Mini review

The Roles of Thiolate-Heme Proteins, Other Than the P450 Cytochromes, in the Regulation of Heme-Sensor Proteins

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Abstract

Cytochrome P450, nitric oxide synthase, and chloroperoxidase are typical thiolate-heme enzymes, in which heme iron coordinated with the cysteine thiolate activates molecular oxygen or hydrogen peroxide. A new group of thiolate-heme proteins is becoming recognized. In these proteins, termed heme-responsive/sensing proteins, or simply heme-sensor proteins, the thiolate-heme iron has a sensor function. All known heme-sensor proteins use a cysteine residue to bind heme. The first question is why cysteine is employed in this capacity. Ligation of heme with thiolate, the presence of redox-dependent ligand switches, fast heme dissociation rates from the heme-sensor proteins, and formation of 5-coordinated NO-Fe(II) heme complexes, appear to be common characteristics of heme-sensor proteins. The Cys-Pro (CP) motif may also be important for heme binding in some heme-sensor proteins. In this minireview, we summarize the inorganic and physicochemical characters of heme-sensor proteins, and include short comments on heme-regulated inhibitor (HRI), and neuronal PAS protein 2 (NPAS2), under study in our laboratory over the last several years. Some gas-sensing heme-sensor proteins, with thiolate-heme complexes, will also be briefly discussed.

Keywords: Thiolate-heme iron, heme-sensor, gas-sensor, nitric oxide, CP motif

1. Introduction

The iron-containing heme complexes found in various types of heme-containing proteins are used for oxygen storage (myoglobin), oxygen transfer (hemoglobin), electron transfer (cytochromes), peroxide activation (peroxidase), activation of molecular oxygen (P450), and for many other functions.[1] Enzymes with thiolate-coordinated heme iron form a special category in the heme protein family. For example, thiolate coordination to the heme iron is important to activate molecular oxygen, bound to the heme, in the monooxidation reactions catalyzed by cytochrome P450[2–8] and nitric oxide synthase.[9–12] Chloroperoxidase also uses the thiolate-heme complex when catalyzing reactions with peroxide, in which halogenated compounds are synthesized.[13–15]

Recently, an important role for thiolate coordination of the heme iron in heme-sensor proteins has been emerging. In this category, heme association (or dissociation) *per se* regulates important biological functions such as protein kinase reactions or transcription of heme-associated proteins (Table 1).[16–29] All heme-sensor proteins described so far have a Cys thiolate as the heme binding site, which may also be termed the heme-sensing site. In this minireview, we summarize the characteristics of heme-sensor proteins with thiolate-coordinated heme iron molecules, and we discuss the role of the thiolate-heme in heme sensing. Gas-sensor proteins with thiolate-heme iron will also be discussed.

2. Heme-Responsive/Sensing Heme-Sensor Proteins

In the heme-sensor proteins, association and/or dissociation of the heme iron regulate protein functions such as catalysis and transcriptional activation (Figure 1).[29] The transcriptional activation is in some cases executed by

Table 1: Thiolate-heme proteins, and redox-dependent ligand switches, of heme-sensor proteins.

	Fe(III)	Fe(II)	Fe(II)–CO	Ref.
Heme-responsive/sensing heme-sensor proteins				
HRI	Cys/His	Cys?/His?	His/CO	16–18
NPAS2 (bHLH-PAS-A)	Cys/His	Cys?/His?	His/CO	19–21
NPAS2+E-boxDNA	Cys/OH^-	Cys?/His?	His/CO	19,49
Bach1	Cys/His		His/CO	22, 23
IRP2	Cys/His	His	His/CO	24
E75	Cys/His?	Cys?/His?	His/CO	25, 26
DGCR8	Cys	Cys		27
Slo BK channel	Cys		His/CO	28, 29
Gas-responsive/sensing heme-sensor proteins				
CooA	Cys/Pro	His/Pro	His/CO	30, 31
CBS	Cys/His	Cys/His	His/CO	32–34
Other thiolate-heme proteins				
SoxAX, SoxXA	Cys/His			35–37
cNP	Cys			37
P450	Cys/OH^-, Cys	Cys	Cys/CO	2–8
NOS	Cys/OH^-, Cys	Cys	Cys/CO	9–12
CPO	Cys/OH^-, Cys	Cys	Cys/CO	13–15

Note that proteins P450, NOS, and CPO, all requiring the anionic thiolate of Cys as the axial ligand of Fe(II) heme, for the activation of molecular oxygen or hydrogen peroxide, do not show redox-dependent ligand switches from Cys to His upon heme reduction from Fe(III) to Fe(II) in the heme complexes.

binding of the heme-sensor protein with a partner protein, and/or with specific sequences of DNA associated with transcription. We briefly summarize inorganic and physicochemical characters critical for heme sensing of the heme-sensor proteins, and we focus on heme-responsive inhibitor (HRI)[16–18] and neuronal PAS protein 2 (NPAS2),[19–21] under study by us over the last several years.

2. 1. Cys is the Binding Site, or Sensing Site, for the Fe(III) Hemin Complex

The best-known heme-sensor proteins are eukaryotic initiation factor 2α kinase (eIF2α kinase) and HRI.[16–18] HRI regulates protein synthesis to balance the molar ratio of heme iron and globin proteins in red blood cells. Under

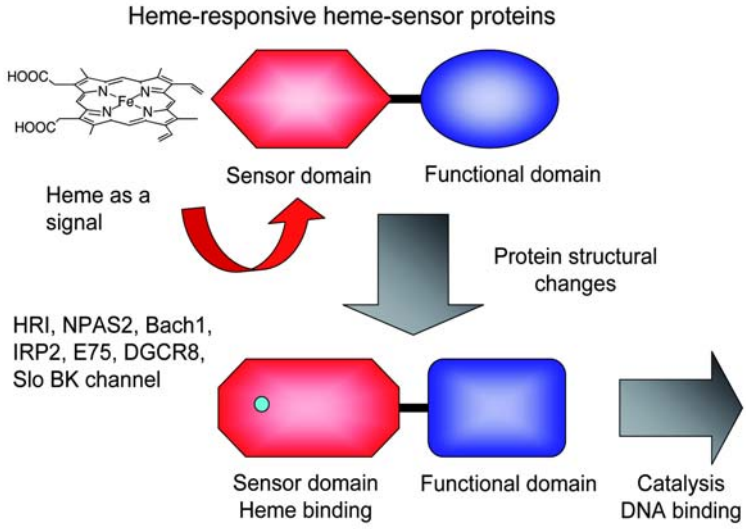

Figure 1: Mechanism of action of heme-sensor proteins. Heme association/dissociation from the heme binding domain changes the structure of the heme-binding sensor domain. The structural change caused by the association/dissociation of the heme is a signal which is then transduced to a protein functional domain. Here, catalysis, transcriptional activation, DNA binding, or a protein-protein interaction, is executed.

normal conditions, the heme binds to the active site of the kinase and blocks the catalytic function of HRI. Under conditions of heme shortage, the heme iron dissociates from the kinase active site, then the kinase active site becomes exposed to the solvent allowing the substrate accessible to the active site, and thus phosphorylation of the α subunit of eukaryotic initiation factor 2 is executed (Figure 2). In HRI, the binding of the heme iron is relatively weak, and the facile association/dissociation of the heme iron, as just described above, regulates the catalytic function of HRI in response to heme concentration. It was found that one of the axial ligands in the binding site of the Fe(III) heme complex in HRI is the thiolate of a Cys residue (18). All other heme-sensor proteins described to date, such as Bach1,[22,23] IRP2,[24] E75,[25,26] DGCR8,[27] and the Slo BK channel protein,[28,29] also have a Cys residue in the binding/sensing sites for the Fe(III) heme complex (Table 1). We therefore strongly suggest, but cannot definitively conclude, that all heme-sensor proteins use a Cys residue as the Fe(III) heme binding/sensing site. Incidentally, NPAS2 has been described as a heme-containing transcriptional factor associated with circadian rhythms.[47] Although it was suggested that NPAS2 was a CO-sensing heme protein, NPAS2 has Cys at the binding/sensing site of the Fe(III) hemin complex, based on several lines of physicochemical evidence. We thus suggest that NPAS2 is also a heme-responsive/heme-sensing transcriptional factor.

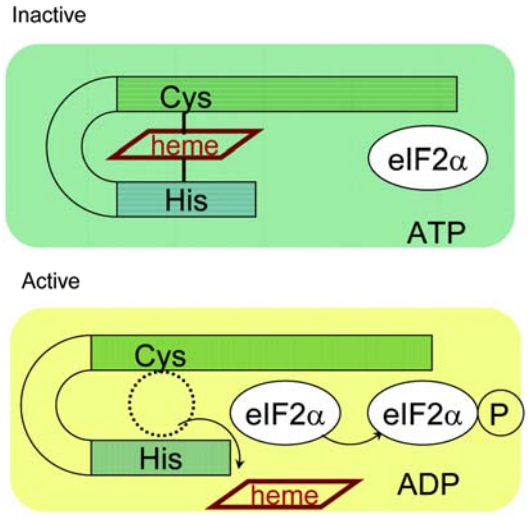

Figure 2: Molecular mechanism of catalytic regulation by the heme-sensing domain in HRI. Under normal conditions, the heme iron binds to the kinase active site and blocks kinase activity (upper panel). Under conditions of heme shortage, however, the heme iron dissociates from the active site, leading to exposure of the active site and upregulation of kinase activity (lower panel).

2. 2. Redox-Dependent Ligand Switching

An interesting feature of the heme-sensor proteins is that Cys, one of the axial ligands for the Fe(III) hemin complex, is replaced by a His residue, or another unknown ligand, upon heme reduction to the Fe(II) heme complex (Table 1). This ligand switch is understandable, because the anionic thiolate group of the Cys residue as a heme axial ligand would repel the relatively less positive Fe(II) heme cation (compared with Fe(III) hemin), and will therefore easily dissociate from the heme iron upon heme reduction. This redox-dependent ligand switch will be important for the functions of heme-sensor proteins because profound structural changes in the heme-sensor domain, caused by the redox changes, may become signals that are transduced to protein functional domains. Alternatively, the heme-sensing functions of heme-sensor proteins with Fe(III) hemin complexes will be substantially different from those of proteins with Fe(II) heme complexes, and such functional differences may well be used to regulate protein functions in cells responding to new redox conditions caused by oxidative stress or hypoxia. It is noteworthy that in P450, NOS, and CPO, Cys coordination to the Fe(II) heme complex is critical for catalytic function, and no redox-dependent ligand switch from Cys to His occurs in these proteins (Table 1).

2. 3. Fast Heme Dissociation Rate Constant

It is reasonable to speculate that heme-sensor proteins have weak heme binding abilities, because association and dissociation of heme must be easily executed. The heme dissociation rate constants of HRI and the isolated PAS-A domain of NPAS were 1.5×10^{-3} s^{-1} and 5.3×10^{-3} s^{-1}, respectively (Table 2).[17,19] These rate constants are substantially higher than those of sperm whale myoglobin and human hemoglobin (8.4×10^{-7} s^{-1} and 7.1×10^{-6} s^{-1}, respectively).[45,46] The fast heme dissociation rate constant of NPAS2 again suggests that NPAS2 is a heme-sensor protein similar to HRI. It is notable that heme association rate constants for HRI and NPAS2 are comparable to those of sperm whale myoglobin and human hemoglobin (Table 2). Because information on heme dissociation rates for heme proteins is limited, more data are required before definite conclusions on possible relationships between

Table 2: Association and dissociation rate constants for heme binding.

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (M)	Ref.
HRI	1.1×10^7	1.5×10^{-3}	1.4×10^{-10}	17
NPAS2 (bHLH–PAS–A)	3.3×10^7	5.3×10^{-3}	1.6×10^{-10}	19
p22HBP	2.1×10^8	4.4×10^{-3}	2.1×10^{-11}	44
SOUL	1.9×10^6	6.1×10^{-3}	3.2×10^{-9}	44
Sw Mb	7.6×10^3	8.4×10^{-7}	1.3×10^{-14}	45, 46
huHb	2.9×10^7	7.1×10^{-6} (α)	2.5×10^{-13}	45, 46
BSA	$5{,}0 \times 10^7$	1.1×10^{-2}	2.2×10^{-10}	45, 46

Note that proteins p22HBP and SOUL have His as axial ligand(s), and, while they may be heme transporter proteins, their functions remain unclear. BSA is not a prototype heme-binding protein.

heme dissociation rates and heme sensing may be drawn. We note here, however, that the heme dissociation rate constants of p22HBP and SOUL are fast, and indeed comparable to those of HRI and NPAS (Table 2)[44]. The p22HBP and SOUL proteins are speculated to be heme-transporter proteins with His, and not Cys, as axial ligands, although this contention remains unproven. Bovine serum albumin, with a high heme dissociation rate constant, is not a prototype heme binding protein.

2. 4. Heme Regulatory Motif (HRM), or the Cys-Pro (CP) Motif

Zhang and Guarente proposed that heme binding proteins, including HRI, commonly have a special motif, termed a heme-regulatory motif (HRM) or a Cys-Pro (CP) motif.[48] For IRP2, the CP motif was suggested to be the heme binding/sensing site, based on catalytic and spectroscopic data.[24] For HRI, one of the CP motifs appears to be an axial ligand for Fe(III) hemin (our unpublished results). Our spectroscopic studies, using oligopeptides, indicated that the Pro residue next to the Cys residue was important to assist with Cys binding to the Fe(III) hemin.[49,50] The adjacent Pro residue appears to make the side chain of the Cys residue stereochemically less flexible. In other words, movement constraint is imposed, to facilitate Cys binding with heme iron, or to cause the Cys to protrude from the protein surface. The CP motif also may be useful for heme sensing by facilitating interaction between the Fe(III) heme and the protein, leading to the dissociation of heme iron from the Cys residue upon heme reduction. The CP motif, thus, appears to be valuable to prevent tight binding of Cys to heme, because non-heme-sensor thiolate-heme proteins that do not have the CP motif, such as P450 or NOS, show relatively tight binding of the Cys residue when the Cys functions as an axial ligand for either Fe(III) hemin or Fe(II) heme. No redox-dependent ligand switch from Cys to His, or to any other ligand, has been described in these proteins. The stable binding of Cys to the Fe(II) heme is critical for electron donation by the Cys residue which, in turn, is crucial in the activation of molecular oxygen bound in trans to Cys. We emphasize that not all heme-sensor proteins may contain the functional CP motif. Other features of protein structure, or characteristics of the ionic or hydrophobic environment, may be more important than the CP motif for heme binding/sensing.

2. 5. 5-Coordinated NO-Fe(II) Complexes

In HRI, a typical 5-coordinated NO-Fe(II) heme complex is formed,[18,51] the formation of this complex was accompanied by a marked enhancement in enzyme catalysis after addition of NO. The NPAS2 protein also has the same 5-coordinated NO-Fe(II) heme complex.[19,49] We do not know, however, if the 5-coordinated NO-Fe(II) heme complex is directly involved in protein function, although circumstantial physicochemical evidence suggests that NPAS2 is also a heme-sensor protein.[19] Further studies may confirm this, and will also determine if the function of NPAS2 is modulated by NO. In addition, the limited available data on NO-heme complexes in heme-sensor proteins indicate that further work is needed to unequivocally show that formation of the 5-coordinated NO-Fe(II) heme complex is critical for the functions of heme-sensor proteins. In this context, it is noted that the formation of the 5-coordinated NO-Fe(II) heme complex is essential for the catalytic function of sGC.[41–43] Several heme-containing proteins also form 5-coordinated NO-Fe(II) heme complexes upon NO addition (Table 3).[30–34,38–40]

Table 3: Soret bands of the 5-coordinated NO-Fe(II) complexes.

Proteins	Soret band (nm)	Ref.
HRI	398	18
bHLH/PAS-A NPAS2	394	19, 49
E75	391	25
CooA	399	30, 31
CBS	390	32–34
cNP	405	38
Cytochrome *c′*	395	39, 40
sGC	398	41–43

Note that the axial ligand of Fe(II) heme in cytochrome *c*' and sGC is His, but that the Cys axial ligand of the Fe(III) hemin in other heme proteins switches to His upon reduction of heme to Fe(II) (see Table 1).

3. Other Thiolate-Heme Proteins

Gas-responsive/sensing heme-sensor proteins are known (Table 4).[52] In general, the gas-sensor proteins have two domains, a heme-bound sensing domain located at the N-terminus, and a functional domain located at the C-terminus (Figure 3). Gas-sensor proteins with thiolate-heme iron as the gas sensor are known (Tables 1 and 4). Protein CooA, a CO-sensing transcriptional factor from *Rhodospirillum rubrum,* uses a thiolate-coordinated heme iron as the CO sensor, and CO binding to the heme iron triggers transcriptional activation.[30,31] Protein CBS, cystathionine β-synthase, has a thiolate-heme iron associated with catalytic regulation.[32–34] Catalytic regulation through the heme iron is achieved by the binding of external ligands such as CO or NO, or by redox change. In addition, redox-dependent ligand switches, similar to those of heme-sensor proteins, occur.

Protein cNP, a nitrophorin from *Cimex tectularius*, also has a thiolate-heme, perhaps used for regulation of NO transport (Table 1).[37] SoxAX and SoxXA are cytochrome *c*-type heme-proteins, with Cys thiolates as axial ligands (Table 1).[35–37]

Table 4: Gas-responsive heme-sensor proteins.

Protein	Gas	Regulated function	Coordination	Ref.
CooA	CO	Transcription	Cys-Fe(III)–N-term	30, 31
CBS	CO	Met synthesis	Cys-Fe(III)–His	32–34
NPAS2	CO	Transcription/Body clock	Cys-Fe(III)–His	19–21, 47
BjFixL	O_2	His kinase/Nitrogen fixation His-Fe(II)		52, 53
HemAT-Bs	O_2	Methylation/Chemotaxis	His-Fe(II)	54
DevS	O_2	His kinase	His-Fe(II)	55
sGC	NO	Smooth muscle relaxation	His-Fe(II)	41–43
Ec DOS	O_2, CO, NO	Phosphodiesterase/c-di-GMP	His-Fe(II)–Met	56–58

See Table 1 for the coordination structures of the Fe(II) complexes in CooA, CBS and NPAS2.

4. Conclusions

In this minireview, we have summarized the inorganic and physicochemical characters of the heme-responsive /sensing heme-sensor proteins. Specific characteristics of heme-sensor proteins include thiolate coordination to the Fe(III) hemin complex in the sensor protein, and a redox-dependent ligand switch, after which the ligand axial to the Fe(II) heme in the sensor protein is no longer thiolate. These redox-dependent ligand switches appear to be important to allow sensor proteins to express heme-sensing functions. Fast, easy, heme association and dissociation are important in this context, although more data are required before unequivocal conclusions may be drawn. It seems logical that a sensor protein would use heme association or dissociation to regulate protein function. The CP motif may be critical for the control of Fe(III) hemin binding in some heme-sensor proteins, although various aspects of protein structure, and features of the ionic or hydrophobic environments, may also significantly affect sensor protein binding to heme iron. The formation of 5-coordinated NO-Fe(II) heme complexes in heme-sensor proteins appears important, but more data are required. Because Hg(II) binds to the cysteine thiolate, the physiological effects of mercury poisoning might be explained in part by impairment of heme-sensing functions.[51] While cytochrome P450 was the first heme protein found to have a thiolate anion as the heme axial ligand, it appears that more thiolate-heme proteins with diverse functions will emerge.[59]

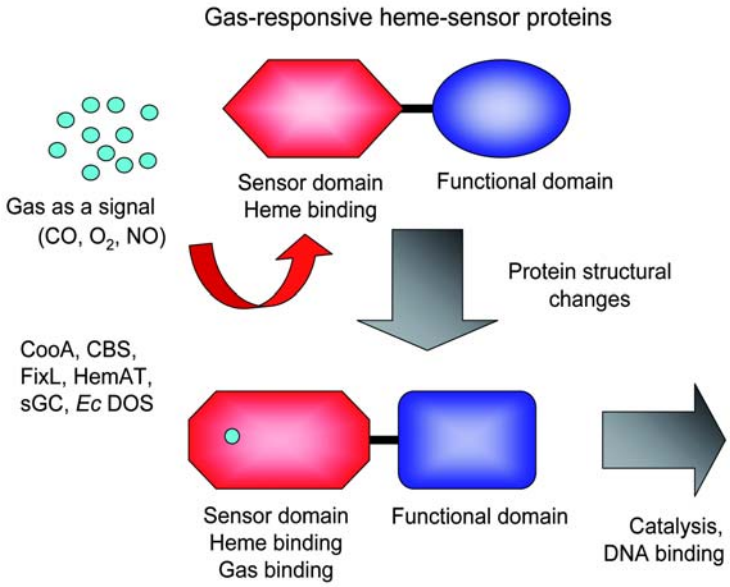

Figure 3: Mechanism of action of gas-responsive heme-sensor proteins. Gas association/dissociation from the heme in the sensor domain changes the structure of the heme-binding sensing domain. The structural change caused by the association/dissociation of gas becomes a signal which is transduced to the functional domain, where catalysis, transcriptional activation, DNA binding, or a protein-protein interaction, is executed.

Abbreviations

Bach1: transcriptional repressor of heme oxygenase-1 and β-globin genes, both of which are transcriptionally induced by heme
BjFixL: a heme-binding oxygen sensor kinase, FixL, from *Bradyrhizobium japonicum*
BSA: bovine serum albumin
CBS: cystathionine β-synthase with a heme as a redox and/or CO sensor
cNP: a nitric oxide transporter protein, nitrophorin, from the blood sucking insect *Cimex tectularius*
CooA: a CO-sensing transcriptional factor from the photosynthetic bacterium *Rhodospirillum rubrum*
CPO: chloroperoxidase
Cytochrome *c'*: cytochrome *c'* from *Alcaligenes xylosoxidans*
DevS: a heme-containing two-component oxygen sensor from *Mycobacterium tuberculosis*
DGCR8: RNA-binding protein, DiGeorge critical region-8, that is essential for premicroRNA processing
E75: *Drosophila* nuclear receptor with heme as a nuclear receptor ligand
*Ec*DOS: a phosphodiesterase toward cyclic-di-GMP from *Escherichia coli*
Fe(III) hemin: Fe(III)-protoporphyrin IX
Fe(II) heme: Fe(II)-protoporphyrin IX
HAP1: a yeast transcriptional activator that activates transcription encoding cytochrome in response to heme
HemAT-Bs: a heme-based oxygen sensor responsible for aerotaxis control in *Bacillus subtilis*
HRI: eukaryotic initiation factor 2α kinase (eIF2α kinase) or heme-regulated inhibitor that is a Ser/Thr kinase and regulates protein synthesis in response to the heme concentration in erythrocytes.
huHb: human hemoglobin
IRP2: iron regulatory protein modulated by heme-mediated ubiquitination and self-degradation.
NOS: nitric oxide synthase.
NPAS2: neuronal PAS protein 2, a heme-bound transcriptional regulatory factor associated with circadian rhythms.
PAS: an acronym formed from the names of proteins in which imperfect repeat sequences were initially recognized: PER (the *Drosophila* period clock protein), ARNT (vertebrate aryl hydrocarbon receptor nuclear translocator), and SIM (*Drosophila* single-minded protein).
p22HBP: An intracellular heme-binding protein that may act as a heme transporter or chaperone for heme insertion into hemoglobin.
sGC: soluble guanylate cyclase
SOUL: a heme-binding protein from mammalian retina and pineal gland with amino acid 45% sequence homology with p22HBP.
SoxAX: a heterodimeric *c*-type cytochrome with thiolate heme that plays an essential role in photosynthetic thiosulfate and sulfide oxidation of the bacterium *Rhodovulum sulfidophilum*.
SoxXA: a heterodimeric hemoprotein with thiolate heme that is essential for lithotrophic sulfur oxidation of the aerobic bacterium *Paracoccus pantotrophus*.
Sw Mb: sperm whale myoglobin

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Povzetek

Citokromi P450, sintaze dušikovega monoksida in klorperoksidaze, so tipični hem-tiolatni encimi, kjer hemsko železo, ki je koordinirano s tiolno skupino cisteina, aktivira molekularni kisik ali vodikov peroksid. V zadnjem času se uveljavlja nova skupina hem-tiolatnih proteinov, imenovanih na hem odzivni/senzorski proteini, ali na kratko hem-senzorski proteini. Pri tej skupini ima hemsko železo vlogo senzorja. Vsi do sedaj poznani proteini te skupine za vezavo hema uporabljajo cisteinski ostanek. Postavlja se vprašanje, zakaj je prav cistein nujen za senzorično sposobnost. Povezava hema s tiolatom, prisotnost redoks-odvisnega obrata liganda, hitra disociacija hema iz hem senzoričnega proteina in tvorba 5-koordiniranega NO–Fe(II) hemskega kompleksa, so skupne značilnosti hem-senzorskih proteinov. Motiv Cys-Pro (CP) je tudi lahko pomemben za vezavo hema v nekaterih hem-senzorskih proteinih. V tem kratkem preglednem članku bomo povzeli anorganske in fizikalno kemijske lastnosti hem-senzorkih proteinov, v kratkem pa bomo opisali tudi s hemom uravnavani inhibitor (HRI) in proteine PAS 2 živčnega sistema (NPAS2), ki jih v zadnjih letih proučujemo v našem laboratoriju. Opisani bodo tudi nekateri hem-senzorski proteini, ki vsebujejo hem-tiolatne komplekse in so sposobni zaznati pline.