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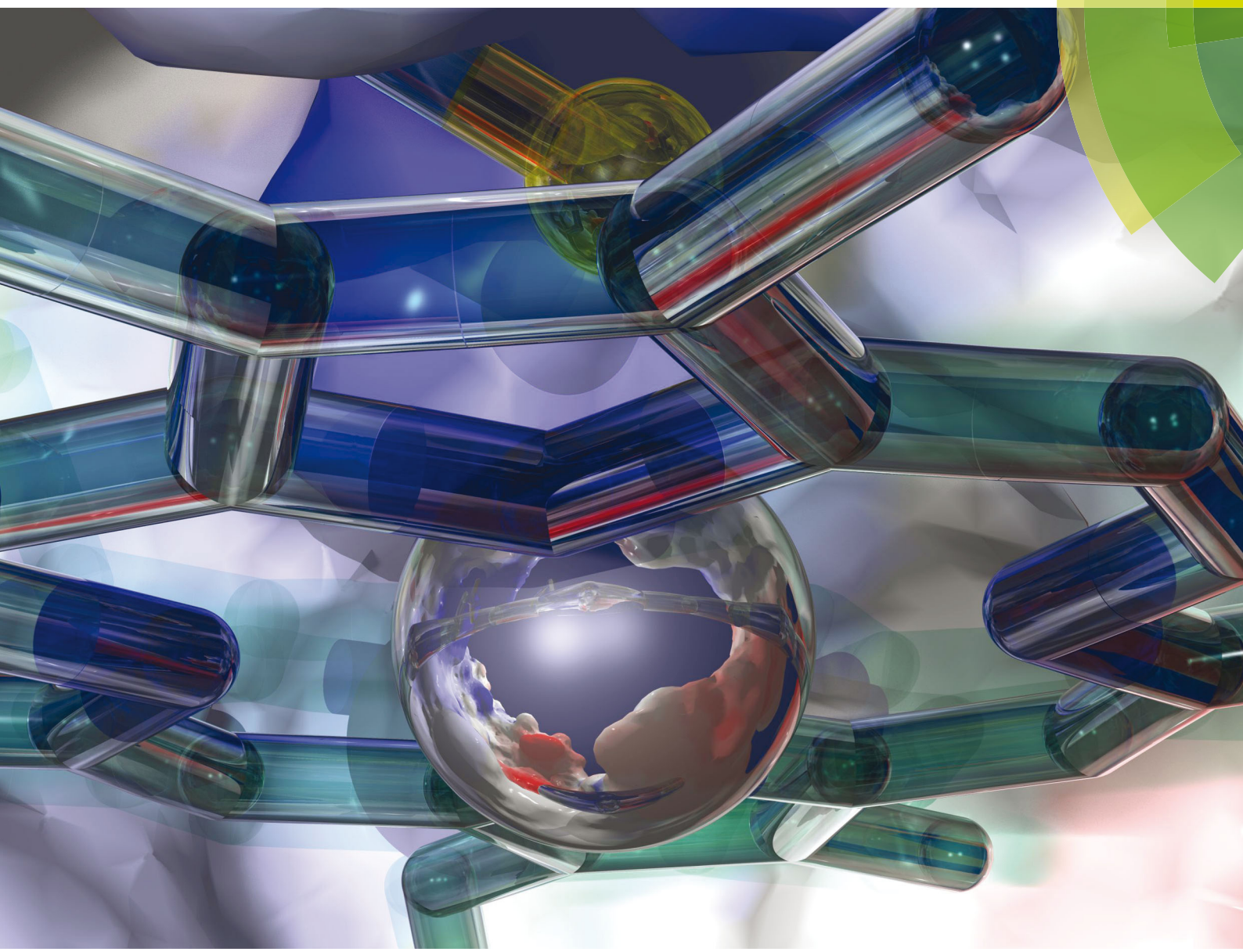

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Heme: emergent roles of heme in signal transduction, functional regulation and as catalytic centres

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Protoporphyrin IX iron complex (heme) is an important cofactor for oxygen transfer, oxygen storage, oxygen activation, and electron transfer when bound to the heme proteins hemoglobin, myoglobin, cytochrome P450 and cytochrome *c*, respectively. In addition to these prototypical heme proteins, there are emergent, critical roles of exchangeable/labile heme in signal transduction. Specifically, it has been shown that association/dissociation of heme to/from heme-responsive sensors regulates numerous functions, including transcription, DNA binding, microRNA splicing, translation, protein kinase activity, protein degradation, heme degradation, K^+ channel function, two-component signal transduction, and many other functions. In this review, we provide a comprehensive overview of structure–function relationships of heme-responsive sensors and describe new, additional roles of exchangeable/labile heme as functional inhibitors and activators. In order to complete the description of the various roles of heme in heme-bound proteins, we also mention heme as a novel chemical reaction centre for aldoxime dehydratase, *cis–trans* isomerase, N–N bond formation, hydrazine formation and S–S formation, and other functions. These unprecedented functions of exchangeable/labile heme and heme proteins should be of interest to biological chemists. Insight into underlying molecular mechanisms is essential for understanding the new role of heme in important physiological and pathological processes.

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1. Introduction

Metal cations are involved in numerous significant functions and make structural contributions to biological substances and proteins, and thus are very important for the survival of living creatures.[1–3]

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Toru Shimizu received his BS, MS and PhD degrees in chemistry from Tohoku University (Sendai, Japan). He held the positions of Associate Professor and Full Professor at Tohoku University before retiring in 2012. He is now a Professor Emeritus of Tohoku University, Visiting Professor of Charles University (Prague, Czech Republic) and Visiting Researcher of AIST (Sendai). He is a recipient of the Academic Award from the Chemical Society of Japan. He has expertise in the structure–function relationships of heme-responsive sensors and heme-based oxygen sensors.

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Alžběta Lengálová received her MS degree in biochemistry from Charles University (Prague, Czech Republic) in 2016. Her Master's thesis was dedicated to anticancer drugs and their transport when incorporated into nanoparticles. Currently, she is a PhD student in the laboratory of Markéta Martínková in the Department of Biochemistry, Charles University in Prague. Her main research interests are heme-containing sensor proteins such as Bach1, HRI, YddV and AfGcHK.

Among important transition metal cations is the iron cation.[4,5] The free iron cation itself is very toxic and unstable.[6,7] Siderophore molecules (*e.g.*, enterobactin, mycobactin, bacillibactin)[8–10] and many proteins (*e.g.*, ferritin, transferrin, hepcidin, ferroportin) are involved in the storage, transfer, export, and/or uptake (acquisition) of iron cations and regulate transcription during iron metabolism and homeostasis, including ferroptosis.[11–14]

Many iron cations exist as an iron-bound protoporphyrin IX (b-type porphyrin) complex called the heme iron complex. The redox state of iron in heme complexes can vary. Among redox states, the two most common are the heme Fe(III) complex (or ferric protoporphyrin IX), termed hemin, and the heme Fe(II) complex (or ferrous protoporphyrin IX). The heme iron complex itself is practically insoluble in aqueous solution and is toxic.[15] This toxicity manifests as reactive oxygen species (ROS) generation, as is the case for the free iron cation. Thus, the concentration of labile hemin is very low in the cytosol (as low as 20–40 nM) of *Saccharomyces cerevisiae*,[16] and even lower in mitochondria and the nucleus (<2.5 nM).[17] By contrast, the concentration of labile cytosolic heme in the malaria parasite is ~1.6 μM.[18]

Some proteins harbor hemin in a manner that precludes hemin contact with O_2 in aqueous solutions. These proteins, which include heme scavengers such as helminth defense molecule (HDM), heme chaperone proteins such as hemopexin and other heme-binding proteins (*e.g.*, DNA protecting protein, PgDps), hold hemin so as to limit its interaction with O_2 and prevent subsequent generation of ROS, thereby protecting the cell against their toxic effects.[12,19–24]

Numerous heme-binding proteins, such as HasA, IsdB, PhuR, ShuA, HRG-1/2 and FLVCR1a/1b, among others, act as heme carriers, and transfer and/or take up proteins that cross the membrane into and/or out of the cytosol and nucleus during heme iron metabolism.[1,25–33]

Prototypical hemeproteins that harbor a bound heme iron complex play numerous important physiological roles as O_2 carriers (hemoglobin), O_2 storage molecules (myoglobin), activators of molecular O_2 (cytochrome P450), mediators of electron transfer (cytochrome *c*) and many other important functions required for cell survival. Heme proteins classified as b-type hemes, such as hemoglobin, myoglobin, cytochrome P450 enzymes, catalases, peroxidases, NO synthases and soluble guanylate cyclase, among others, are major players in physiology; other proteins with non b-type heme include cytochrome *c* (heme *c*), cytochrome *c* oxidase (heme *a3*), cytochrome *d* (heme *d1*), and cytochrome *o* oxidase (heme *o*).[34–36]

In addition to these prototypical and better-known roles of the heme iron complex in physiological functions, new roles of the heme iron complex are emerging. Two prominent non-prototypical roles of the heme iron complex include (1) a "heme-responsive sensor" function, where the exchangeable/labile heme iron complex acts as the first signal for subsequent successive signal transduction, and (2) a "heme-based gas sensor" function, in which the heme iron complex acts as the sensing site of a gas (O_2, NO, CO).[37]

For most "heme-based gas sensors", functional domain activities, including phosphodiesterase, diguanylate cyclase and histidine kinase, among others, are switched on/off in response to gas (O_2, NO and CO) binding to the heme iron complex in the sensing domain.[37–41] In the present review, we provide an in-depth description of "heme-responsive sensors". For heme-responsive sensors, exchangeable/labile hemin (Fe(III) protoporphyrin IX complex) plays a significant role in regulating important physiological functions, such as transcription, microRNA processing, translation, protein phosphorylation, protein degradation, heme iron degradation, K^+ channel function and many others. Specifically, association/dissociation of exchangeable/labile hemin switches these functions on/off; thus, impairment of these sensing functions in eukaryotes and even in (patho)bacteria may be linked to serious diseases.

In Section 2. Heme-responsive sensors, we emphasize the various heme-sensing motifs that exist beyond the prototypical,

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Markéta Martínková

Markéta Martínková received her PhD degree from Charles University (Prague, Czech Republic) in 2003 for her work on the cytochromes P450 and peroxidases from an enzymology viewpoint. In 2004, she was awarded a postdoctoral fellowship by the JSPS to work on heme-containing sensor proteins in the laboratory of Toru Shimizu at Tohoku University (Sendai, Japan). In 2006 she returned to Charles University. Her research interests lie in the characterization of various hemoproteins. Currently, she is an Associate Professor of Biochemistry in the Department of Biochemistry, Faculty of Science, Charles University and a Vice-Dean of the same faculty.

canonical Cys-Pro (CP) motif, and highlight how they differ from classic concepts of heme sensing. Importantly, we discuss overlapping or duplicate roles of heme-responsive sensors and heme-based CO sensors that remain unresolved. These distinctions, which tend to have been obscure in previous papers, often misconstrue differences between changes caused by CO binding and those induced by alterations in the heme redox state. In the Section 3. Heme-regulated inhibition, activation and non-canonical heme active sites of heme proteins, we describe the exchangeable/labile heme iron complex's inhibitory and regulatory functions and heme proteins that contain a non-canonical heme active site; in these latter proteins, heme serves unprecedented functions that are totally unlike those of the well-known prototypical heme proteins, such as hemoglobin, cytochrome *c*, and cytochrome P450s. In Conclusions, we summarize the significance of heme-responsive sensors as well as expected outcomes and future directions of heme-responsive sensors in terms of their potential pharmaceutical and medical benefits, focusing particularly on understanding the molecular mechanisms of heme-stimulated signaling functions.

2. Heme-responsive sensors (Tables 1 and 2)

2.1. General concepts, principles and mechanisms of heme-responsive sensors, and their heme iron complex sensing/binding sites (Fig. 1)

[1] Concept of heme sensing: hemin (heme Fe(III) complex) acts as the first signal in a heme-responsive sensor in that the association/dissociation of hemin to/from the heme-sensing/binding site of the protein regulates important physiological functions, including transcription, translation, protein degradation, heme degradation, ion channel function, and other important functions operating at other sites/domains within the same protein.

[2] Cys-Pro motif (CP motif) as the sensing/binding site for hemin (Fig. 1A and Tables 1, 2): the Cys thiolate of the CP motif is the prototypical sensing/binding site for hemin in many heme-responsive sensors. The importance of the Pro residue adjacent to the Cys residue lies probably in its steric regulation of the protein structure in the neighborhood of the heme-sensing/binding site, which serves to facilitate sensing/binding of hemin by the adjacent Cys residue.[42–47]

[3] Stand-alone Cys of non-CP motifs as a sensing/binding site for hemin (Fig. 1B and Tables 1, 2): There are several cases of heme-responsive sensors in which a stand-alone Cys residue in a non-CP motif performs heme sensing/binding.[47] These include Cys612/His616 (612CXX615C616H motif) in the Ca^{2+}-sensitive large-conductance K^+ (BK) channel,[48,49] Cys13/His16 + His35 (13CXX16H motif) in the voltage-dependent K^+ ($K_V1.4$) channel,[50] Cys628/His648 (628CXXHX648H motif) in the K_{ATP}-channel[51] and His119/Cys170 in NPAS2 (neuronal PAS domain protein 2).[52]

[4] His or other non-Cys amino acid residues can serve as the sensing/binding site for hemin (Fig. 1C and Table 1): it is important to note that Cys and/or the CP motif may not always be the binding site for hemin in the heme-responsive sensor. Instead, His or another non-Cys amino acid residue can serve this function, for example, in HrtR,[53,54] Rev-erbα,[55–57] Rev-erbβ,[56,58–62] CLOCK,[63] TRpRS[64] and PGRM1.[65,66] Although the most common protein arrangement for heme binding is a helical scaffold, other conformations are also possible. For example, the β-hairpin conformation is a possible heme-binding site, as demonstrated by artificially constructed heme-binding β-hairpin peptides.[67]

[5] The affinity of hemin for the heme-responsive sensor varies depending on the cell and subcellular environment: For the heme-sensing nuclear receptor Rev-erbβ to regulate transcription, its affinity for hemin should be very high, with a K_d value on the same order as the concentration of heme in the nucleus ($\sim 10^{-9}$ M).[17,60] In contrast, the affinity of hemin for heme-regulated eukaryotic initiation factor 2α kinase (HRI), a heme-responsive sensor in red blood cells, where the hemin concentration is approximately 10^{-6} M,[68] is rather low, with K_d values around 10^{-5} M.[69]

[6] The hemin-binding/sensing site in a heme-responsive sensor is generally very flexible: global rearrangement of the heme-responsive sensor protein occurs upon binding of hemin to HRI,[43] just as in the case of heme binding to Gis1.[70] In addition, in the heme-regulated transcription factors Bach1 and Bach2, the binding site(s) of the whole protein molecule is (are) very flexible.[71,72] The hemin-binding sites at the thiol/disulphide switching regions of HO2,[73,74] the BK channel,[49,74] ALAS[75] and CLOCK[63] are very flexible in the absence of hemin, but are likely to become structured in the presence of hemin. However, note that most of the heme-regulated-motif region of heme sensors remains unstructured, although it is true that a local structure develops around the heme binding site.

[7] In some heme-responsive sensors, a stand-alone Cys or CP motif ceases to serve as the binding site for the heme Fe(II) complex—the reduced form of hemin (Fig. 1A and B): Importantly, the axial ligand (sensing/binding site) Cys thiolate for hemin is dissociated from the heme Fe(II) complex upon reduction of the heme Fe(III) complex (hemin), because the interaction of the anionic Cys thiolate with the heme iron complex is hampered when hemin is reduced to values that are less positive than those of the heme Fe(III) complex.[44,76,77] Therefore, the heme Fe(II) complex binds to sites different from those for hemin, or the coordination structure of the heme Fe(II) bound to the heme-responsive sensor differs from that of hemin in the heme-responsive sensor. Thus, for such heme-responsive sensors, a heme-redox-dependent ligand switch occurs in that the Cys thiolate bound to hemin is switched to the His imidazole, neutral thiol (protonated Cys)[76] or another amino acid residue upon reduction of hemin to the heme Fe(II) complex. Accordingly, several studies have emphasized that this type of heme-responsive sensor should instead be considered a heme redox sensor[38,78–81] (see Section 2.6. Heme redox sensors).

This situation is in contrast to other heme proteins containing a Cys-bound heme Fe(III) complex, such as cytochrome P450 enzymes and NOS (nitric oxide synthase).[35,36,44] In these heme proteins, Cys thiolate is still the axial ligand for the heme Fe(II) complex, even when the heme Fe(III) complex is reduced to the

Table 1 Heme-responsive sensors associated with transcriptional regulation, DNA binding, tRNA synthesis, miRNA processing and translational regulation. Note that DnrF is also a heme-based NO sensor, E75s of *D. melanogaster, B. mori* are heme-based NO/CO sensors, and E75 of *O. fasciatus* is possibly a heme-based NO sensor

Name	Functions	Hemin-sensing/binding site	Hemin K_d, k_{off} or redox potential	Partner	Origin	Ref.
Hap1	Hemin binding activates transcription of genes encoding cytochromes	5-Coordinated Cys1193 (7th of seven CP motifs, located at the C-terminus distant from the zinc-cluster motif and dimerization element)	$K_d < 20$ μM (with peptide)	Hsp90	*S. cerevisiae* (yeast)	42 and 82
NPAS2	Hemin and heme Fe(II) regulate NPAS2-BMAL1 heterodimer formation and DNA binding in association with transcription of circadian rhythm-related genes; hemin binding to NPAS2 facilitates NPAS2 DNA binding	6-Coordinated His119/Cys170 for hemin (non-CP motif) 6-Coordinated His119/His171 for heme Fe(II), located in the PAS-A domain downstream of the N-terminal bHLH domain	$k_{off} \approx 5.3 \times 10^{-3}$ s^{-1} $K_d \approx 10^{-4}$ μM	BMAL1	Mouse	52 and 83–87
All4978	Hemin binding facilitates DNA binding (heme redox sensor)	6-Coordinated Cys92/His97 or His99 (CP motif: Cys92-Pro93-X-His95-X-His97-X-His99, in 1st of three GAF domains) for hemin and His95 for heme Fe(II)	$K_d < 20$ μM (redox potential: −445 to −453 mV)		*Nostoc* sp. PCC7120 (cyanobacterium)	78
DnrF	Binding of heme Fe(II) or 5-coordinated Fe(II)–NO enhances DNA binding, leading to transcriptional activation of the NO reductase gene and repression of the nitrate reductase gene (heme-based NO sensor)	Not identified	$K_d < 28$ μM for heme Fe(II)	RNA polymerase	*D. shibae* DFL12^T (marine bacterium)	88
Bach1	Hemin inhibits DNA binding, leading to initiation of transcription of HO1, ferritin and ferroportin, and ultimately inducing nuclear export and polyubiquitination	5-Coordinated CP3, CP4, CP5 and CP6 (C-terminal side) out of 6 total CP motifs	$K_d \approx 0.1$ μM	MafK, HOIL-1	Human	89–93
Bach2	Hemin binding regulates immune response signaling cascades; also regulates transcription of Bach1	Not known which of the five CP motif(s) senses/binds hemin		MafK	Human	71, 72 and 92–95
p53	Hemin interferes with DNA binding and triggers nuclear export and cytosolic degradation	5-Coordinated Cys275-Ala-Cys277-Pro (C-terminal DNA-binding domain; one of three total CP motifs)	$K_d \approx 1.2$ μM	PER2	Human	96 and 97
Gis1	Hemin enhances demethylase and transcription	5-Coordinated binding of two hemin complexes to two CP motifs: Cys250-Pro (N-terminal JmjN + JmjC domain) and Cys859-Pro (C-terminal Zn-finger domain)	$K_d > 20$ μM	Unknown proteins	Yeast	70
PpsR	Hemin inhibits DNA binding and increases transcription of a subset of target genes involved in photosynthesis and tetrapyrrole biosynthesis	6-Coordinated His275 (2nd PAS)/Cys424 (C-terminal HTH DNA-binding domain) (non-CP motif; one heme per protein)	$K_d \approx 1$ μM		*Rhodobacter sphaeroides* (purple photosynthetic bacterium)	98
HrtR	Hemin prevents DNA binding and increases the expression of heme-efflux system proteins, HrtA and HrtB, which alleviate heme toxicity (cytoplasmic heme-sensing system)	6-Coordinated His72 (DNA binding domain)/His149			*Lactococcus lactis*	53 and 54
Rev-erbα	Hemin binding facilitates interaction with NCoR-HDAC3 and suppresses the expression of glucose metabolism- and circadian rhythm-related genes	6-Coordinated Cys418-Pro419/His602 (CP motif) or X/His602 for hemin; contribution of Cys418 to heme binding has not been directly confirmed	$K_d \approx 2$–3 μM	NCoR-HDAC3) complex	Human	55 and 56
Rev-erbβ	Hemin binding has functional effects similar to those for Rev-erbα; also promotes degradation through the ubiquitin-proteasome pathway; disulphide/free thiol redox switch in the CP motif regulates heme status, suggesting that the CP motif acts as a redox sensor	6-Coordinated Cys384-Pro385/His568 (CP motif) or X/His568 for hemin; CP motif acts as a redox sensor	Reduced heme binding domain: $K_d < 0.1$ nM and >10 nM for hemin and heme Fe(II), respectively; oxidized heme binding domain: $K_d > 10$ nM for hemin; full-length protein: $K_d \approx 0.1$ nM for hemin, $k_{off} \approx 10^{-6}$ s^{-1}	NCoR-HDAC3 complex	Human	56, 58–62 and 74

Table 1 (*continued*)

Name	Functions	Hemin-sensing/binding site	Hemin K_d, k_{off} or redox potential	Partner	Origin	Ref.
FurA	Hemin binds to FurA bound to DNA sequences (iron-boxes) of the promoter of iron-responsive gene and dissociates its DNA binding; disulphide/free thiol redox switch might regulate hemin binding	6-Coordinated Cys141 (CP motif)/X for hemin	$K_d < 1$ μM	HU, Alr4123, All1140	*Anabaena* sp. PCC 7120 (cyanobacterium)	99 and 100
SbnI	Hemin binding prevents DNA binding and decreases synthesis of staphyloferrin B (a siderophore)		$K_d < 1$ μM		*S. aureus*	101 and 102
E75	Hemin or heme Fe(II) forms a heterodimer with DHR3, leading to DNA binding of DHR3 and suppresses activation of target genes; NO and CO can abolish the inhibitory effect of heme Fe(II) (heme-based NO/CO sensor)	His364/His574 for hemin for *D. melanogaster*; 6-coordinated Cys/His (non-CP motif) for hemin for *B. mori*		DHR3	*D. melanogaster, B. mori*	61, 103 and 104
E75	Heme-based NO sensor, c-type heme	E75 has covalently bound heme and possibly acts as an NO sensor			*O. fasciatus*	105
DHR51	*Drosophila* hormone receptor, homologous to human photoreceptor cell-specific nuclear receptor	6-Coordinated Cys/His (non-CP motif)	$K_d \approx 0.43$ μM		*D. melanogaster*	106
Per2	Transcriptional regulator associated with circadian rhythms; hemin binding leads to ubiquitin-dependent protein degradation	5-Coordinated Cys215 (non-CP motif in PAS-A domain) and His454 (in PAS-B) for mouse Per2; 5-coordinated Cys841/Pro842 (1st of two CP motifs) for human Per2		CRY, p53	Mouse, human	84, 97 and 107–110
CLOCK	Transcriptional regulator associated with circadian rhythms. Hemin binding disrupts binding CLOCK to its E-box DNA target	6-Coordinated His/His144 Additional His/Cys at 20k	$K_d \approx 1.05$ μM, 4.2 μM	BMAL1	Human	63 and 111
CRY1	Transcriptional regulator associated with circadian rhythms	5-Coordinated Cys414/Pro415 (CP motif)		Per	Mouse	112
TrpRS	Hemin induces activation of aminoacylation *Hc*ArgRS	5-Coordinated His Hemin induces oligomerization and inhibits catalysis in the N-end role pathway	5-Coordinated Cys115 (non-CP motif)		Human Human	64 113
Pf RRS	Hemin induces dimerization and inhibits catalysis		$K_d \approx 2$ μM		*P. falciparum*	114
GluRS	Hemin inhibits catalysis			Glu-t-RNA-reductase (GluTR)	*Acidithiobacillus ferrooxidans*	115
GDCR8	Hemin promotes dimerization and activates mRNA splicing, or induces a conformational switch that enables binding to the terminal loop with high specificity	5-Coordinated Pro351–Cys352 (CP motif) for hemin; no cysteine interactions with heme Fe(II)	$k_{off} \ll 10^{-6}$ s^{-1} for hemin; $k_{off} > 10^{2}$ s^{-1} for heme Fe(II)	Drosha	Human	46 and 116–120
HRI	Hemin deficiency activates Ser/Thr/Tyr kinase activity, thereby suppressing translation of globin	6-Coordinated His119 (or His120)/Cys409–Pro410 (1st of two CP motifs)	$k_{off} \approx 10^{-3}$ s^{-1}	eIF2α		43, 69 and 121–126

Table 2 Heme-responsive sensors associated with protein degradation, heme degradation, cation channel, two-component signal transduction, redox sensing and protein–protein interaction. Proteins in [] are already described above in Table 1. Note that the isolated AA584-717 linker region of the BK channel is also a heme-based CO sensor

Name	Functions	Hemin sensing/binding site	Hemin K_d, k_{off} or redox potential	Partner	Origin	Ref.
IRP2	Hemin binding triggers ubiquitination and proteasome-mediated degradation; hemin-dependent oxidative modification triggers protein degradation	5-Coordinated Cys201-Pro-Phe-His204 (single CP motif); hemin binds Cys201, heme Fe(II) binds His204		HOIL-1, FBXL5	Human	5, 128 and 130–132
ALAS1	Hemin-dependent oxidative modification triggers protein degradation	5-Coordinated Cys108–Pro109 (3rd of three CP motifs)		ClpXP, LONP1	Human	133
Arginyl transferase (N-end rule pathway)	Hemin inhibits arginyl-transferase, induces proteasome-dependent degradation, and inhibits UBR1	5-Coordinated Cys71–Cys72–Pro73 (2nd of five CP motifs)		UBR1	Mouse *S. cerevisiae*	134–137
UBR1	Hemin inhibits E3 ubiquitin ligase activity (inhibition of protease activity within the N-end rule pathway of protein degradation)			CUP9	Mouse *S. cerevisiae*	134–137
[Bach1]	Proteosome-dependent protein degradation	5-Coordinated CP3, CP4, CP5 and CP6 motifs (C-terminal side) out of six total CP motifs		MafK HOIL-1	Mouse	90
[Rev-erbβ]	Proteosome-dependent protein degradation	6-Coordinated Cys384–Pro385/His568 (CP motif) or X/His568 for hemin; CP motif acts as a redox sensor		NCoR-HDAC3	Human	59 and 60
[Per2]	Proteosome-dependent protein degradation	5-Coordinated Cys841–Pro842 (1st of three CP motifs)		CRY, p53	Human	97 and 108
Irr	Heme degradation triggers protein degradation	Two hemin binding sites: 5-coordinated Cys (Cys29-Pro-X-His; CP motif), and 6-coordinated bis His-bound low-spin complex			*B. japonicum* (nitrogen-fixing bacterium), Gram-negative bacteria generally	138–140
Slo1 BK channel	Both hemin and heme Fe(II) binding inhibit channel activity	6-Coordinated Cys615 and/or His616 (Cys612-X-X-Cys615-His616 non-CP motif) for hemin	$K_d \approx$ 45–120 nM		Human Slo1 BK channel	48
Isolated AA584-717 linker region of the BK channel	Hemin binding inhibits channel activity and CO recovers channel activity (heme-based CO sensor)	5-Coordinated His616 (Cys612-X-X-Cys615-His616 non-CP motif)	$K_d \approx$ 2.8 μM for oxidized disulphide form, $K_d \approx$ 0.21 μM for reduced disulphide form	HO2	Human BK channel	49
$K_V1.4$	Hemin binding enhances channel activity	6-Coordinated bis-His with His16 (Cys13-X-X-His16) and His35	$K_d \approx$ 20 nM		Rat	50
K_{ATP} channel	Hemin binding enhances channel activity	6-Coordinated Cys628/His648 (Cys628-X-X-His(X_{16})-His648 non-CP motif)	$K_d \approx$ 100 nM based on K_{ATP} currents; $K_d \approx$ 8 μM for isolated SUR2A subunit		Human	51 and 141
HssS	TCS: hemin phosphorylates HssS-HssR and increases transcription of HrtAB (P-HssR binds to the *hrtAB* promoter) and alleviates heme toxicity	Localizes to the extracellular part of the transmembrane protein		HssR	*S. aureus*	142 and 143
ChrS	TCS: hemin binding influences ChrS-ChrA, increasing transcription of HO and ABC-type heme exporter			ChrA	*C. diphtheria*	144 and 145
Fre-MsrQ	TCS (non-classical): heme-mediated electron transfer system, but not a heme-responsive sensor	5-Coordinated b-type; two hemes through histidine residues		MsrP	*E. coli*	146

Table 2 (*continued*)

Name	Functions	Hemin sensing/binding site	Hemin K_d, k_{off} or redox potential	Partner	Origin	Ref.
MA4561	TCS: heme redox sensor (c-type heme); active with hemin, inactive with heme Fe(II)	5-Coordinated Cys656 (2nd GAF domain, non-CP motif); heme is covalently bound *via* a vinyl side chain	Redox potential: −95 to −75 mV	Not known	*M. acetivorans*	79
NtrY	TCS: Heme redox sensor; inactive with hemin, active with heme Fe(II)	Hemin binds to the PAS domain of NtrY		NtrX	*Brucella* spp.	80
Tll0287	Redox sensor (*c*-type heme)	6-Coordinated Cys68/His145 (PAS domain, Cys-X-X-Cys-His non-CP motif)	Redox potential: −255 mV		*T. elongatus* (thermophilic cyanobacterium)	81
[All4978]	Redox sensor: hemin binding facilitates DNA binding, heme Fe(II) binding does not	6-Coordinated Cys92/His97 or His99 (Cys92-Pro93-X-His95-X-His97-XHis99 CP motif in one of 3 GAF domains) for hemin or His95 for heme Fe(II)	K_d < 20 μM; redox potential: −445 mV to −453 mV		*Nostoc* sp. PCC7120 (cyanobacterium)	78
PGRM1	Hemin binding recruits EGFR and cytochrome P450 enzymes	5-Coordinated Tyr113	Redox potential: −331 mV; $K_d \approx 50$ nM	EGFR, cytochrome P450 enzymes, ferrochelatase	Human	65, 66 and 147
HO2	Disulphide/free thiol redox switch in CP motifs regulates hemin affinity in association with hemin degradation	5-Coodinated His45 at the catalytic core under oxidative conditions 6-Coordinated His256/Cys265 (CP motif) and 5-coordinated Cys282 (CP motifs) at regulatory sites under reduced conditions; CP motifs act as redox sensors	$K_d \approx 0.014$ μM (His45) under oxidative conditions $K_d \approx 0.09$ μM (His256/Cys265) and $K_d \approx 0.9$ μM (Cys282) under reduced conditions	Slo1 BK channel	Human	73, 74 and 148–153

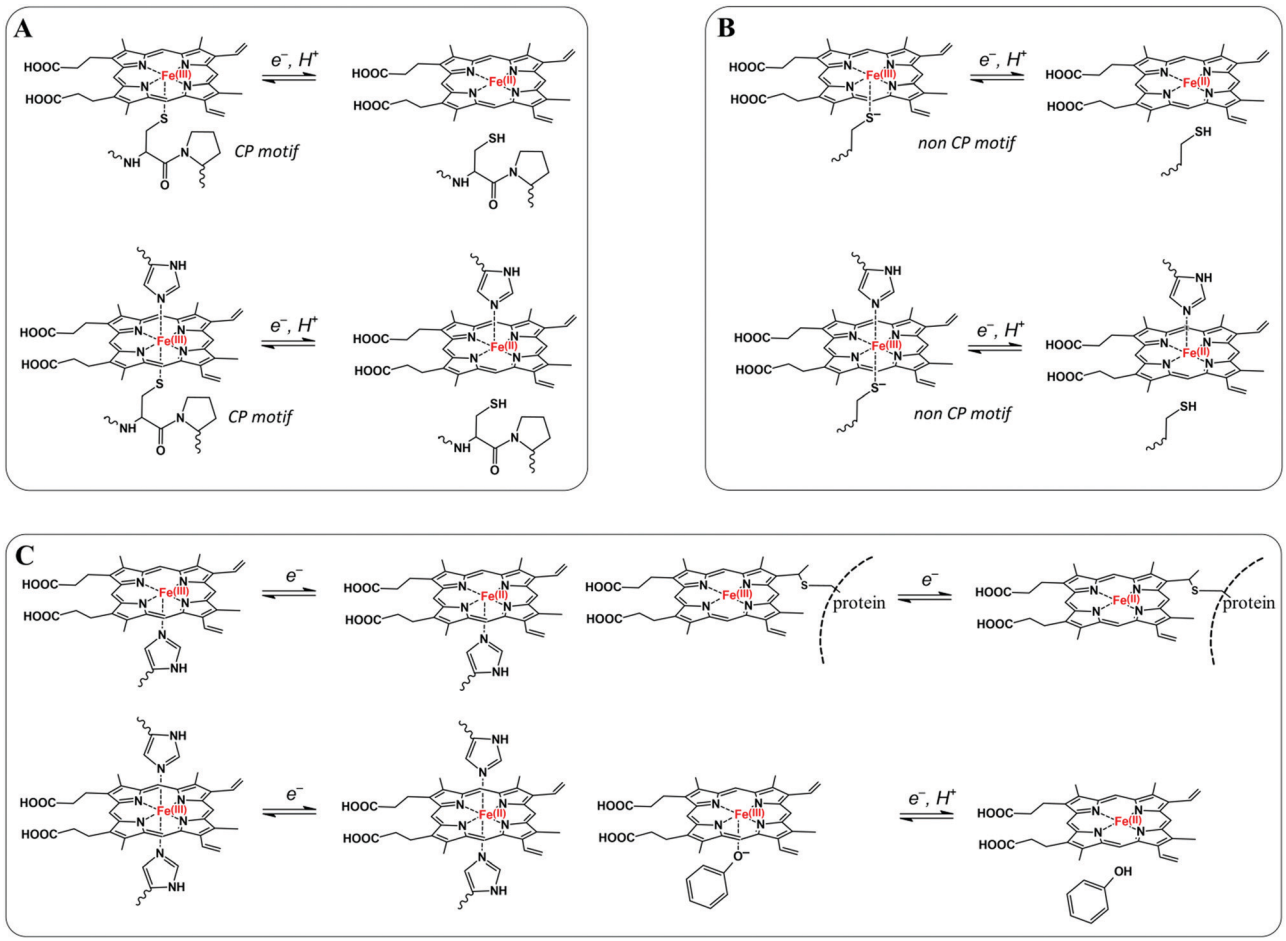

Fig. 1 Coordination structures of heme iron/protoporphyrin IX iron complexes bound to heme-sensing/binding sites in heme-responsive sensors. (A) 5-Coordinated CP-hemin complex (upper): Hap1, Bach1, p53, Gris1, Per2 (human), CRY1, GDCR8, IRP2, HO2 (sensing site under reduced conditions with low affinity), ALAS1, N-end rule pathway/arginyl transferase and Irr (1st site). 6-Coordinated CP-hemin-His complex (lower): All4978, Rev-erbα, Rev-erbβ, HO2 (sensing site under reduced conditions with low affinity) and HRI. (B) 5-Coordinated Cys (non-CP)-hemin complex (upper): Per2 (PAS-A domain, mouse), HcArgRS, porphobilinogen deaminase, PgDps and STEAP1 (1st site). 6-Coordinated Cys (non-CP)-hemin-His complex (lower): NPAS2, PpsR, E75 (*D. melanogaster*), DHR51, Slo1 BK channel, K_{ATP} channel, ALAS and Z-ISO (1st site). (C) First reaction (left-handed upper: 5-coordinated His-hemin complex: TrpRS, BK channel (isolated linker), HO2 (catalytic core under oxidized conditions with high affinity), Fre-MsrQ, OxdB and KtzT. Second reaction (left-handed lower): 6-coordinated His-hemin-His complex: HrtR, CLOCK, Irr (2nd site), Kv1.4, OxdA, Z-ISO (2nd site), STEAP1 (2nd site), STEAP3 and Dcyb. Third reaction (right-handed upper): cytochrome *c*-type heme covalently bound to the protein *via* vinyl or Met and used in redox sensors: E75 (*O. fasciatus*), MA4561, Tll0287, TsdA and hydrazine synthase. Fourth reaction (right-handed lower): 5-coordinated Tyr-hemin complex: PGRM1. Note that Cys residues of CP and non-CP motifs bound to hemin dissociate from the heme iron complex upon heme reduction; thus, the thiolate does not bind to the heme Fe(II) complex. Also, coordination structures for heme Fe(II) complexes and functions of sensors with bound heme Fe(II) described here are, in most cases, not well characterized, although those for hemin have been the focus of considerable research.

heme Fe(II) complex. This is because axial ligation of the Cys residue to the heme Fe(II) complex is supported by hydrogen bonds from neighboring amino acids, preventing the anionic Cys thiolate from dissociating from the less positive heme Fe(II) complex.

Importantly, the redox-dependent ligand switching of a heme-responsive sensor casts significant doubt on the proposed eukaryotic heme-based CO sensors, as described later (see Section 2.9).

2.2. Heme-responsive sensors involved in DNA binding, transcriptional regulation, tRNA synthesis, microRNA splicing and protein synthesis (Table 1)

Binding of hemin to a transcriptional regulatory protein switches on/off transcription of various enzymes and proteins that are critical for cell survival.

2.2.1 Hemin binding facilitates transcription or DNA binding by Hap1, NPAS2, All4978, and DnrF (Fig. 2A). HAP1 is a heme-sensing transcriptional regulator involved in heme iron metabolism; upon hemin binding, its DNA binding is facilitated and its mitochondrial import of δ-aminolevulinate synthase is inhibited.[42] It was originally proposed that the CP motif constituted the consensus binding site for hemin among all heme-responsive sensors that had been identified.

The 7th of seven CP motifs in the HAP1 protein was found to be the single binding site for hemin that activated transcription.[82] HAP1 forms a higher-order complex composed of HAP1 and other cellular proteins, mainly heat shock proteins, such as Hsp90. Upon hemin binding, Hsp90 interacts with HAP1, causing structural changes that are optimal for full activation of HAP1.[82]

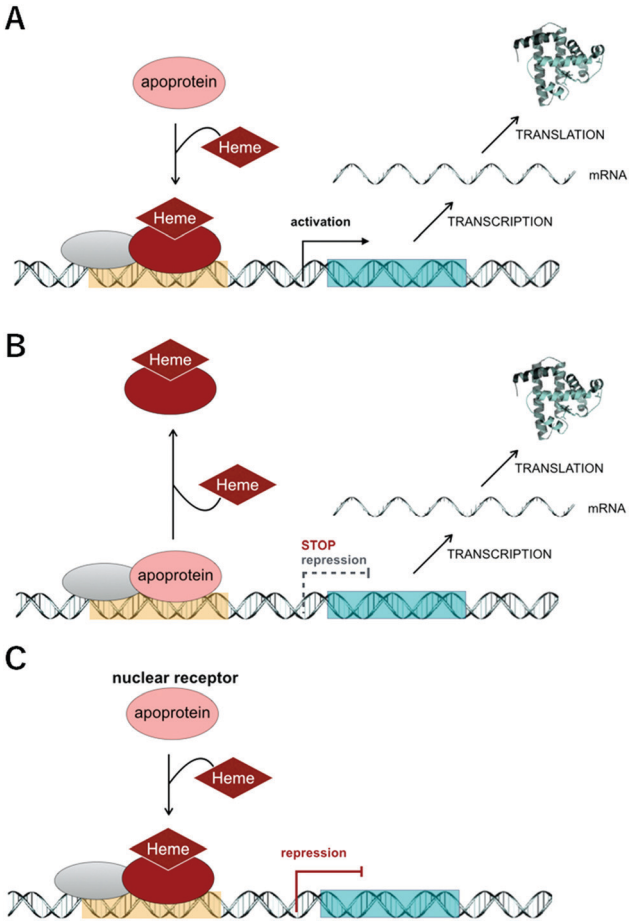

Fig. 2 (A) Hemin binding facilitates transcription or DNA binding: Hap1, NPAS2, All4978 and DnrF. Hemin binds to the free apoprotein, promoting binding of the heme-bound protein to DNA and stimulating transcriptional activation. (B) Hemin binding interferes with DNA–protein interactions, leading to transcriptional activation: Bach1, Bach2, p53, Gis1, PpsR, HrtR, Rev-erbα, Rev-erbβ, FurA and CLOCK. The apoprotein is constitutively bound to DNA and represses transcription; hemin binding to the apoprotein activates transcription by dissociating the protein from DNA. (C) Hemin binding inhibits transcription through binding to a nuclear receptor: E75, DHR51, Per1, Per2, CLOCK and possibly CRY. Hemin binds to a free nuclear receptor protein and regulates gene transcription by promoting binding of the heme-bound protein to DNA.

NPAS2 is a transcriptional regulator associated with circadian rhythms.[83] It is composed of N-terminal bHLH, PAS-A and PAS-B domains, the latter two of which both bind hemin. His119 and Cys170 were found to be the hemin-binding sites for the isolated PAS-A domain of NPAS2.[52] A heterodimeric protein composed of NPAS2 and BMAL1 (brain and muscle Arnt-like protein-1) interacts with E-box DNA.[83,84] Both heme-free and heme-bound forms of the NPAS2–BMAL1 heterodimer bind to DNA in the presence of 3–5 mM NADPH.[83] Binding of hemin to the isolated basic helix-loop-helix (bHLH)-PAS domains of NPAS2 significantly facilitates NPAS2 DNA binding ability in the absence of BMAL1, suggesting that NPAS2 is a prototypical heme-responsive sensor.[85] It was also proposed that NPAS2 is a heme-based CO sensor, since CO binding to the heme-bound heterodimer in the presence of NADPH disrupts its DNA binding.[83] Although CO binds only to the heme Fe(II) complex, and not to hemin, the conversion of hemin, bound to the isolated PAS domain of the NPAS2 protein, to the heme Fe(II) complex significantly changes the heme binding site or the heme coordination structure from the axial ligand His119/Cys170 to His119/His171.[52] This has led to questions about whether CO binding is solely responsible for disrupting its DNA binding. Consistent with this latter concern, mutations at His119 or His171 in the binding site of the heme Fe(II) of NPAS2 were shown to impair NPAS2–BMAL1 heterodimer formation and remarkably reduce its DNA binding activity in the absence of CO, suggesting that the heme redox state and/or heme binding status is involved in the DNA binding and transcriptional activity of the NPAS2–BMAL1 heterodimer.[86]

Moreover, NADPH significantly enhances the DNA-binding ability of the NPAS2–BMAL1 heterodimer even in the absence of heme iron.[87,127] The molecular mechanism underlying the function of NPAS2 in response to CO will be discussed in detail later (see Section 2.9).

All4978 of cyanobacterium *Nostoc* sp. PCC7120, encoding a putative heme-binding GAF (cGMP-specific phosphodiesterase, adenylyl cyclase, and FhlA) protein, has three GAF domains; Cys92 and His97 or His99 in the Cys92XXHis95XHis97XHis99 motif in one of the three GAF domains constitutes the hemin binding site in All4978.[78] Binding of hemin to All4978 facilitates its DNA binding at a helix-turn-helix motif.[78] However, since reduction of hemin to the heme Fe(II) complex switches the axial ligand or heme binding site from the Cys residue to a His residue, thereby abolishing DNA binding, it has also been suggested that All4978 is a redox sensor, as described later (see Section 2.6. Heme redox sensors).

Under oxygen-limiting conditions, the marine bacterium *Dinoroseobacter shibae* DFL12^T generates energy through denitrification, and the resulting accumulation of nitric oxide (NO) may cause cytotoxic effects. The response to this nitrosative (NO-triggered) stress is controlled by the Crp/Fnr-type transcriptional regulator, DnrF,[88] which in its dimeric form binds one molecule of hemin per subunit. DnrF senses NO through its bound cofactor, heme Fe(II), by forming a 5-coordinated NO–heme Fe(II) complex. Addition of a heme Fe(II) or heme Fe(II)–NO complex to apo-DnrF protein, together with the aid of RNA polymerase, induces up to a 5-fold increase in the affinity of the holo-DnrF for its specific binding motif on the NO reductase gene promoter, leading to activation of NO reductase genes and promotion of NO consumption. The same treatment also represses the expression of nitrate reductase genes, hampering NO production. Whether the result is transcriptional activation or repression depends on the position of the DnrF-binding site within the corresponding promoter.

2.2.2 Hemin binding interferes with protein–DNA interactions of the heme-responsive transcription factors Bach1, Bach2, p53, Gis1, PpsR, HrtR, Rev-erbα, Rev-erbβ, FurA and CLOCK and siderophore regulator SbnI, leading to transcriptional activation and decreased siderophore synthesis, respectively (Fig. 2B). Many heme-responsive transcriptional regulators form a heterodimer or hetero-oligomeric complex with partner proteins that bind to

specific DNA sequences, resulting in transcriptional suppression of genes. Binding of hemin to a regulator changes interactions with partner proteins and disrupts protein–DNA interactions, leading to initiation of transcription and subsequent protein expression.

Bach1 (BTB domain and CNC homolog 1) is the first mammalian transcription factor recognized as being regulated by hemin. Bach1 forms a heterodimer with small Maf family proteins, such as MafK. The Bach1/MafK heterodimer binds to the Maf-recognition element (MARE) in DNA and suppresses the expression of HO1 (heme oxygenase-1), ferritin and ferroportin genes.[89,91,92]

Hemin binds to four of six CP motifs in the C terminus of Bach1, causing a marked decrease in DNA binding affinity and dissociation of the heterodimer, leading to expression of *HMOX1* and other genes.[89,91] Hemin binding to Bach1 also induces its nuclear export and polyubiquitination, after which Bach1 binds to a ubiquitin-protein ligase or HOIL-1 (heme-oxidized IRP2 ubiquitin ligase-1) and is subsequently degraded *via* the proteasome.[90,128] However, it is not clear which specific CP motif(s) is (are) involved in the regulation of Bach1 DNA binding and/or polyubiquitination, although CP motifs CP3 and CP4 are likely responsible for inducing nuclear export. Consistent with this, there are two modes of heme binding to Bach1: one with six-coordinated hemin and one with five-coordinated hemin.

Bach2 has five CP motifs and its transcriptional regulation is similar to that of Bach1.[94] However, binding of hemin to Bach2 may additionally regulate immune responses through its involvement in a signaling cascade that induces plasma cell differentiation.[92,93,95] Results of a charge-stage-distribution analysis suggest that the binding region for hemin is intrinsically disordered.[71,72]

The tumor-suppressor protein p53, which suppresses tumorigenesis and regulates DNA-damage repair, cell-cycle arrest and tumor responses to chemotherapy,[96,97] also binds hemin. Similar to the case for Bach1 and Bach2, hemin binding interferes with p53–DNA interactions and triggers both nuclear export and cytosolic degradation of p53.[92,96] A Cys residue in the third of three CP motifs (CACP motif: C275AC277P) in the C-terminus of the p53 protein is involved in binding hemin. The ubiquitin-dependent degradation of p53 is prevented by binding to period 2 (Per2), a circadian transcriptional regulatory factor that also binds heme,[97] as described in detail below. Interestingly, iron deprivation suppresses the growth and tumorigenicity of human colon carcinoma cells in a p53-dependent manner,[96] suggesting that the involvement of hemin in the p53–Per2 interaction is associated with heme iron metabolism.

The yeast Gis1 protein is a transcriptional regulator that belongs to the JMJD2/KDM4 subfamily of histone demethylases.[70] Gis1 contains two Jmj domains – JmjN and JmjC – in the N-terminus, and a zinc finger domain (ZnF) in the C-terminus.

Each domain contains a CP motif to which hemin binds. At low concentrations, hemin first partially binds to the high-affinity site in the CP motif of the paired JmjN + JmjC domains, enabling Gis1 to exhibit partial demethylase activity. At increased concentrations, hemin binds to the CP motif in ZnF, inducing profound conformational changes and oligomerization of Gis1 and causing dissociation of unidentified cellular proteins that probably inhibit the transcriptional activity associated with their DNA binding. As a consequence, Gis1 histone demethylase and transcriptional activities are fully activated by hemin.[70] It has also been suggested that Gis1 is oxygen sensitive; consistent with this, oxygen signaling can be mediated by heme.[70]

PpsR controls the synthesis of heme and bacteriochlorophyll in purple photosynthetic bacteria.[98] Both His275 in the second PAS domain and Cys424 (in a non-CP motif) in the C-terminal helix-turn-helix DNA-binding domain of PpsR are the hemin axial ligands; one molecule of heme interacts with a single molecule of PpsR. Binding of hemin to PpsR inhibits the ability of PpsR to form a higher-order PpsR–DNA complex, with hemin binding at Cys424 appearing to be more important in the hemin-induced DNA dissociation. Hemin binding to PpsR differentially induces the expression of a subset of PpsR-controlled photosynthetic and tetrapyrrole biosynthesis genes.[98]

The transcriptional regulator HrtR of *Lactococcus lactis* interacts with a specific sequence in the promoter region of *hrtA* and *hrtB* genes (encoding the heme-regulated transporters, HrtA and HrtB) that is needed for transcriptional repression of *hrtA* and *hrtB*. HrtR binds hemin at His72 and His149, forming a 6-coordinated low-spin complex.[53,54] Binding of hemin to HrtR abolishes HrtR–DNA interactions, allowing transcription and subsequent translation of the HrtA–HrtB transporters. Binding of hemin to HrtR causes a profound coil-to-helix transition of the α4 helix in the heme-sensing domain that triggers structural changes in the DNA-binding domain of HrtR, causing it to dissociate from the target DNA.[54] Therefore, HrtA–HrtB permease controls heme toxicity by directly and specifically controlling its efflux.

Rev-erbα, a nuclear receptor also known as NR1D1 (nuclear receptor subfamily 1, group D, member 1), acts as a constitutive transcriptional repressor that regulates circadian rhythm, glucose metabolism and energy metabolism in a tissue-specific manner.[55,56,62] Given the high homology with the structurally better characterized Rev-erbβ it can be assumed that hemin binds to Rev-erbα at Cys418/His602 and regulates its function by promoting its assembly with two proteins: nuclear receptor co-repressor (NCoR) and histone deacetylase 3 (HDAC3). The resulting complex represses the expression of genes encoding phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, both of which control glucose metabolism. Heme also augments transcriptional repression of the core clock gene *Bmal1* by Rev-erbα. A spectral study of the binding of hemin to the isolated heme-binding domain of Rev-erbα revealed that His602 is the axial ligand for the hemin complex. However, the contribution of Cys418 to heme-binding affinity has not been directly confirmed. Moreover, it is not clear how hemin influences the interaction of Rev-erbα, alone or in a NCoR–HDAC3 complex, with the specific DNA sequence that is also bound by the retinoic acid receptor-related orphan receptor (ROR).

Rev-erbβ, a nuclear receptor similar to Rev-erbα, is believed to function as a transcriptional silencer and negative regulator

of glucose metabolism and circadian rhythms.[56,62] Hemin binds to Cys384/His568 of the isolated heme-binding domain of Rev-erbβ.[61,62] Crystal structures of the isolated heme-binding domain with hemin or heme Fe(II) have been determined.[62] NO and CO also interact with the heme Fe(II) bound to Rev-erbβ, additionally suggesting that Rev-erbβ is a gas sensor.[58]

Comprehensive biochemical studies have elegantly demonstrated a number of functional features of Rev-erbβ.[58–60] (1) The affinity of hemin for the reduced form of the isolated heme-binding domain of Rev-erbβ is very high, with a $K_d < 0.1$ nM. Free Cys374 and Cys384 serve as the axial ligand in the reduced form, but in the oxidized form, the isolated heme-binding domain contains a Cys374–Cys384 disulphide bridge that changes the coordination structure of hemin, decreasing its affinity for the isolated heme-binding domain by nearly 100-fold. These observations suggest that oxidative stress is linked to circadian and/or metabolic imbalances and that Rev-erbβ is a redox sensor. Hemin dissociation from this protein de-represses the expression of target genes in response to changes in intracellular redox conditions. (2) Changes in the coordination structure of the isolated heme-binding domain accompany the 100-fold lower affinity of heme Fe(II) that occurs upon reduction of hemin to heme Fe(II); thus, a Cys residue is no longer available to bind heme Fe(II) as the axial ligand, additionally implying that Rev-erbβ is a redox sensor. (3) The rate constant and K_d of hemin dissociation from full-length Rev-erbβ were found to be extremely slow ($\sim 10^{-6}$ s^{-1}) and very low (0.1 nM), respectively, values that are in accord with the very low concentration (<2.5 nM) of labile hemin in the nucleus.[17] By contrast, the corresponding values for heme Fe(II) or the oxidized form of Rev-erbβ are greater than the concentration of hemin. Thus, heme reduction or conversion to the disulphide form causes dissociation of heme. (4) Hemin binding to full-length Rev-erbβ acts indirectly through unidentified cellular components to facilitate Rev-erbβ interactions with HDAC3-bound NCoR, resulting in repression of Rev-erbβ target genes. (5) Hemin binding to Rev-erbβ leads to Rev-erbβ degradation *in vivo*, likely through the proteasome-dependent pathway, and indirectly regulates its interaction with NCoR1. (6) These data support the hypothesis that Rev-erbβ binds hemin too tightly to function as a direct heme-responsive sensor. However, the authors also expand on this idea by noting that Rev-erbβ possesses qualities of a redox sensor, such that heme binding is coupled to the redox-sensing function and acts to poise the receptor for NCoR1 binding or degradation.

The cyanobacterial transcriptional repressor FurA (ferric uptake regulator) from *Anabaena* sp. PCC 7120 regulates iron metabolism.[99] FurA binds to DNA sequences (iron-boxes) in the promoter of iron-responsive genes. *In vitro*, one molecule of hemin binds to one molecule of FurA with high affinity ($K_d < 1$ μM) and inhibits FurA–DNA interactions.[100] Cys141 within the CP motif of FurA should be an axial ligand for 6-coordinated low-spin hemin. On the other hand, FurA also exhibits disulphide reductase activity. FurA has two redox CXXC motifs. Cys101 and Cys104 in one redox motif form a disulphide that acts as a thiol/disulphide switch to regulate reductase activity.[99] In the second motif, interactions between hemin and Cys141 of FurA are likely regulated by a thiol/disulphide switch, as observed for Rev-erbβ (as shown above) and HO2 (as shown later). However, the physiological function of the FurA-hemin complex remains to be explored.

SbnI of *Staphylococcus aureus* is a hemin and iron sensor that controls the expression of genes involved in the synthesis of siderophores (mainly staphyloferrin B), which are involved in iron uptake during times of iron scarcity in infectious diseases.[101,102] Under conditions of low hemin and low iron, the Sbn1 protein forms dimers in solution and binds to DNA within the *sbnC* coding region, promoting the expression of genes *sbnD-H*, which are involved in staphyloferrin B synthesis. Under conditions of high hemin and high iron, hemin binds to Sbn1 and prevents its DNA binding, thereby resulting in decreased expression of *snbD-H* genes. Thus, it is possible that SbnI senses both the hemin complex and iron ion.

CLOCK, a transcriptional regulatory protein associated with circadian rhythms,[84] has a domain structure similar to that of NPAS in that its N-terminal site is composed of bHLH, PAS-A, and PAS-B domains. Spectroscopic studies of the characteristics of hemin binding to the isolated PAS-A domain of CLOCK[111] showed that hemin binds to the protein to form a 6-coordinated low-spin complex, whereas heme Fe(II) binds to the protein in an equilibrium between 5-coordinated high-spin and 6-coordinated low-spin states. In a separate study, hemin addition to CLOCK containing bHLH-PAS-A domains disrupted its bHLH domain-mediated DNA binding.[63] The hemin binding site was a low-spin complex with bis-His residues (His144 as one of the axial ligands) in the PAS-A domain. EPR spectra of the hemin bound to CLOCK indicate that additional His/Cys coordination exists at 20k, suggesting a conformationally mobile protein framework within the hemin binding site.

2.2.3 Hemin inhibits transcription by binding to the nuclear receptors E75, DHR51, Per1, Per2 and possibly CRY (Fig. 2C). The *Drosophila* transcriptional nuclear receptor, E75 (ecdysone-induced protein 75), acts as a repressor that tightly binds hemin to its His364 and His574 residues.[103] A second nuclear receptor, DHR3 (*Drosophila* hormone receptor 3), acts as a transcriptional activator that increases transcription by ~10-fold over basal levels. Cotransfection of E75 with DHR3 reduces DHR3-induced activity by 3- to 4-fold. Providing supplemental hemin further reduces the level of reporter gene expression to near background levels owing to an increase in E75 stability and accumulation, suggesting that E75 is a heme-responsive sensor.[103] Addition of an NO donor to the culture media restores the activity of DHR3 to the levels observed in the absence of E75, suggesting that E75 is also a heme-based NO sensor in which NO acts to antagonize E75 repressor activity. The interaction of E75 with the DHR3-derived peptide, HR3 AF2, increases E75 stability, resulting in an increase in the denaturation transition temperature, suggesting that E75 directly interacts with DHR3. Interestingly, this thermostabilization only occurs when binding is carried out with the reduced Fe(II)-bound form of E75, suggesting that E75 is a redox sensor. Taken together, these observations indicate that E75 interacts with DHR3 and interferes with its activation. Reduction of

hemin in the E75–DHR3 dimer to heme Fe(II), a conversion that might occur in cells, reduces the transcriptional activity of E75–DHR3, probably because of significant structural changes in the protein caused by heme Fe(II). NO interacts with heme Fe(II) and restores the activity of DHR3, likely owing to a decrease in the interaction of E75 with DHR3. It has also been suggested that E75 is a CO sensor, given that addition of CO to E75 containing heme Fe(II) increases the transition temperature. However, roles of hemin and heme Fe(II) in functions were unclear, because both chemicals were not strictly distinct in the study. Further studies are needed to clarify the problem. We will discuss the CO sensor function of E75 later (see Section 2.9).

Spectrometric studies of hemin binding to the isolated heme-binding domains of E75 proteins from *Drosophila melanogaster* and *Bombyx mori* have shown that hemin bound to the heme binding domain forms a 6-coordinated low-spin complex with the axial ligands Cys and His.[61,104,105] The Cys binding site for hemin is analogous to that in other heme-responsive sensors. Significant changes in the coordination structure are observed following reduction of hemin to heme Fe(II). It should be noted that the heme iron complex of E75 from *Oncopeltus fasciatus* is covalently bound to the protein, similar to the case in cytochrome *c*.[105] Biochemical and biophysical data suggest that E75 is a heme-based NO sensor.[105]

DHR51, a *D. melanogaster* hormone receptor, is the homologue of the human photoreceptor cell-specific nuclear receptor that controls neuronal differentiation in the developing retina. Spectral characterization of the isolated ligand-binding domain of this protein revealed that hemin is bound to the protein through the axial ligands His and Cys, forming a 6-coordinated low-spin complex.[106]

Period circadian protein (Per) is an important transcriptional regulatory factor involved in circadian rhythms. Per binds to cryptochrome (CRY) and the resultant Per/CRY heterodimer interacts with the NPAS2–BMAL1 heterodimer to inhibit the transcription of *Per* and *Cry*.[84] Heme shuttling between mouse Per2 (mPer2), which is a heme-binding protein, and NPAS2 may regulate the transcriptional activity of NPAS2–BMAL1.[107] Hemin was found to bind the isolated PAS-A domain of Per2 through Cys215.[109] Hemin is transferred from the isolated holo bHLH-PAS 2 domain of NPAS2 to the isolated apo PAS-A domain of mPer2, suggesting that mPer2 is a heme-responsive sensor.[109] The site in the isolated PAS-B domain of mPer2 to which hemin binds is found to be His454.[110]

Importantly, hemin binds to one of the two CP motifs located in the C-terminus of human PER2 and destabilizes the PER2/CRY heterodimer, leading to ubiquitin-dependent degradation of the protein.[108] In contrast, hemin binding to the N-terminal PAS domain leads to the formation of a stable PER2/CRY heterodimer. These observations have implications for the association of PER2 with p53, suggesting that the spatiotemporal organization of PER2–p53 interactions and the nature of their chemical modifications are critical for their time-dependent subcellular redistribution and potential biological functions.[97] Additional studies of the involvement of the heme iron complex in these functions are warranted.

As described above, CRY is an important transcriptional regulatory protein associated with circadian rhythms.[84] CRY contains a CP motif that is evolutionarily well conserved among divergent animals.[112] Transgenic mice overexpressing CRY1 containing a mutation in the CP motif were shown to display abnormal entrainment behavior, exhibiting markedly modified circadian activity. Surprisingly, this mutant also caused diabetes mellitus.[112] Although this study did not provide direct evidence for hemin binding to the CP motif of CRY, its results strongly suggest that hemin plays a significant role in circadian rhythms.

2.2.4 tRNA synthases as heme-responsive sensors (Fig. 3A and Table 1). Mammalian tryptophanyl-tRNA synthases (TrpRSs) are Zn^{2+}-binding proteins that catalyze the aminoacylation of $tRNA^{Trp}$.[64] Biochemical analyses using purified human full-length TrpRS have revealed that hemin interacts strongly with Zn^{2+}-depleted TrpRS, with a stoichiometric hemin:protein ratio of 1 : 1, and enhances aminoacylation activity by more than 10-fold.[64] His residues were suggested to be the axial ligand for the hemin complex bound to human TrpRS.[64] This study further showed that, in contrast, the Zn^{2+}-bound form of TrpRS does not bind hemin. The enhancement of catalytic activity caused by association of heme is similar to that caused by Zn^{2+}. Because Trp is the least abundant amino acid in humans, regulation of TrpRS activity by hemin could serve as a mechanism for protecting the cell against Trp starvation, reflecting hemin-mediated activation of the Trp degradation pathway through activation of indoleamine 2,3-dioxygenase. On the other hand, TrpRSs from mouse, zebrafish and *Arabidopsis* do not bind hemin.

Human cytoplasmic arginyl-tRNA synthase (HcArgRS) was reported to bind hemin, resulting in a decrease in its catalytic activity.[113] This study showed that the addition of hemin did not influence K_m values for t-RNAArg, arginine or ATP, but dramatically reduced k_{cat} values. Hemin was shown to induce oligomerization of the enzyme, an effect that could account for the inhibition of its catalytic activity. Cys115 was identified as the specific binding site for hemin; however, a Cys115Ala mutant was also inhibited by hemin, suggesting that binding of hemin at Cys115 is not solely responsible for the inhibitory effect.

An analysis of the structure of arginyl-tRNA synthase (*Pf*RRS) of *Plasmodium falciparum*[114] showed that hemin drives *Pf*RRS dimerization and inhibits its catalysis. Hemin interferes with interactions between *Pf*RRS and its cognate $tRNA^{Arg}$. Excessive amounts of hemin in chloroquine-treated malaria parasites result in significantly reduced levels of charged $tRNA^{Arg}$, suggesting deceleration of protein synthesis.

Glutamyl-tRNA, formed by Glu-tRNA synthase (GluRS), is a substrate for protein biosynthesis and tetrapyrrole formation by the C_5 pathway.[115] In *Acidithiobacillus ferrooxidans*, an acidophilic bacterium that expresses two forms of GluRS (GluRS1 and GluRS2) with different tRNA specificities, intracellular heme levels vary depending on growth conditions. Under respiration conditions, in which high concentrations of heme are required, the levels of GluRS and Glu-tRNA-reductase (GluTR), an enzyme responsible for transforming Glu-tRNA to

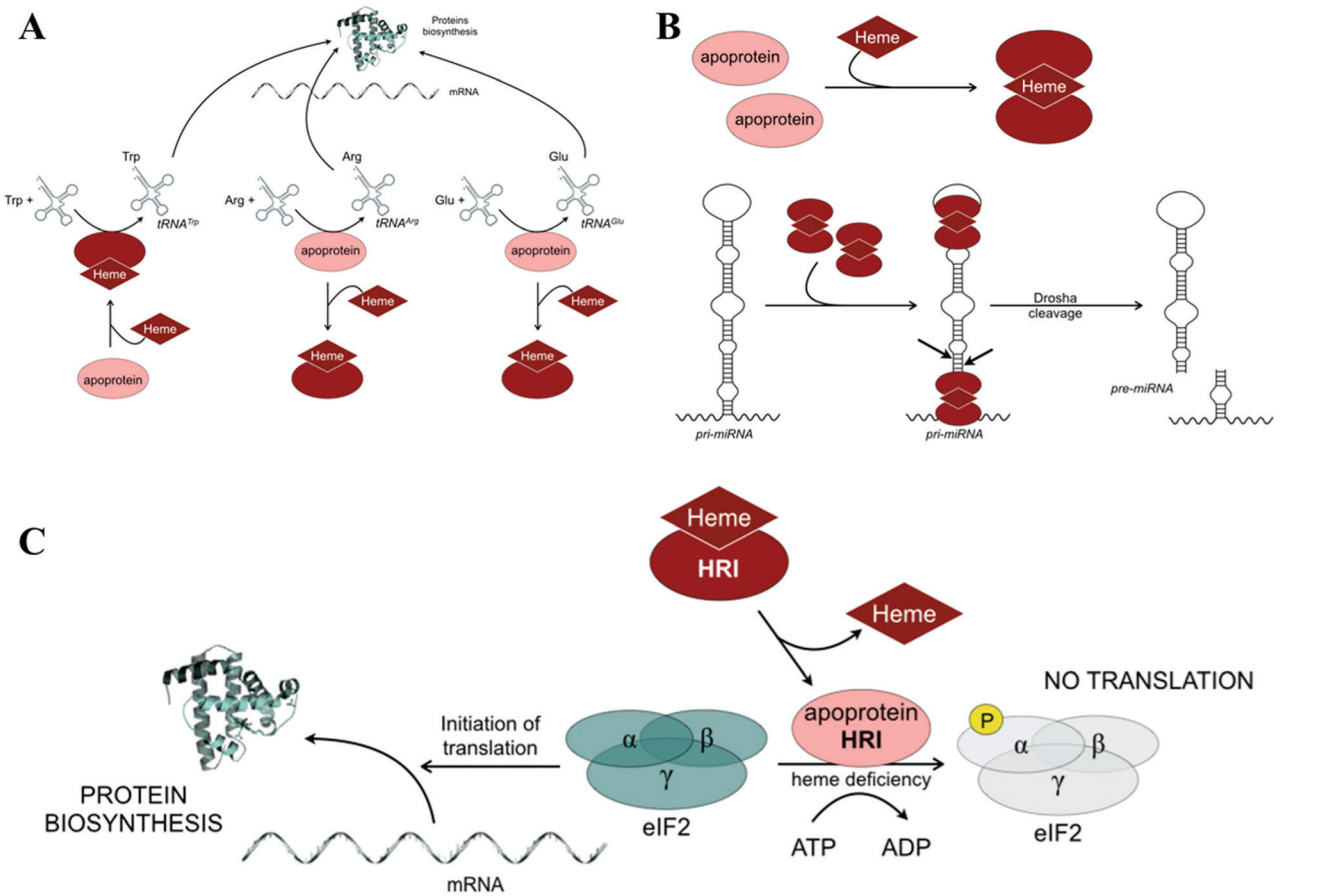

Fig. 3 (A) Hemin binding regulates tRNA synthase: TrpRS, *Hc*ArgRS, *Pf*RRS, GluRS. Hemin binds to Trp tRNA synthase (TrpRS), leading to enhancement of its aminoacylation activity and thus protein biosynthesis, whereas for Arg t-RNA synthases (*Hc*ArgRS, *Pf*RRS) and Glu t-RNA (GluRS) synthase, hemin binding results in a decrease of catalytic activity. (B) Hemin binding regulates miRNA splicing: GDCR8. Hemin promotes dimerization of GDCR8 and activates mRNA splicing. (C) Hemin binding regulates protein translation: HRI. Hemin-bound HRI has no kinase activity, allowing initiation of translation (left side). In contrast, under conditions where hemin concentrations are low (*e.g.*, in blood diseases) hemin dissociates from HRI, thereby activating HRI and allowing it to phosphorylate eIF2α and thus terminate translation of globin in erythroid cells (right side). Note that GCN2, PERK, and PKR operate in the same fashion as HRI in that those sensor kinases phosphorylate Ser51 of eIF2α and stop translation in response to various stimuli.

delta-aminolevulinic acid—the universal precursor of tetrapyrroles, including heme—are increased. However, when intracellular heme levels are in excess, the cells respond by dramatically decreasing GluRS activity and GluTR levels. These results suggest that GluRS plays a major role in regulating the cellular level of heme. Indeed, GluRS1 activity is inhibited by hemin, but is restored by NADPH. Further biochemical studies are needed to elucidate the role of hemin and heme Fe(II) in GluRS catalysis.

2.2.5 GDCR8: a heme-responsive sensor involved in microRNA (miRNA) splicing (Fig. 3B and Table 1). The RNA-binding protein DGCR8 (DiGeorge critical region 8) and its ribonuclease partner Drosha are essential for cleaving primary microRNA (pri-miRNAs) transcripts as part of the canonical miRNA-processing pathway.[116] Mammalian DGCR8 forms a highly stable, active, dimeric complex with hemin in which two Cys residues serve as the axial ligands, one from each monomer.[46,116] In addition to its double-stranded RNA-binding domain, DGCR8 has been reported to act through a dimeric heme-binding domain to directly contact pri-miRNA.[118] This RNA-binding heme domain directs two DGCR8 dimers to bind each pri-miRNA hairpin. However, another group suggested that hemin induces a conformational change in DGCR8 that serves to activate DGCR8 and allows it to recognize pri-miRNAs by specifically binding the terminal loop near the 3′ single-stranded segment.[117] Incidentally, reduction of hemin to heme Fe(II) abolishes pri-miRNA processing activity, accompanied by a marked decrease in affinity, implying that DGCR8 is a redox-sensitive regulator.[119] Accordingly, the coordination structure of heme Fe(II) is significantly different from that of hemin in that Cys thiolate is no longer an axial ligand for heme Fe(II) in DGCR8.[120]

2.2.6 HRI: a heme-responsive sensor involved in protein synthesis in red blood cells (Fig. 3C and Table 1). Eukaryotic initiation factor 2α (eIF2α) kinases are sensors that are activated under various stress conditions. These kinases, which include PKR (a double-stranded RNA sensor involved in detecting virus infection and mitochondria RNA), PERK (a sensor of endoplasmic reticulum (ER) stress associated with accumulation of denatured proteins) and GCN2 (a sensor for amino acid starvation), act by phosphorylating Ser51 in the α subunit of eIF2, a key regulatory translational initiation factor, thus terminating translation at the

initiation stage.[122,123,129] In erythroid precursors, hemin controls the translation of globins by modulating the activity of the heme-regulated eIF2α kinase, HRI.[121] The translational regulation of HRI is essential for reducing excessive synthesis of globin proteins and decreasing the severity of phenotypes associated with iron deficiency anemia, erythropoietic protoporphyria, and β-thalassemia.[121]

Under normal healthy conditions, the concentration of free hemin in erythroid cells—between 0.1 and 10 μM[31,68]—is sufficient to allow hemin binding to HRI, resulting in blockade of the catalytic site and prevention of eIF2α phosphorylation. However, in the context of a hemin deficiency caused by impaired heme biosynthesis (*e.g.*, in blood diseases), hemin dissociates from HRI, leading to opening of the catalytic site, followed by autophosphorylation, subsequent phosphorylation of eIF2α, and ultimately a halt in globin translation that serves to balance the concentrations of hemin and globin. An examination of the molecular mechanism of this heme-regulated function for the full-length HRI enzyme[43,69,124–126] revealed that (1) hemin binds to HRI with a hemin : HRI ratio of 1 : 1 and inhibits catalysis; (2) hemin binding to the protein through one of two axial ligands, Cys409, located at the C-terminus (the other axial ligand, His119/120, is located at the N-terminus), causes global structural changes in the protein; (3) the axial ligand, Cys409, is part of a CP motif in the protein in which Pro410 is important for the hemin binding, as evidenced by abolition of hemin binding to mutant HRI containing a Pro410Ala substitution; (4) added NO forms a 5-coordinated NO-heme Fe(II) complex in which catalysis inhibited by hemin is restored; (5) the dissociation rate of hemin from HRI (k_{off} 10^{-3} s^{-1}) is much higher than that of myoglobin and hemoglobin (10^{-6}–10^{-7} s^{-1}), suggesting that hemin is not tightly bound to HRI, which probably has a K_d of about 10^{-5} M; (6) Hg^{2+} strongly inhibits the function of HRI (IC_{50} ≈ 0.6 μM), but NO fully reverses this inhibition, suggesting that a free Cys residue is involved in the inhibition and restoration of catalysis; and (7) autophosphorylation at Tyr193, Thr485, and Thr490 of HRI is important for the initial stage of catalysis.

2.3. Heme-responsive sensors involved in protein degradation *via* ubiquitination: IRP2, ALAS1, N-end rule pathway components, Bach1, Rev-erbβ, and Per2 and a heme-responsive sensor involved in heme degradation and subsequent protein degradation, Irr (Fig. 4A and Table 2)

IRP2 (iron regulatory protein 2) is a post-transcriptional regulator of iron metabolism.[5,130] When iron is depleted, IRP2 binds to mRNAs that encode proteins involved in iron homeostasis, promoting translation of the transferrin receptor and blocking translation of ferritin, with the net result being an increase in cytosolic iron levels through stimulation of iron uptake and use and mobilization of iron stores.

When iron levels are sufficient, IRP2 is targeted for degradation by the iron-stabilized E3 ligase component FBXL5 (F-Box and leucine-rich repeat protein 5) or HOIL-1.[128] As a result, translation of the transferrin receptor is blocked and translation of ferritin is facilitated, ultimately leading to a decrease in the cytosolic iron level. Similar sequential reactions apply to the IRP2 homolog, IRP1.[5,130] Hemin binding to IRP2 triggers ubiquitination and degradation of IRP2.[128,131] Both Cys201 and His204 residues in the Cys201-Pro-Phe-His204 cluster (CP motif) of the iron-dependent degradation domain are required for the heme-dependent degradation of IRP2. Oxidative modification of IRP2 mediated by heme binding triggers IRP2 ubiquitination, and the oxidized IRP2 is selectively recognized by FBXL5 (or HOIL-1). Cys201 binds hemin, whereas His204 binds to the heme Fe(II) complex and might generate ROS when the heme Fe(II) complex is bound. Pulse radiolysis experiments on IRP2 have shown that a transient 5-coodinate His-liganded heme Fe(II) is a prerequisite for oxidative modification of hemin-bound IRP2.[132] Incidentally, IRP1 binds two molecular equivalents of hemin, but does not have the Cys-Pro-X-His motif or the iron-dependent degradation domain ascribed to oxidative modification.[132] Interestingly, bacterial iron regulatory protein (Irr) is degraded following heme degradation in a heme-dependent manner similar to IRP2 and has a similar heme-binding motif Cys-Pro-X-His,[138,139] as described later.

In eukaryotic cells, heme production is tightly controlled by heme itself through negative feedback-mediated regulation of ALAS1, a nonspecific 5-aminolevulinate synthase that is the rate-limiting enzyme for heme biosynthesis.[5,25,34,133] ALAS1 expression is suppressed by heme at the transcriptional, translational and post-translational levels. ALAS1 forms a complex with mitochondrial ATP-dependent protease (ClpXP) in a heme-dependent manner.[133] Hemin binds to Cys108 and Pro109, the third of three CP motifs, located at the N-terminus of the mature ALAS1 protein, and forms an ALAS1/ClpXP complex within the mitochondrial matrix, leading to heme-dependent degradation of ALAS1. Hemin binding to the CP motif is also necessary for the hemin-dependent oxidative modification of ALAS1, which enables the recruitment of LONP1 (lon peptidase 1, mitochondrial), another ATP-dependent protease in the mitochondrial matrix, into the ALAS1/ClpXP complex. Thus, the degradation of ALAS1 maintains appropriate intracellular heme levels.

The N-end rule pathway is a set of proteolytic systems whose function is to recognize and polyubiquitylate proteins containing N-terminal degradation signals, thereby causing the proteasome-mediated degradation of these proteins.[134–137] Regulated degradation of specific proteins by the N-end rule pathway mediates a legion of physiological functions, including heme, O_2 and NO sensing, and selective elimination of misfolded proteins. The conjugation of arginine by arginyl-transferase to N-terminal aspartate, glutamate, or oxidized Cys is a part of the N-end rule pathway of protein degradation. Hemin functions in multiple ways: (1) it binds and inhibits arginyl-transferase of mice and the yeast, *Saccharomyces cerevisiae*.[134,136] This inhibition by hemin is mediated by a redox mechanism that involves the formation of a disulphide bond between Cys71 and Cys72 residues of the Cys71–Cys72–Pro73 sequence by hemin. (2) It induces the proteasome-dependent degradation of arginyl-transferase *in vivo*. (3) It interacts with UBR1 (an E3 ubiquitin ligase of the N-end rule pathway) and blocks the activation of one of its substrate-binding sites, which targets the transcriptional

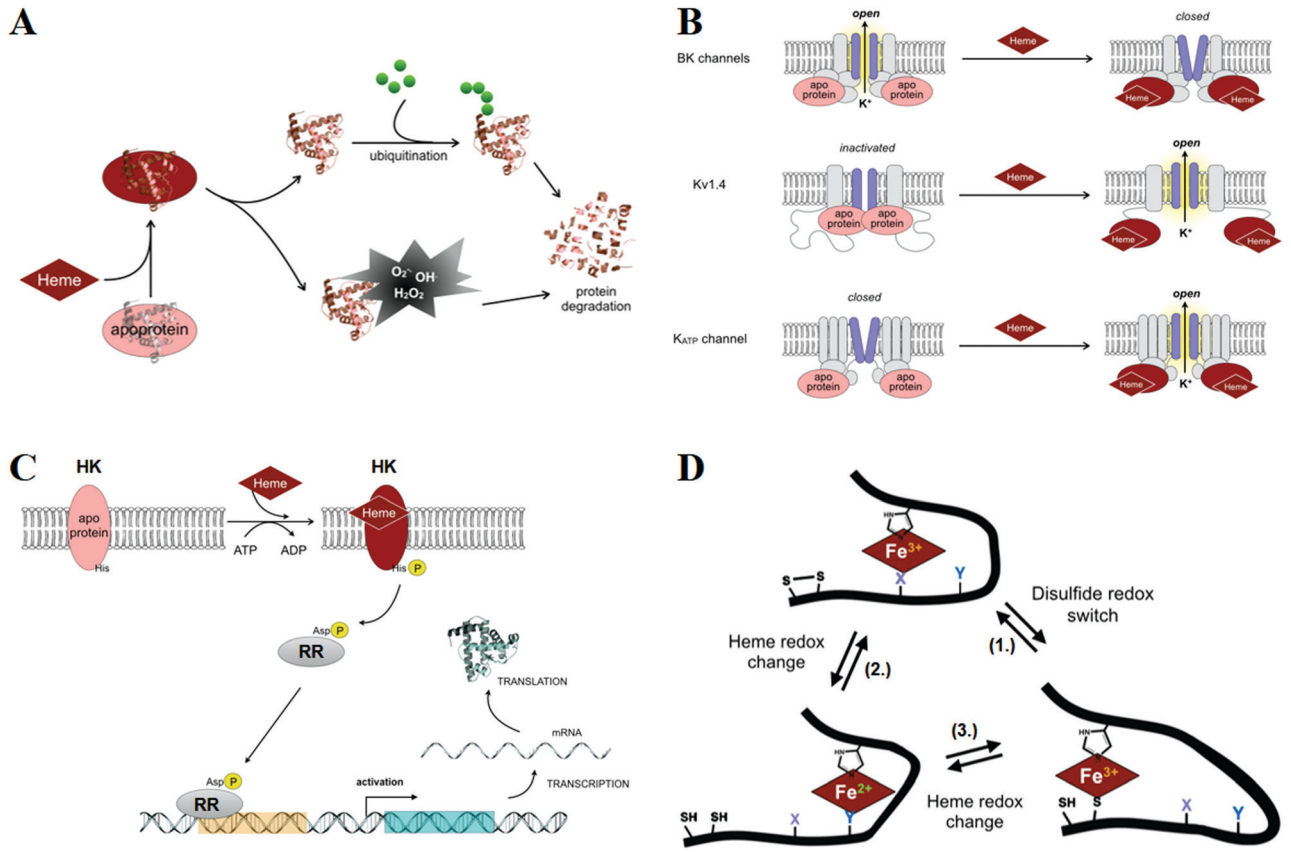

Fig. 4 (A) Hemin binding regulates protein degradation: IRP2, ALAS1, N-end rule pathway systems, [Bach1], [Rev-erbβ], [Per2], and Irr. For IRP2, hemin triggers ubiquitination, leading to proteasome-dependent degradation (upper route); for Irr, hemin triggers formation of ROS, leading to protein degradation (lower route). (B) Hemin binding regulates K^+ channel function: BK channels, Kv1.4 channels, and K_{ATP} channels. In the case of Kv1.4 and K_{ATP} channels, hemin binding activates the channels by causing their opening; in the case of BK channels, hemin binding deactivates the channel by decreasing the frequency of channel opening. (C) Hemin binding regulates TCSs: HssS-HssR, ChrS-ChrA, Fre-MsrQ-MsrP, MA4561, and NtrY–NtrX. Hemin triggers autophosphorylation of histidine kinase (HK), then the activated HK transfers the phosphate group to Asp residues of a response regulator (RR), which further interacts with a transcriptional activator, leading to activation of transcription. (D) Hemin binding regulates heme-redox sensors: [MA4561], [NtrY–NtrX], Tll0287, and All4978. A thiol/disulphide redox switch (1) regulates heme affinity/redox and/or heme redox-mediated ligand switching (2), subsequently leading to heme degradation (HO-2) or transcriptional regulation (Rev-erbβ). Reduction of hemin to heme Fe(II) (3) changes the heme coordination structure and triggers various physiological functions, including autophosphorylation (MA4561, NtrY–NtrX), H_2S oxidation (Tll0287), and DNA binding (All4978) (see Fig. 2).

repressor, CUP9. Hemin binds UBR1 at a site different from the CUP9 binding site and thus allosterically blocks its interaction with CUP9, resulting in inhibition of protease reactions of CUP9. Thus, hemin acts as both a "stoichiometric" and "catalytic" down-regulator of the N-end rule pathway. The molecular mechanism of the hemin-induced degradation of arginyl-transferase remains to be elucidated.

As noted above, the hemin-regulated mammalian transcriptional factor, Bach1, is degraded after hemin-bound Bach1 interacts with the ubiquitin-protein ligase, HOIL-1, in a hemin-dependent manner.

Rev-erbβ is a heme-binding transcriptional regulatory protein, as described above (see Section 2.2).[56,58–62] Hemin binding to Rev-erbβ triggers its proteasome-mediated degradation *in vivo* and indirectly regulates its interaction with the partner protein NCoR1, probably resulting in a decrease in the repressor activity of the Rev-erbβ–NCoR1 complex.[59,60]

Per2 is a transcriptional regulator associated with circadian rhythms, as described above (see Section 2.2). Hemin binding to the first of two CP motifs in the C-terminal domain of human PER2 triggers ubiquitination-dependent degradation of PER2 in the absence of interaction with CRY.[108] However, when human PER2 first interacts with CRY, hemin or heme Fe(II) is bound to the PAS domain at the C-terminal side of PER2, leading to a stable complex. A model of this process proposes that heme-mediated oxidation triggers human PER2 degradation, thereby controlling heterodimerization and ultimately gene transcription.[108] The tumor-suppressor p53 is a heme-responsive sensor, as described above. The spatiotemporal organization of PER2–p53 interactions and the nature of their chemical modifications are critical for their time-dependent subcellular redistribution and potential biological functions.[97] Involvement of the heme iron complex in these interactions is anticipated.

Irr from the nitrogen-fixing bacterium, *Bradyrhizobium japonicum*, is a key transcriptional regulator of iron homeostasis that binds to target genes and represses the translation of genes encoding enzymes involved in heme biosynthesis.[138–140]

In the presence of iron or hemin, Irr is degraded, thereby initiating the transcription of target genes. Irr has two heme binding sites, one with a 5-coordinated, Cys29 (CP motif)-bound high-spin complex, and the other with a 6-coodinated bis His (in a His cluster)-bound low-spin complex. Hemin binding to Irr and subsequent hemin degradation trigger Irr protein degradation. Irr protein degradation triggered by hemin proceeds according to the following steps: (1) hemin binding to the two hemin-binding sites, (2) reduction of hemin to heme Fe(II), (3) O_2 binding to heme Fe(II), (4) H_2O_2 generation at heme Fe(II) binding sites, (5) heme degradation and free iron release, (6) ''active site conversion'' from heme iron to non-heme iron, (7) H_2O_2 activation to generate ROS such as $^{\bullet}OH$, and (8) oxidative protein modification at the iron binding site.[139] This heme-dependent Irr protein degradation mechanism is significantly different from that of IRP2 in which protein degradation is mediated by the ubiquitin-proteasome system.[131]

2.4. Heme-responsive sensors involved in K^+ channel function: BK channels, K_V1.4 channels and K_{ATP} channels (Fig. 4B and Table 2)

The large-conductance Ca^{2+}- and voltage-activated K^+ channel (BK channel) complex contains four Slo1 (KCNMA1) subunits, each of which possesses the putative transmembrane segments, S0–S6.[154] Human and rat Slo1 BK channels bind both hemin and heme Fe(II), which profoundly inhibit transmembrane K^+ currents by decreasing the frequency of channel opening.[48] The affinity of hemin for these channels is high, with IC_{50} values of 45–120 nM. The inhibition by hemin persists at higher, more physiological concentrations of Ca^{2+}. Hemin appears to bind to the heme-binding motif, CXXCH (specifically, C612KACH616), located between cytoplasmic putative RCK1 and RCK2 (regulators of conductance for K^+) subdomains near the C-terminus. His616 is probably the heme axial ligand. It has been suggested that hemin binding to the channel interferes with allosteric interactions among the channel's gate, voltage sensors and Ca^{2+}-binding segments.[154] However, it is still puzzling how heme Fe(II) interacts with BK channels, since the coordination structure for heme Fe(II) is, in general, significantly different from that for hemin.

Heme binding studies have been conducted for an isolated, putative heme-binding domain (HBD; residues 584–717) that forms a linker segment between RCK1 and RCK2.[49] This study focused on the control of HBD affinity for heme by a thiol/disulphide redox switch, as described above for Rev-erbβ[58–60] and below for HO2.[73,149] The HBD contains a CXXCH motif in which His616 serves as the axial ligand; the two Cys residues in the Cys612XXCys615His616 motif can form a reversible thiol/disulphide (Cys612–Cys615) redox switch. The reduced dithiol state binds hemin ($K_d \approx 210$ nM) ~14-fold more tightly than the oxidized disulphide state, suggesting that a thiol/disulphide redox switch system also operates in the BK channel system. The HBD was shown to tightly bind CO ($K_d \approx 50$ nM) with the Cys residues in the CXXCH motif regulating the affinity of HBD for CO. The normoxic/hypoxic-dependent affinity of heme and CO for HBD were rationalized based on an intrinsic mechanical interaction between the human BK channel and HO2.[49,155] Relationships between CO binding and hemin will be discussed later (see Section 2.9).

Inactivation of the K_V1.4 channel, an A-type voltage-gated K^+ channel, is mediated by a ''ball-and-chain'' mechanism in which the distal N-terminal structure (ball) occludes the ion permeation pathway.[50] K_V1.4 channels are potently regulated by intracellular free hemin. Hemin binds to the N-terminal ball-and-chain inactivation domain and thereby impairs the inactivation process, thus enhancing K^+ currents with an apparent EC_{50} value of ~20 nM. The ball-and-chain domain contains a heme-responsive binding motif involving Cys13XXHis16 and a secondary His35. The N-terminus containing the heme-binding motif is predicted to be highly flexible and disordered, whereas the helix containing His35 is well defined. Binding of hemin to the N-terminal domain through formation of bis His coordination (His16/His35) might reduce the flexibility of the ball-and-chain machinery and induce a partial secondary structure that would make it impossible for the peptides comprising the ball to reach the channel's cavity.[50]

Members of the K_{ATP} family of ion channels respond to intracellular ATP and play a pivotal role in linking cellular metabolism to excitability. Functions of cardiac K_{ATP} channels, which regulate the excitability of cardiac ventricular myocytes, are regulated by hemin.[51] Hemin binds a cytoplasmic Cys628XXHis(X_{16})His648 motif on the sulphonylurea receptor subunit (SUR2A) of the K_{ATP} channel by forming a Cys/His complex and increases cardiac K_{ATP} single-channel activity. The hemin-binding motif is located in an unstructured region between the first transmembrane domain and the first nucleotide-binding domain. Spectral evidence that the heme-binding form is composed of a 5-coordinated high-spin complex and a 6-coordinated low-spin complex suggests the flexibility or intrinsic mobility of the heme binding site or the protein structure. His648 is a potential ligand for the low-spin hemin complex, whereas spectroscopic data for the Cys628 variant do not unambiguously confirm this residue as a ligand for the low-spin heme species. CO activates K_{ATP} channels, and hemin binding to the Cys628XXHis(X_{16})His648 motif in SUR2A is required for the CO-dependent increase in channel activity.[141] Molecular mechanisms of this CO-induced function will be discussed later (see Section 2.9).

2.5. Heme involvement in two-component signal transduction systems: HssS-HssR, ChrS-ChrA, Fre-MsrQ-MsrP, MA4561 and NtrY–NtrX (Fig. 4C and Table 2)

Organisms constantly sense and respond to extracellular signals so as to adapt to environmental changes and survive. For this, bacteria employ a large number of two-component signal transduction systems (TCSs), which consist of paired sensor and response regulator (RR) proteins specific to different stimuli.[156,157] The sensor protein of a typical TCS is a histine kinase (HK), most often membrane-bound,[158,159] that autophosphorylates a His residue at the C-terminal domain upon stimulation and then transfers its phosphate group to its connate RR. The sensing mechanism should be coupled with the autophosphorylation from ATP and the phospho-transfer reaction from HK and RR.

Heme-based gas (O_2, NO)-sensor kinase TCSs, such as FixL and *Af*GcHK, have been reported.[38] In addition to these gas sensors, several TCSs are reported in which hemin binding or the heme redox state regulates HK function.[158]

Hemin is toxic at high concentrations and kills bacteria. On the other hand, the human pathogen *S. aureus* requires hemin as a vital source of nutrient iron during infection. To maintain cellular heme homeostasis, *S. aureus* employs the coordinated actions of the heme-sensing two-component heme-responsive sensor system, HssS-HssR, and the heme-regulated transporter efflux pump, HrtAB.[142] Binding of extracellular hemin to the heme-responsive sensor, HssS, causes autophosphorylation (activation) of a bacterial membrane-localized HK, which transfers its phosphate group to the cognate RR, HssR. Phosphorylated HssR then binds to a direct-repeat DNA sequence within the heme-regulated transporter (hrt) efflux pump promoter genes, *hrtA* and *hrtB*, leading to expression and translation of the heme efflux pump proteins, HrtA and HrtB. Therefore, HssS-HssR dependent expression of HrtA and HrtB results in the alleviation of heme toxicity and tempered staphylococcal virulence. Since a role for bacterial NOS in bacterial survival in the host has been noted, NO synthesis and heme sensing are intertwined in *Staphylococci*.[143]

The pathogen *Corynebacterium diphtheria* employs a TCS composed of the heme-sensing kinase, ChrS, and the cognate RR, ChrA, to regulate degradation and transport of heme.[145] ChrA consists of an N-terminal regulatory domain, a long linker region, and a C-terminal DNA-binding domain. When ChrS protein is incorporated into proteoliposomes, it catalyzes hemin-dependent autophosphorylation by ATP.[144] The phosphorylated and activated ChrA specifically binds to either *hmuO* or *hrtAB* promoter regions to promote the transcription of genes encoding heme oxygenase and the ABC-type heme exporter, respectively. The crystal structure of ChrA revealed that the structural flexibility of the linker could be an important feature in rearranging the domain orientation to create a dimerization interface that binds DNA during heme-sensing signal transduction.[145]

Fre-MsrQ-MsrP, a methionine sulphoxide reductase system found in *Escherichia coli*,[146] appears to be specifically involved in repairing the periplasmic protein, MsrP, for example following oxidation by hypochlorous acid (HOCl). Ferric reductase (Fre) is a cytosolic NAD(P)H flavin reductase and a potential soluble physiological dehydrogenase partner for MsrQ that delivers electrons to MrsQ. MsrQ is an integral membrane-spanning, b-type heme protein containing two b-type hemes, coordinated through His residues, that acts as a specific electron donor for MsrP. MsrP, a periplasmic protein, is a methionine sulphoxide reductase that shows structural similarities to the sulphide oxidase molybdenum-enzyme family. Thus, Fre and MsrQ proteins form a prokaryotic TCS for electron transfer through the membrane that could be structurally related to the eukaryotic NADPH oxidase system.[146] This system is not a typical heme-responsive sensor, but is a new heme-mediated two-component electron transfer system containing methionine sulphoxide reductase. Further detailed studies remain to be done in order to establish the molecular mechanism.

The protein MA4561 from the methanogenic archaeon *Methanosarcina acetivorans* contains a heme iron complex that is covalently attached to Cys656 *via* a vinyl side chain in the second GAF domain,[79] marking the first report of a covalently attached heme cofactor in a cytoplasmic sensor protein. MA4561 containing hemin possesses strong autophosphorylation activity, whereas MA4561 containing heme Fe(II) does not show any such activity. It has been suggested that heme is bound to a large pocket in the protein that enables imidazole and dimethyl sulphoxide to bind to the heme iron complex. The intrinsic coordination structure of the heme is heterogeneous; thus, a clear identification of the axial ligands has not been feasible. Moreover, potential phosphorylated residues in the protein have not been identified. Given its genomic localization, it has been suggested that MA4561 is a redox-sensor kinase component of a TCS that affects the regulation of a set of three homologous corrinoid/methyltransferase fusion protein isoforms involved in methyl sulphide metabolism.[79] Further detailed studies remain to be done in order to establish the molecular mechanism.

The NtrY–NtrX TCS of *Brucella* spp. acts as a redox sensor to regulate the expression of the nitrogen respiration system.[80] *Brucella* spp. are facultative intracellular bacteria that are pathogenic for many mammalian species, including humans, causing a disease called brucellosis. The bacterium contains the TCS, NtrY–NtrX, in which hemin is bound to the PAS domain of the NtrY HK and a phosphate group in a His residue of the kinase domain is transferred to the cognate RR, NtrX. NtrY containing heme Fe(II) exhibits autophosphorylation activity, whereas that containing hemin does not. NO or CO binding to the enzyme containing heme Fe(II) does not change catalytic activity.

2.6. Heme redox sensors: [MA4561], [NtrY–NtrX], Tl10287, [All4978] (Table 2)

It has been suggested that some heme-responsive sensors are heme-redox sensors. Generally speaking, the coordination structure of the heme iron complex and the protein structures of the heme binding site are substantially changed upon reduction of hemin to heme Fe(II) (Fig. 1). Accordingly, the functions of heme-responsive sensors that respond to hemin would be significantly changed or abolished, when hemin is reduced to heme Fe(II). Thus, it would be reasonable to suggest that the functions of heme-responsive sensors should overlap with those of heme-redox sensors.

As shown above, MA4561 contains a covalently-attached heme iron complex and is suggested to be the redox-heme sensor kinase of a TCS[79] (third reaction in Fig. 1C and 4C). MA4561 containing hemin autophosphorylates, whereas MA4561 containing heme Fe(II) does not, suggesting that this enzyme is a heme-based redox sensor.

As described above (Fig. 4C), the heme-bound kinase, NtrY, of the *Brucella* NtrY–NtrX TCS system is a heme redox sensor in that NtrY containing heme Fe(II) exerts autophosphorylation activity, whereas that containing hemin does not.[80] NO and CO binding to NtrY containing heme Fe(II) does not alter NtrY

activity. O_2 binding to NtrY containing heme Fe(II) rapidly oxidizes the heme iron and generates NtrY containing hemin, ruling out the possibility that NtrY is a heme-based O_2 sensor.

Hemin binds to Tll0287, which has a CXXCH motif characteristic of *c*-type cytochromes (third reaction in Fig. 1C), from the thermophilic cyanobacterium *Thermosynechococcus elongatus* in which Cys68 and His145 in the PAS domain serve as the axial ligands.[81] The redox potential at pH values greater than 7.5 (−255 mV) dramatically increases to 57 mV at lower pH values, suggesting the involvement of a protonatable group with a $pK_{red} \approx 7.2$. It has been suggested that Tll0287 acts as a redox sensor under microaerobic conditions or as a cytochrome subunit of an H_2S-oxidizing system.[81]

As described above and shown in Table 1, hemin binds to All4978 and facilitates binding of its helix-turn-helix motif to DNA.[78] Reduction of hemin to heme Fe(II) abolishes DNA binding, suggesting that All4978 is not only a heme-responsive sensor associated with transcriptional regulation, but also a heme redox sensor.[78]

Although other heme-redox sensors containing a covalently bound *c*-type heme, such as GSU582/GSU935, DcrA and insect E75, have been reported (third reaction in Fig. 1C), their intrinsic functions in relation to redox or electron transfer reactions are not well understood.[38]

2.7. PGRMC1: a heme-responsive sensor involved in protein–protein interactions (Table 2)

PGRMC1 (progesterone receptor membrane component 1), a member of the membrane-associated progesterone receptor family, contains an N-terminal transmembrane domain and a putative C-terminal cytochrome b_5 domain.[65,66,147] Apo-PGRMC1 exists as an inactive monomer. On binding to hemin, PGRMC1 forms a stable dimer through stacking interactions between the two protruding heme moieties. The hemin is 5-coordinated with Tyr113 as the proximal axial ligand, and the open surface of the heme mediates dimerization[66] (fourth reaction in Fig. 1C). Hemin binding to PGRMC1 enables the protein to interact with the epidermal growth factor receptor (EGFR) and cytochrome P450 enzymes, leading to enhanced proliferation and chemo-resistance of cancer cells. Binding of progesterone to hemin-bound PGRMC1 has also been suggested,[65] and CO binding to heme Fe(II) significantly decreases these functions.[66] However, heme coordination structures and the heme binding environment of heme Fe(II)-bound PGRMC1 are expected to be significantly different from those of hemin-bound PGRMC1. Therefore, there is a reason to doubt that CO acts through CO-sensor functions to affect PGRMC1, as discussed later (see Section 2.9).

On the other hand, the redox potential of heme-bound human PGRMC1 is very low—as low as −331 mV.[65] The axial ligand of many heme (hemin) transfer proteins is a Tyr residue, reflecting the fact that tyrosine as the axial ligand for hemin, similar to that for many heme-transfer proteins, would play a significant role in decreasing the redox potential so as to avoid heme reduction, which hampers the heme (hemin) transfer reaction.[27,29] The low redox potential of the heme-bound PGRMC1, taken together with several lines of experimental evidence, has led to a number of suggested potential roles of PGRMC1. These include the possibility that PGRMC1 serves as (1) a heme chaperone to deliver newly synthesized heme to hemoproteins in different cellular locations, (2) a heme-responsive sensor that directly interacts with ferrochelatase and decreases ferrochelatase activity, (3) a heme-responsive sensor that regulates endosomal trafficking of iron to the mitochondria for heme synthesis, and (4) a heme-responsive sensor that regulates localization of the mitochondrial heme biosynthesis complex to inner and outer membrane junction points.[147]

2.8. HO2: a hemin-degrading enzyme containing a redox switch at two CP motifs, which act as a redox sensor to regulate the affinity of hemin (Fig. 4D and Table 2)

Heme oxygenase (HO), the only known mammalian enzyme capable of degrading heme, is a key player in heme homeostasis that catalyzes the conversion of hemin to biliverdin, CO, and free ions.[73,148–152] Mammals contain two isoforms of this enzyme, HO1 and HO2, which share the same α-helical fold that forms the catalytic core and heme-binding site. Unlike HO1, HO2 contains three CP motifs: one CP motif includes Cys127, and the other two CP motifs include Cys265 and Cys282 near the C-terminus. These latter two CP motifs are located at positions distinct from the heme-binding site, His45, which serves as the axial ligand on the proximal side where heme degradation occurs at the catalytic core. They also act as a thiol/disulphide redox switch to regulate hemin binding. For the oxidized form of HO2, in which two thiolates form the disulfide bond S(Cys265)–S(Cys282), heme Fe(III) binds only at the catalytic core with high affinity ($K_d \approx 0.014$ μM; His45/H_2O). On the other hand, for the reduced form of HO2 in which two free Cys residues are formed, two heme Fe(III) bind with lower affinities to regulatory sites in the protein different from the catalytic core (His256/Cys265, $K_d \approx 0.09$ μM; Cys282, $K_d \approx 0.9$ μM).[73,149]

Both K_d values (0.09 μM and 0.9 μM) of heme for HO2 under reducing conditions are higher than the normal concentration[17] of labile heme in the cytoplasm of most cells (0.02–0.04 μM); thus, the disulphide switch of HO2 responds to cellular oxidative stress and reducing conditions.[148] Upon reduction of the C-terminal disulphide bond (Cys265 and Cys282) to free thiolates, the two Cys residues become available to ligate heme, suggesting that the CP motifs act as a redox sensor.[149,150] However, heme bound to the C-terminus is not a substrate of HO2; thus, only heme bound to His45 in the N-terminal catalytic site is a substrate of HO.[151] Spectroscopic studies have revealed that the heme regulatory motif of HO2 (containing a disulphide bond) is dynamically disordered under oxidative conditions and lacks heme binding, whereas under reducing conditions, the C-terminal region gains some structure in association with heme binding.[150] Note again that heme-regulatory CP motifs are located in an intrinsically disordered region. The main importance of the S–S bond is that the two Cys residues in the motif are involved in heme binding. Therefore, formation of the S–S bond causes a loss of the Cys ligand and destabilizes heme binding to the two heme-regulatory CP motifs.[152] The disordered structure of the hemin binding site of HO2 is similar to that of Bach1, as described above. Functional

regulation through thiol/disulphide switches acting as redox sensors is also found in other motifs, such as the CXXC motif in the heme-binding domain of FurA[99] and the BK channel,[49] as described above.

2.9. Unresolved issues of heme-responsive sensors with a CO-sensing function or *vice versa*: NPAS2, CLOCK-BMAL1, E75, Rev-erbα, Rev-erbβ, K^+ channels, PGRMC1, and sGC/PKG

The heme iron complex serves as the sensing site for gaseous molecules (O_2, NO and CO) in gas sensors.[38] "CO sensor" is a very attractive term for both physiologists and pathologists. Numerous papers about CO sensors have been reported.[38] But the molecular mechanism of CO sensing is controversial, and several issues related to its biological chemistry remain unresolved.

For heme-responsive sensors, association/dissociation of hemin to/from the heme binding site of the sensor proteins regulates various important functions, such as transcription, DNA binding, tRNA synthesis, microRNA processing, protein translation, protein degradation, heme degradation, K^+ channel activity, autophosphorylation and redox switching, among others (Table 1). In contrast, for CO sensors, CO binding to heme Fe(II) bound to the heme-responsive sensor further up- or down-regulates similar functions already observed for the heme-responsive sensor or allows new functions to be switched on. Importantly, CO binds only to heme Fe(II), and never binds hemin.[160–162]

Most CO sensors hitherto reported are suggested to be both heme-responsive sensors and heme-based CO sensors. The exceptions include heme-based CO sensors such as CooA, RcoM and CBS, in which the heme iron complex is constitutively bound to the heme binding domain/site through distinct coordination, and CO binding to the heme Fe(II) complex regulates functions.

CO binds only to heme Fe(II), forming a stable heme Fe(II)–CO complex. This characteristic of CO is in contrast to that of O_2[39,163–165] and NO.[41,166–178] Specifically, O_2 binds only to heme Fe(II), forming a heme Fe(II)–O_2 complex, but the heme Fe(II)–O_2 complex is often oxidized and converted into hemin through generation of ROS, such as superoxide.[38–40] ROS further interact with amino acid residues on the protein surface. NO binds to both hemin and heme Fe(II), forming the corresponding NO complexes. Additionally, NO and its oxidative derivatives further interact with amino acid residues on the protein surface and regulate numerous reactions.[38] Thus, there is rather straightforward rationale for understanding the role of CO in CO sensing within heme-responsive sensors.

The coordination structure of hemin bound to proteins differs significantly from that of heme Fe(II) bound to the same proteins. In particular, many heme-responsive sensors, though not all of them, have a cysteine or CP motif as the hemin binding/sensing site. Usually, thiolate residues that serve as the axial ligand or binding/sensing site for hemin are converted to His or other amino acid residues when hemin is reduced to heme Fe(II). This is partly because of ionic repulsion between the anionic character of the thiolate residue and the less positive character of heme Fe(II) compared with hemin. Thus, it is possible that heme redox changes in heme-responsive sensors are accompanied by significant changes in the protein structure surrounding the heme. Those redox-dependent heme coordination or protein structural changes should occur before CO is bound to the heme Fe(II) in the heme-responsive sensor. In the case of the RNA-binding protein, GDCR8, redox-dependent changes in the coordination structure of the heme iron complex that accompany the loss of cysteines as axial ligands abolish its function.[119]

The presence of a reductant/reductase (or oxidant/oxidase) poses significant issues for the conversion from hemin to heme Fe(II) as well as changes in coordination and protein structure caused by the conversion from hemin to heme Fe(II), even in the absence of CO. Studies reported to date have provided circumstantial evidence that fails to unequivocally demonstrate that heme-responsive sensors are simultaneously heme-based CO sensors.

Let us consider each heme-responsive sensor with CO-sensing characteristics in turn.

NPAS2. NPAS2 is a transcriptional regulatory protein associated with circadian rhythms, as described above in Section 2.2.1 (Table 1). The heterodimer of NPAS2, both hemin-free and hemin-bound, with BMAL1 binds to DNA in the presence of 3–5 mM NADPH.[83] Importantly, NADPH alone facilitates the DNA-binding ability of the NPAS2–BMAL1 heterodimer, even in the absence of hemin, suggesting that hemin is not a prerequisite for DNA binding of the heterodimer.[87,127] Addition of CO to the hemin-bound NPAS2–BMAL1 heterodimer in the presence of NADPH under anaerobic conditions abolishes the DNA-binding ability of the heterodimer.[83] CO binds only to heme Fe(II), and not to hemin, thus NADPH might help heme reduction. Mutations at the binding site of heme Fe(II) abolish the DNA-binding ability of the heterodimer as a result of cleavage of the NPAS2–BMAL1 heterodimer, suggesting that heme Fe(II) binding or the protein structure of the heme Fe(II)-bound pocket is important for these functions.[86] In contrast, mutations at the hemin binding site do not alter heterodimer formation or DNA-binding ability, suggesting that hemin is not important for these functions.

Taken together, these observations indicate that NADPH, but not hemin, is required for NPAS2–BMAL heterodimer formation and the DNA binding of the heterodimer. If it is assumed that heme iron is involved in heterodimer formation and DNA-binding ability, then hemin, which is bound to the NPAS2–BMAL1 heterodimer, would be reduced to heme Fe(II) with the aid of CO in the presence of NADPH under anaerobic conditions. CO-Stimulated heme reduction in the presence of electron donors has been reported.[179] Redox changes in the heme iron cause striking changes in heme coordination and accompanying protein structures in the heme-binding pocket,[52] leading to heterodimer dissociation and loss of DNA-binding ability[86] prior to CO binding to heme Fe(II). Unfortunately, however, spectral changes caused by adding CO to the heterodimer solution in the presence of NADPH under anaerobic conditions have not been reported, although spectral changes caused by adding CO to the heterodimer containing dithionite-reduced

heme Fe(II) have been observed.[83] Therefore, the NPAS2–BMAL1 system is unlikely a prototypical CO sensor.

Chelation of endogenous CO in mice by intraperitoneal administration of a selective CO scavenging agent promotes the binding of NPAS2 and CLOCK to E-box sites in DNA in the murine liver and causes disruption of circadian rhythms.[180] However, when CO is purposely removed from the cells or body, the heme-degrading enzyme, HO1, is abruptly overexpressed in cells, serving to compensate for the diminished intracellular CO concentration. Thus, free hemin (a substrate of HO1), biliverdin and free Fe(II) (products of HO1), the concentration of NADPH (an essential cofactor for HO1 catalysis), and ROS (byproducts of uncoupled HO1 reactions) play significant roles in the effects of the CO scavenging system on circadian rhythms. Thus, the possibility that effects of these species account for the disruption in circadian rhythm caused by the CO scavenger cannot be ruled out.

CLOCK–BMAL1. HO1 depletion globally alters CLOCK-controlled transcription in hepatocytes.[181] CO, a heme-degradation product, suppresses CLOCK–BMAL1 binding and transactivation of target genes. CLOCK binds both hemin and heme Fe(II) (Section 2.2.3, Table 1).[111] Since CO binds to heme Fe(II), it is reasonable to suggest that heme Fe(II) is involved in those functions that are associated with circadian rhythms. However, questions similar to those that arose with respect to NPAS2 remain to be addressed. Specifically, how is hemin converted into heme Fe(II)? How are CO, hemin and heme Fe(II) involved in CLOCK–BMAL1 heterodimer formation? And how does the heterodimer interact with DNA?

E75. It has been claimed that the heme-binding nuclear receptor E75 from *Drosophila* is a gas (NO or CO) sensor,[103] as described above in Section 2.2.3 (Table 1). E75 containing heme Fe(II), but not hemin, interacts with the peptide HR3 AF2 derived from a second nuclear receptor, DHR3. Addition of CO to E75 containing heme Fe(II) stabilizes the protein, as evidenced by an increase in its transition denaturation temperature. Interestingly, however, addition of HR3 AF2 to E75 containing heme Fe(II) has no further effect on E75 stability beyond the effect of CO alone. This lack of an additional effect suggests that E75 containing heme Fe(II) cannot interact with DHR3 in the presence of CO. Thus, although CO stabilizes the E75 protein, it appears to prevent binding of the DHR3-derived peptide. However, the authors of this study did not assess the effects of CO on transcriptional activity. Thus, these findings do not provide direct evidence that E75 is a heme-based CO sensor, since they only showed that the protein–peptide interacts with heme Fe(II)–CO. In an interesting study, the same group reported a significant effect of NO on the function of E75, although the involvement of heme and CO in function was not described.[182]

Rev-erbα and Rev-erbβ. Hemin and heme Fe(II) bind to the human nuclear receptors Rev-erbα and Rev-erbβ[62] both of which are suggested to be heme-responsive sensors (see Section 2.2.2; Table 1). NO and CO bind to the heme Fe(II) incorporated in Rev-erb proteins, suggesting that Rev-erb proteins are heme-based NO and CO sensors. However, it has been found that both Rev-erb proteins are transcriptional repressors whose activities are reversed by NO binding; by contrast, the effects of CO are marginal. Transcriptional repression by Rev-erb proteins is augmented by binding NCoR, but this repression too is reversed by NO. Interactions of Rev-erb proteins with NCoR peptides, derived from the interaction interface of Rev-erb and NCoR proteins, are weakened by hemin, an effect that is mitigated by NO. Here again, there is no direct evidence that Rev-erb proteins are heme-based CO sensors, although effects of NO on the transcriptional activity of these proteins and on Rev-erb–NCoR protein interactions are clear.

In a separate work, it was suggested that a thiol-disulphide switch between Cys374 and Cys384 controls the interaction of heme Fe(III) (bound *via* Cys384/His568) with the heme-binding domain of Rev-erbβ.[58] The CO affinity for heme Fe(II) of the reduced switched state of Rev-erbβ was found to be very high ($K_d \approx 60$ nM). Nevertheless, the authors of this report did not suggest that Rev-erbβ is a heme-based CO sensor.

K^+ channels. Ca^{2+}-Dependent K^+ channels have been reported to be heme-responsive sensors, such that binding of either hemin or heme Fe(II) to human Slo1 BK channels profoundly inhibits transmembrane K^+ currents,[48] as described above in Section 2.4 (Table 2). It has further been reported that HO2 is part of the BK channel complex and enhances channel activity under aerobic conditions. HO2 knockdown reduces channel activity, but CO, a product of HO2, rescues this loss of function.[155] Although these authors showed that CO binds to heme Fe(II), they did not propose that the K^+ channel is a combined heme-responsive sensor and heme-based CO sensor; instead, they carefully avoided referring to the possibility of the involvement of heme iron in the functional inhibition by CO by suggesting that HO2 is an O_2 sensor.[155] There is clearly no direct evidence that the heme-bound K^+ channel is a heme-based CO sensor. Under aerobic conditions, heme is degraded into Fe(II), CO, and biliverdin with the aid of HO2, but left unanswered is the question of how CO binds to heme Fe(II) when the heme iron complex is degraded by HO2. Moreover, the authors of this paper did not discuss the role of heme Fe(II) in CO-associated functions or how hemin is reduced to heme Fe(II).

A group led by Hoshi, which first reported the important role of heme iron in the function of Slo1 BK channels,[48] examined the role of CO in the function of the same channel.[183] They reported that CO reliably and repeatedly activates Slo1 BK channels in excised membrane patches in the absence of Ca^{2+} in a voltage-sensor-independent manner. The stimulatory action of CO on the Slo1 BK channel requires an aspartate and two His residues located in the cytoplasmic RCK domain. Taking into account the involvement of heme iron, they examined the effects of mutations at the putative heme binding site, Cys612-Lys-Ala-Cys-His616, on CO-induced functional enhancement, but found no such effects. In addition, the current-enhancing effect of CO was completely absent when the intracellular Ca^{2+} concentration was increased to 120 μM, suggesting that the effect of CO is dependent on the Ca^{2+} concentration. Mutations of His365, Asp367, and/or His394 at the putative Ca^{2+} binding site abolished the CO sensitivity of the channel, but failed to alter the sensitivity of the channel to hemin. These results suggest that CO-induced

functional enhancement is mechanically different or exhibits distinct local features compared with heme-induced functional inhibition.

Yi *et al.*[49] identified a thiol/disulphide redox switch in the human BK channel that controls its affinity for heme and CO. The heme-binding domain of the BK channel has a Cys612XXCys615His motif in which histidine serves at the axial heme ligand and the two Cys residues form a reversible thiol/disulphide redox switch that regulates affinity of this domain for heme. This heme-binding domain was shown to interact with HO2. The reduced thiol state binds heme Fe(III) with a K_d of ~210 nM, which is 14-fold more tightly than the oxidized disulphide state binds heme Fe(III). Furthermore, heme Fe(II) in the domain tightly binds CO ($K_d \approx 50$ nM). Accordingly, it was proposed that a thiol/disulphide switch in the heme-binding domain is a mechanism by which the activity of the BK channel can respond quickly and reversibly to changes in the redox state of the cell, especially as it switches between hypoxic and normoxic conditions. Nevertheless, it remains unclear how CO regulates the function of the channel.

Al-Owais *et al.*[184] reported that CO mediates the anti-apoptotic effects of HO1 in medulloblastoma DAOY cells through inhibition of K^+ channel activity. The voltage-gated rectifier K^+ channel, $K_V2.1$, plays a significant role in neuronal apoptosis by permitting the K^+ efflux necessary to initiate caspase activation. Induction of HO1 markedly increases the resistance of DAOY cells to oxidant-induced apoptosis. CO generated from CORM-2 (30 μM) was shown to inhibit voltage-gated K^+ currents in DAOY cells and largely reverse the oxidant-induced increase in K^+ channel activity. Thus, it was demonstrated that CO can protect central neurons against oxidative stress by inhibiting $K_V2.1$, thereby suppressing the pro-apoptotic effect of the loss of intracellular K^+. Nevertheless, it is again unclear how CO inhibits the function of $K_V2.1$ and whether heme Fe(II) is involved in the process.

CO activates K_{ATP} channels, and hemin binding to the Cys628XXHis631(X_{16})His648 motif of the SUR2A K_{ATP} channel subunit is required for the CO-dependent increase in channel activity, as described above in Section 2. Heme-responsive sensors (Table 2). The affinity of hemin for SUR2A, the kinetics of CO binding to heme Fe(II), and the resonance Raman spectrum of CO-heme Fe(II) bound SUR2A have been carefully examined, and the results themselves are not in doubt.[141] However, in these studies, hemin was reduced to heme Fe(II) in solution by adding sodium dithionite, but this does not automatically result in the reduction of hemin. It is not clear why or how hemin added to the K_{ATP} channel solution is converted into heme Fe(II), only that it accepts CO as its axial ligand. A spectroscopic study is needed to demonstrate that hemin bound to the K_{ATP} channel is converted into heme Fe(II) under certain conditions to generate CO-accepting heme Fe(II). Is a reductase or reducing agent needed for heme reduction? Or is it possible that CO facilitates the heme reduction?

At this point, an interesting and important proposal regarding the "heme-independent interplay between iron and CO in Slo1 BK channels" is worth noting.[185] In this critical review article, the author emphasized the importance of considering whether contaminating free iron in media used for overexpressing the channel plays a role in the CO-dependent functional regulation of the channel. The author claims that free iron is always present in an overexpressed, purified protein. CO repeatedly stimulates the human Slo1 BK channel, possibly by binding to an iron site. It has been suggested that CO acts as a small, strong iron (or Fe(II)) chelator that disrupts a putative iron bridge in ion channels, thereby tuning their activity. *In silico* structural models of Slo1 BK channels have been generated based on structures of the channel from human and *Aplysia californica*. It was assumed that in the human Slo1 BK channel, Fe(II) is located in a bowl containing the putative motifs, Trp524XXTyrTyr/Phe528 and Phe391XXHisPhe395, and further suggested that dynamic binding of His365/Asp367 to the first Fe(II) bowl or Gln970/Cys911 binding to the second Fe(II) bowl promotes channel closure. Accordingly, CO might act by outcompeting His365/Asp367 and Gln970/Cys911 for the first and second Fe(II) bowls to promote Ca^{2+}-independent and Ca^{2+}-dependent channel opening, respectively. Washout followed by CO stimulation was shown to reverse channel activity, indicating that the CO effect is repeatable. The author claimed that, because CO may target other metalloproteins to which transition metals are bound, the heme-independent regulation mode of CO may have significant and extensive implications for these metalloproteins.

On the assumption that the above-proposed role of free iron cations in functions impacted by CO is appropriate, we proposed an additional role of Fe(II) generated by constitutively expressed or overexpressed HO under aerobic conditions in the interaction with CO. The degradation products of heme by HO are free Fe(II), CO and biliverdin, the latter of which is further degraded to bilirubin, which has antioxidative properties. A number of papers have emphasized the critical effect of CO on important functions, making the claim that a protein acts as a CO sensor in cases where knockout of HO significantly up- or down-regulates these functions.[155] However, such effects do not rule out the idea that free Fe(II) generated from hemin plays a role in interacting with CO, implying that free Fe(II) binds to numerous enzymes, including K^+ channels, and that Fe(II) bound to the protein further rebinds CO and exerts numerous CO-dependent functions. Whereas overexpression of HO in cells generates free Fe(II), the endogenous HO transcription/expression system regulated by Bach1/MafK is accompanied by expression of the iron-capturing protein ferritin, and other iron-removing proteins with a high affinity for free Fe(II).[92] Under appropriate conditions, timely and synergistic expression of ferritin and other iron-removing proteins with HO might not occur, allowing cells to accumulate enough free Fe(II) for rebinding to proteins such as K^+ channels. Thus, free Fe(II) generated by HO, in addition to free iron present in culture medium, could significantly influence numerous CO effects that are interpreted as effects of CO and hemin (or heme Fe(II)).

PGRMC1. The crystal structure of the hemin-bound homodimeric form of PGRMC1 and the role of hemin-bound PGRMC1 in recruiting proteins that interact with PGRMC1

were described above in Section 2. Heme-responsive sensors (Table 2). In a study reporting that CO binding to hemin-bound PGRMC1 dimers disrupts protein–protein interactions, it was claimed that the binding of hemin is essential for PGRMC1 dimer formation and interactions with several proteins.[66] However, although CO binds only to heme Fe(II), no results related to protein–protein interactions for the heme Fe(II)-bound protein were reported. The spectral features of the heme Fe(II)-bound protein generated by adding sodium dithionate, a strong reductant, are significantly different from those of the hemin-bound protein. In addition, the gel-filtration chromatography pattern of the protein containing sodium dithionate-induced heme Fe(II) is significantly different from that containing hemin in terms of the background level. Thus, it is possible that the protein structure and function of the heme Fe(II)-bound form are already significantly different before addition of CO to the solution. Importantly, the redox potential of hemin-bound PGRMC1 is very low (−331 mV),[65] suggesting that the redox state of hemin-bound PGRMC1 is very stable and difficult to reduce to heme Fe(II) under aerobic conditions. Thus, it is not clear how CO *per se* influences the structure and function of PGRMC1.

sGC/PKG. Yuan *et al.*[186] reported that CO inhibits the catalysis of cystathionine-γ-lyase (CSE), a non-heme-binding protein, through activation of sGC and subsequent stimulation of protein kinase G (PKG)-dependent phosphorylation of Ser377 in CSE. It has additionally been reported that an exogenously added excess of CO (concentration not reported) activates sGC. However, the affinity of CO for sGC is very low ($K_d \approx 10^{-4}$ M) compared with that of NO ($K_d \approx 10^{-12}$ M),[187,188] and CO only enhances the catalytic activity of sGC by 4–5-fold[189] compared with the nearly 200-fold increase induced by NO.[168,169,173,190,191] Therefore, the effects of CO on these functions will require further investigations.

3. Heme-regulated inhibition and activation, and non-canonical heme active sites of heme proteins (Table 3)

3.1. Heme-regulated inhibition and activation

Labile hemin *per se* acts as an inhibitor or an activator of several catalytic functions, although the roles of hemin in inhibition/activation may not be strictly distinct from those of heme-responsive sensors described in Section 2. Heme-responsive sensors. As described in that previous section, the N-end rule pathway is involved in regulating the *in vivo* half-life of a protein based on the identity of its N-terminal residue.[136] Hemin inhibits arginyl-transferase through a redox mechanism that involves the formation of a disulphide bond between the enzyme's Cys71 and Cys72 residues in the CP motif (Cys71Cys72Pro73).[134] Hemin also interacts with yeast and mouse E3 ubiquitin ligases (UBR1) of the N-end rule pathway.

YybT family proteins contain an N-terminal PAS domain and a C-terminus composed of a DHH/DHHA1 phosphodiesterase (PDE) domain that exhibits PDE activity toward c-di-AMP, and a GGDEF domain that possesses weak ATPase activity.[192] Hemin binds to the PAS domain of YybT, forming a typical heme-based gas sensor in which the enzyme is composed of an N-terminal heme-binding domain and a C-terminal functional domain.[38] Interestingly, the heme-free form has high PDE activity toward c-di-AMP, with a k_{cat}/K_m value of 0.42 s^{-1} μM^{-1}, whereas the hemin-bound form has a 276-fold lower k_{cat}/K_m value (0.0015 s^{-1} μM^{-1}) than the heme-free form. YybT protein containing the heme Fe(II) complex has similarly low activity. However, YybT proteins containing a hemin–CN complex or heme Fe(II)–NO complex have 2–3-fold higher activity than those in a ligand-free form. This is a unique case in that heme binding significantly reduces the PDE activity of YybT, which has a domain structure typical of a heme-based sensor.[38,192]

Elevated hemin levels impair the control of bacterial proliferation independently of heme-iron acquisition by pathogens. Hemin strongly inhibits phagocytosis and the migration of human and mouse phagocytes by disrupting actin cytoskeletal dynamics through activation of the GTP-binding Rho family protein, Cdc42, by the guanine nucleotide exchange factor, DOCK8.[193] Details of the molecular mechanisms involved in this inhibition remain to be determined.

Heme binds to porphobilinogen deaminase from *Vibrio cholera* with a dissociation constant of 0.33 μM and decreases its activity by ∼15%.[194] Spectral data suggest that the heme in this protein is a mixture of 5- and 6-coordinated states. Mutational studies have shown that the axial ligand of the 5-coordinated heme is Cys105 (part of a non-CP motif) and the axial ligand for the 6-coordinated heme is His227. The crystal structure of this protein suggests that coordination of His227 to heme induces reorientation of the domain containing Cys105, leading to a decrease in catalysis.

The activity of aminolevulinic acid synthase (ALAS) is negatively regulated by hemin through repression of *ALAS* gene expression, degradation of ALAS mRNA, and inhibition of mitochondrial translocation of the mammalian precursor protein, as shown above (see Section 2.3). It has been found that hemin directly binds to His340 and Cys398 of ALAS from *Caulobacter crescentus* (*c*ALAS) and inhibits its activity by releasing the important coenzyme, pyridoxal phosphate (PLP), from the protein.[75]

Hemin has been described as a potent proinflammatory molecule that is able to induce multiple innate immune responses. One cellular response induced by hemin is the formation of p62/SQTM1 aggregates containing ubiquitinated proteins in structures known as aggresome-like induced structures (ALISs), which are ultimately degraded by autophagy.[195] Heme degradation by HO1 is required for ALIS formation; thus, the free Fe(II) ion released upon heme degradation is necessary and sufficient to induce ALIS formation. Moreover, ferritin, a key protein in iron metabolism, prevents excessive ALIS formation. Thus, hemolysis delivers a free heme iron complex, which promotes an increase in ALIS formation in target tissues. This action is part of a defensive response to the excessive generation of ROS induced by heme iron, driven by the transcription factor NRF2 (nuclear factor erythroid 2-related factor 2).[195]

Inflammation induces stress erythropoiesis through heme-dependent activation of the transcriptional factor SPI-C.

Table 3 Heme-regulated inhibition, activation and non-canonical heme active sites of heme proteins. Proteins in [] are already described above in Table 2

Name	Functions	Hemin-sensing/binding site	Hemin K_d or k_{off}	Partner	Origin	Ref.
[Arginyl-transferase]	Hemin inhibits activity (inhibition of arginylation within the N-end rule pathway of protein degradation)	5-Coordinated Cys71Cys72Pro73 (2nd of five CP motifs)		UBR1	Mouse, *S. cerevisiae*	134 and 136
[UBR1]	Hemin inhibits E3 ubiquitin ligase activity (inhibition of protease activity within the N-end rule pathway of protein degradation)			CUP9	Mouse, *S. cerevisiae*	134 and 136
YybT	Hemin inhibits PDE activity	Phe107 (in the PAS domain)			*S. aureus, Listeria monocytogenes, Bacillus subtilis, Geobacillus thermodenitrificans*	38 and 192
Cdc42	Hemin inhibits phagocytosis			DOCK8		193
Porpho-bilinogen deaminase	Hemin inhibits activity	5-Coordinated Cys105 (non-CP motif); 6-coordinated Cys105/His227	$K_d \approx 0.33$ μM		*V. cholerae*	194
ALAS	Hemin inhibits ALA synthesis	6-Coordinated His340/Cys398 (non-CP motif)			*C. crescentus*	75
ALIS	Hemin induces formation of p62/SQTM1 aggregates containing ubiquitinated proteins, aggresome-like induced structure (ALIS), which is degraded by autophagy in response to excessive generation of ROS formed by heme iron			NRF2		195
Inflammation	Erythrophagocytosis, heme-regulated transcriptional factor SPI-C				Mice	196
Fibrinogen	Hemin assists in diTyr cross-linking of fibrinogen, leading to blood coagulation					68
PgDps	Hemin tightly binds PgDps and protects DNA from ROS formed by hemin or heme Fe(II)	5-Coordinated Cys101 (non-CP motif)	$K_d \approx 0.037$ μM		*P. gingivalis*	22
Amyloid-β	Hemin suppresses amyloid-β-mediated inflammatory activation by reducing expression of proinflammatory cytokines				Mouse	197
Heme-DNAzyme	Hemin catalyzes carbene insertion into styrene					198
DOG1	Hemin regulates dormancy and germination of plants	6-Coordinated His245/His249		AHG1, PP2C	*Arabidopsis*	199
Oxd (aldoxime dehydratase: OxdA from *Pseudomonas chlororaphis* B23; OxdB from *Bacillus* sp. Oxd-1; OxdRE from *Rhodococcus* sp. N-771)	Heme Fe(II) is the catalytic centre of aldoximes; dehydrated to form R–C≡N	OxdA: 5-coordinated His299 (assisted with His320) OxdB: 5-coordinated His306 OxdRE: 5-coordinated His299			*Pseudomonas chlororaphis* B23, *Bacillus* sp. OxB-1, *Rhodococcus* sp. N-771	200–202
TsdA	c-Type dihemes, catalyze tetrathionate S–S bond formation	6-Coordinated Cys138/His99 and Met255/His207 (*C. jejuni*); 6-coordinated His53/Cys96 and His164/Lys208 (*A. vinosum*); Cys330 (putative) (*M. purpuratum*) (non-CP motifs)			*C. jejuni*, *A. vinosum*, *M. purpuratum*, *Cupriavidus metallidurans*, *Thiomonas intermedia*, *Sideroxydans lithotrophicus*	203–206
HZS	c-Type heme, catalyzes N–N bond formation in hydrazine synthesis	Tyr591			*Kuenenia stuttgartiensis*	207 and 208
KtzT	Hemin catalyzes N–N bond formation in the biosynthesis of piperazate	5-Coordinated His65			*Kutzneria* sp. 744	209
Z-ISO	Heme Fe(II) is involved in *cis–trans* isomerization of 9,15,9′-*cis*-ζ-carotene	6-Coordinated His150/Cys263 (non-CP motif); 6-coordinated His150/His266			*Zea mays* (plant)	210

Table 3 (*continued*)

Name	Functions	Hemin-sensing/binding site	Hemin K_d or k_{off} Partner	Origin	Ref.
STEAP3 and STEAP1 (ferric reductase)	Electron transfer *via* heme iron to reduce free Fe(III) to Fe(II)	6-Coordinated His316/His409 for STEAP3; 5-coordinated Cys57 (non-CP motif); 6-coordinated His175/His268 for STEAP1		Human, rabbit	211 and 212
Dcytb	Electron transfer *via* heme iron to reduce free Fe(III) to Fe(II)	6-Coordinated His50/His120, His86/His159		Human	213
Myoglobin (artificial system, directed evolution)	Heme catalyzes carbene transfer to olefin in the synthesis of cyclopropanes				214
Cytochrome *c* (artificial system, directed evolution)	Heme catalyzes carbene transfer in the silylation reaction			*Rhodothermus marinus*	215
Cytochrome *c* (artificial system, directed evolution)	Heme catalyzes chiral organoborane synthesis			*Rhodothermus marinus*	216
Cytochrome P450s (artificial system, directed evolution)	Heme catalyzes direct anti-Markovnikov alkene oxidation			*Labrenzia aggregata*	217
Cytochrome P450s (artificial system, directed evolution)	Heme catalyzes syntheses of highly strained carbocycles, such as bicyclobutane and cyclopropene			*Bacillus megaterium*	218
Cytochrome P450s (artificial system, directed evolution)	Heme catalyzes C–C bond formation *via* iron-catalyzed sp^3 C–H functionalization			*Bacillus megaterium*	219
Cytochrome P450s (artificial system, directed evolution)	Heme catalyzes C–H amidation for synthesis of diverse lactams			*Bacillus megaterium*	220
Cytochrome P450s (substrate misrecognition by decoy)	Heme catalyzes hydroxylating reactions toward non-native substrates with the aid of decoy molecules			*Bacillus megaterium, Bacillus subtilis*	221 and 222
SOUL (HEBP2)	Heme metabolism, heme insertion into hemoglobin, import of coproporphyrinogen into mitochondria	His	$K_d \approx 0.003$ μM	Rat, mouse	223–225

Note: the functions exerted by the exchangeable/labile heme iron complex are, in general, believed to be conceptually different from those of heme-responsive sensors. However, strictly speaking, the functions partially overlap and thus are difficult to clearly differentiate.

Specifically, activation of Toll-like receptors (TLRs) by zymosan A results in an increase in erythrophagocytosis. Increased erythrophagocytosis leads to an increase in intracellular heme through breakdown of hemoglobin. This increase in heme, in turn, promotes induction of *Spi-c*, which encodes the heme-regulated transcription factor SPI-C.[196]

Hemin assists dityrosine cross-linking of fibrinogen upon non-thermal plasma exposure and facilitates blood coagulation.[68] It has been suggested that fibrinogen, the most important coagulation protein, binds to hemin, and the resulting protein–hemin complex exhibits pseudo-peroxidase activity such that, in the presence of H_2O_2, the complex can induce dityrosine formation between fibrinogen molecules, leading to the formation of the fibrin network necessary for blood coagulation.

The widely expressed Dps (DNA-protective protein from starved-cells) family proteins are major contributors to prokaryotic resistance to stress.[22] Dps from *Porphyromonas gingivalis* (PgDps), which was previously described as an iron-storage and DNA-binding protein, binds hemin with a high affinity (K_d = 0.037 μM). Hemin forms a 5-coordinated high-spin complex in which Cys101 serves as the axial ligand. Recombinant PgDps protein exists as a stable dodecamer that oligomerizes upon binding of hemin. Since the heme Fe(II) and/or free Fe(II) generated from hemin by HO promotes the production of ROS, which damage DNA, the ability of PgDps to sequester heme iron sequestration would protect DNA against ROS-mediated degradation and confer resistance to heme toxicity.[22]

Hemin and hemoglobin act through dual mechanisms to function as potent modulators of astrocyte immune activity.[197] Hemin and hemoglobin suppress the immune activity of primary mouse astrocytes by reducing astrocyte expression of several proinflammatory cytokines and the scavenger receptor CD36, and by reducing fibril growth/aggregation of amyloid-β (1–42). Specifically, hemin (and hemoglobin) directly binds to highly inflammatory amyloid-β (1–42) oligomers and suppresses inflammation. Hemin also up-regulates phosphoprotein signaling in the phosphoinositide 3-kinase (PI3K)/Akt pathway, which regulates immune functions such as cytokine expression and phagocytosis.

Guanine-rich single-stranded DNAs and RNAs that fold into G-quadruplexes (GQs) are known to bind tightly to hemin and heme Fe(II). Heme–GQ (DNA) complexes, known as heme–DNAzymes, catalyze a variety of one-electron (peroxidase) and two-electron (peroxygenase) oxidation reactions.[198] Complexes of heme Fe(II) with GQ also catalyze carbene insertion into styrene.[198]

Seed dormancy and germination of plants are controlled by a DOG1 (delay of germination1)–AHG1 (abscisic acid hypersensitive germination1)–PP2C (type 2C protein phosphatase) complex through binding of heme. DOG1 is an α-helical heme-binding protein. His245 and His249 are suggested to be axial ligands for 6-coordinated low-spin heme bound to DOG1.[199]

3.2. Emergent novel roles of heme in non-stereotypical hemoprotein functions

In addition to classical heme-containing enzymes, such as cytochrome P450 and peroxidase, heme iron complex bound in non-canonical heme-containing proteins acts as an active centre for several unique catalytic functions (Fig. 5 and Table 3).

Aldoxime dehydratase (Oxd), a heme-bound protein, catalyzes the dehydration of aldoximes (R–CH═N–OH) to their corresponding nitrile (R–C≡N)[200–202] (Fig. 5A). Unlike other heme enzymes, the substrate is directly bound to the heme iron complex in Oxd. OxdA from *Pseudomonas chlororaphis* B23 contains heme Fe(II) with His299 as the proximal axial ligand and receives aldoxime as the substrate and catalyzes its conversion to nitrile and water with the aid of His320 located on the distal side of the heme iron complex. This distal His residue is a prerequisite for the catalysis, and its direct interaction with the OH group of aldoxime[200,202] or the C atom of the C═N bond of aldoxime[201] facilitates the dehydration reaction. In contrast, Oxd containing hemin does not catalyze the dehydration reaction. The X-ray crystal structure of the Michaelis complex of OxdRE from *Rhodococcus* sp. N-771 demonstrates the unique substrate binding and activity-regulating properties of Oxd.[202]

Bifunctional thiosulphate dehydrogenases/tetrathionate reductases (TsdAs) are a distinct type of diheme *c*-type cytochromes that catalyze the reversible formation of a sulphur–sulphur bond between the sulphane atoms of two thiosulphate molecules ($^-O_3S–S^-$), yielding tetrathionate ($^-O_3S–S–SO_3^-$) and releasing two electrons[203–206] (Fig. 5B). Both hemes (heme 1 and heme 2) are covalently bound to the protein through Cys residues. Heme 1 of TsdA from *Campylobacter jejuni* is a 6-coordinated complex with Cys138/His99 as the axial ligands, whereas heme 2 is a 6-coordinated complex with Met255/His207 as the axial ligands. Replacement of one (Cys138 for heme 1, Met255 for heme 2) of these axial ligand residues with another amino acid results in loss of heme binding and a virtually inactive enzyme. Heme 1 acts as the active centre, whereas heme 2 acts as an electron relay centre that wires the active site to the enzyme's redox partner.[203] In TsdA from *Allochromatium vinosum*, heme 1 and heme 2 have His53/Cys96 and His164/Lys208, respectively, as the axial ligands.[205] Cys96 is essential for catalysis, as evidenced by the fact that mutations at Cys96 completely abolish catalysis. A ligand switch from Lys208 to Met209 occurs upon reduction of heme 2. Binding of thiosulphate to hemin and reduction of hemin to heme Fe(II) cause dissociation of the axial ligand Cys96 from the heme iron complex.[205] The crystal structure of a fusion protein composed of TsdA and TsdB (an electron receptor) from *Marichromatium purpuratum* revealed that Cys330, the putative axial ligand for heme 1 of TsdA in the fusion protein, is situated 2.9 Å from the heme iron, a distance that is too far for direct ligation. Importantly, thiosulphate is covalently bound to Cys330.[204]

Hydrazine synthase (HZS) is a multiprotein complex, each subunit of which contains two unique *c*-type heme-containing active sites, as well as an interaction point for a redox partner. HZS produces hydrazine (N_2H_4) from NO and ammonium (NH_3)[207,208] (Fig. 5C). Electron transfer processes involving the c-type heme of HZS play a significant role in N–N bond formation. The crystal structure of HZS implies a two-step mechanism for hydrazine synthesis: (1) three-electron reduction

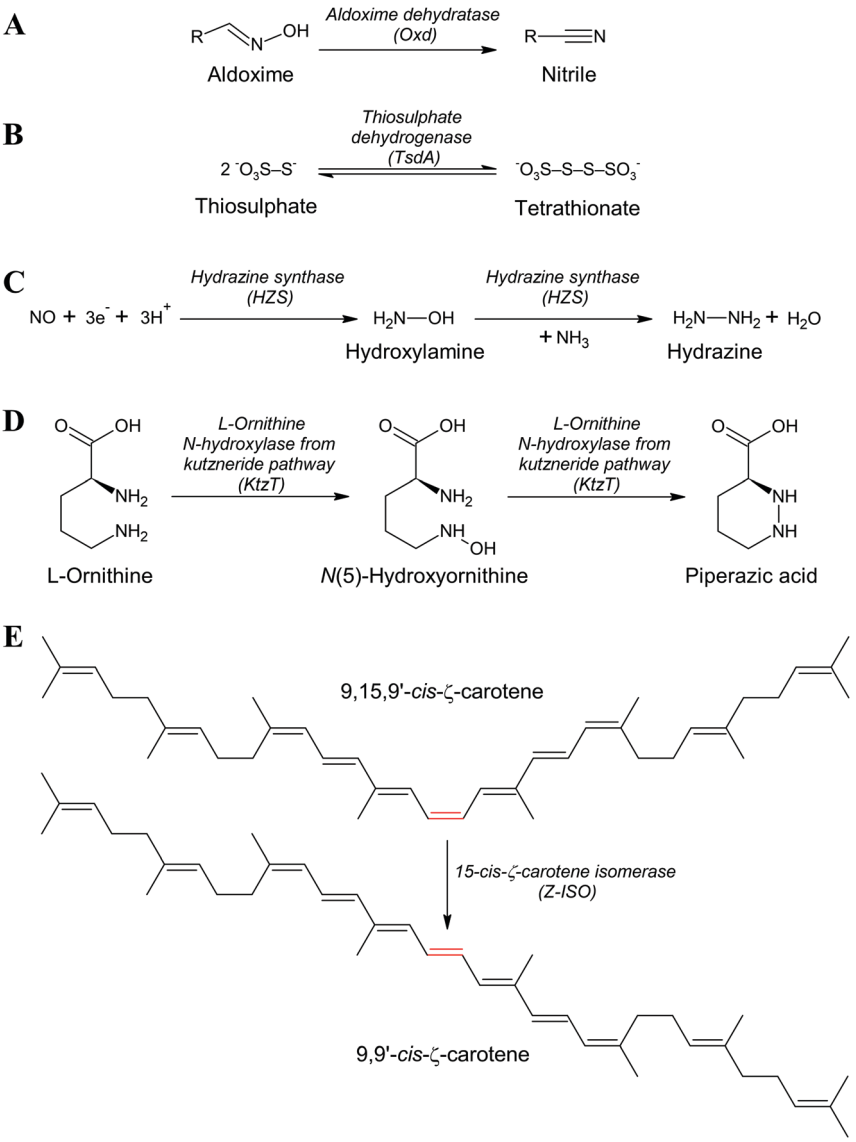

Fig. 5 Novel chemical reactions catalyzed by the heme iron complex of heme proteins. (A) OxdA, OxdB and OxdRE catalyze dehydrogenation of aldoxime to the corresponding nitrile. (B) TsdA catalyzes the reversible formation of an S–S bond between the sulphane atoms of two thiosulphate molecules. (C) HZS produces hydrazine from NO and ammonium. (D) KtzT catalyzes N–N bond formation in the biosynthesis of piperazate. (E) Z-ISO catalyzes *cis–trans* isomerization of 9,15,9′-*cis*-ζ-carotene.

of NO to hydroxylamine (NH_2OH) at the active site of the γ-subunit, and (2) subsequent condensation with NH_3, yielding hydrazine in the active centre of the α-subunit.[208]

KtzT from *Kutzneria* sp. 744 catalyzes heme-dependent N–N bond formation in the biosynthesis of piperazate, a building block for non-ribosomal peptides[209] (Fig. 5D). His65 appears to be the axial ligand for the heme iron complex, and the fact that a His65Ala mutant loses both heme-binding and catalytic ability suggests that heme iron binding is a prerequisite for catalysis. The ability of this heme-dependent enzyme to catalyze N–N bond formation is independent of oxygen and oxidation state of the heme iron complex. This suggests that the reaction mechanism might involve direct coordination of the substrate to the heme iron, polarizing the N–O bond. Such a coordination to the heme iron could then facilitate a nucleophilic attack by the α-amine of the N^5 atom, leading to N–N bond formation.[209]

The biosynthetic pathway for plant carotenoids that occurs in chloroplasts and other plastids requires 15-*cis*-ζ-carotene isomerase (Z-ISO).[210] Z-ISO is a heme-bound membrane protein that catalyzes *cis–trans* isomerization of the 15–15′ carbon–carbon double bond in 9,15,9′-*cis*-ζ-carotene to produce the substrate required by the subsequent enzyme in the biosynthetic pathway (Fig. 5E). The enzyme containing heme Fe(II) has catalytic activity, whereas that containing hemin does not. It has been suggested that heme Fe(II) with a His residue as the proximal axial ligand provides its empty orbital to the delocalized π electrons of the 15–15′-*cis* carbon–carbon double bond of 9,15,9′-*cis*-ζ-carotene. The resulting carbon–carbon bond adopts a single bond-like character and therefore is

able to rotate to the thermodynamically more favorable *trans* orientation.

STEAP (six-transmembrane epithelial antigen of prostate metalloreductases) are the major ferric reductases in developing erythrocytes, where they are critical for metal homeostasis; they are also linked to multiple diseases.[211,212] The single b-type heme, flavin-adenine dinucleotide (FAD), and iron-binding sites are located in the transmembrane domain. Bis-His residues are axial ligands for hemin, and two Tyr residues bind a free Fe(III) atom adjacent to heme.[211] STEAP3 functions as a transmembrane electron shuttle, moving cytoplasmic electrons derived from NADPH across the lipid layers to the extracellular face, where they reduce free Fe(III) to free Fe(II) and potentially Cu^{2+} to Cu^{+}. STEAP3 functions as a homodimer and utilizes an intrasubunit electron-transfer pathway through the single heme moiety rather than an intersubunit electron transfer pathway through a potential domain-swapped dimer. STEAP1 is likely to form a homotrimer and forms a heterotrimer when co-expressed with STEAP2. Heme Fe(II) of STEAP1 reacts readily with O_2.[212] The sequence motifs in the transmembrane domain that are associated with the FAD and metal binding sites are present among STEAP1–STEAP4.

Duodenal cytochrome *b* (Dcytb), a ferric Fe(III) reductase, is an integral membrane protein that catalyzes reduction of non-heme Fe(III) by electron transfer from ascorbate across the membrane.[213] The resultant ferrous iron Fe(II) would be transported by divalent metal ion transporter 1 (DMT1). The function of Dcytb is similar to that of STEAPs. Crystal structures of Dcytb have revealed that the free metal cation, in this case Zn(II) instead of Fe(III), coordinates to two hydroxyl groups of apical ascorbate and a His residue, suggesting that Fe(III) uptake is promoted by ascorbate or relevant reducing agents.

Heme-based catalysis also operates in artificial synthetic pathways created by engineering prototypical heme proteins, such as myoglobin, cytochrome *c* and cytochrome P450, through directed evolution. Catalytic carbene transfer to olefins is a useful approach for synthesizing cyclopropanes, which are key structural motifs of many drugs and biologically active natural products.[214] Myoglobin promotes carbene-mediated cyclopropanation reactions with excellent catalytic activity and selectivity.[214] The crystal structure of a reactive iron porphyrin carbine intermediate for the carbine-transferring silylation reaction in the heme binding pocket of an engineered cytochrome *c* protein has been determined.[215] Taken together with computational methods, such structural insights into key catalytic intermediates should advance our understanding of the reaction mechanism of the "carbine transferase" of heme proteins.[214,215] Effective chiral organoborane syntheses have also been achieved through directed evolution of cytochrome *c* mutants.[216] Applying directed evolution to engineering of cytochrome P450, a well-known monooxygenase,[35,36] makes anti-Markovnikov alkene oxidation,[217] synthesis of highly strained carbocycles, such as bicyclobutane and cyclopropane,[218] C–C bond formation[219] and C–H amidation possible.[220] Furthermore, hydroxylating reactions toward non-native substrates such as propane, butane, cyclohexane, benzene, styrene and ethylbenzene mediated by cytochrome P450 with the aid of decoy molecules have been reported.[221,222]

SOUL (heme-binding protein 2, also known as HEBP2) homologs constitute a superfamily of heme-binding proteins involved in heme biosynthesis/metabolism that are associated with a number of physiological processes.[223–225] The SOUL protein is expressed in the retina and pineal gland in chicken and is solely expressed in the retina in mice. Recombinant murine SOUL exists as a dimer in the absence of heme iron, but it specifically binds one hemin per monomer unit and becomes a hexamer upon binding hemin.[223] Murine p22HBP (p22 heme-binding protein), also known as HEBP1, is a heme protein ubiquitously expressed in numerous tissues that has been reported to exhibit 27%[224] or 40%[223,226] identity to murine SOUL. It also binds one hemin per monomer, but its coordination to hemin uses no specific axial ligand. SOUL binds hemin through coordination with a His residue.[223,224] Dissociation rate constants of hemin from both SOUL and p22HBP are very high, with k_{off} values of 1.2×10^{3} s^{-1} and 4.4×10^{-3} s^{-1}, respectively, compared with 8.4×10^{-7} s^{-1} for myoglobin, suggesting that hemin has a high affinity for these SOUL proteins.[223] In contrast, similarly low affinity of hemin for p22HBP ($K_d \approx 0.03$ μM) has been demonstrated using a fluorescence quenching method.[224] SOUL and p22HBP share what is likely to be a conserved tertiary fold, but NMR spectroscopy suggests that they bind hemin at different sites within this fold.[224] SOUL and p22HBP could either function as heme transporters or chaperones for heme insertion into hemoglobin or as mediators of coproporphyrinogen import into mitochondria.[224]

Note that a helical scaffold is not essential for hemin binding, as hemin can bind to a peptide composed predominantly of β-scaffold secondary structures.[67]

4. Conclusions

Biochemistry textbooks describe the important role of the heme iron complex prosthetic group in physiology. In particular, it is said that the heme iron complex manifests its capabilities in hemeproteins, rather than as a free heme iron complex. For example, the heme iron complex in prototypical hemeproteins plays a key role in O_2 storage, O_2 transfer, O_2 activation, peroxide activation and electron transfer at the heme active site. However, new and totally different types of heme proteins—heme-responsive sensors—have emerged. In these proteins, the association/dissociation of hemin regulates numerous intrinsic functions that are important physiologically and pathologically. In the present review, we have provided comprehensive coverage of heme-responsive sensors, with a focus on molecular mechanisms.

Heme-responsive sensors are critically involved in numerous important physiological functions, such as transcriptional regulation, tRNA syntheses, mRNA splicing, translational regulation, proteasome-dependent and -independent protein degradation, K^{+} channel regulation, autophosphorylation, and protein–protein interactions. Some functions are associated with the biosynthesis and metabolism of the heme iron complex. Interestingly, some

heme-sensing transcriptional regulations are associated with circadian rhythms. In addition, heme-responsive sensors act as scavengers that bind detrimental heme iron complexes so as to prevent their participation in the generation of ROS.

There are additional novel, emergent roles of the free heme iron complex and heme iron complexes in hemeproteins. For example, hemin inhibits PDE and deaminase activity, phagocytosis, and amyloid β-mediated inflammation, whereas hemin recruits EGFR and cytochrome P450 enzymes, forms aggregates with ubiquitinated proteins, and induces diTyr cross-linking of fibrinogen. Furthermore, aldoxime dehydration, *cis–trans* isomerization, N–N bond formation, S–S bond formation, and carbine-transfer reactions are directly catalyzed by the heme iron complex in heme proteins.

It is claimed that some heme-based CO sensors simultaneously act as heme-responsive sensors or *vice versa*. However, the molecular mechanisms of overlapping or duplicate roles of these sensors still remain unresolved, but hopefully will be adequately addressed in the near future.

Dysfunction of heme-responsive sensors is critical in various diseases. In particular, if functions associated with heme synthesis and heme metabolism are impaired, serious blood-related diseases can ensue. On the other hand, in pathobacteria, the heme iron complex is degraded to provide free iron for use as a bacterial nutrient. Since heme-sensing systems tend to precede heme-degradation reactions, impairment of the heme-sensing ability of bacteria would help cure infectious bacterial diseases.

It is certain that, in future, numerous physiologically important heme-sensing functions and heme-associated catalysis/reactions will be discovered. Many spectroscopic methods can be used to characterize the structure and functions of heme-responsive sensors. This will prove beneficial for biological chemists in advancing our understanding of the molecular mechanisms of numerous important reactions involving heme and heme-responsive sensors, and ultimately lead to clinically meaningful therapeutic targets in diverse diseases associated with ROS, NO, CO, iron, heme biosynthesis and metabolism, and/or bacteria.

Abbreviations

ALAS	Aminolevulinic acid synthase
ALAS1	Nonspecific 5-aminolevulinate synthase
ALIS	Aggresome-like inducted structure
All4978	Protein from cyanobacterium *Nostoc* sp. PCC7120
Arnt	Aryl hydrocarbon receptor nuclear translocator
Bach1	BTB domain and CNC homolog 1
Bach2	BTB domain and CNC homolog 2
bHLH	Protein domain with a basic helix-loop-helix motif
BK channel	Ca^{2+}-Sensitive large-conductance K^+ channel
BMAL1	Brain and muscle Arnt-like 1
cALAS	ALAS from *Caulobacter crescentus*
ChrA	Response regulator (RR) of ChrS protein
ChrS	Heme-sensing kinase of *Corynebacterium diphtheria*
CLOCK	Transcriptional regulatory protein associated with circadian rhythms
ClpXP	Mitochondrial ATP-dependent protease
CP motif	Cys-Pro motif
CRY	Cryptochrome
CSE	Cystathionine-γ-lyase
CUP9	Homeodomain-containing transcriptional repressor or homeobox protein
Dcytb	Duodenal cytochrome *b*
DGCR8	DiGeorge critical region 8
DHR3	Drosophila hormone receptor 3
DHR51	Drosophila hormone receptor 51
DnrF	Crp/Fnr-type transcriptional regulator
DOG1	Delay of germination1
Dps	DNA-protective protein from starved-cells
E75	Ecdysone-induced protein 75
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic initiation factor 2α
FAD	Flavin-adenine dinucleotide
FBXL5	F-Box and leucine-rich repeat protein 5
Fre	Ferric reductase
Fre-MsrP-MsrQ	Methionine sulphoxide reductase system from *Escherichia coli*
FurA	Ferric uptake regulator from cyanobacteria *Anabaena* sp. PCC 7120.
GAF	Protein domain named after three proteins, cGMP-specific phosphodiesterase, adenyl cyclase and FhlA
GCN2	General control nonderepressible 2
Gis1	Yeast histone demethylase
GluRS	Glutamyl-tRNA synthase
GluTR	Glutamyl-tRNA reductase
GQ	Guanine-rich single-stranded DNA and RNA that fold into G-quadruplex
HAP1	Heme-sensing transcriptional regulator
HBD	Heme binding domain
HcArgRS	Human cytoplasmic arginyl-tRNA synthase
HDAC3	Histone deacetylase 3
HDM	Helminth defense molecule
HEBP1	Heme-binding protein 1 or p22HBP
HEBP2	Heme-binding protein 2 or SOUL
heme	Protoporphyrin IX iron complex
heme Fe(II) complex	Ferrous protoporphyrin IX
hemin	Heme Fe(III) complex or ferric protoporphyrin IX
HK	Histidine kinase
HO1	Heme oxygenase-1
HO2	Heme oxygenase-2
HOIL-1	Heme-oxidized IRP2 ubiquitin ligase-1
HR3 AF2	Peptide derived from DHR3 protein
HRI	Heme-regulated inhibitor (eukaryotic initiation factor 2α kinase)

hrt	Gene for heme-regulated transporter
HrtA, HrtB	Heme-regulated transporters of *Lactococcus lactis*
HrtAB	Heme-regulated transporter efflux pump
HrtR	Transcriptional regulator of *Lactococcus lactis*
Hsp90	Heat shock protein 90
HssS-HssR	Heme-sensing two-component heme-responsive-sensor system
HZS	Hydrazine synthase
IRP1	Iron-responsive element-binding protein-1 or iron regulatory protein 1
IRP2	Iron-responsive element-binding protein-2 or iron regulatory protein 2
Irr	Ferric uptake regulation protein from *Bradyrhizobium japonicum*
KtzT	Protein from *Kutzneria* sp. 744
$K_V1.4$ channel	Voltage-dependent K^+ channel
LONP1	Lon peptidase 1, mitochondrial
MA4561	Kinase protein from *Methanosarcina acetivorans*
Maf	Musculoaponeurotic fibrosarcoma
MafK	bZip Maf transcription factor protein
mPer2	Mouse period circadian protein homologue 2
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCoR	Nuclear receptor co-repressor
NOS	Nitric oxide synthase
NPAS2	Neuronal PAS domain protein 2
NRF2	Nuclear factor erythroid 2-related factor 2
NtrY–NtrX	Redox sensor system of *Brucella* spp.
Oxd	Aldoxime dehydrase
p22HBP	p22 heme-binding protein or HEBP1
p53	Tumor-suppressor protein
PAS	Protein domain named after three proteins (Per-Arnt-Sim)
PDE	Phosphodiesterase
Per	Period circadian protein
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
PfRRS	*Plasmodium falciparum* arginyl-tRNA synthase
PgDps	DNA protecting protein from *Porphyromonas gingivalis*
PGRMC1	Progesterone receptor membrane component 1
PKG	Protein kinase G
PKR	Protein kinase R
PpsR	Protein from *Rhodobacter sphaeroides*
pri-miRNA	Primary microRNA
RCK	Regulator of conductance for K^+
Rev-erbα	Nuclear receptor also known as NR1D1 (nuclear receptor subfamily 1, group D, member 1)
Rev-erbβ	Nuclear receptor also known as NR1D2 (nuclear receptor subfamily 1, group D, member 2)
ROR	Retinoic acid receptor-related orphan receptor
ROS	Reactive oxygen species
RR	Response regulator
SbnI	Protein from *Staphylococcus aureus*
sGC	Soluble guanylate cyclase
Sim	Single-minded protein
SOUL	Heme-binding protein 2 or HEBP2
STEAP	Six-transmembrane epithelial antigen of prostate metalloreductase
SUR2A	Sulphonylurea receptor subunit
TCS	Two-component signal transduction system
Tll0287	Protein from *Thermosynechococcus elongatus*
TrpRS	Tryptophanyl-tRNA synthase
TsdA	Thiosulphate dehydrogenase/tetrathionate reductase
UBR1	E3 ubiquitin ligase of the N-end rule pathway
YybT	Stress signaling proteins from *Bacillus subtilis*
Z-ISO	15-*cis*-ζ-Carotene isomerase
ZnF	Zinc finger domain

Conflicts of interest

There are no conflicts to declare.

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