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Focussed review

Binding of cysteine thiolate to the Fe(III) heme complex is critical for the function of heme sensor proteins

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ABSTRACT

Heme sensor proteins function in response to the availability of the heme iron complex. The heme iron complex *per se* becomes the first signal for various important physiological functions of these proteins. The role of the heme iron complex in heme sensors is distinct from those of prototype heme proteins, such as hemoglobin, cytochromes *c* and P450, in which the heme iron complex is the functional center. For heme sensor proteins, association/dissociation of the heme iron complex regulates physiological processes, including catalysis, transcription, and other functions essential for cell survival. Importantly, the main binding/sensing site of the heme iron complex in heme sensor proteins is cysteine thiolate, which is critical for the heme sensing function. The role of the cysteine thiolate in heme sensor proteins differs from that of the P450 system, in which the cysteine thiolate donates an electron to activate molecular oxygen bound to the heme iron complex *trans* to cysteine thiolate to facilitate the monooxygenase reaction. In this review, we discuss heme proteins with cysteine thiolate as the heme axial ligand, and summarize recent studies on heme sensor proteins and their molecular mechanisms. In particular, we focus on the controversial role of the heme iron complex in transcriptional regulation associated with circadian rhythms.

1. Introduction

1.1. Heme proteins with the cysteine thiolate–Fe(III) heme complex

Cytochrome P450, P450 and nitric oxide synthase, NOS, enzymes contain the cysteine thiolate–Fe(III) heme complex at the active site. These enzymes require the cysteine thiolate-coordinated Fe(II) heme complex to activate bound molecular oxygen *trans* to cysteine thiolate. The cysteine thiolate is supported by hydrogen bond networks on the heme proximal side in the proteins, resulting in tight coordination to the Fe(II) heme complex (with Soret absorption of the Fe(II)–CO complex at around 450 nm), although external stimuli, such as pH variations, easily disrupt this bond, generating the denatured P420 form (with Soret absorption of the Fe(II)–CO complex at around 420 nm). However, in general, cysteine thiolate displays weak coordination to the Fe(III) heme complex. Additionally, the Fe(II) heme–thiolate bond is considerably weaker (does not coordinate or dissociates more rapidly) than the Fe(III) heme–thiolate bond if the cysteine thiolate is not supported by proximal amino acids on the protein surface or other factors, as shown for cytochrome P450, P450, and nitric oxide synthase, NOS, enzymes [1,2].

Table 1 summarizes heme proteins with the cysteine thiolate-coordinated Fe(III) heme complex. The Fe(II) heme complexes of heme–thiolate peroxidases, such as chloroperoxidase, CPO, and aromatic peroxidase, APO, also contain cysteine thiolate as the axial ligand, suggesting that the Fe(II)-thiolate complex is necessary for these enzyme to generate the oxo-ferryl radical (Compound I) and/or oxo-Fe(V) complexes to express peroxidase functions, similar to P450 and NOS enzymes [3]. SoxAX cytochromes, are a novel type of heme-copper protein, with SoxAX-mediated S–S bond formation involving both the copper and heme centers [4]. Alternatively, additional heme proteins listed in Table 1 contain amino acids other than cysteine thiolate as the axial ligand for the Fe(II) heme and Fe(II)–CO complexes. In these cases, cysteine coordination to the Fe(III) heme complex as the axial ligand is altered to another amino acid in the Fe(II) heme complex. Therefore, the cysteine residue appears to play a critical role in exerting heme redox-dependent coordination switches and/or concomitant protein structural changes in the heme-binding domain critical for protein function [1,2]. In cystathionine β-synthase, CBS, binding of CO to

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Table 1
Proteins with the thiolate heme complex that are not heme sensors. The heme iron complex is firmly bound to the protein with the aid of the coordination bond via cysteine thiolate. The thiolate heme complex is the catalytic active center or gas binding site for gas sensor or NO transfer proteins [1,2].

Proteins	Axial ligands			Absorption	Functions
	Fe(III)	Fe(II)	Fe(II)CO	Fe(II)CO	
P450	Cys/	Cys/	Cys/CO	450 nm	Monooxygenation
NOS	Cys/	Cys/	Cys/CO	450 nm	Monooxygenation
CPO	Cys/Glu	Cys/	Cys/CO	443 nm	Halogenation, Peroxygenation
APO (AaP)[a]	Cys/Glu	Cys/	Cys/CO	445 nm	Peroxygenation
SoxAX[b]	Cys/His				Thiosulfate oxidation
CBS	Cys/His	Cys/His	His/CO	420 nm	Cystathione synthesis
CooA	Cys/Pro	His/Pro	His/CO	420 nm	Transcription
Nitrophorin	Cys/				NO transfer
*Bx*RcoM-2	Cys/His	Met/His	His/CO	420 nm	Transcription

[a], ref. [3]; [b], ref. [4].

the Fe(II) heme complex leads to dissociation of cysteine, although the function of heme in CBS remains unclear.

2. Critical role of cysteine thiolate binding to the Fe(III) heme complex in heme sensing

As specified earlier, association/dissociation of the heme iron complex, mostly Fe(III) heme, to and from the heme sensor protein switches on/off functions, such as catalysis and transcription. Interestingly, the cysteine residue is the heme sensing/binding site for most heme sensor proteins. Here, we have provided a summary on several heme sensor proteins with relatively well characterized structural and spectroscopic data [5–11]. Publications from 2008 onwards reviewing the heme sensing mechanism have been cited. Refs. [1] and [2] provide a comprehensive review of heme sensor as well as heme-based gas sensor proteins.

The following characteristics are important in the molecular mechanism of heme sensing:

2.1. Fast heme dissociation rate constants

Since association/dissociation of the heme iron complex to/from the heme sensor protein is critical for heme sensing, weak binding of the complex to the protein is necessary. It appears unlikely that the heme iron complex coordinates to or covalently binds heme sensor proteins in a manner comparable to prototype heme proteins, such as hemoglobin, cytochrome *c* and P450. Experimental evidence on weak binding of the heme iron complex to heme sensor proteins is provided by the rapid dissociation rate constants of the Fe(III) heme complex from these proteins. Specifically, the dissociation rate constants of the Fe(III) heme complex from heme sensor proteins have been evaluated as 10^7 fold higher than those of the prototype heme proteins, hemoglobin and myoglobin [1,2,6]. Histidine imidazole is the heme binding site for hemoglobin and myoglobin, while cysteine thiolate appears to be the optimal heme sensing site due to weak binding of the Fe(III) heme complex to heme sensor proteins. The electron donating character of the cysteine thiolate group, which is used to activate the molecular oxygen for P450 and NOS, is appropriate for heme sensing, even if the iron metal cation in the Fe(III) heme complex is electrically positive. Furthermore, the contribution of protoporphyrin IX in heme sensing of the Fe(III) heme complex via promotion of hydrophobic interactions between heme and protein cannot be ruled out. Structural characteristics of heme sensor proteins, such as flexibility, are also different from those of hemoglobin and myoglobin, as discussed below.

2.2. Heme redox-dependent ligand switching

In general, binding of the electron-rich cysteine thiolate to the Fe(II) heme complex as the axial ligand is difficult, since the Fe(II) heme complex has more electrons than the Fe(III) heme complex, leading to stronger electrostatic repulsion. Hydrogen networks on the heme proximal side or other factor(s) to facilitate anchoring of the complex to the protein surface are required to support binding of the Fe(II) heme complex to the protein, as observed for P450 and NOS. The heme sensor protein does not supply structural and/or ionic and/or hydrophobic characteristics on the surface to support binding to the Fe(II) heme complex. Therefore, redox-dependent ligand switching may easily occur in that when the Fe(III) heme complex is reduced to Fe(II) heme, cysteine thiolate dissociates, and is replaced with another residue, such as histidine imidazolate, as the axial ligand (Fig. 1). Heme-redox dependent ligand switching would induce significant alterations in the heme sensor protein structure, leading to change/initialization/termination of function. Accordingly, normoxia/oxidative conditions and/or hypoxia/reductive conditions influencing the bias of the heme redox state or equilibrium between Fe(III) and Fe(II) heme complexes may regulate the functions of heme sensor proteins to a significant extent [1,2].

2.3. Thiol-disulfide redox dependence of heme sensing (binding)

Recent studies have suggested an attractive proposal to clarify the critical role of cysteine thiolate in the heme sensing mechanism. The thiol disulfide redox dependence of heme binding and heme ligand switching that were first proposed and experimentally proved by

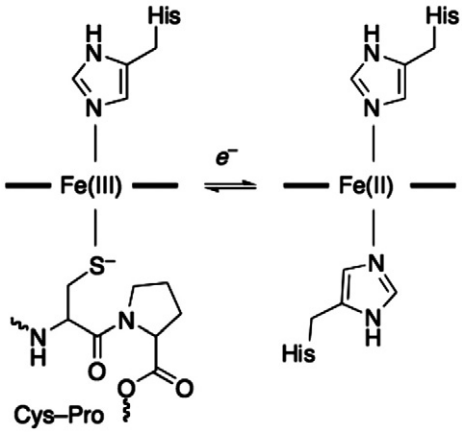

Fig. 1. Heme redox-dependent axial ligand switching from cysteine thiolate in the Fe(III) heme complex to histidine imidazolate (or another residue) in the Fe(II) heme complex for heme sensor proteins [1,2]. Since the Fe(II) heme complex is not supported by hydrogen networks on the proximal side, cysteine thiolate of the axial ligand tends to dissociate and is replaced with histidine imidazole. The CP (Cys-Pro) motif is important for the binding of cysteine thiolate in some cases (Table 2), since the proline residue adjacent to cysteine assists in cysteine thiolate coordination to the Fe(III) heme complex. CO binds to the Fe(II) heme from either site, producing a Soret absorption peak at 420 nm as the CO–Fe(II)–imidazole complex. Adopted from Ref. [2].

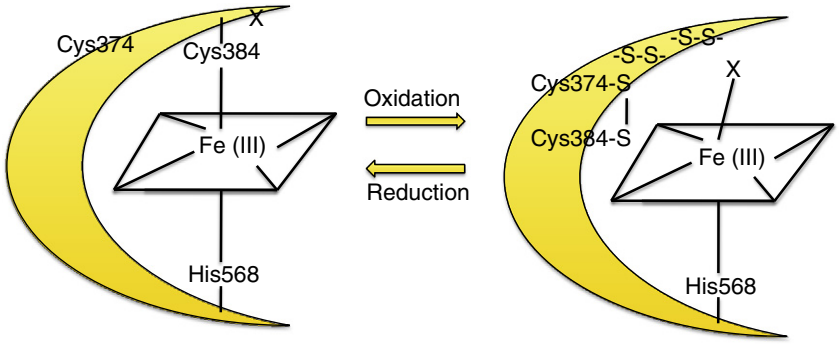

Fig. 2. Thiol-disulfide redox-dependent formation of the heme-cysteine thiolate complex. Thiol-disulfide redox switch of the protein surface regulates binding of cysteine thiolate to the Fe(III) heme complex of the nuclear hormone receptor, Rev-erbβ. Protein redox-dependent heme binding regulation is another critical factor that determines cysteine binding to the heme iron complex in addition to heme redox-dependent heme binding regulation (Fig. 1). Adopted from Ref. [17].

the Ragsdale group may regulate the heme sensing functions of arginyl-transferase, the N-end rule pathway [12], heme oxygenase-2 [13–15], human Slo BK channel [16], a nuclear receptor, Rev-erbβ [17] (Fig. 2). Under oxidative (or normoxic) conditions, cysteine thiolate tends to form disulfide bonds with another cysteine thiolate within the same protein (or in the other subunit), thus hampering cysteine thiolate–Fe(III) heme coordination. In contrast, under reductive (or hypoxic) conditions, free cysteine thiolate is generated, which coordinates with the Fe(III) heme complex more easily. These redox-dependent differences in the thiolate bond status on the protein surface may therefore be important for the regulation of heme binding (or sensing) of these heme sensor proteins.

2.4. CP (Cys-Pro) motif and HRM (heme regulatory motif)

The CP motif, present in the heme binding/sensing sites of many heme sensor proteins, is proposed to be important for function (Fig. 1) [1,2,13–15]. In particular, the proline residue adjacent to cysteine appears critical for heme sensing. Mutation of this residue led to a significant decrease in the heme binding capability and consequently to a decline in heme sensing capability, as demonstrated for heme-regulated inhibitor, HRI [6], an RNA-binding protein, DGCR8 [8] and an iron-responsive regulator, *Rj*Irr [9]. Thus, Pro within the CP motif plays an important role in supporting the coordination of cysteine thiolate to the Fe(III) heme complex in heme sensor proteins. Some heme sensor proteins contain multiple CP motifs within the protein. However, it is not clear whether these proteins use one or more CP motifs for function, which may be dependent on the stress conditions. Moreover, some heme sensor proteins contain the cysteine residue, but not a CP motif, suggesting that this sequence is not absolutely essential for heme sensing. Cys/His coordination for the Fe(III) heme complex with DGCR8 has also been proposed [18].

2.5. Protein flexibility/plasticity and global structural changes of heme sensors

Since heme association/dissociation regulates the functions of heme sensor proteins and heme affinity to these proteins is weak, high protein flexibility to accommodate heme binding is assumed. Limited studies have focused on the flexibility of heme sensor proteins. However, structural studies on the heme sensor protein, mouse HRI, strongly suggest the requirement of the global protein structure for the heme sensing function, in view of the findings that the overall conformation appears different before/after heme binding to the protein and significant structural changes are induced by heme [6] (Fig. 3). It is suggested that one of the heme axial ligands (His119/His120) is located at the N-terminus, whereas another (Cys409) is positioned at the C-terminus. To demonstrate global intramolecular protein-protein interactions between the N- and C-termini in the heme iron complex-bound protein, isolated N-terminal C-terminal domains were generated. SDS-PAGE experiments disclosed that the isolated N-terminal and C-terminal domains interacted with each other only in the presence of the Fe(III) heme complex. This finding is consistent with the proposal that the two axial ligands (or sensing sites) of the Fe(III) heme complex in full-length HRI proteins are scattered, specifically, His119/His120 in the N-terminal domain and Cys409 in the C-terminal domain. However, to our knowledge, this is the only report to demonstrate that flexibility or global movement of heme sensor proteins is critical for the heme sensing function. Global structural changes caused by heme binding for a heme transfer protein, HemS, will be discussed later in Section 5.

Site-directed mutagenesis of the Cys residue of the heme binding (or sensing) site of heme sensor proteins may not lead to unequivocal spectral changes [7,8]. Flexibility of the heme sensor protein and/or redox-dependent equilibrium of bond formation/breakage between free thiol and disulfide may contribute significantly to the ambiguous results obtained from site-directed mutagenesis experiments. Thus, further studies are needed to validate the proposed critical role of protein flexibility and/or global structural changes in the heme sensing function.

2.6. Roles of heme chaperones or heme carrier proteins

Hemin (the Fe(III) heme complex) is toxic, since it can generate reactive oxygen species, such as the superoxide or hydroxyl radical, under all circumstances. Therefore, free hemin does not appear to exist in cells, but may be harbored in proteins within cells. Heme chaperone or heme carrier proteins may facilitate the interactions of heme with the apo form of heme-bound proteins [19].

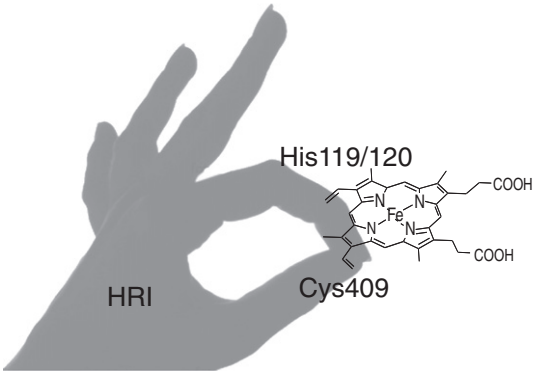

Fig. 3. Flexibility of the heme sensor protein. Imagine that the HRI protein acts as a hand in motion and uses the index finger and thumb to pick up the heme. Adopted from Ref. [2].

3. Involvement of heme in circadian rhythms

This section summarizes the publications on heme binding to the transcriptional regulatory factors associated with circadian rhythms from both biological and chemical viewpoints, and introduces a report asserting that heme regulation of the transcriptional factors associated with circadian rhythms is biologically irrelevant.

3.1. Cell biology/molecular biology studies

Accumulating molecular biological and biochemical evidence on heme involvement in transcriptional regulation associated with circadian rhythms suggests that heme binding to transcriptional regulatory factors and/or heme transfer between regulatory factors control many important transcriptional functions associated with circadian rhythms [1,2,20–28]. For example, it is suggested that neuronal PAS protein 2, NPAS2, is a heme binding protein and binding of CO to the Fe(II) heme bound to NPAS2 inhibits its transcriptional regulatory function [20]. Kinase reaction and protein degradation of transcriptional regulatory factor proteins associated with circadian rhythms are additionally significantly regulated by heme iron binding to human Period 2, Per2 [23].

3.2. Biochemical studies

The heme binding characteristics of purified transcriptional regulatory factors associated with circadian rhythms have been analyzed. Transcriptional regulatory factors, such as NPAS2, Period 1, Per1, Per2 and Period 3, Per3, are composed of PAS-A and PAS-B domains at the N-terminal site and a longer domain the C-terminal site, except for NPAS2, which contains the basic loop-helix-loop (b-LHL) subdomain as a direct DNA interaction site [29]. Fe(III) heme binding to the isolated N-terminal domain containing the b-LHL and PAS-A subdomains significantly facilitates DNA binding affinity (to the E-box), as observed with the quartz-crystal microbalance method [31]. DNA interactions with the isolated N-terminal domain bound to the Fe(III) heme complex clearly induce alterations in optical absorption and EPR spectra [2]. These biochemical and biophysical findings corroborate that DNA binding affinity, at least to the isolated N-terminal domain containing the b-LHL and PAS-A subdomains, is significantly enhanced by binding of the Fe(III) heme complex. The heme binding (or sensing) characteristics of the isolated PAS-A subdomain of these transcriptional factors have been examined. It is suggested that cysteine thiolate in the isolated PAS-A subdomain is the Fe(III) heme binding (or sensing) site [30,31]. The Fe(III) heme complex binds to the isolated PAS-B subdomains of NPAS2 and Per2, but the heme binding (or sensing) site appears to be His and not Cys, suggesting that the role of the heme of the PAS-B subdomain is distinct from that of the PAS-A subdomain [33,34]. The PAS-B subdomain is generally involved in homodimer formation of PAS proteins. The C-terminal domain of Per2 contains two CP motifs. Molecular biology and biochemical findings suggest that the CP motif (C841P) in the N-terminal region is the heme binding (or sensing) site of human Per2 [23]. However, a separate spectroscopic study on the isolated C-terminal domain of mouse Per2 has suggested that another CP motif (C956P) at the N-terminal region is the heme binding (or sensing) site [27], distinct from that of human Per2. These findings imply differences in the heme sensing mechanism of Per2 between species. Notably, mutations at His119 and His171 as the axial ligand(s) for the Fe(II) heme complex of NPAS2 resulted in significantly decreased transcriptional activity, suggesting that the Fe(II) heme complex regulates transcription in NPAS2 [35].

3.3. Heme binding to mouse Per2 is non-specific

Airola and co-workers suggested that heme binding to mouse Per2 is non-specific and that heme is not involved in all transcriptional regulation processes associated with circadian rhythms, based on results of a study on mouse Per2 and other proteins [36]. These conclusions were based on the following findings: (a) mouse Per2 is overexpressed as a heme-free and not heme-bound form in *E. coli*, (b) site-directed mutagenesis at His, but not Cys as the putative heme binding site does not alter the spectrum of mouse Per2, (c) the heme dissociation process in mouse Per2 is composed of two phases, implying multiple heme binding sites, (d) heme dissociation rate constants of other PAS proteins are lower than that of mouse Per2, (e) an electrochemical experiment suggests that heme binds non-specifically to the protein surface of mouse Per2, (f) the hydrophobic cavity inside the protein is absent in mouse Per2, based on the model constructed from protein structures of other proteins, since the crystal structure of the mouse protein is yet to be solved. In view of the collective findings, the authors expressed reservations about the proposal that heme binding to transcriptional regulatory factors associated with circadian rhythms is specific, and ultimately claimed that heme is physiologically invaluable in all transcriptional processes involving NPAS2 and Per2.

However, the following points are significant:

(a) The majority of heme sensor proteins have been overexpressed as heme-free forms in *E. coli*. Since the heme binding affinities to heme sensor proteins with cysteine as the binding (or sensing) site are low, the culture medium was supplemented with hemin (Fe(III) protoporphyrin IX complex), prior to overexpression of *Drosophila* hormone receptor 51, DHR51 [7], Rev-erbα [24,25], Rev-erbβ [11,26], *Drosophila* nuclear receptor, E75 [11], and human ion channel, BK channel [13] to obtain heme-bound proteins (listed in Table 2). Alternatively, hemin was added to *E. coli* homogenates before purification or purified proteins of HRI [6], NPAS2 [17,21,30,35], mouse Per2 [21–23,29,31], Rev-erbβ [14], human cytoplasmic arginyl-tRNA synthetase, hcArgRS [10], N-end rule pathway [12], and *Rj*Irr [9] to form heme-bound sensor proteins (Table 2). For overexpression in *E. coli*, heme sensor proteins are pretreated with hemin *per se*, in contrast to prototype heme proteins, such as myoglobin, P450 and cytochrome *c*, which are firmly

Table 2
Heme sensor proteins with cysteine thiolate as the heme sensing (or binding) site. The axial ligand for the Fe(II) heme complex is not the cysteine residue for these proteins, and the Fe(II) heme-CO complex displays an absorption peak at 420 nm. Note that the heme binding sites of the N-end rule pathway, heme oxygenase-2, and BK channel (the lowest three proteins in brackets) are histidine, but heme binding to the protein is regulated by a thiol/disulfide redox switch.

Proteins	Axial ligands for the Fe(III) heme complex			Ref.
HRI	Cys409/His119 (His120) (CP motif)			[6]
DHR51	Cys/His			[7]
DGCR8	Cys352 (CP motif)			[8]
*Rj*Irr	Cys29 (CP motif)			[9]
hcArgRS	Cys115			[10]
E75	Cys396/His574			[11]
Rev-erbα	Cys418/His602			[24,25]
Rev-erbβ	Cys384/His568	disulfide and free thiolate		[11,17,25,26]
NPAS2	Cys170/His119			[20,21,30,32,35]
Per2	Cys215, Cys841 (human) (CP motif), Cys956 (mouse) (CP motif)			[21–23,29,31]
[N-end rule pathway	His	Cys71/Cys72	disulfide and free thiolate	[12]]
[heme oxygenase-2	His	Cys265/Cys282	disulfide and free thiolate	[13–15]
[BK channel	His	Cys612/Cys615	disulfide and free thiolate	[16]

bound to heme and thus overexpressed as the heme-bound forms in the presence of ALA (a heme synthetic precursor) in the incubation medium or even in the absence of ALA [1,2]. Hemin supplementation for overexpression of heme sensor proteins is not surprising since relatively weak binding of heme is important to allow heme association/dissociation to act as a trigger for heme sensor proteins, as will be stated later in (d). Extensive *in vitro* biophysical and biochemical studies have been performed to characterize the heme binding properties and environmental structures of heme sensor proteins, and the involvement of heme in physiological functions has been unequivocally established. Note that the dissociation constant, K_d, for heme needs to be in the physiological range as has been pointed out by the Ragsdale group [13–17].

(b) The cysteine residue has been identified as the axial ligand of mouse Per2, based on the finding that chemical modification with mercury and iodoacetimide alters the optical absorption spectrum of the protein [31,36].

(c) The cysteine residue has been suggested as the axial ligand of mouse Per2 from the MCD spectrum of the Fe(III) heme complex-bound protein, which is similar to that of the histidine-bound form of P450cam [36].

(d) A hydrophobic cavity at the heme binding site is not necessary for the heme sensing function. Facile heme association/dissociation of heme at the protein surface is required for heme sensing as stated in (a) and thus, profound or global protein structural changes would occur concomitant with heme binding to facilitate the process [1,2,6].

It should be emphasized that there are proteins (such as Rev-erbα and Rev-erbβ) other than Per2 and NPAS that bind heme. The physiological data clearly show that heme is important in the circadian process, even if heme does not bind to Per2 and NPAS.

4. Heme transfer

The role of heme transfer between proteins in the regulation of important physiological functions has been documented [21,39,40]. Pathogenic bacteria possess efficient heme transfer/uptake/acquisition/trafficking systems for extraction of the heme iron complex from red cells of the host body, which is essential for their nutrition [39,40]. For this purpose, heme transfer processes are rapid (within 1 s) and well-coupled for pathogenic bacterial systems [41]. Histidine imidazolate and/or tyrosine phenolate and/or methionine, but not cysteine thiolate, may be the heme binding site for these well-coupled direct heme transfer systems. Moreover, global protein structural changes occur, accompanied by heme binding and flexibility/plasticity of bacterial heme trafficking proteins [40]. A molecular biological study has proposed that heme transfer from NPAS2 to mouse Per2 is critical for eukaryotic transcriptional regulation of circadian rhythms [21]. Moreover, heme transfer from NPAS2 to mouse Per2 is suggested to occur indirectly *in vitro* [31]. Specifically, differences in the equilibrium dissociation constants for the Fe(III) heme complex between the two proteins (NPAS2 and mouse Per2) may determine heme transfer from one protein to another. Heme transfer has also been observed between NPAS2 and mouse Per1 [42]. These indirect heme transfer processes observed *in vitro* for the transcriptional regulatory factors associated with circadian rhythms were slow and occurred in minutes, in contrast to the rapid direct heme transfer observed for pathogenic bacterial systems [40,41]. It is unlikely that toxic heme *per se* exists over a long time-period in cells, as specified in Sections 2–6. If the heme transfer reaction is slow and indirect, another protein, such as a heme chaperone or heme carrier, must mediate the heme transfer reaction. For example, it was suggested that heat shock protein 90, Hsp90, binds to heme-free induced NOS monomer and the complex interacts with a heme carrier protein [19]. Hsp90 would use its ATPase activity to help drive heme insertion into the heme binding site of the enzyme. Further intensive studies are required to address the molecular mechanism of the heme transfer process in the eukaryote system.

5. Concerns that need to be addressed

One of the important remaining issues in determining the specificity of heme binding for heme sensor proteins is that heme binding characteristics must be examined for the whole protein, and not the isolated putative heme binding domain. The characteristics of the isolated putative heme-binding domain may be distinct from those of the full-length protein, since heme binding (or sensing) sites may be distributed (or scattered) in both N- and C-terminal domains, as observed for HRI, resulting in the occurrence of global structural alterations for heme sensing (Fig. 3) [1,2,6]. For example, the Fe(III) heme complex binds to the cysteine residue of the isolated central domain of HRI, which is not observed for the whole HRI protein [37]. However, stepwise truncation mutation from the N-terminus of the HRI protein has facilitated the identification of the two axial ligands (His119/120 and Cys409) of heme. Specifically, truncation of the protein up to a certain point (position 145 from the N-terminus) led to a coordination change from the 6-coordinated (His and Cys) low-spin complex with the Soret band at 423 nm to the 5-coordinated (Cys) high-spin complex with the Soret band at 370 nm (Fig. 4) [38]. Note that global protein conformational changes in a heme transporter protein, HemS, accompanied by the heme binding to the protein, were shown [43]. However, it is generally difficult to overexpress large eukaryotic protein molecules in *E. coli* (even less than 50 kDa) without the formation of inclusion bodies or precipitates. Moreover, the study must be conducted under various conditions, taking special consideration of the presence and absence of oxygen. Importantly, the thiol/disulfide redox switch on the protein surface should contribute significantly to the heme sensing function, depending on the oxidative/reductive conditions (Fig. 2) [12–14]. Thus, heme binding behavior of heme sensor proteins under anaerobic conditions may be significantly different from that under aerobic conditions. Lastly, post-translational modification of the protein would also critically influence the heme sensing function. For example, phosphorylations at Thr and Tyr residues of HRI change the heme-mediated intramolecular interaction and the interaction between the heme and the protein [44].

6. Summary

In summary, the heme binding (or sensing) site of heme sensor protein is most likely to be the cysteine residue, and its role in heme sensing activity may be validated, even for transcriptional regulatory factors associated with circadian rhythms. However, further comprehensive studies are required to address unresolved issues, including heme binding specificity, protein structural flexibility/plasticity, thiol/disulfide

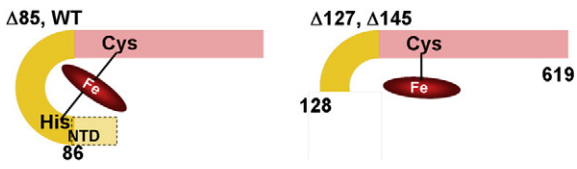

Fig. 4. Stepwise truncation from the N-terminus of the HRI protein is useful to identify axial ligands (or heme sensing sites) of HRI. The Fe(III) heme-bound forms of wild-type and Δ85 (deletion of amino acids 1–85) mutants produced Soret absorption spectra around 420 nm of a 6-coordinated low-spin complex with His and Cys as axial ligands. On the other hand, the Fe(III) heme-bound forms of Δ127 (deletion of amino acids 1–127) and Δ145 (amino acids 1–145 removed) mutants produced Soret absorption spectra around 370 nm of a 5-coordinated high-spin complex with Cys as the axial ligand. These results suggest that His is one of the axial ligands located between amino acids 86 and 127, whereas Cys, another axial ligand, is positioned between amino acids 128 and 619.

formation and post-translational protein modification for heme sensor proteins including transcriptional regulatory factors.

Abbreviations

ALA 5-aminolevulinic acid, δALA
APO (AaP) Fungal heme peroxidases, aromatic peroxidases of Agaricomycetes
bHLH A basic-loop-helix domain critical for DNA binding
BK channel An ion channel; the large-conductance calcium-dependent potassium channel
*Bx*RcoM-2 A heme binding protein from *Burkholderia xenovorans*; a regulator of CO metabolism and promoter of CO-dependent transcription
CBS Cystathionine β-synthase, a pyridoxal phosphate-dependent enzyme that catalyzes the condensation of homocysteine with serine to yield cystathionine and water. This enzyme also forms hydrogen sulfide and lanthionine or homolanthionine.
CooA A heme-regulated CO-sensing transcriptional factor from the photosynthetic bacterium *Rhodospirillum rubrum*
CP motif The Cys-Pro motif or heme-responsive motif critical in heme sensing
CPO Chloroperoxidase of *Caldariomyces fumago*
DGCR8 An RNA-binding protein (DiGeorge critical region-8) essential for processing of primary transcripts
DHR51 *Drosophila* hormone receptor 51; the protein has affinity for heme
E-box The "enhanced box" in DNA that contains specific nucleotide sequences important for binding of proteins regulating transcriptional patterns associated with circadian rhythms
E75 *Drosophila* nuclear receptor containing heme
hcArgRS human cytoplasmic arginyl-tRNA synthetase
Heme Iron protoporphyrin IX complex
HemS Heme transport proteobacteria protein
HRI Heme-regulate inhibitor, heme-regulated eukaryotic initiation factor 2α kinase, or eIF2α kinase
HRM Heme-responsive motif or CP motif critical in heme sensing
Hsp90 Heat shock protein 90
N-end rule pathway Conjugation of arginine, via the action of arginyl-transferase, to N-terminal aspartate, glutamate, or oxidized cysteine
Nitrophorin NO transporter protein from the blood sucking insect, *Cimex lectularius*
NOS Nitric oxide synthase
NPAS2 Neuronal PAS protein 2; a regulatory factor associated with control of circadian rhythm-specific transcription
Per2 Period 2; a regulatory factor associated with control of circadian rhythm-specific transcription
P450 Cytochrome P450
Rev-erbα A human homolog of E75 associated with control of the circadian clock, glucose homeostasis, and energy metabolism.
Rev-erbβ A synonym of Rev-erbα.
*Rj*Irr Iron-responsive regulator of heme biosynthesis in *Bradyrhizobium japonicum*
SoxAX a heme copper protein involved in bacterial energy generation from sulfur compounds

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