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Original Research Communication

Heme Oxygenase-2 Interaction with Metalloporphyrins: Function of Heme Regulatory Motifs

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ABSTRACT

Heme oxygenase-2 (HO-2) degrades heme [Fe-protoporphyrin IX (Fe-PP)] to CO and bilirubin. The enzyme is a hemoprotein and interacts with nitric oxide. HO-2 has two copies of heme regulatory motif (HRM) with a conserved core of Cys^{264}-Pro^{265} and Cys^{281}-Pro^{282}. We examined interaction of HO-2 HRMs with Fe-PP, Zn-protoporphyrin IX (Zn-PP; HO-2 inhibitor), and protoporphyrin IX (PP IX). Spectral analyses, using 1:4 or 1:1 molar ratio of the heme to 10-residue peptides, corresponding to HRM containing HO-2 sequences, revealed specific interactions as indicated by a shift in the absorption spectrum of heme. Five residue peptides qualitatively produced similar results. Substitution of cysteine with alanine in either peptide eliminated interactions, and substitution of proline with alanine reduced the peptides' affinity for heme. Neither Zn-PP nor PP IX absorption spectrum was affected by HRM peptides. The circular dichroism spectra confirmed heme–HRM peptides interactions. An astounding 4,000–6,000-fold higher concentrations of KCN were required at pH 7.5 to displace HRM peptides from heme. Data suggest (a) each HRM can contribute to HO-2–heme interaction, (b) heme iron interacts with cysteine thiol, (c) charged residues upstream of Cys^{264}-Pro^{265} result in its high-affinity heme binding, and (d) inhibition of HO-2 activity by synthetic metalloporphyrins does not involve HRMs. We suggest that heme bound to HRMs may serve as a binding site/reservoir for gaseous signal molecules. Antioxid. Redox Signal. 3, 685–696.

INTRODUCTION

THREE ISOZYMES OF HEME OXYGENASE (HO), referred to as HO-1, HO-2, and HO-3 (8, 31, 34, 37, 41, 49), have been identified to date. HO-2 and HO-3 are related proteins, whereas, HO-1 is a distinctively different protein and shares a mere 43% overall similarity with HO-2 in the predicted primary protein structure (44, 49). Both proteins, however, are active in heme catalysis and oxidatively cleave the molecule to form CO and biliverdin. Biliverdin is subsequently reduced to the bile pigment, bilirubin. The chelated Fe is released subsequent to opening of the porphyrin ring (for review, see 29). HO-3 is only marginally active (37). The products of HO activity are biologically active; CO is believed to function as nitric oxide (NO) in cyclic GMP cell signaling (30, 32, 51) and bilirubin is an antioxidant (38). The HO proteins have a stretch of 24 amino acids that forms the heme catalytic pocket (8, 33, 45). Except for a single amino acid that is conservatively substituted between HO-1s and HO-2s (leucine in HO-1 → methionine in HO-2), the sequence is evolutionarily conserved among all HO-1s and HO-2s (29). One half of this domain (Pro-Glu-Leu-Leu-Val-Ala-His-Ala-Tyr-Thr-Arg-Tyr) is

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designated as the "HO signature" (GenBank) and is made of mostly hydrophobic residues surrounding the histidine. This histidine is essential for the catalytic activity of HO-2 (33). Binding of synthetic metalloporphyrins to this domain inhibits HO activity (21, 28), and this principle has been used for treatment of neonatal jaundice and in investigating the role of CO as a signal molecule for generation of cyclic GMP (5, 12, 30, 57).

Cysteine residues are not present in HO-1; HO-2 and HO-3, both of which are hemoproteins (36, 37), contain cysteine. When flanked by a proline residue, the dipeptide forms the core of a heme binding sequence called "heme regulatory motif" or HRM (60). HO-2 and HO-3 have two copies of HRM (36, 37, 44). In HO-2 they correspond to Cys^{264}-Pro^{265} and Cys^{281}-Pro^{282}. In addition to HO-2 and HO-3, five other proteins have been identified to have the HRM sequence, and all have heme-dependent regulatory functions in the cell. These proteins include rabbit heme-regulated initiation factor (HRI) kinase (6, 43), and yeast human apurinic endonuclease (HAP1), which activates transcription of genes in response to oxygen/heme (7); the others are cytochrome *c* heme lyase of *S. cerevisiae* and *N. crassa*, human erythroid δ-aminolevulinate synthase (10, 11, 25, 52), the rate-limiting enzyme in heme biosynthesis (46), and *E. coli* catalase (55). In HRI kinase, cytochrome *c* heme lyase, and catalase, one to three copies of the HRM are present. It is not known whether synthetic metalloporphyrins interact with HO-2 HRMs. Also, although postulated (9, 36), it is not known whether both HO-2 HRMs bind to heme, and if so, whether the relative affinity of HRMs for heme is the same. Moreover, characteristics of the amino acids that surround HRMs could be of importance to their reactivity with heme. However, to date, the significance of residues flanking the core (Cys-Pro) to the relative affinity of HRMs for heme binding has not been examined.

A recent study has inquired into the functional significance of HRMs in HO-2 and has shown that HO-2 binds NO generated by various NO donors; binding of NO/NO derivatives attenuates HO-2 catalytic activity (9). This has led to the suggestion that HO-2 may function as an intracellular "sink" for NO. The suggestion also has been made that HO-2 functions as an intracellular reservoir for heme for regulation of HO-1 and as an "oxygen sensor" (30). There is evidence that a heme complex(es) serves as an oxygen sensor for hypoxic gene expression (1). Considering these possibilities, it would be of biological significance to examine the affinity and interaction of HO-2 HRMs with metalloporphyrins.

The present study used peptides encompassing the HO-2 HRMs to characterize their interaction with metalloporphyrins. The study has identified the astonishingly high affinity of Cys^{264}-Pro^{265} and Cys^{281}-Pro^{282} containing peptides corresponding to the HO-2 sequence for heme. Data suggest that HO-2 HRMs do not bind to synthetic metalloporphyrins, and that the formation of an iron-cysteine thiol coordinate is critical for formation of a high-affinity HRM–heme complex. Data are supportive of the potential function of HO-2 HRMs in the regulation of cellular levels of gaseous heme ligands such as oxygen and nitrogen radicals.

MATERIALS AND METHODS

Materials

Peptides for heme binding studies were obtained in HPLC purified (>95%) form from Primm Laboratories (Cambridge, MA, U.S.A.). Fe-protoporphyrin IX (Fe-PP) and protoporphyrin IX (PP IX) were from Sigma (St. Louis, MO, U.S.A.). Zn-PP was obtained from Porphyrin Products (Logan, UT, U.S.A.). All other reagents were of the highest quality commercially available.

Heme binding assays

Three synthetic peptides, one consisting of 12 residues representing "HO signature" and two 10 residues long encompassing the HRMs of HO-2 (aa261Val-Arg-Lys-Cys-Pro-Phe-Tyr-Ala-Ala-Gln) and (aa278 Gly-Ser-Asn-Cys-Pro-Phe-Arg-Thr-Ala-Met), were used. The sequence of peptides is based on human HO-2 cDNA (35). In addition, the HRM peptides were also obtained substituting alanine for the conserved cysteine residues, or substituting alanine for proline. HRM peptides were prepared as 400 μM

stocks in water and stored at −80°C. Due to its hydrophobic nature, the "HO signature" peptide was dissolved in 5% acetonitrile in water, and the assay system (see below) was also supplemented with a comparable concentration of acetonitrile. Hemin solution (500 μM) was prepared fresh before use as follows: 3.26 mg of hemin chloride was dissolved in 100 μl of 1 M NH_4OH/methanol (1:1, vol/vol). Thereafter, a 1:100 dilution with 0.1 M Tris-HCl (pH 7.5) buffer containing 0.01% Tween 20 was made. The final concentration of heme was 1.2 μM. Heme binding was determined by difference absorption spectroscopy between the heme solution in the sample cuvette and the same buffer in the reference cuvette. First, the absorption spectrum of hemin solution against the buffer was recorded, then various concentrations of peptides, as noted in the appropriate figure legends, were added, and spectra were recorded. Scans were performed at 2 nm/s, using an SLM Aminco DW-2C spectrophotometer. Binding assays were also carried out using peptides consisting of five residues. These peptides were Arg-Lys-Cys-Pro-Phe and Ser-Asn-Cys-Pro-Phe. Conditions for the binding assays were identical to those for the 10-residue peptides. For assays utilizing pp IX or Zn-PP, the complexes were prepared as a 500 μM stock solution as described for heme, and used for peptide binding assays. To analyze the pH effect, the heme:peptide spectrum was also obtained in 0.1 M Tris HCl (pH 9.0) containing 0.01% Tween 20. In these assays, the stock hematin solution was also brought to volume using the same buffer used for the assay.

Spectral analyses were also carried out in the presence of KCN, prepared immediately before use as a 400 mM stock solution in water. For each peptide and pH (7.5 and 9.0), the molar ratio of heme:peptide required to obtain a complete shift of the Soret band was established and the difference spectrum recorded. Subsequently, stock KCN was added incrementally to the heme:peptide solution and the spectrum was recorded following each addition. The molar absorption of heme present in the heme–peptide complex (ΔOD between 369 and 500 nm) and heme-CN (ΔOD between 422 and 500 nm) was measured. Calculation of K was carried out as described by Hogness *et al*. (19) for the cyanide hemochromogen reaction.

Circular dichroism (CD) spectra measurements were obtained as described by Zentz *et al*. (59) using a Jasco J710 spectropolarimeter, scanning at 100 nm/min from 350 to 200 nm. The path length was 1 cm, and the cell volume was 360 μl. Data on the peptides were determined at a concentration of 44 μg/ml (40 μM) in 25 mM sodium phosphate (pH 7.5) in the absence or presence of 10 μM heme (4:1 molar ratio). Each spectrum represents the average of four scans. The values for peptide plus heme were corrected by subtraction of data for heme alone in the same buffer, whereas those for peptide in the absence of heme were corrected for buffer.

RESULTS

Both HO-1 and HO-2 share a conserved 24 residue, called "heme catalytic pocket" (45), that is essential for heme catalysis. In addition, HO-2 contains two copies of HRM. HRMs are not required for heme catalysis by HO-2, but appear to be responsible for the hemoprotein nature of HO-2. Because of the curious aspects of HO-2 tissue/cell distribution and high levels of its expression in some unlikely organs, *e.g*., brain and testis, we undertook the present study to examine the function of HO-2 HRM sequences in metalloporphyrin binding. The study used HO-2-based peptides, and peptide interaction with heme was assessed by shift in the absorption spectrum of heme. The results of stepwise addition of 10-residue Cys^{264}-Pro^{265} or Cys^{281}-Pro^{282} containing peptides to a 1.2 μM heme solution are shown in Fig. 1a and b, respectively. Heme by itself (dotted line) produced the expected spectrum with absorption of the Soret band components at 389 nm and Soret′ (shoulder). Addition of either peptide at an equimolar ratio to heme had virtually identical effects on the spectrum of heme, with both causing a major (∼15 nm) shift to a lower wavelength (blue shift) of the Soret band to 374 nm and disappearance of the Soret′. The addition of either peptide at fourfold molar excess to heme caused a further blue shift of ∼20 nm to 369 nm with an increase in the intensity

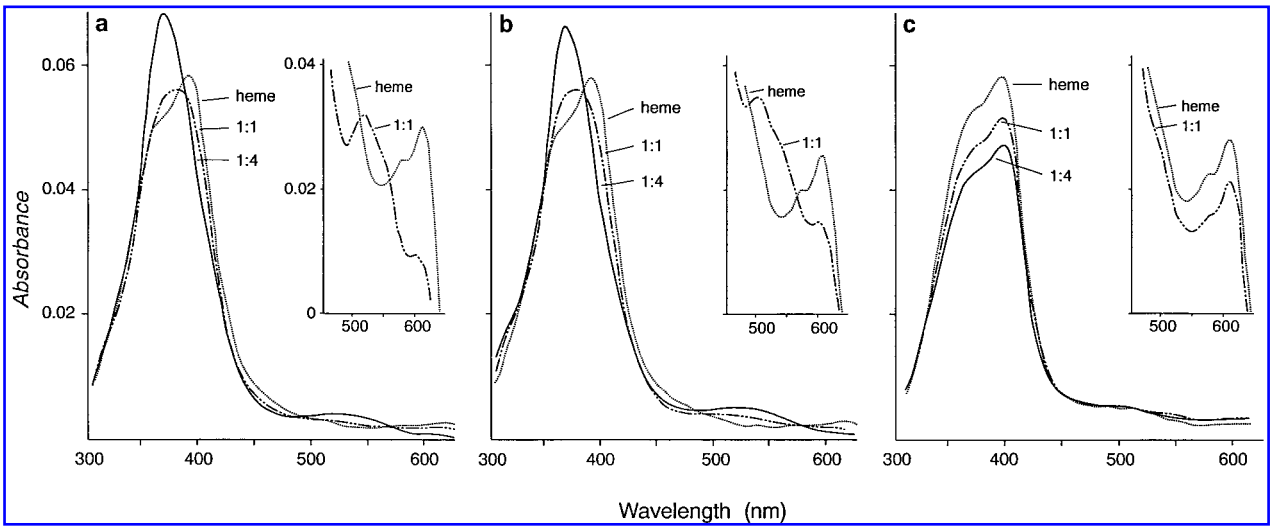

FIG. 1. Heme binding spectra of peptides corresponding to HO-2 HRMs or a fragment of heme catalytic domain. The difference absorption spectrum of a 1.2 μM solution of heme in 0.1 M Tris-HCl (pH 7.5) containing 0.01% Tween 20 was measured over the range of 350–650 nm at a scanning rate of 2 nm/s. Subsequently, 10-residue-long peptides corresponding to Cys^{264}-Pro^{265} or Cys^{281}-Pro^{282} containing HRMs, or a 12-residue-long peptide corresponding to a fragment of the 24-residue heme catalytic site (45) was added to obtain a final molar ratio of 1:1 heme:peptide and the spectrum recorded. Subsequent addition of peptides was continued to achieve a molar ratio of 1:4 (heme:peptide), and the spectrum was again recorded. The α and β bands (**inset** in each panel) are those of 4.8 μM heme at a 1:1 molar ratio of heme to peptide. Peptide used: (**a**) Cys^{264}-Pro^{265}; (**b**) Cys^{281}-Pro^{282}; (**c**) "HO signature."

of the Soret absorption and change in extinction coefficient from 37 mM^{-1} cm^{-1} to 42 mM^{-1} cm^{-1}. Major shifts to lower wavelength of the α and β bands were also seen with both peptides (insets in Fig. 1a and b). The spectral shifts observed were not caused by a change in pH of the assay systems by the peptides. The interaction of the peptides with heme was specific because addition of the 12-residue "HO signature" peptide that corresponds to half of the heme catalytic domain to heme did not affect the position or the shape of the heme Soret, α, or β bands and only caused some decrease in absorption (Fig. 1c). The quenching may involve interaction of the porphyrin ring with side chains of amino acid residues of the peptide, although slight changes in the concentration of heme in the assay system cannot be ruled out.

The involvement of the chelated iron of heme in the interaction of metalloporphyrins with HRM-containing peptides was indicated by the absence of a shift in the absorption spectrum when either zinc replaced iron or when the protoporphyrin nucleus alone was used (Fig. 2). In these experiments, a 1:4 molar ratio of the porphyrin complex to Cys^{264}-Pro^{265} was used. The findings strongly suggest that the chelated iron of heme is involved in interaction with HO-2 HRM peptides.

The formation of heme iron cysteine coordinate was further substantiated by competition binding with another heme ligand, cyanide (CN). Heme iron reacts with π-bonding heme ligands, such as cyanide, histidine, and imidazole, which results in a shift to higher wavelengths (red shift) of the Soret absorption spectrum (2, 22, 23, 27). Moreover, the interaction of side chains of amino acids with the porphyrin ring itself, as well as with the bound iron, can be affected by a change in pH (19, 23). Thus, we assessed the effects of pH on HRM peptide–heme affinity; these analyses were carried out at pH 7.5 and 9.0. This experiment also allowed us to assess the relative affinity of HRMs for heme. The results are shown in Fig. 3.

At pH 7.5, incremental additions of KCN to the heme:peptide solution split the single absorption band into two bands at 369 and 422 nm. As the KCN concentration increased, the 422 nm peak increased. Additions were con-

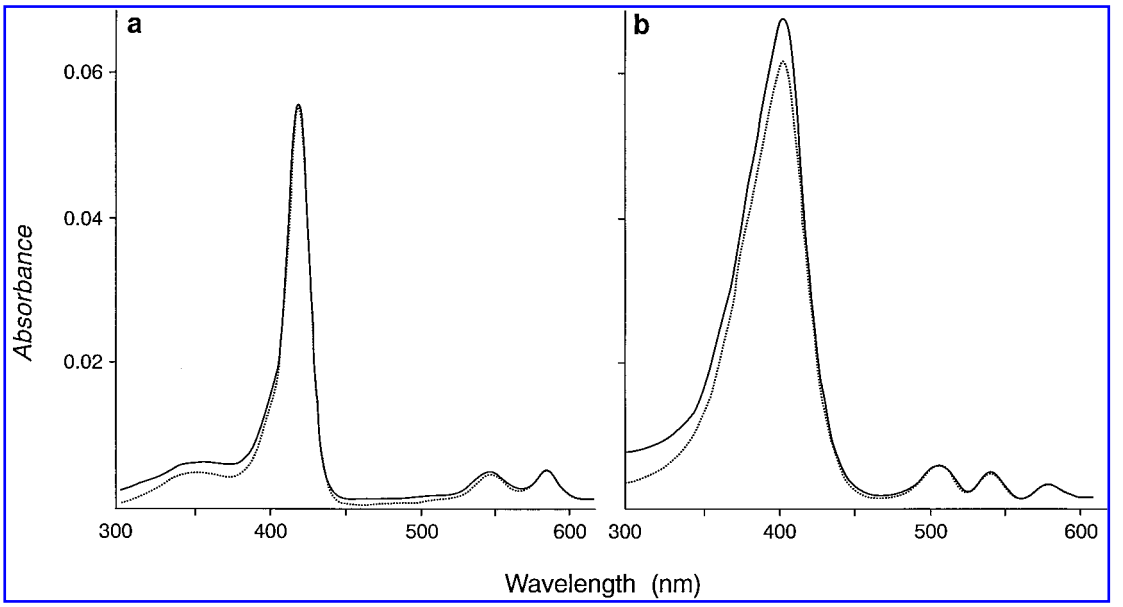

FIG. 2. Zn-PP and PP IX do not interact with HO-2 HRMs. The difference absorption spectra of Zn-PP or PP IX (1.2 μM) were obtained for the porphyrin complexes alone and subsequently after the addition of Cys^{264}-Pro^{265} HRM peptide at a fourfold molar excess. (**a**) Zn-PP; (**b**) PP IX. Solid line = in the absence of the peptide; dotted line = in the presence of the peptide.

tinued until absorption was not affected. As indicated by the apparent isosbestic point, there is a direct conversion of one species, *i.e.*, heme iron-cysteine, to another, *i.e.*, heme iron-CN, with both HRM peptides. The spectrum of heme-CN at the completion of titration is that of dicyanoheme. The maximum absorption to the higher wavelength was identical to that obtained when KCN was added to heme in the absence of peptide, and was obtained at a remarkably great excess of KCN: 25 m*M* KCN for Cys^{264}-Pro^{265} containing peptide and 15 m*M* KCN for Cys^{281}-Pro^{282} HRM peptide. This amounted to a 6,000-fold excess of KCN to Cys^{264}-Pro^{265} peptide, and a nearly 4,000-fold excess to Cys^{281}-Pro^{282} peptide, for KCN to displace HO-2 HRMs.

Difference in affinity for heme of the two HRM peptides persisted at pH 9.0. At this pH value, although both peptides were displaced from heme more readily by CN, molar ratios of 1:1,250 and 1:125 of Cys^{264}-Pro^{265} and Cys^{281}-Pro^{282} HRM peptides, respectively, were required. It is noteworthy that in all cases the final absorption peak is observed at the wavelength of peptide:heme, *i.e.*, 369 nm, or heme:CN alone, *i.e.*, 422 nm, and not at intermediate wavelengths. In addition, the isosbestic points at pH 7.5 and 9.0 are the same at all KCN concentrations. These observations are consistent with the direct conversion of one species into another with no intermediate form. Essentially identical results were obtained at each ratio of heme:peptide:KCN when the order of addition of KCN and peptide was reversed, consistent with a competition between CN and peptides for heme binding. As a quantitative measure of the relative affinity of the peptides and CN for heme, the equilibrium constants for the KCN-mediated dissociation of the heme–peptide complexes at pH 7.5 and 9.0 were measured and are shown in Table 1.

Next, the role of the conserved cysteine and proline residues of the HRMs separately in heme binding was investigated. This study used peptides in which these residues were individually replaced by alanine. Figure 4a shows the results obtained with $Cys^{264} \rightarrow$ Ala peptide. A Soret band shift was not observed following addition of up to 32-fold molar excess of this peptide to heme. Identical results were obtained using $Cys^{281} \rightarrow$ Ala peptide (data not shown). The findings suggest that cysteine thiol forms an axial ligand of the heme iron and that the replacement of the cysteine disrupts heme–peptide interaction. A modest

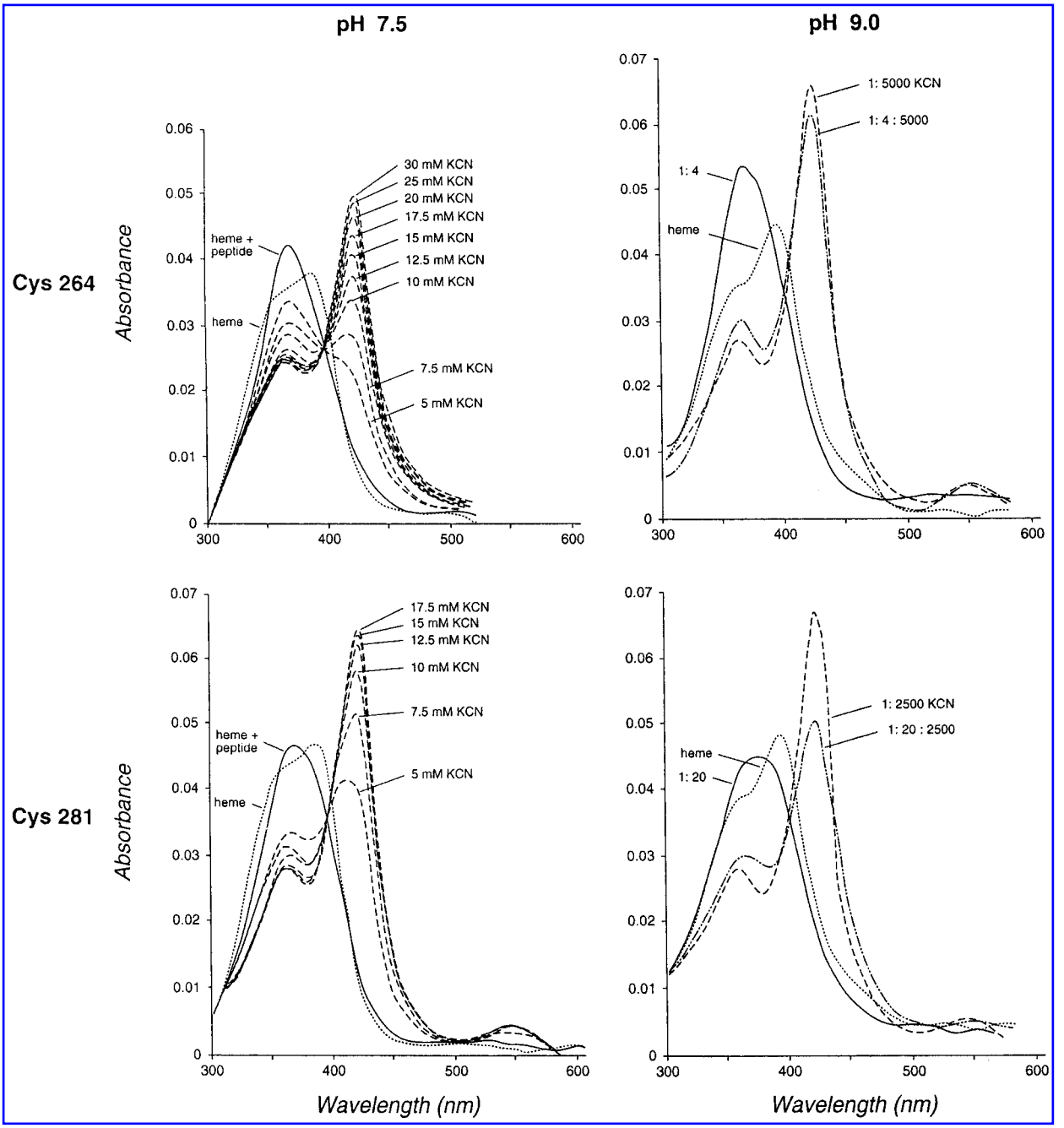

FIG. 3. Displacement of peptide bound to heme by cyanide. The difference absorption spectrum for a 1.2 μM heme solution was determined as described in the legend to Fig. 1 at pH 7.5 or 9.0. A 10-residue peptide corresponding to Cys^{264}-Pro^{265} or Cys^{281}-Pro^{282} containing HRM was then added in a stepwise manner to obtain a complete shift of the Soret band to the lower wavelength. Incremental additions of KCN solution were then made, and the absorption spectrum was determined following each addition until the complete red shift of the Soret band to 422 nm was reached. When KCN was added to heme in the absence of peptide, a 422-nm absorption peak was observed. The absorption spectrum for heme alone is shown in each panel. Shown for each peptide at pH 9.0 are the spectra obtained at the ratio of heme:peptide required for a complete blue shift and at the ratio of heme:peptide:KCN necessary to obtain the maximum red shift. The spectra for heme alone and for heme plus cyanide are shown in each panel for reference. **Upper panels** used the Cys^{264}-Pro^{265} containing HRM peptide, and **lower panels** used the Cys^{281}-Pro^{282} containing HRM peptide.

TABLE 1. EQUILIBRIUM CONSTANTS FOR THE KCN-MEDIATED DISSOCIATION OF HEME–PEPTIDE COMPLEXES

	K (Heme-peptide Heme-CN)	
Peptide	*pH 7.5*	*pH 9.0*
Cys^{264}-Pro^{265} HRM	6.6×10^{-11}	1.8×10^{-9}
Cys^{281}-Pro^{282} HRM	7.9×10^{-11}	4.7×10^{-8}

The equilibrium constant values for the shift from heme-peptide to heme-CN were obtained at pH 7.5 and 9.0 as described by Hogness *et al.* (19) and detailed in Materials and Methods.

decrease in intensity of absorption was observed.

The proline residue of the HRM apparently facilitates cysteine thiol interaction of the porphyrin nucleus and the peptide, but is not indispensable for coordination with heme iron. Specifically, when using $Pro^{265} \rightarrow$ Ala HRM peptide at 1:1 or 1:4 molar ratio of heme to peptide, a shift in the Soret absorption band was not observed. But when the concentration of the peptide was increased to 1:32 (Fig. 4b), a clear shift to the lower wavelength of the absorption band was observed. To examine the contribution of cysteine alone to the shift in the absorption spectrum of heme, the amino acid was added to a heme solution at pH 7.5, and at 32-fold molar excess, the amino acid alone had no effect on the spectrum (data not shown).

To narrow down the effect of flanking residues, additional binding assays were carried out using five-residue peptides containing the invariant Cys-Pro dipeptide (Arg-Lys-Cys-Pro-Phe and Ser-Asn-Cys-Pro-Phe). These peptides also caused shift of the heme spectrum to the lower wavelength (data not shown). A five-residue peptide in which methionine was substituted for the cysteine residue of the Cys^{264} containing HRM (Arg-Lys-Met-Pro-Phe) failed to cause a shift in the Soret band consistent with the suggestion that the binding is specific for the cysteine.

CD spectroscopy was used to examine whether residues flanking the core interact with heme. Based on results shown in Fig. 5, the HO-2 peptide structure is responsive to heme. The CD scans were carried out with a fourfold molar excess of peptide to heme. Due to the relatively high contribution of heme alone at higher wavelengths (59), only the 200–250-nm range data are presented. Values for peptide plus heme have been corrected for readings obtained for heme alone at the same concentration, and those for peptide alone have been corrected for buffer. Therefore, changes in CD bands are solely due to changes in the structure of peptides in the presence of heme. In-

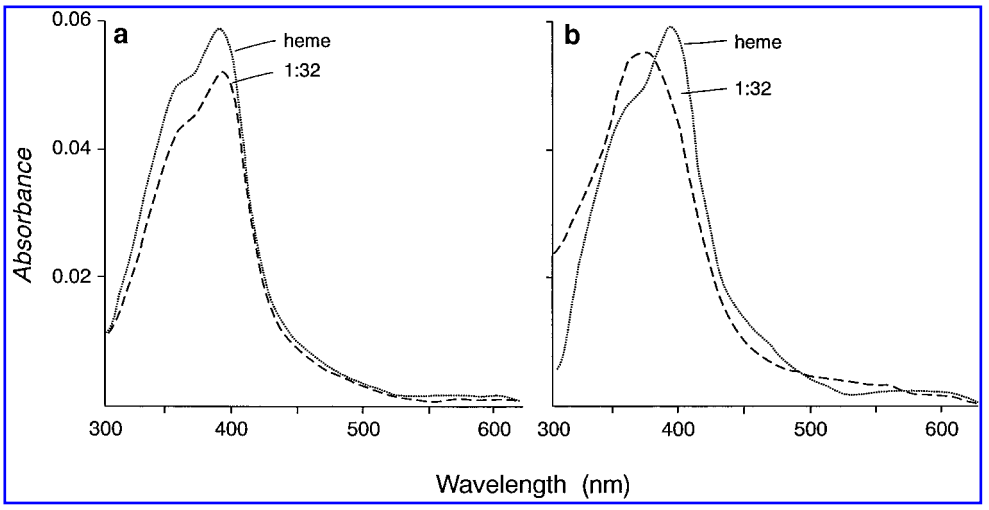

FIG. 4. Role of cysteine and proline residues in the Soret band shift by HRM containing peptides. Spectral analyses were carried out as described in the legend to Fig. 1. HO-2 HRM peptides in which alanine was substituted for cysteine or for proline of the Cys^{264}-Pro^{265} were used. Stepwise addition of the peptide was continued up to a 1:32 molar ratio of heme:peptide. The final spectra are shown. HRM peptide used: (**a**) $Cys^{264} \rightarrow$ Ala substituted; (**b**) $Pro^{265} \rightarrow$ Ala substituted.

teraction with heme causes a significant alteration in the secondary structure of the Cys^{264}-Pro^{265} HRM peptide as suggested by the appearance of a positive peak of molecular ellipticity in the CD spectrum of the peptide at ~228 nm and by spectral changes between 200 and 205 nm range (Fig. 5a, dashed line vs. solid line). The CD spectrum of Cys^{281}-Pro^{282} HRM peptide also was modified in response to the presence of heme (Fig. 5b); the change was most notable at ~210 nm. The CD spectrum changes, however, were less dramatic than those observed with Cys^{264}-Pro^{265} HRM peptide. The observation is consistent with the lower affinity of Cys^{281}-Pro^{282} HRM peptide for heme compared with Cys^{264}-Pro^{265} HRM peptide.

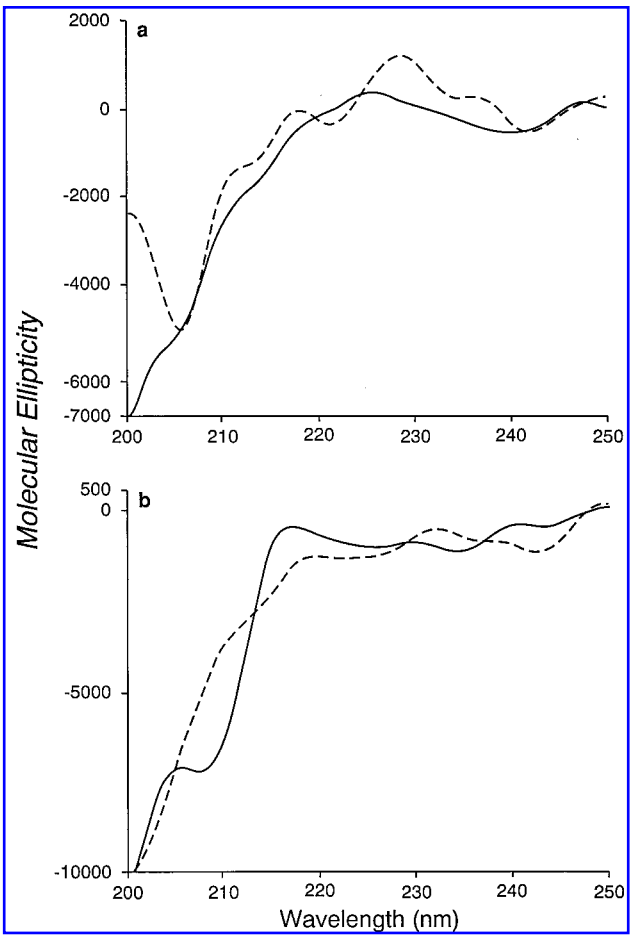

FIG. 5. CD spectra of HO-2 HRM containing peptides in the presence or absence of heme. CD spectra of peptides (40 μM) in 25 mM sodium phosphate buffer were determined before and after addition of heme (10 μM) according to Zentz *et al.* (59) as described in Materials and Methods. The molar ratio of heme to peptide was 1:4. Solid line = in the absence of heme; dashed line = in the presence of heme. HRM peptide used: (**a**) Cys^{264}-Pro^{265} containing; (**b**) Cys^{281}-Pro^{282} containing.

DISCUSSION

Recent reports have suggested that, in addition to catalyzing the heme molecule and generating bilirubin and CO, the HO enzymes may have other functions in the cell (30). This study has further examined, using spectral analysis, the potential additional functions of HO-2 in the cell by comparing and characterizing interactions of the two HO-2 HRM sequences with porphyrin complexes. Heme and porphyrin compounds have a distinct absorption spectrum characterized by the Soret region absorption (27). The position and intensity of the Soret band are dependent on the electronic field of the fifth and sixth axial ligands of heme iron. These criteria have been used effectively for examining heme interaction with amino acids and various ligands (22, 23, 27). In addition, the porphyrin ring binds to side chains of valine, phenylalanine, tyrosine, glycine, lysine, and arginine (15, 16, 23). Also, terminal amine is a candidate for interaction, therefore, the environment of the heme binding domain can make a large contribution to the affinity of peptides and proteins for heme.

The present study has shown that the two HO-2 HRMs differ in their affinity for heme, and that the HRMs bind Fe-PP, but not Zn-PP or protoporphyrin. In addition, the findings of this study reveal the significance of residues flanking the core Cys-Pro dipeptide to the interaction of a peptide with heme. This suggestion is supported by the higher affinity of Cys^{264}-Pro^{265} containing HO-2 HRM peptide for heme than that of Cys^{281}-Pro^{282} containing peptide. In the high-affinity peptide, a pair of positively charged residues, arginine and a lysine, are upstream of cysteine, whereas in Cys^{281}-Pro^{282} HRM peptide two uncharged amino acids, asparagine and serine, are in this position. It is likely that the positively charged arginine and lysine interact with negatively charged propionate carboxylic groups of heme and strengthen the electrostatic field of interaction (16, 23). It follows that the contribution of flanking amino acids to heme binding of

HRMs most likely accounts for differences between the present study and a previous report (60) in the concentration of the heme ligands that is required to displace an HRM peptide from heme. Specifically, whereas up to a 6,000-fold excess of KCN is required for the displacement of HO-2 peptide (Fig. 3), only a 25-fold excess of imidazole is necessary to displace the DNA binding domain of HAP1 from heme (60). In HO-2 HRMs downstream of proline are two hydrophobic residues, phenylalanine and tyrosine, or a hydrophobic and a positively charged residue, phenylalanine and arginine, whereas in HAP1, valine and the negatively charged aspartic acid are in this position. HAP1 is a transcriptional activator of genes that encodes cytochromes in response to oxygen/heme (7).

Noteworthy is the absence of a shift in the Soret band with the 12-residue fragment of the catalytic domain (Fig. 1). This domain consists of 24 residues that form a hydrophobic heme binding pocket (33, 45). This finding suggests specificity of heme–HRM peptide interaction. It also suggests that the conserved heme catalytic domain in its entirety is required for heme interaction. The possibility cannot be ruled out that the fragment has a significantly lower K_d for heme than the HRM peptides. As noted earlier, His^{151} is essential for HO-2 activity (33).

Based on experiments with peptides in which cysteine is replaced by an alanine or methionine, it is most likely that the cysteine residue of the HRMs is an axial ligand for heme iron. It is noteworthy that when methionine is substituted for cysteine in HRMs, interaction of peptides with heme is hindered. Interestingly, methionine is the heme ligand in bacterial and eukaryotic cytochrome *c* types (18). In animals, including the mammals, cytochrome P450 heme binds to cysteine residue (4, 56), and in globins a histidine is bound to one side of chelated iron (27). Clearly, proline plays a significant role in HRM interaction with heme. The role of proline not only involves electrostatics, but also its conformational rigidity, which even in the small peptides appears to be of importance. It is notable that in plant P450 CYP73AI (48) a proline C-terminal to the heme binding cysteine has been shown to be essential for the correct insertion of heme in apoprotein.

The present study has indicated that cysteine is the fifth axial ligand of heme; however, the study does not provide direct evidence for identity of the sixth axial ligand. Based on the wavelength shift, as a function of peptide concentration, it may be reasonable to speculate on the identity of the sixth ligand. As was shown in Fig. 1, shift to a lower wavelength of absorption at the Soret region continued until the concentration of the peptide to heme reached a 4:1 ratio. This indicates that the sixth axial ligand for heme at the lower ratio of peptide to heme (1:1) is different from that at the higher ratio. This phenomenon is described for the coordination of cyanide with heme; mono- or dicyano complexes are formed depending on cyanide concentration (22, 27). In HO-2 HRMs, possibly at low peptide concentrations, a solvent water molecule would form the sixth ligand of heme; and, at high peptide concentrations, the peptides would occupy this position.

Aside from their differences in primary structure, HO-1 and HO-2 proteins differ significantly in cellular distribution and, likely, function. Whereas HO-1 is the product of a stress-responsive gene (13, 14, 17, 20, 24, 26, 29, 31, 39, 40, 42, 47, 50, 54, 58), HO-2 is constitutively expressed in all cell types and is present at impressively high levels in tissues and cells that do not have a function in hemoglobin heme degradation, such as the brain (53). It would then be reasonable to propose that the additional function of HO-2 within the cell relates to its HRMs. We suggest that this isozyme of HO serves to sequester and bind all types of heme ligands in the cell, including the various gaseous ligands. This suggestion is based on findings of the present study and the previous findings that HO-2 is a constitutive hemoprotein (36) and interacts with heme ligands, such as NO (9). Heme bound to proteins is not only an effective target for NO (3), but also a target for other gaseous ligands, including O_2 and CO. Such interactions would potentially have significant consequences for the cell, such as the activation of stress protein genes, including HO-1 and NO synthase, and modulation of the biological activities of CO and NO signaling pathways. Therefore, the findings of the pres-

ent study would be of relevance to understanding gaseous ligand–heme–HO-2 interaction, albeit that the isolated 10-residue peptides would not be expected to fold in the same configuration as when present in the intact HO-2 protein.

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ABBREVIATIONS

aa, amino acid; CD, circular dichroism; Fe-PP, Fe-protoporphyrin IX; HO, heme oxygenase; HRM, heme regulatory motif; NO, nitric oxide; PP IX, protoporphyrin IX; Zn-PP, Zn-protoporphyrin IX.

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