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# Regulatory $Fe^{II/III}$ Heme: The Reconstruction of a Molecule's Biography[+]

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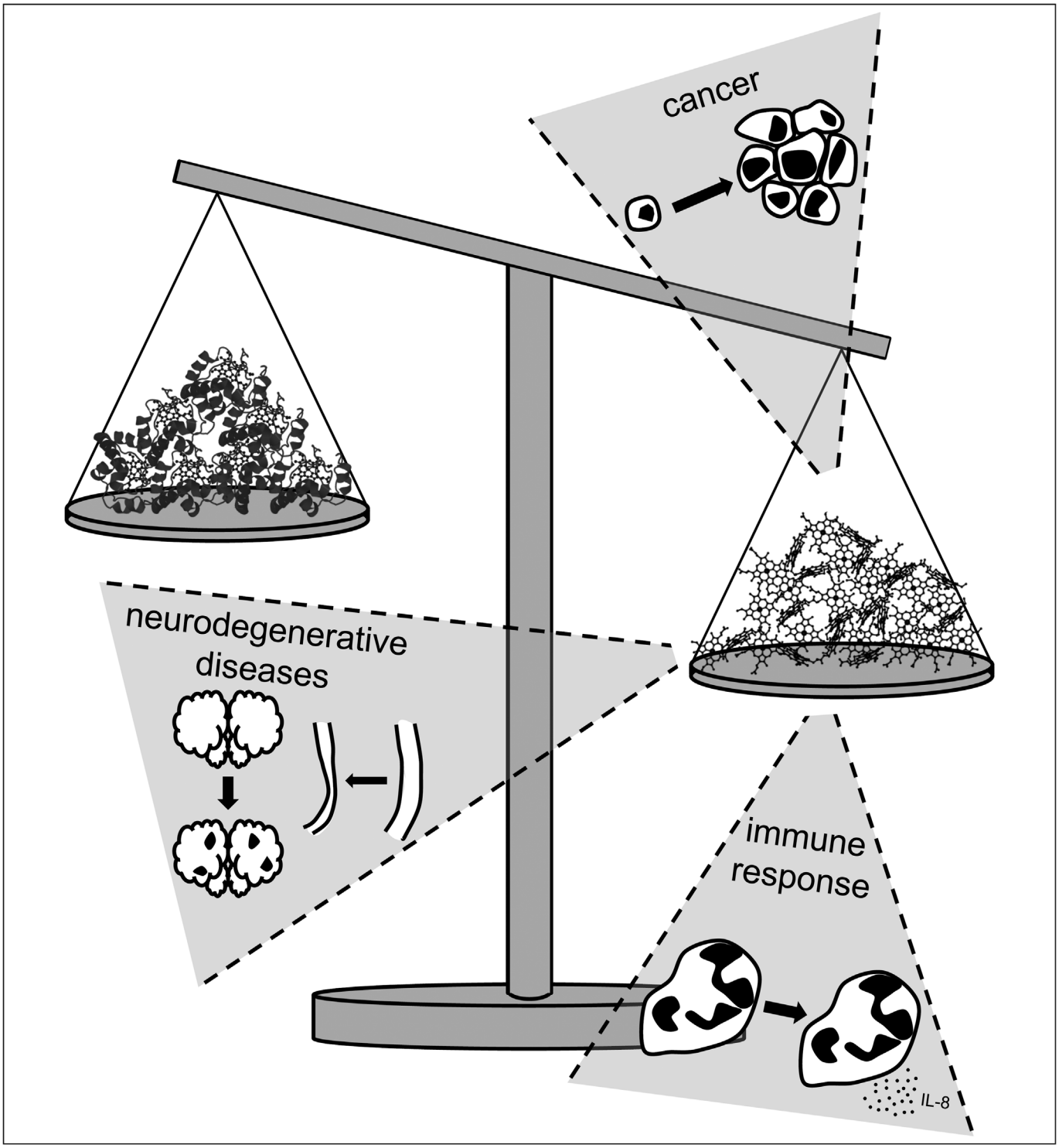


  

More than 20 years of research on heme as a temporary effector molecule of proteins have revealed its widespread impact on virtually all primary functions in the human organism. As our understanding of this influence is still growing, a comprehensive overview of compiled data will give fresh impetus for creativity and developing new strategies in heme-related research. From known data concerning heme-regulated proteins and their involvement in the development of diseases, we provide concise information of $Fe^{II/III}$ heme as a regulator and the availability of "regulatory heme". The latter is dependent on the balance between free and bound $Fe^{II/III}$ heme, here termed "hemeostasis". Imbalance of this system can lead to the development of diseases that were not always attributed to this small molecule. Diseases such as cancer or Alzheimer's disease highlight the reawakened interest in heme, whose function was previously believed to be completely understood.

## Introduction

Approximately 80% of heme (i.e., heme b: $Fe^{II}$–protoporphyrin IX) in the human body is produced and found in red blood cells, 15% is in the liver, and the remaining 5% is synthesized in other tissues.[1] In contrast to its rather limited sites of production, the role of $Fe^{II/III}$ heme in proteins is multifunctional. Basically, two aspects of action in heme–protein interactions need to be stressed. Firstly, there is a great variety of hemoproteins containing $Fe^{II/III}$ heme as an essential component for the protein architecture, as well as for the individual biological functions. Secondly, studies demonstrating the occurrence and impact of $Fe^{II/III}$ heme regulation of proteins have become an increasingly major focus in $Fe^{II/III}$ heme research over the last decade. This is supported by the steadily growing number of such proteins.

Recently, comprehensive and excellent reviews on heme sensor proteins and their importance in the development of distinct diseases have been published; these focus on issues such as the correlation between cancer and changes in $Fe^{II/III}$ heme levels.[2] For example, Girvan and Munro review the role of $Fe^{II/III}$ heme in the context of distinct proteins and discuss their binding parameters (if available). The central point is the function of $Fe^{II/III}$ heme-based gas sensor proteins, which represent a rather huge class of $Fe^{II/III}$ heme-binding proteins.[2a] A review by Hooda et al. highlights the importance of $Fe^{II/III}$ heme in the development of diseases such as anemia, porphyrias, Alzheimer's disease (AD), and, as the primary focus, cancer.[2b]

$Fe^{II/III}$ heme is known to be involved in several regulation processes; these can cause serious trouble if misregulated (hemolysis, cancer, AD, cerebral vasospasm, etc.). Therefore, a fine-tuned balance is required between free (heme that is not yet associated with apo-hemoprotein, and non-degraded heme liberated from hemoproteins)[3] and bound $Fe^{II/III}$ heme. Processes to correct disturbances of this balance are also required to maintain the correct heme homeostasis. Here, heme homeostasis is termed "hemeostasis", inspired by portmanteau words such as "proteostasis" and "biochemistry".

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[+] Parts of this review are available in German from the Digitale Bibliothek Thüringen (http://www.db-thueringen.de/servlets/DocumentServlet?id=24017).

This review outlines the progress and significant results obtained concerning proteins whose biological activity/function is modulated by temporary interaction with free $Fe^{II/III}$ heme ("regulatory heme"). We describe the current knowledge of $Fe^{II/III}$ heme-regulated proteins in the context of their cellular/biochemical process, and highlight the consequences of imbalanced hemeostasis, in particular how $Fe^{II/III}$ heme-regulated proteins are influenced by this and participate in $Fe^{II/III}$ heme-induced diseases. The fundamental role of $Fe^{II/III}$ heme as a prosthetic group in proteins and its impact on important biochemical processes is first summarized, as the availability of free $Fe^{II/III}$ heme also depends on the proper functioning of hemoprotein synthesis and degradation.

## Heme as an Essential Component in Hemoproteins

The integration of $Fe^{II/III}$ heme into hemoproteins is usually essential for their function in the context of specific biochemical processes, such as binding and transport of gases (e.g. oxygen, nitric oxide), catalytic processes, and electron transfer reactions (Scheme 1 A).

### Gas transport and storage

The ubiquitous and well-studied globins hemoglobin, myoglobin, neuroglobin, and leghemoglobin are the most common proteins to use $Fe^{II/III}$ heme for gas transport.[4] More specifically, they are responsible for the transport, removal, and storage of essential molecules such as oxygen within the cell. A common feature is the incorporation of one to four $Fe^{II}$ heme molecules with oxygen binding to the central $Fe^{II}$ ion.[4b,c] In humans, $Fe^{II}$ heme is predominantly found in the hemoglobin of erythrocytes (80%).

### Heme-based gas sensors

Another common function of $Fe^{II/III}$ heme is as a" heme-based gas sensor" (Scheme 2 A). These proteins carry $Fe^{II/III}$ heme as a permanently bound prosthetic group in a regulatory domain responsible for the recognition and binding of gases (e.g. CO, NO, $O_2$).[5] In response to this, the activity of the catalytic domain is changed. Depending on the structure, four main types of heme-based gas sensor can be distinguished: 1) Per-Arnt-Sim (PAS) domain sensors, 2) globin-coupled sensors, 3) CooA-heme binding domain sensors, and 4) heme-NO-bind-

ing proteins. Each can be coupled to various functions of the catalytic domain.[5] Typical heme-based gas sensors are neuronal PAS-domain-containing protein 2 (NPAS2, *Mus musculus*; involved in circadian rhythm)[6] and CO-sensing transcriptional activator (CooA, *Rhodospirillum rubrum*; regulation of expression of coo-operons).[7] Both are transcription factors that contain a $Fe^{II/III}$ heme molecule in their characteristic heme-binding pocket. By offering gas molecules such as CO, their function is modulated in a $Fe^{II/III}$ heme-dependent manner.[6,8]

Human proteins such as soluble guanylate cyclase (sGC) and cystathionine β-synthase (CBS) with their $Fe^{II/III}$ heme can be similarly regulated by gases.[9] $Fe^{II/III}$ heme-binding CBS is a lyase catalyzing the condensation of serine and homocysteine to a cystathionine intermediate in sulfur metabolism. Modified conditions, for example, changed pH or the application of gases (CO, NO) lead to significant changes in enzyme activity. In this context, $Fe^{II}$ heme is less active than oxidized heme. In addition, NO and CO lead to enzyme inhibition exclusively for $Fe^{II}$ heme.[9a]

Soluble guanylate cyclase (sGC) is activated by attachment of NO or CO to its $Fe^{II/III}$ heme.[9b–e] The resulting increase in cGMP (second messenger) leads to activation of protein kinase G; subsequent phosphorylation of downstream proteins influences signaling pathways that are involved in the cardiovascular and the nervous systems.[9b] In addition to NO, (lower) sensitivity of sGC for CO has been reported;[9c,d] sGC is also interesting as the function of $Fe^{II/III}$ heme is not completely solved. Recently it was reported that this protein can be regulated by $Fe^{II/III}$ heme, and that it also exists in an apo form in vivo.[9e] This is another example of a particular protein for which the link between heme-binding and heme-regulation are not fully understood.

Toni Kühl studied biochemistry at the Friedrich-Schiller-University Jena. During his PhD he moved from Jena to Bonn to continue his studies under the supervision of Diana Imhof. He specialized in heme and peptide chemistry and received his PhD in Biochemistry from the Friedrich-Schiller-University in 2013. He is currently working at the Pharmaceutical Institute of the University of Bonn as a postdoctoral fellow. His research interests include heme–peptide and heme–protein interaction, protein biochemistry, peptide and protein folding, and peptide ligation reactions.

Diana Imhof studied chemistry and biology at the University of Jena and Dublin City University, respectively. She received her PhD in biochemistry in 1999. She performed postdoctoral research at the University of Jena and Ohio State University (Columbus). From 2005, she was head of the Peptide Chemistry and Biology research group at the Center for Molecular Biomedicine in Jena. She joined the University of Bonn as Professor for Medicinal Chemistry and Drug Synthesis in 2011. Her research interests cover all aspects of peptide synthesis and characterization, peptide-based drug design, and peptides as tools to study molecular basis of biochemical phenomena.

### Heme-mediated enzyme catalysis

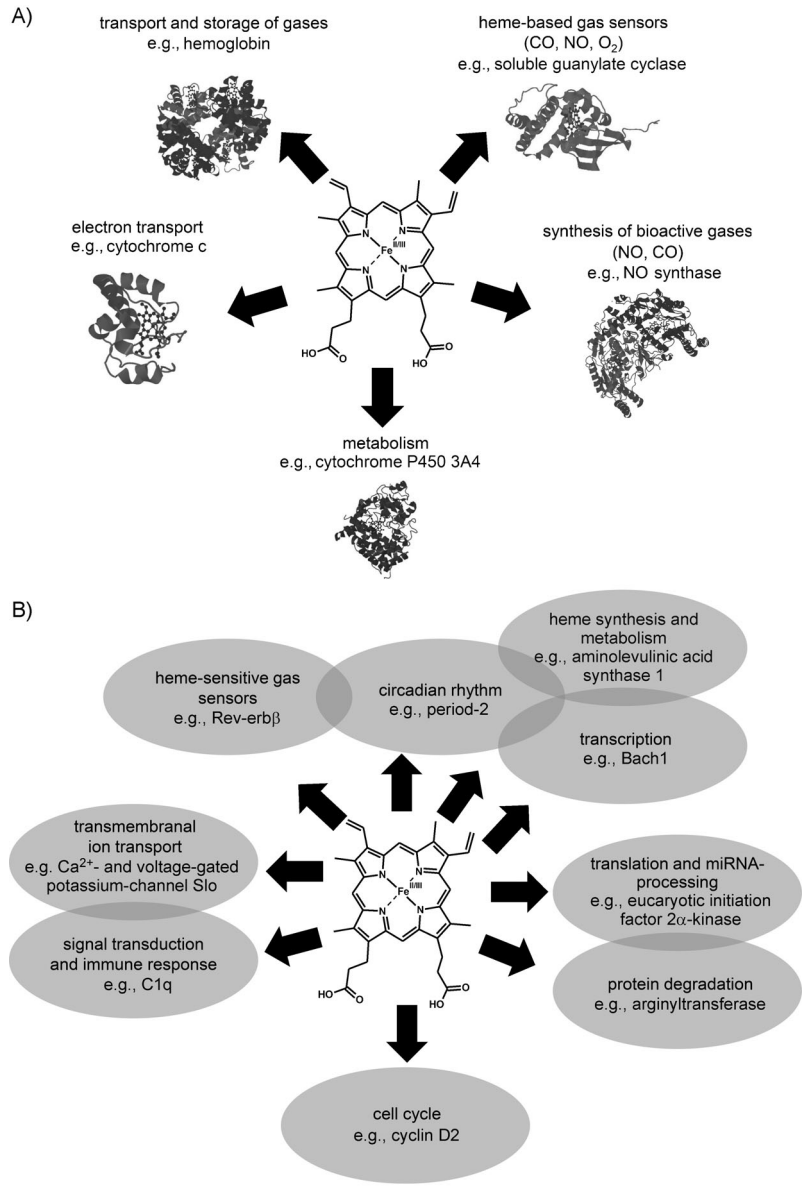
NO (required for the activation of various heme-based gas sensors) is synthesized by the $Fe^{II/III}$ heme-containing enzyme nitric oxide synthase (NOS). Three isoforms can be distinguished: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS).[10] These enzymes catalyze the conversion of arginine to citrulline with concomitantly released NO.[11] Within the reaction mechanism, both reduced ($Fe^{II}$) and oxidized ($Fe^{III}$) heme appear in complex with NO, whereas the release of NO is induced by $Fe^{III}$ heme only. In contrast, primarily $Fe^{II}$ heme triggers oxidation of NO to nitrate (by using oxygen).[10]

Other enzymes in which $Fe^{II/III}$ heme plays an important catalytic role are peroxidases, cytochrome P450 proteins, nitrate reductase, and heme oxygenase.[12] For these enzymes, (unlike sGC) the binding of $Fe^{II/III}$ heme and the mechanism of action are quite well understood in most cases.[13a–c] Especially for cytochrome P450, a huge set of representatives has allowed in-depth analysis of the structural features of $Fe^{II/III}$ heme binding and substrate conversion.[12a,c] P450, nitrate reductase, and heme oxygenase significantly influence the levels of free regulatory $Fe^{II/III}$ heme and thus hemeostasis. These enzymes and their impact for the organism are summarized below.

In the liver and certain areas of the brain, the main class of hemoprotein is cytochrome P450.[13] These monooxygenases have a strong influence on oxidative, peroxidative, and reductive metabolism of numerous endogenous substances (steroids, bile acids, fatty acids, etc.). For many of these enzymes, conversion of a broad spectrum of exogenous substances (e.g. drugs, alcohol, environmental toxins) is essential for health.[14] Recruitment of oxygen for substrate oxidation as well as electron transfer is performed by the $Fe^{III}$ heme molecule of the enzyme. Different iron oxidation states (II, III, IV) have been identified during the reaction cycle; $Fe^{III}$ is the "resting" state.[12a,b]

Peroxidases and catalases include a huge number of enzymes that carry $Fe^{III}$ heme at the catalytic site. These convert hydrogen peroxide to less reactive products;[12c,15] whereas catalases release water and oxygen, peroxidases oxidize a further substrate (electron donor) in addition to releasing water.[15,16] The significance of $Fe^{III}$ heme is its function in recruiting hydrogen peroxide and, in this context, with the formation of high valency iron–oxygen intermediates that lead to oxidation of the substrate.[17]

Additionally, heme oxygenases are responsible for $Fe^{II/III}$ heme degradation to biliverdin, CO, and ferrous iron. $Fe^{II/III}$




**Scheme 1.** A) $Fe^{II/III}$ heme as a permanently bound prosthetic group and functional unit of proteins plays a significant role in different biochemical reactions and processes (PDB IDs: 1GZX, 1HRC, 1U56, 2NSE, 1W0E). B) $Fe^{II/III}$ heme as a regulatory effector of proteins is involved in a variety of biochemical pathways and processes that partially overlap or influence each other.

heme binds to the protein and leads, in a stepwise manner, to the recruitment of oxygen and its degradation.[18]

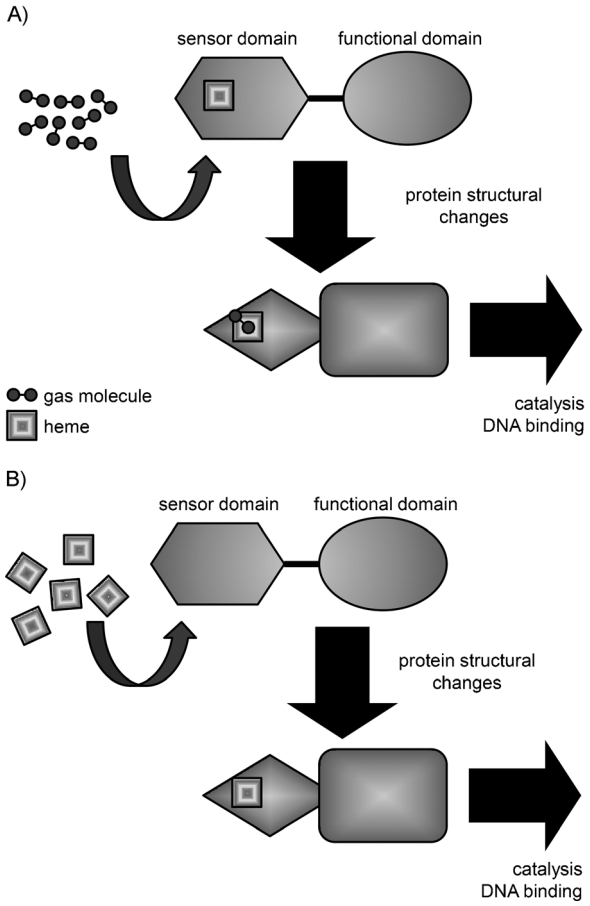
### Electron transport

Cytochrome $b_5$ is an essential protein in electron transport and involves oxidation/reduction of its heme group.[19] Cytochrome $b_5$ also forms part of other proteins (e.g., nitrate reductase, mitochondrial sulfite oxidase, flavocytochrome $b_2$), where the $Fe^{II/III}$ heme is also responsible for electron transfer.[12d,20] For cytochrome $b_5$ and others (e.g. flavocytochrome $b_2$), regeneration of $Fe^{III}$ heme and, thus the whole protein, is carried out by electron transfer to $Fe^{III}$ heme-containing cytochrome c.[20,21] By using the redox activity of the heme iron in cytochrome $b_5$, cytochrome c takes up and transports electrons along the membrane to the cytochrome c oxidase complex (respiratory chain).[21] However, these cytochromes differ in their binding of $Fe^{III}$ heme: cytochrome $b_5$ carries non-covalently bound heme b, whereas heme c in cytochrome c is covalently attached at the propionate side chains to cysteine residues of the protein.[22]

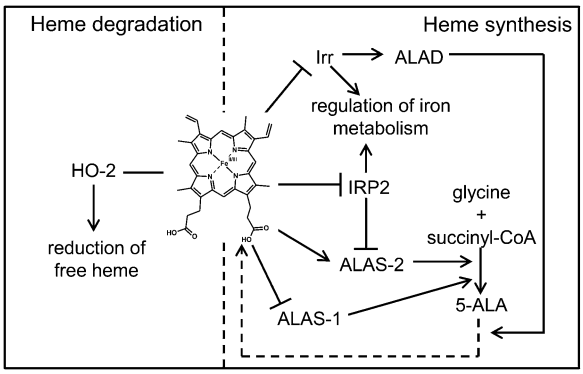
Thus, the character (e.g. binding mode, consensus sequence) and consequences of $Fe^{II/III}$ heme binding are obviously influenced by multiple factors, particularly protein architecture and environment. Similarity of hemoprotein biochemical function is not necessarily indicative of a similar construction. This implies an even more complicated situation for $Fe^{II/III}$ heme-regulated proteins, because less-distinctive features with respect to binding determinants and characteristics are expected.

### Heme as a regulatory effecter of protein function

There are several reports of a pool unbound cellular $Fe^{II/III}$ heme (~0.1–1.0 μM).[3] The significance of this free $Fe^{II/III}$ heme to the proteins mentioned above has not been take into account. However, the unbound $Fe^{II/III}$ heme does not necessarily account solely for the synthesis of hemoproteins; over the past two decades it role as a regulator of numerous proteins has gained importance.[23] Extracellular $Fe^{II/III}$ heme is usually bound to proteins such as hemoglobin, albumin, and hemopexin, but various conditions (e.g., bleedings after injury) can lead to increased levels of free $Fe^{II/III}$ heme in tissues. In hematomas, the concentration of (mainly) oxidized heme ($Fe^{III}$ heme) can exceed 350 μM.[24] After injury, release of hemoglobin from erythrocytes leads to oxidation of heme ferrous iron to form methemoglobin and subsequent liberation from the protein. This oxidation correlates with the formation of reactive oxygen species (ROS). Similar processes are found for other intra- and extracellular hemoproteins after cell lysis. Thus, for the surroundings of heme in several proteins, the binding capacity for $Fe^{III}$ heme is significantly lower.[3b] In contrast, $Fe^{III}$ heme (rather




**Scheme 2.** A) Modulation of the activity of a heme-based gas sensor (CooA, NPAS2, cystathionine β-synthase, or soluble guanylate cyclase) by diatomic gases (CO, NO, $O_2$). Upon binding of gas molecules to $Fe^{II/III}$ heme at the sensor domain, structural changes in the protein lead to changes in the functional domain thereby resulting in a modified protein activity. B) Similarly, heme-responsive protein perceives the presence of $Fe^{II/III}$ heme, which upon binding mediates structural changes and, again, alters protein activity. (Modified from ref. [6])




**Scheme 3.** $Fe^{II/III}$ heme reduces its own availability in a cell-type-specific way. In erythrocytes, $Fe^{II/III}$ heme induces primarily ALAS2 expression, whereas ALAS1 biosynthesis in most tissues (e.g. liver) is inhibited. Inhibition of IRP2 (*Homo sapiens*) and Irr (*B. japonicum*) is achieved by $Fe^{II/III}$ heme-induced proteasomal degradation of these proteins. This highlights the conservation of the regulatory function of $Fe^{II/III}$ heme in different organisms. Through binding of $Fe^{II/III}$ heme to heme oxygenase 2 (HO-2), the available amount of $Fe^{II/III}$ heme in the cell is reduced. (5-ALA: 5-aminolevulinic acid).

than $Fe^{II}$ heme) shows increased binding for cysteine in cytochrome P450 proteins and NOS. Assuming no other amino acids significantly increase the binding capacity for $Fe^{II}$ heme, for these proteins an inverse effect with respect to binding of $Fe^{II}$ heme or $Fe^{III}$ heme is observed compared to hemoglobin.[25]

The availability of free $Fe^{II}$ and $Fe^{III}$ heme occurs under different conditions, both intracellularly and extracellularly, thus demonstrating that a regulatory impact on proteins is possible. These effects can be observed at different stages of protein synthesis and degradation, in signal transduction, and in ion channel regulation, as well as in immune responses, in the reaction to diatomic gases, and many other processes (Scheme 1 B). The modes of action of such heme sensors are shown in Scheme 2 B.[6]

### Heme biosynthesis and metabolism

High levels of free $Fe^{II/III}$ heme (1.0–10.0 μM) are toxic due to the formation of ROS.[3a] Consequently, the synthesis and degradation of $Fe^{II/III}$ heme need to be strictly controlled. The biosynthesis of $Fe^{II}$ heme is performed in eight steps in mitochondria and the cytoplasm. It is controlled in nearly all cells (Scheme 3) by regulating the levels of the first enzyme in the synthesis (non-specific aminolevulinic acid synthase, ALAS1) in a $Fe^{II/III}$ heme-inducible negative feedback loop. High concentration of $Fe^{II/III}$ heme prevents transcription, translation, and translocation of ALAS1 into mitochondria.[26] In contrast, the erythrocyte-specific isoform ALAS2 is controlled completely differently with respect to $Fe^{II/III}$ heme. Here, an increase in the levels of ALAS2 is observed upon application of $Fe^{II/III}$ heme.[26a] Translocation into mitochondria is also different.[23e,26b] One reason for the increase in ALAS2 levels is associated with the regulation of iron-regulatory protein 2 (IRP2). In the case of ALAS2, its translation is inhibited by binding of IRP2 to iron-responsive elements (IREs) in the 5′ untranslated region.[2a,27] Binding of $Fe^{II/III}$ heme to IRP2 induces oxidation of the protein and subsequent ubiquitylation and proteasomal protein degradation,[2a,23b] which, in turn, allows translation of ALAS2 mRNA.[27]

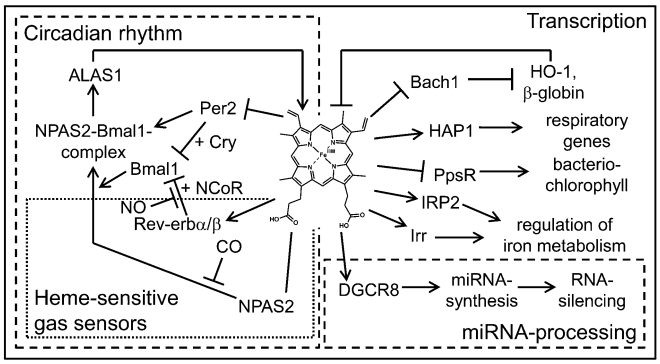
But degradation of IRP2 can also decrease protein synthesis, as for example in the case of the transferrin receptor.[27] IREs of the receptor mRNA are in the 3′-untranslated region; binding of IRP2 stabilizes the mRNA thereby supporting its translation. Thus, $Fe^{II/III}$ heme-induced degradation of IRP2 increases the probability of transferrin receptor mRNA degradation thus reducing protein synthesis.[27] IRP2 is obviously involved in the maintenance of iron homeostasis.[27] Another protein that regulates iron metabolism similarly to IRP2 is the iron-response-regulator (Irr) protein from *Bradyrhizobium japonicum*. Binding of $Fe^{II/III}$ heme also induces degradation of Irr.[2a,28] Additionally, iron deficiency leads to inhibition of aminolevulinic acid dehydratase in this bacterium (essential for $Fe^{II/III}$ heme biosynthesis).[29]

The binding of regulatory $Fe^{II/III}$ heme to the $Fe^{II/III}$ heme-degrading enzyme heme oxygenase 2 (HO-2) has also been de-

scribed, although no change in enzyme activity was observed. HO-2 is suggested to control the levels of free $Fe^{II/III}$ heme in the cell, as its $Fe^{II/III}$ heme-binding capacity is modulated by the redox state of its cysteine residues.[30] In contrast, heme oxygenase 1 (HO-1) is one of the most highly induced acute-phase proteins (stress reaction) for neutralizing released $Fe^{II/III}$ heme, which acts as a pro-oxidative and proinflammatory signal in wound healing.[31] Upregulation of HO-1 is achieved by the binding of $Fe^{III}$ heme to transcription regulator Bach1, which forms heterodimers in complex with proteins of the Maf-like oncoprotein family. These dimers mediate binding to Maf-recognition elements in the regulatory region of genes encoding proteins such as HO-1, globins, and ALAS2.[2a,32]

### Transcription, circadian rhythm, and heme-sensitive gas sensor

For a broad range of proteins, transcription in yeast, photosynthetic bacteria, and mammals is regulated by $Fe^{II/III}$ heme, for example, heme activator protein 1 (HAP1), Period 2 (Per2), transcriptional repressors Rev-erbα and Rev-erbβ, and transcription factor PpsR (Scheme 4). Bach1 belongs to this list and




**Scheme 4.** Versatile influences of $Fe^{II/III}$ heme on transcriptional (transcription repression and activation, miRNA-processing) are indicated for prokaryotes (e.g. Irr from *B. japonicum*, PpsR from *Rhodobacter sphaeroides*) and eukaryotes (e.g. HAP1 from *Saccharomyces cerevisiae*, Bach1 from *Homo sapiens*). Rhythmically oscillating $Fe^{II/III}$ heme levels are reflected in regulatory processes by $Fe^{II/III}$ heme during circadian rhythm. Therefore, binding of $Fe^{II/III}$ heme in a first step allows sensing concentration changes of CO and NO. (Cry: cryptochrome; DGCR8: DiGeorge syndrome critical region 8).

shows significant reduction in affinity for DNA upon binding of $Fe^{III}$ heme; this allows the transcription of genes for β-globins and HO-1.[32,33] Proteasomal degradation of Bach1 is induced by $Fe^{III}$ heme,[34] a regulatory mechanism that has also been observed for Irr and the human proteins IRP2, arginyltransferase 1 (ATE1), and Per2.[28]

Regularly fluctuating levels of $Fe^{II/III}$ heme led to investigations of proteins that are regulated by $Fe^{II/III}$ heme in the context of the circadian clock (Scheme 4).[35] Per2, as an example, contains the key function for connecting circadian rhythm to $Fe^{II}$ heme biosynthesis, by positively regulating the transcriptional activity of the Bmal1/NPAS2-complex (Bmal1: brain and muscle ARNT-like 1) that induces the expression of ALAS1 (among others).[35a] Simultaneously, in complex with cryptochromes, it inhibits expression of Bmal1 and, in a negative feedback loop, Bmal1/NPAS2 induces Per2 expression.[36] The $Fe^{III}$ heme-induced degradation processes of Per2 decrease activation of the Bmal1/NPAS2-complex and thus ALAS1 expression.[23d,35a] This appears to be a negative feedback loop as well, thus explaining the periodic increase of $Fe^{II}$ heme concentration.

With respect to circadian rhythm, human nuclear receptors Rev-erbα and Rev-erbβ are also known to bind transcription repressor NCoR and inhibit gene expression of proteins, for example Bmal1, in a $Fe^{II/III}$ heme-induced manner.[2a,35b] Additionally, Rev-erbβ was shown to act as a heme-based gas sensor for NO and CO upon $Fe^{II/III}$ heme application.[2a,37] For this reason this protein and others (e.g. NPAS2) have dual-role characteristics: heme sensor and, after $Fe^{II/III}$ heme recruitment, heme-based gas sensor.[4c,25]

HAP1 (*Saccharomyces cerevisiae*) is a transcription factor that is responsible for the activation of the synthesis of different respiration proteins, by a chaperone (Hsp70/Hsp90)-coupled mechanism. Excess $Fe^{II/III}$ heme and/or oxygen activates HAP1 and increases the transcription of various genes.[23c,34]

Recently, regulation of transcription repressor PpsR (*Rhodobacter sphaeroides*) by $Fe^{II/III}$ heme was reported: for example, $Fe^{II/III}$ heme binding is known to increase the synthesis rate of bacteriochlorophyll.[33]

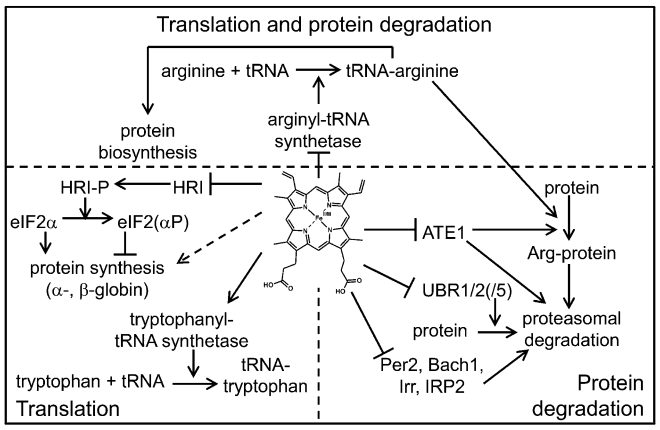
These examples demonstrate that a finely tuned balance of the levels of free regulatory and bound $Fe^{II/III}$ heme is of great importance for cellular homeostasis—in the case of initiation of gene expression, to control basic processes such as circadian rhythm, hemoglobin synthesis, and $Fe^{II/III}$ heme degradation. Changes in free $Fe^{II/III}$ heme levels can be immediately sensed and responded to.

### Translation and miRNA processing

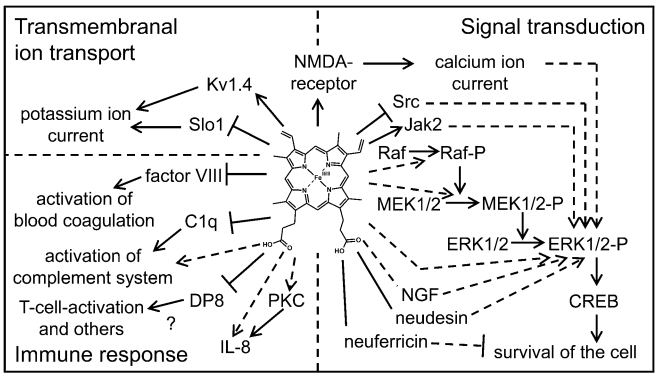
Translation is regulated in numerous ways (Scheme 5), and $Fe^{II/III}$ heme interacts at various points of the process including, for example, stability control of synthesized mRNA by miRNA and its processing (Scheme 4), as well as regulation of translation initiation (Scheme 5).[13,38]

During miRNA processing, binding of $Fe^{III}$ heme to DGCR8 (DiGeorge syndrome critical region 8 protein, *Homo sapiens*) is essential, to form the complex for synthesizing miRNA. Increased protein activity leads to increased formation of the miRNA that degrades complementary transcripts.[13,39] Apart from this silencing process, there are several further key processes that are regulated by $Fe^{II/III}$ heme and, thereby, protein translation is controlled.

Eukaryotic initiation factor 2 α (eIF2α) has a significant role in translation initiation. Phosphorylation at Ser51 decreases its activity, and protein synthesis is consequently inhibited.[38] This process has been observed in erythrocytes, and is mainly relevant for the synthesis of α- and β-globins. It can be triggered by eIF2 α kinase (HRI, heme-regulated inhibitor), which was downregulated upon $Fe^{II/III}$ heme application. For the latter, an intrinsic phosphorylation event in the HRI molecule was miss-




**Scheme 5.** Translation and proteasomal protein degradation are regulated and cross-linked by $Fe^{II/III}$ heme at different levels. Inhibition of HRI in erythrocytes increases synthesis of α- and β-globins; these integrate $Fe^{II}$ heme during the formation of hemoglobin. Arginyl-tRNA synthetase cross-links processes of translation and proteasomal protein degradation. Through attachment of $Fe^{II/III}$ heme to proteins Per2, Bach1, Irr, and IRP2, their own proteasomal degradation is induced. In contrast, degradation processes that are mediated by ATE1, UBR1, UBR2, and arginyl-tRNA synthetase are inhibited by $Fe^{II/III}$ heme. (HRI-P: phosphorylated HRI; eIF2(αP): phosphorylated eIF2α).




**Scheme 6.** Influences of $Fe^{II/III}$ heme on signal transduction pathways, immune responses, and transmembrane ion transport. $Fe^{II/III}$ heme binds directly to ion channels, which, in the case of hSlo1, leads to increased vasoconstriction in the brain.[49a,b] In most reports, changes in signal transduction levels and immune responses are general reactions upon application of $Fe^{II/III}$ heme to whole cells or from growth of cells on $Fe^{II/III}$ heme-deficient media. Neudesin and neuferricin are the first extracellular, temporary $Fe^{III}$ heme-binding proteins, and show different effects upon $Fe^{III}$ heme application.[3b] Reports on the cross-link of PKC and MAPK-pathway raise the suggestion of further crossovers not mentioned here. (ERK1/2-P: phosphorylated extracellular signal-regulated kinase 1/2; Raf-P: phosphorylated Raf; MEK1/2: phosphorylated MAP/ERK kinase 1/2; NGF: nerve growth factor).

ing, and intermolecular disulfide bridge formation between monomers in the HRI homodimer was observed, thereby resulting in loss of kinase activity.[38] This, in turn, allowed the synthesis of globin molecules to bind excess $Fe^{II}$ heme to form hemoglobin.[38]

The influence of $Fe^{III}$ heme on translation has also been reported by affecting aminoacylation of amino acids to their corresponding tRNAs. This process is catalyzed by specific aminoacyl tRNA synthetases. For example, arginyl tRNA synthetase (ArgRS) oligomerizes in vitro upon binding to $Fe^{III}$ heme, thereby losing its activity.[40] In contrast, tryptophanyl tRNA synthetase is activated by interacting with $Fe^{III}$ heme.[41]

In addition, there are numerous proteins that are degraded upon interaction with regulatory $Fe^{II/III}$ heme (Scheme 5), such as Irr, IRP2, Bach1, and Per2 (see above).[28] Another is ATE1, which increases the degradation probability of various proteins according to the N-end rule (by attaching arginine to their N termini).[23f] Binding of $Fe^{III}$ heme to ATE1 decreases enzyme activity by forming an intramolecular disulfide bridge as well as by initiation of protein degradation.[23f] For the synthesis of arginine-loaded tRNA, activity of $Fe^{III}$ heme-sensitive ArgRS is required (see above). Therefore, ArgRS and its sensitivity for $Fe^{III}$ heme are important for both protein synthesis and protein degradation.[40] Finally, ubiquitin-protein E3 ligases (e.g. UBR1, UBR2) have been shown to lose activity after binding to regulatory $Fe^{III}$ heme, thus further demonstrating the influence of $Fe^{II/III}$ heme on protein degradation.[23f]

### Signal transduction, immune response, and transmembrane ion transport

In addition to these regulatory effects of $Fe^{II/III}$ heme on metabolism, protein synthesis, and protein degradation, an impact on immune response and inflammatory processes is known (Scheme 6).[42] For example, C1q (the recognition unit of the first compound of the classical complement pathway) plays an important role for activation of the complement system. Upon binding of $Fe^{III}$ heme to C1q the mechanism of recognition between immunoglobulin G and C-reactive protein changes. This leads to reduced binding and thus prevents activation of further complement compounds.[42a] In vitro studies of the interaction of recombinant blood coagulation factor VIII and $Fe^{II/III}$ heme revealed an inhibitory effect.[43] Similarly, the activity of dipeptidyl peptidase 8 (DP8), which is known to play a role as a mediator of T-cell activation, was inhibited upon application of $Fe^{III}$ heme in vitro.[44] In addition to structural investigations, we reported the effect of $Fe^{III}$ heme on DP8 by using an enzyme assay employing the modified dipeptide Ala-Pro-*para*-nitroanilide as the substrate.[44a] Inhibition by $Fe^{III}$ heme occurred at the low-micromolar level ($IC_{50} = 11.5 \pm 1.2$ μM), which is higher than the concentration of free $Fe^{II/III}$ heme in the heme pool. This indicates that inhibition of dipeptidyl peptidase 8 is most important under pathophysiological conditions of increased levels of $Fe^{II/III}$ heme (e.g. hematomas). All these data emphasize the role of $Fe^{II/III}$ heme in the regulation of pathways and cascades that are closely connected to the immune system.

Concerning inflammatory processes, an increase of interleukin-8 (IL-8), both mRNA and protein, was observed upon $Fe^{III}$ heme application in human neutrophils.[42b] This was caused by increased activation of protein kinase C (PKC).[42b] However, inhibition of PKC still resulted in increased IL-8 expression following $Fe^{III}$ heme application. This led to the suggestion that further modes of regulation by $Fe^{III}$ heme exist, although these have not been fully explored or described.[42b] Regarding PKC, several reports have demonstrated a link to the mitogen-activated protein kinase (MAPK) pathway (Scheme 6).[45] This signal

transduction pathway to the nucleus via ERK1/2, MEK1/2, and Raf can also be activated by $Fe^{II/III}$ heme.[46] Usually, activation of the MAPK pathway is induced by ligand binding to a protein tyrosine kinases (e.g. epidermal growth factor receptor, platelet-derived growth factor receptor).[47] However, in HeLa cells, $Fe^{II/III}$ heme-regulated proteins such as Jak2 and Src were also substantially influenced by $Fe^{II/III}$ heme, as was the MAPK pathway.[48]

Motivated by studies on cerebral vasospasm, ion channels have gained increased interest concerning protein regulation by $Fe^{II/III}$ heme in recent years (Scheme 6).[23a,49] One of the most intensively studied proteins is the human large conductance calcium-dependent potassium channel Slo1 (hSlo1).[23a,49b,c] Intracellular binding of $Fe^{II/III}$ heme to the channel yielded a reduction in $K^+$ influx by (reversible) inhibition of the ion channel.[23a] In addition, a voltage-gated potassium channel ($K_V$; subtype $K_V1.4$ from *Rattus norvegicus*) showed intracellular interaction with $Fe^{III}$ heme; this impaired the inactivation process by the "ball-and-chain" N terminus of the channel protein induced by $Fe^{III}$ heme thus prolonging the opening of the channel pore.[50] This apparently contradictory influence of $Fe^{II/III}$ heme on ion channel activity highlights the complex network in which $Fe^{II/III}$ heme influences not only ion channels but also many different proteins in a wide variety of cellular processes.

## Pathophysiological Consequences of Heme-Associated Dysregulation

The ubiquitous presence and this diversity of function renders $Fe^{II/III}$ heme a central anchor in numerous essential processes. Dysregulation and interruptions in the typical $Fe^{II/III}$ heme-associated pathways can result in severe problems.

### Porphyria and anemia

Complete termination of $Fe^{II}$ heme biosynthesis is lethal for the organism. Several types of $Fe^{II}$ heme deficiency are caused by reduced biosynthesis, for example, anemia and porphyria.[26a,32]

Anemia can develop under various conditions, such as iron deficiency, defective globin synthesis, and dysfuntion of ALAS2, and is characterized by dramatically reduced hemoglobin synthesis.[38] Phenotypic changes in erythrocytes are typically observed, with sickle cell anemia (mutation in β-globin) as the most familiar variant.[51] Sideroblastic anemia is closely linked to $Fe^{II}$ heme biosynthesis, and results from less-active ALAS2.[26a,32] This enzyme catalyzes the rate-limiting step of the covalent attachment of glycine to succinyl-coenzyme A to form 5-aminolevulinic acid. Hindrance of this reaction (or its extent) causes an increase in toxic iron. Classical symptoms of anemia are pallor, breathlessness, exhaustion, and general weakness.[26a] However, synthesis of $Fe^{II}$ heme is an eight-step process, and thus malfunctions can also arise in the other seven enzymes (for an overview see Zhang and Sessoms.)[26a]

Defects and reduced rates of the relevant enzyme activities lead to accumulation of $Fe^{II}$ heme precursors such as porphyrin (the origin of "porphyria").[32] Typical symptoms are photosensitivity and neurological disturbance. Accumulation of $Fe^{II}$ heme precursors can also lead to red teeth and skin lesions upon light exposure, because of the light sensitivity of porphyrin precursors and subsequent ROS formation. Acute attacks have the characteristic symptoms abdominal pain, vomiting, occlusion, hypertension, tachycardia, and dysfunction of the bladder.[26a,32] Mental problems often also appear (depression, anxiety, sleep disorders, hallucination, and paranoia).[26a,32] Porphyrias are divided into inherited and acquired, as well as hepatic and erythropoietic.[26a] For erythropoietic porphyrias, light sensitivity is often the only characteristic symptom, whereas in most hepatic porphyrias acute attacks and neurodegenerative lesions are described (e.g., AD as discussed in the next section).[26a]

### Neurodegenerative diseases

Alzheimer's disease (AD), Parkinson's disease and (age-related) dementia are a selection of severe neurodegenerative disorders. In recent years, an increasing number of studies have been performed to clarify the correlation between altered $Fe^{II/III}$ heme levels and the occurrence of $Fe^{II/III}$ heme-related diseases. In general, in most of the affected cells, increased levels of HO-1 are observed.[3b] Degradation of $Fe^{II/III}$ heme, which is catalyzed by HO-1, leads to the formation of bilirubin, which exhibits antioxidative properties, and to CO, which has an important role as a neurotransmitter. These positive effects, however, contrast with the release of ferrous iron, which is toxic in high doses. Therefore, a balanced system is essential for $Fe^{II/III}$ heme degradation,[3b] and this can be is exemplified by the classical neurodegenerative diseases AD and Parkinson's disease. In AD, increased levels of HO-1 cause a decrease in Tau protein expression, and this leads to a reduced activation of the MAPK pathway.[52] In 2004, $Fe^{III}$ heme interaction with human amyloid-β protein (Aβ) was shown in vitro for the first time, thus shedding light on how $Fe^{III}$ heme is involved in the development of AD.[53] In addition, a complex formed between Aβ and $Fe^{III}$ heme displayed peroxidase activity; this was suggested to cause additional oxidative stress in AD affected cells. Thus, the disease-related, typically elevated oxidative stress levels are further elevated.[54] In the initial phase, the Aβ–$Fe^{III}$ heme complex prevents the formation of cytotoxic Aβ aggregates.[54,55] In parallel, reduction in the levels of ALAS1 and porphobilinogen deaminase (and, subsequently, of $Fe^{II}$ heme biosynthesis) have also been observed in the early phases of AD. This was suggested to induce $Fe^{II/III}$ heme deficiency, which does not prevent the development of the characteristic plaques in contrast to the formed Aβ-$Fe^{III}$ heme complexes at non-deficient cells for heme biosynthesis.[3b,53]

In Parkinson's disease, increased levels of HO-1 are accompanied by the appearance of "Lewy bodies" in affected dopaminergic neurons.[56] This suggests a therapeutic approach in which increased expression of HO-1 recovers the cell's redox homeostasis to achieve neuroprotective and anti-inflammatory effects.[57]

Of relevance for dementia (as well as other neurodegenerative diseases), application of extracellular $Fe^{III}$ heme to cortical neurons induced rescue of the affected cells, thus preventing degradation of neurites and apoptosis.[3b,58] A survival-promoting signal is induced by recovering ERK1/2 activity upon $Fe^{III}$ heme application (Scheme 6).[3b,58] Cortical neurons that grow under $Fe^{II/III}$ heme depletion showed significantly decreased neurite generation.[58] Additionally, a dependence of ERK1/2 activity on activation of membrane-localized *N*-methyl-D-aspartate (NMDA) receptors was observed. $Fe^{II/III}$ heme depletion caused a decrease in calcium influx into the cell, and this reduced ERK1/2 activity.[3b,58] There was a simultaneous decrease in expression, phosphorylation level, and functionality of NMDA receptors; these are indispensable for the processes of learning and memory, as well as for synaptic plasticity.[3b,58,59]

### Aging

A decrease in $Fe^{II/III}$ heme synthesis is found with increasing age.[3b] The resulting $Fe^{II/III}$ heme deficiency causes problems such as neurodegeneration and reduction of Complex IV (respiratory chain).[32,60] The latter is accompanied by an increase in oxidative stress and malfunctions in calcium homeostasis.[61] Medication use increases with age, yet the drugs need to be converted by cytochrome P450 proteins.[3b] Synthesis of these proteins is indispensable in most cases where increased amounts of pharmaceuticals need to be managed. But, usually decreased $Fe^{II/III}$ heme biosynthesis is caused by decreased levels of ALAS1 and heme oxygenase. Consequently, $Fe^{II/III}$ heme depletion leads to reduced availability of cytochrome P450. Cytochrome P450 CYP1A1, for example, is reported to require $Fe^{II/III}$ heme for its transfer from the cytosol to the endoplasmic reticulum to be finally converted into its active form.[62]

All these reports clearly identify $Fe^{II/III}$ heme as a risk factor that has a significant influence on aging processes of cells; however, in most cases the molecular basis leading to these conditions has yet to be elucidated.

### Hemolysis and cerebral vasospasm/subarachnoid hemorrhage

In contrast to porphyria and anemia, increased levels of $Fe^{II/III}$ heme can also cause physiological disturbances. Uncontrolled release of $Fe^{II/III}$ heme (concentrations above physiological) by lesions in cells and tissues can arise for very different reasons. Hemolytic anemia, sickle-cell anemia, malaria, trauma, and bleeding are typical causes of increased concentration of regulatory $Fe^{II/III}$ heme.[42b,51a] Subsequently, the organism responds by stimulating a proinflammatory signal to initiate an immune response. Degradation of $Fe^{II/III}$ heme is then an essential step that is realized by a HO-1-induced switching off of the original trigger of inflammation.[31]

Hemolysis is an $Fe^{II/III}$ heme-releasing processes that induces degradation of erythrocytes in the liver and spleen under controlled conditions, when erythrocytes are approximately 120 days old.[63] If this process is initiated earlier (i.e., pathophysiological conditions), excess cytotoxic $Fe^{II/III}$ heme is released from hemoglobin, and this urgently needs to be bound and degraded.[3a,26a] In the case of defects in $Fe^{II/III}$ heme degradation, hyperbilirubinemia can develop; this can cause liver dysfunction and jaundice among other disorders.[26a] A classical dysfunction of this type is neonatal jaundice, which is induced by insufficient activity of UDP-glucuronosyl transferase. It can be treated by application of blue light to convert excess bilirubin to non-toxic products.[26a] The consequences of untreated, repeatedly occurring hemolysis are ubiquitous and can result in severe disorders ranging from arteriosclerosis to kidney failure.[32,42b]

Bleeding caused by injuries results in increased concentrations of $Fe^{II/III}$ heme ($>350\ \mu M$) in the resulting hematomas.[24] Typically, the heme oxygenase system and efficient $Fe^{II/III}$ heme-binding proteins (hemopexin, albumin) serve as scavengers to buffer the action of free $Fe^{II/III}$ heme.[51a] However, $Fe^{II/III}$ heme levels that exceed the normal have negative consequences due to free regulatory $Fe^{II/III}$ heme, both intracellularly and extracellularly.[3b] For the latter, $Fe^{II/III}$ heme uptake is a prerequisite and can occur in different ways. Ebert et al. discussed passive diffusion across the membrane within 30 min for heme (i.e. hematin),[64] recent reports emphasize the existence of distinct transport proteins for $Fe^{II/III}$ heme transfer.[65] Imported $Fe^{II/III}$ heme induces inflammation processes, for example, activation of IL-8 by PKC (Scheme 6).[42b] Additional effects are activation of vasoconstriction and of the complement system.[31] This contrasts with the reports on inhibition of C1q-mediated initiation of the complement system by $Fe^{III}$ heme (see above).[42a] Another proinflammatory signal is the formation of ROS caused by $Fe^{II/III}$ heme; this leads to activation of transcription factors NF-κB, AP-1, and SP-1.[31,51a] Independently, there was an increase in the expression of adhesion proteins and selectins in endothelial cells: this in turn recruits leucocytes.[51a] The combination of these and other factors (for details see ref. [51a]) enables an adequate response by the immune system to increased regulatory $Fe^{II/III}$ heme after injury.

Hemorrhagic infarct, intracerebral bleeding, and subarachnoid hemorrhage (SAB) are responsible for an increase in free $Fe^{II/III}$ heme levels and thus toxicity.[3b] Typical symptoms of SAB are pathological vasoconstriction and cerebral vasospasm.[49a,66] After bleeding in the central nervous system, hemoglobin is released from erythrocytes within a few hours.[67] In addition to the formation of cytotoxic ROS by $Fe^{II/III}$ heme and $Fe^{II/III}$ heme degradation products such as iron, accumulation of these can cause brain atrophy.[3b] In an alternative route, $Fe^{II/III}$ heme degradation leads to bilirubin oxidation products A and B (BOX A, BOX B) and 4-methyl-3-vinylmaleimide (MVM), which are suggested to be involved in the development of vasoconstrictive processes in cerebral vasospasm.[66] Also, $Fe^{II/III}$ heme might directly affect ion channel proteins, for example, hSlo1 (Scheme 6) in cerebral tissue; these are modulated by $Fe^{II/III}$ heme binding to an intracellular site on the channel.[23a,49b] Following SAB, this regulation was suggested to initiate vasoconstriction of vessels and vasospasm that, in turn, can lead to an infarct.[49a,b]

**Cancer**

Growth and apoptosis are significant in a cell's fate. Growth is regulated by the cell cycle, but apoptotic mechanisms are controlled by proteins that can be regulated in different ways, thus causing events ranging from cell-cycle arrest to specific differentiation and/or cell degradation.[32] The impact of $Fe^{II/III}$ heme is cell-type-specific,[32] as was demonstrated with PC12 neurons and K562 erythroid cells, which differentiate upon treatment with $Fe^{III}$ heme. In contrast, in HeLa cells increased cell growth and cell-cycle activity was reviewed.[32] Different proteins are responsible for this process, for example, p18, p21, and p53 as negative regulators of cell cycle, and cyclin-dependent kinase 1 (Cdk1) and cyclin-dependent kinase 4 (Cdk4) as positive regulators. In K562 cells, differentiation was initiated after $Fe^{II/III}$ heme application through induction of p18 and p21, and these was a simultaneous decrease in cyclin D2 expression.[32,46] In contrast, in HeLa cells $Fe^{II/III}$ heme depletion led to an increase in p21 and p53 and inhibition of Cdk1 and Cdk4, thus forcing the cell into cell-cycle arrest and eventually apoptosis.[3b,32]

Detailed analysis of HeLa cells identified $Fe^{II/III}$ heme as regulator of the phosphorylation activity of various proteins of the MAPK pathway (Scheme 6): activation and phosphorylation of Raf, MEK1/2, and ERK1/2.[46] In addition, protein tyrosine kinases (e.g., Jak2 and Src) showed significantly changed phosphorylation activity upon application of $Fe^{II/III}$ heme.[48] Jak2, for example, was activated and phosphorylated on tyrosine residues 1007/1008, whereas phosphorylation of tyrosine 530 of Src inhibited its protein tyrosine kinase activity. Src is a well-known oncogene and is thus a promising target for a tumor therapy.[48,68]

Derived from their studies, Ye et al. postulated that inhibition of $Fe^{II/III}$ heme biosynthesis in cancer cells would be a starting point for blocking cell proliferation and, moreover, for inducing senescence and apoptosis in affected tissue.[46] Yao et al. suggested the use of $Fe^{II/III}$ heme analogues to mimic or change the reactions of protein tyrosine kinases upon application of $Fe^{II/III}$ heme or its analogues.[48] Finally, a recent comprehensive overview by Hooda et al. extensively underlines the direct correlation between dietary $Fe^{II/III}$ heme intake and the risk of different types of cancer, type 2 diabetes, and coronary heart diseases.[2b]

## Summary

Today $Fe^{II/III}$ heme is recognized as a temporary regulator of protein activity and as a key player in a complex network that interacts with a broad spectrum of cellular processes (circadian rhythm, transcription, translation, signal transduction, immune response, etc.). The implications of such processes for the development of diseases such as AD, cerebral vasospasm, and cancer caused by dysregulation of the level of free $Fe^{II/III}$ heme are obvious. This emphasizes the necessity for a detailed understanding at the molecular level of processes affected by regulatory $Fe^{II/III}$ heme in a cellular context; this is, in our opinion, still missing. The intention of this review is to combine the current knowledge on $Fe^{II/III}$ heme-regulated proteins and related diseases in an ambitious attempt to support closing this gap.

Although there is a well-tuned mechanism for discriminating free and bound $Fe^{II/III}$ heme in an organism, the level of free $Fe^{II/III}$ heme significantly influences the proportion of regulatory free $Fe^{II/III}$ heme. Because of the influence of $Fe^{II/III}$ heme on a broad repertoire of $Fe^{II/III}$ heme-regulated proteins, the disturbance of homeostasis is a clear indicator for the development of the aforementioned disorders.

## Outlook

The selection here of $Fe^{II/III}$ heme-regulated proteins and pathways is not intended as a complete registration of $Fe^{II/III}$ heme-binding and $Fe^{II/III}$ heme-regulated proteins; nor do we provide an in-depth view on the molecular (structural) basis of the specific $Fe^{II/III}$ heme–protein interactions. Instead, we chose these examples to provide indicators as to where the topic is likely to head in the next few years. $Fe^{II/III}$ heme is far from being one of the molecules checked off the list of valuable research topics; its involvement in a broad variety of biochemical processes as a regulator illustrates the new opportunity for researchers to participate in the exploration of its involvement in the development and progression of diseases. Thus, in our opinion, novel diagnostic and therapeutic strategies and pharmaceuticals in the near future can be derived by exploiting research related to regulatory $Fe^{II/III}$ heme.

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