wild type peptide (line 1, the same fractions as in line 2 of A) and the Cys to Ala peptide (line 2, the same fractions as in line 3 of A).

the elution profiles of heme alone (line 1), heme mixed with the wild type peptide prior to loading (line 2), and heme mixed with the Cys to Ala mutant peptide (line 3). The presence of heme or heme–peptide complexes in the fractions was monitored by absorption at 362 nm. As shown by line 1, in the presence of the wild type peptide, heme eluted in fractions 3, 4 and 5. The Cys to Ala mutant peptide was inert; heme remained in the column even after extended elution just as in the case of heme alone (see line 1 and 3). All the peak fractions in line 1 showed maximum absorption at 362 nm, indicating that they all contained heme–peptide complexes.

We also monitored the presence of peptides in the fractions by detecting absorbance at 208 nm. Figure 3B shows the elution profiles of wild type (line 1) and Cys to Ala mutant peptide (line 2) in the same columns as shown in Figure 3A. The Cys to Ala mutant peptide readily eluted from the column in fractions 4–7. The elution profile of the wild type peptide showed two peaks, one in fraction 3 due to the heme–peptide complex, and the other in fractions 4–7. The latter peak was very similar to that of the Cys to Ala mutant, suggesting that it comprised the unbound peptide. Although heme is able to absorb light at 208 nm, this absorption is slight compared with that of the peptide.

The spectral and chromatographic studies show that the peptide containing a HRM binds to heme and changes its physical and chemical properties. We suggest that the cysteine of the HRM serves the critical function of donating electrons to the Fe of heme. The basic residues which are usually found at the first position in the HRM and the hydrophobic residues found in the fourth position may enhance binding by interacting with the carboxyl and vinyl side chains of heme, respectively (Scheidt, 1979). The proline in the HRM may serve a structural role by exposing the cysteine residue for bonding to the Fe of heme.

A single HRM modulates the activity of a transcriptional activation domain

Earlier experiments showed that the domain containing HRM1–6 repressed the DNA binding activity of HAP1 in the absence of heme (Pfeifer *et al.*, 1987, 1989). When this domain was deleted, DNA binding by HAP1 was totally independent of heme (Fytlovich *et al.*, 1993; Zhang and Guarente, 1994). Inspection of the HAP1 sequence

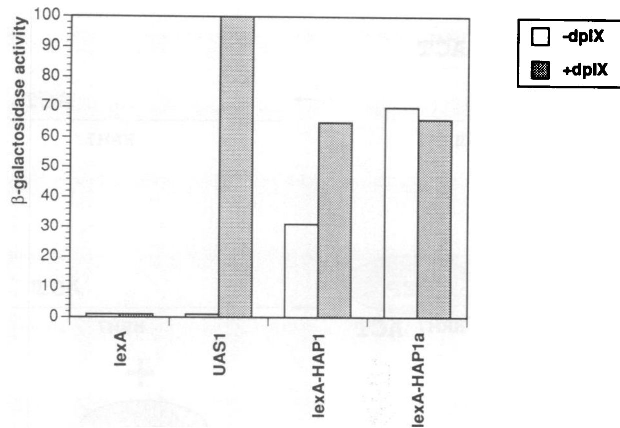

Fig. 4. Heme regulation of the HAP1 transcriptional activation domain. A lexA–HAP1 chimera was constructed containing the DNA binding and dimerization domains of lexA (residues 1–202) fused to the activation domain of HAP1 (residues 946–1483) or HAP1 Cys1193 to Ala, and expressed from the ADH1 promoter. In order to demonstrate whether heme regulated this chimera, constructs borne on a 2μ plasmid were transformed into strain MHY100 (*MATa ura3-52 leu2-3, 112 his4-519 ade1-100 hem1-Δ100;* Haldi and Guarente, 1989) also bearing a β-galactosidase reporter driven by a lexA binding site (Brent and Ptashne, 1985). Cells were grown on limiting amounts of δ-aminolevulinate (0.4 μg/ml), the cultures split in half and 0.4 μg/ml deuteroporphyrin IX (dpIX) added to one of the two cultures. After an induction period of another 7 h, cells were harvested for determination of β-galactosidase activity (Rose *et al.*, 1988). lexA–HAP1 displayed a higher activity when induced by dpIX (■) than when not induced (□). The level of induction varied between 2- and 2.5-fold in two experiments each done in triplicate. The lexA–HAP1 Lys1193 → Ala construct (lexA–HAP1a) did not show induction by heme in either experiment, but was constitutively active. Unfused lexA (lexA) was not active and the UAS1 reporter gave a measure of the high level of inducibility by heme of the intact HAP1.

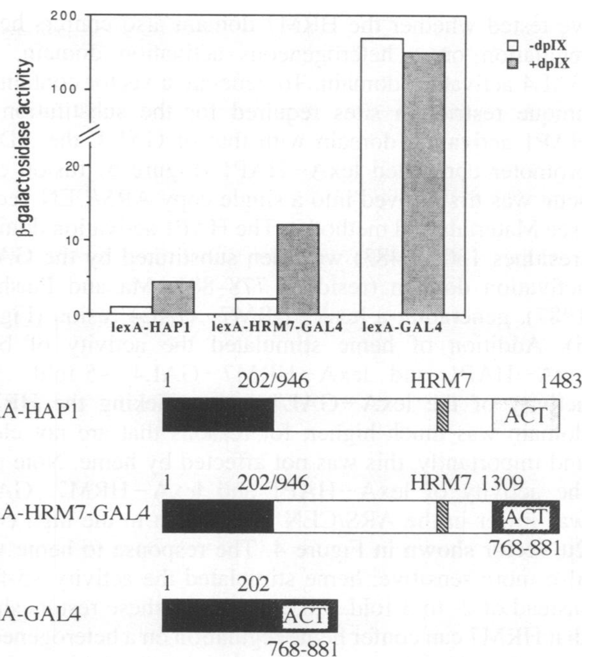

Fig. 5. HRM7 also confers heme regulation on the GAL4 activation domain. ARS/CEN plasmids containing the lexA fusion proteins, lexA–HAP1, lexA–HRM7–GAL4 and lexA–GAL4 expressed from ADH1 promoter were transformed into strain MHY100 (*MATa ura3-52 leu2-3, 112 his4-519 ade1-100 hem1-Δ100;* Haldi and Guarente, 1989) also bearing a β -galactosidase reporter driven by a lexA binding site (Brent and Ptashne, 1985). Cells were grown in the presence (+dpIX, ■) or absence (−dpIX, □) of deuteroporphyrin and their β-galactosidase activities assayed as described in the legend to Figure 4. The data plotted here are averaged from six independent transformants. The HAP1 and GAL4 activation domains are labeled 'ACT'. The lexA–HAP1 fusion contains residues 1–202 of lexA and residues 946–1483 of HAP1. The lexA–HRM7–GAL4 fusion contains residues 1–202 of lexA, residues 946–1309 of HAP1, and residues 768–881 of GAL4. The lexA–GAL4 fusion contains residues 1–202 of lexA and residues 768–881 of GAL4.

revealed a seventh copy of the HRM (HRM7) at a location distant from the other six, spanning residues 1192–1197 of the protein (Figure 1). While this motif does not regulate DNA binding it may affect HAP1 activity due to its proximity to the acidic activation domain [residues 1309 and 1483 (Pfeifer *et al.*, 1989)]. However, we were not previously able to test this possibility because the HAP1 derivative missing HRM1–6 was toxic when cells were starved for heme (Pfeifer *et al.*, 1989).

In order to test whether HRM7 regulated the activity of the transcriptional activation domain of HAP1, we fused a fragment of HAP1 containing residues 946–1483 (including HRM7 and the acidic activation domain) to a lexA DNA binding and dimerization moiety (residues 1–202). This chimera expressed from the ADH1 promoter (Becker *et al.*, 1991) was introduced into a *hem1⁻* mutant (deficient in ALAS; MHY100; Guarente *et al.*, 1984; Haldi and Guarente, 1989) bearing a lacZ reporter driven by a single lexA site (Brent and Ptashne, 1985). Levels of β-galactosidase were determined in cells grown either in the presence of limiting concentrations of ALA (resulting in starvation for heme) or in the presence of the heme supplement, deuteroporphyrin IX (dpIX). Activation by the lexA–HAP1 chimera was observed in the absence of heme, and, importantly, activity was stimulated by the presence of heme by ~2- to 3-fold (Figure 4). This magnitude of regulation by a single HRM is significant although it is much less than that observed for the intact HAP1, as revealed by the heme-responsive reporter, UAS1. The smaller effect of heme on the activity of the chimera is not surprising, however, because it is missing the region including HRM1–6, which regulates DNA binding of HAP1 by at least 10-fold *in vitro* (Pfeifer *et al.*, 1987; Fytlovich *et al.*, 1993; Zhang and Guarente, 1994).

To confirm that the regulation by heme of the HAP1 activation domain was mediated by HRM7, we constructed a cysteine to alanine mutation in HRM7 of the chimera and assayed the activity of the mutant in heme-sufficient (+dpIX) or heme-starved (−dpIX) cells. The regulation by heme was abolished in the mutant, and activity in both cases resembled that observed in the wild type construct in the presence of heme. The constitutively high activity in this mutant may suggest that the heme binding site may overlap a repressor binding site, as discussed below. Thus, we conclude that HRM7 modulates the activity of the HAP1 transcriptional activation domain in response to heme and that this regulation can be separated from regulation of DNA binding by heme.

HRM7 confers heme regulation on the GAL4 activation domain

To investigate further the molecular mechanisms governing heme regulation of the HAP1 activation domain by HRM7,

we tested whether the HRM7 domain also confers heme regulation on a heterogeneous activation domain, the GAL4 activation domain. To generate a vector containing unique restriction sites required for the substitution of HAP1 activation domain with that of GAL4, the ADH1 promoter controlled lexA–HAP1 (Figure 5) fusion cassette was first moved into a single copy ARS/CEN vector (see Materials and methods). The HAP1 activation domain (residues 1309–1483) was then substituted by the GAL4 activation domain (residues 778–881; Ma and Ptashne, 1987), generating a lexA–HRM7–GAL4 fusion (Figure 5). Addition of heme stimulated the activity of both lexA–HAP1 and lexA–HRM7–GAL4 ~5-fold. The activity of the lexA–GAL4 fusion lacking the HRM7 domain was much higher, for reasons that are not clear, and importantly, this was not affected by heme. Note that the activity of lexA–HAP1 and lexA–HRM7–GAL4 was lower in the ARS/CEN vector than in the high copy 2μ vector shown in Figure 4. The response to heme was also more sensitive: heme stimulated the activity ~5-fold instead of 2- to 3-fold. Taken together, these results show that HRM7 can confer heme regulation on a heterogeneous activation domain, suggesting that the repression of the HAP1 activation domain by HRM7 does not involve any specific interactions between these two domains.

Discussion

HRMs represent a new class of heme binding module different from those in cytochromes or globins

A peptide containing one HRM motif from the HAP1 heme domain binds to heme with an affinity in the micromolar range. This interaction between heme and the HRM is completely different from the interactions in cytochromes or globins (Bock *et al.*, 1978). In cytochromes, the axial iron ligands are commonly a histidine/methionine pair or bis-histidine, which bind to heme and shift the Soret peak to a longer wavelength due to the change of electron distribution on the porphyrin ring (Bock *et al.*, 1978; Choma *et al.*, 1994). Recently, a heme binding four-helix bundle has been synthesized and shown to bind to heme tightly via bis-histidine coordination, also shifting the Soret peak to a longer wavelength (412 nm, like imidazole; Figure 2E; Choma *et al.*, 1994; Robertson *et al.*, 1994). The fact that the HRM shifts the heme absorption peak to a shorter wavelength (362 nm) suggests that the HRM–heme interaction is qualitatively different from that of heme and cytochrome. The HRM peptide may interact with heme to distort the porphyrin ring and increase the energy gap of the Π–Π^* transition (Smith and Williams, 1970). HRMs thus represent a new class of heme binding module which binds heme reversibly and transiently, allowing changes in heme concentration to be sensed.

Implications of heme binding to the HRM

The different roles of heme as a regulatory molecule can now be understood in terms of heme binding to the HRM. In the case of HAP1, the region between residues 244 and 445 containing HRM1–6 has been shown to cause binding to a cellular repressor (Fytlovich *et al.*, 1993; Zhang and Guarente, 1994) in the absence of heme. We

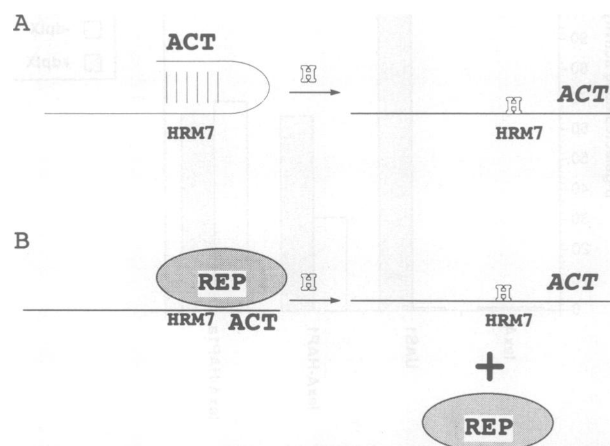

Fig. 6. Models for regulation of the HAP1 transcriptional activation domain (ACT) by heme (H). Both models rest on the finding that the Cys1193 → Ala mutation in HRM7 gives rise to constitutively high activity. In (**A**), HRM7 is included in a region of HAP1 which masks the activation domain in the absence of heme. When heme binds to HRM7 masking is prevented. In (**B**), a repressor binds to sequences including HRM7 and the activation domain in the absence of heme. When heme binds to HRM7 it prevents the repressor from binding.

propose that this region contains a sequence recognized by the repressor which is interspersed with the HRMs. The binding of heme to the HRMs would prevent the repressor from binding to HAP1. Indeed, HRM1–3 are part of an extended repeat encompassing an additional 17 amino acids (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). These additional residues could be a part of the repressor recognition sequence.

In the case of the mammalian ALAS, heme probably binds to the HRMs in the leader when the protein is still in the cytoplasm, thus preventing unfolding and translocation across the mitochondrial membrane (Lathrop and Timko, 1993). In model experiments using DHFR, methotrexate was shown to prevent unfolding and concomitant translocation into mitochondria (Eilers and Schatz, 1986). In the case of the HRI, heme most likely binds to the HRMs and inhibits the catalytic activity of the enzyme.

A database search for HRM sequences identified regions in heme lyase (which attaches heme to cytochromes; Dumont *et al.*, 1987; Drygas *et al.*, 1989), heme oxygenase-2 (which degrades heme; Rotenberg and Maines 1991) and catalase (which degrades hydrogen peroxide; Triggs-Raine *et al.*, 1988; Figure 1B). The first two enzymes bind heme as a substrate, and the third uses heme as a cofactor. It will be interesting to see whether the HRMs serve as substrate (or cofactor) binding sites or function as regulatory domains in these proteins.

Heme regulation of HAP1 activity: a consecutive heme binding model

The heme responsive motif HRM7 confers heme regulation on the HAP1 activation domain and the GAL4 activation domain. We envision two possible ways by which heme could exert its effects on the HAP1 activation domain. Both models propose that HRM7 mediates repression of the HAP1 activation domain in the absence of heme, because of the constitutively high activity of the Cys to Ala mutant. One possibility is that sequences including

HRM7 inhibit the activation domain, perhaps by intramolecular masking (Figure 6A). Heme would then bind to HRM7 and prevent masking, potentiating the full activity of the activation domain. However, intramolecular masking probably involves specific interactions between HRM7 and the HAP1 activation domain in the absence of heme. The fact that HRM7 confers heme repression on the GAL4 activation domain suggests that the repression does not require specific interactions between HRM7 and the activation domains.

Another possibility is that sequences around HRM7 bind to a cellular factor which represses the acidic activation domain, and heme dislodges the repressor by binding to HRM7 (Figure 6B). This is analogous to regulation of DNA binding of HAP1 (Fytlovich *et al.*, 1993; Zhang and Guarente, 1994). Are the HRM1–6 region and sequences around HRM7 bound by the same sequence? One reason for believing that the repressors might be different is the presence of an extended repeated sequence of 23 amino acids including HRM2, HRM3 and HRM4 (Pfeifer *et al.*, 1989; Creusot *et al.*, 1988). This extended region, not present at HRM7, suggests that HRM2, 3 and 4 may be bound by a repressor different from that which binds sequences around HRM7. There are other examples in which the ability of transcription factors to activate is influenced by small molecules. Examples include members of the steroid hormone receptor family (Yamamoto, 1985; Evans, 1988), the yeast LEU3, which is activated by αIPM (Friden and Schimmel, 1987; Sze *et al.*, 1993) and the bacterial merR, which is activated by mercury (O'Halloran *et al.*, 1989). Activation of merR by mercury does not involve any repressor (O'Halloran *et al.*, 1989) while activation of steroid hormone receptor involves HSP90 (Picard *et al.*, 1988, 1990).

Our data raise the possibility that heme may control HAP1 activity at two levels, HRM1–6 regulating the DNA-binding domain, and HRM7 regulating the activation domain. It is possible that the multiple copies of HRMs near the DNA binding domain allow heme to bind there with a higher affinity than to HRM7. Thus, we suggest a speculative model for heme regulation of HAP1 activity, proposing that heme binds consecutively to the HRMs of HAP1. This would allow a stepwise induction of HAP1 target genes (cytochromes and other aerobic functions) as a function of oxygen/heme concentrations. When cells grow in a limiting supply of oxygen, the low concentration of heme synthesized would bind to HRM1–6, and release the repressor of HAP1 DNA binding. A low to moderate level of induction of HAP1 target genes would result. In a more aerobic environment, heme would bind to HRM7, eliciting full induction of target genes. Separate regulation of the DNA binding and activation functions by the same inducer may be a more general mechanism to fine tune the activity of transcriptional activators.

Materials and methods

Yeast strains and β-galactosidase assays

Saccharomyces cerevisiae strain used was MHY100 (*MAT***a** *ura3-52 leu2-3, 112 his4-519 ade1-100 hem1-Δ100*). Cells were grown in YPD or synthetic complete media. For β-galactosidase assays, cells were grown on synthetic complete media containing 2% glucose with limiting amounts of δ-aminolevulinate (0.4 μg/ml) to OD 0.3, the cultures were then divided in half, and deuteroporphyrin IX (dpIX) added (0.4 μg/ml) to one of the two cultures. After an induction period of another 7 h, cells were harvested for determination of β-galactosidase activity (Rose *et al.*, 1988). At least three independent transformants were assayed and average values were plotted.

Peptides, spectrophotometric analysis, and G50–80 columns

The wild type (Ala-Lys-Arg-Cys-Pro-Val-Asp-His-Thr-Met), the Cys to Ala, and Pro to Ala mutant peptides were synthesized at the MIT facility. The peptides were highly purified by HPLC and amino acid composition was confirmed. For spectrophotometric analysis, 20 μM hemin was mixed with buffer or various concentrations of peptide prior to the measurement of absorbance. All reactions were carried out in phosphate-buffered saline (PBS), pH 7.3.

All the columns used to separate heme and heme–peptide complexes were packed with Sephadex-G50–80 (Sigma) in PBS using 1 ml syringes. The quantity of beads was controlled so that all the columns were of the same height and volume (~1 ml). The columns were washed with PBS before loading with 40 μl of samples containing 0.25 mM hemin or 0.25 mM hemin + 0.5 mM peptide. Fractions, each containing 2 drops of liquid, were eluted by PBS.

Mutagenesis of Cys to Ala

The Cys1193 to Ala mutation was generated by an oligonucleotide-directed *in vitro* mutagenesis system from Bio-Rad. Single-stranded uracil DNA was generated using Stratagene Bluescript II KS+ vector in *dut ung* strain CJ236. A 33 bp oligonucleotide encoding 11 amino acids at HRM7 with TGC (Cys) changed to GCG (Ala) was annealed to the single-stranded uracil DNA. Double-stranded DNA was synthesized and transformed to the wild type MV1190 strain. The mutation created a new *Hae*II site and this was later used to identify the mutant. Mutant DNA was then transferred from KS vector to the lexA vector used in the β-galactosidase assays (Pina *et al.*, 1993). Mutant DNA from several independent colonies was transferred and used for the assay and identical results were obtained.

Construction of the lexA–HRM7–GAL4 fusion

The DNA fragment containing the lexA–HAP1 fusion in the ADH promoter/terminator cassette (Becker *et al.*, 1991; Pina *et al.*, 1993) was cut out of the 2μ vector shown in Figure 4 and inserted into the ARS/CEN PRS316 vector (Sikorski and Hitter, 1989) lacking *Kpn*I and *Xba*I sites. A GAL4 fragment containing residues 768–881 (Ma and Ptashne, 1987) as well as a stop codon was then inserted into the PRS316–lexA–HAP1 plasmids cut with *Kpn*I and *Xba*I. This construction created a fusion protein in which the HAP1 activation domain was substituted by that of GAL4. Multiple correct clones were used to detect β-galactosidase activity and all gave the same results.

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