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Reversible Binding of Heme to Proteins in Cellular Signal Transduction

SHANGWEI HOU,[†] MARK F. REYNOLDS,[§] FRANK T. HORRIGAN,[†] STEFAN H. HEINEMANN,[‡] AND TOSHINORI HOSHI*,[†]

Department of Physiology, University of Pennsylvania, 3700 Hamilton Walk, Philadelphia, Pennsylvania 19104, Department of Chemistry, Saint Joseph's University, 5600 City Avenue, Philadelphia, Pennsylvania 19131, and Institute of Molecular Cell Biology, Molecular and Cellular Biophysics, Medical Faculty, Friedrich Schiller University, Jena, Germany

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ABSTRACT

Heme plays critical roles in numerous biological phenomena. Recent evidence has uncovered a new role of heme in cellular signal transduction, and its mechanism involves reversible binding of heme to proteins. This Account highlights the novel function of heme as an intracellular messenger in the regulation of gene expression and ion channel function.

Heme Structure

Heme consists of an iron atom and an organic protoporphyrin macrocycle. The protoporphyrin is made of four pyrrole rings linked by methene bridges to form a tetrapyrrole ring, and the four pyrrole nitrogens bind iron in the center of the porphyrin. The heme iron can exist in either the reduced ferrous (Fe^{2+}) or the oxidized ferric (Fe^{3+}) state with the pyrrole nitrogens sharing a −2 charge. The oxidation state of the heme iron is often critical in its biological roles.

Hemes are classified according to the type of groups attached to the periphery of their tetrapyrrole macrocycle. The *c*-type hemes have four methyl groups, two propionic acids, and two vinyl-thioether groups attached to their porphyrin periphery. The *b*-type hemes, as found in hemoglobin and myoglobin, have two vinyl groups and are referred to as iron protoporphyrin IX. The *a*-type hemes have a formyl group instead of the methyl group and a farnesylated group instead of a vinyl group. In *c*-type heme, the two vinyl thioether side chains are covalently attached to cysteine residues of the hemoprotein, as in cytochrome *c*. In contrast, the porphyrin periphery of *a*- and *b*-type hemes is not covalently bound to the hemoprotein. Consequently, *a*- and *b*-type hemes are more readily removed from hemoproteins than *c*-type hemes.

Coordination of Heme in Hemoproteins

The iron center in free heme is bound by four nitrogens of the porphyrin macrocycle. One or two additional axial ligand(s) may be present when heme is coordinated by proteins. The fifth or proximal ligand is typically provided by histidine, cysteine, or tyrosine, forming a five-coordinate heme. In many five-coordinate hemoproteins, the distal position may accommodate a water or a specific gas molecule such as O_2, NO, or CO.[1] Six-coordinate hemes with two amino-acid ligands bound to the heme iron are often found in electron-transfer proteins.

Biosynthesis and Degradation of Heme

Heme is synthesized in all nucleated cells in the human body (Figure 1). The starting substrates glycine and succinyl coenzyme A in the cytoplasm are eventually converted to protoporphyrin IX in mitochondria. In the final step, ferrous iron (Fe^{2+}) is incorporated by mitochondrial ferrochelatase (FC) to form heme (Fe^{2+} protoporphyrin IX).[2]

The main catabolic breakdown pathway for heme (Figure 1) typically involves heme oxygenase (HO; E.C. 1.14.99.3),[3,4] but an additional family of heme-degrading enzymes without significant sequence similarity to HO has been discovered in bacteria.[5] In humans, at least three isoforms of HO (HO1, HO2, and HO3) are known.[4] Expression of HO1 is induced by many of stimuli, including heme itself.[3,4] HO cleaves heme at the α-methene carbon atom, leading to formation of carbon monoxide (CO), biliverdin, and Fe^{2+} in an O_2-dependent manner.[3,6] Without O_2, HO is unable to catalyze the reaction, and this dependence may have an implication in cellular O_2 sensing.[7] The main catabolic byproduct biliverdin is converted by the enzyme biliverdin reductase (BVR) to bilirubin, which is transported to the liver to form conjugated bilirubin and excreted from the body.

The degradation process of heme is notable because many catabolic products appear to have a cellular signaling effect. For instance, CO may be a modulator of neuronal and cardiovascular functions.[4] Biliverdin and bilirubin have anti-inflammatory and antioxidants effects.[3]

Shangwei Hou obtained a B.S. from Shanxi Medical University, Shanxi, China, in 1998 and a Ph.D. from Fudan University, Shanghai, China, in 2004 and is a postdoctoral fellow at the University of Pennsylvania.

Mark Reynolds earned a B.S. from Grinnell College in 1993 and a Ph.D. from University of Wisconsin, Madison, in 1999 and is Assistant Professor of Chemistry.

Frank T. Horrigan received a B.S. from the University of Michigan in 1984 and a Ph.D. from Stanford University in 1991 and is currently Assistant Professor of Physiology.

Stefan H. Heinemann was awarded both undergraduate and Ph.D. (1990) degrees from the University of Göttingen, Germany, and is now Professor of Biophysics.

Toshinori Hoshi obtained a B.A. from the University of New Hampshire in 1981 and a Ph.D. from Yale University in 1985 and is Professor of Physiology.

* To whom correspondence should be addressed. Mailing address: Department of Physiology, Richards D100, 3700 Hamilton Walk, University of Pennsylvania, Philadelphia, Pennsylvania 19104. Phone: 215-573-7305. E-mail: hoshi@hoshi.org.

[†] University of Pennsylvania.

[§] Saint Joseph's University.

[‡] Friedrich Schiller University.

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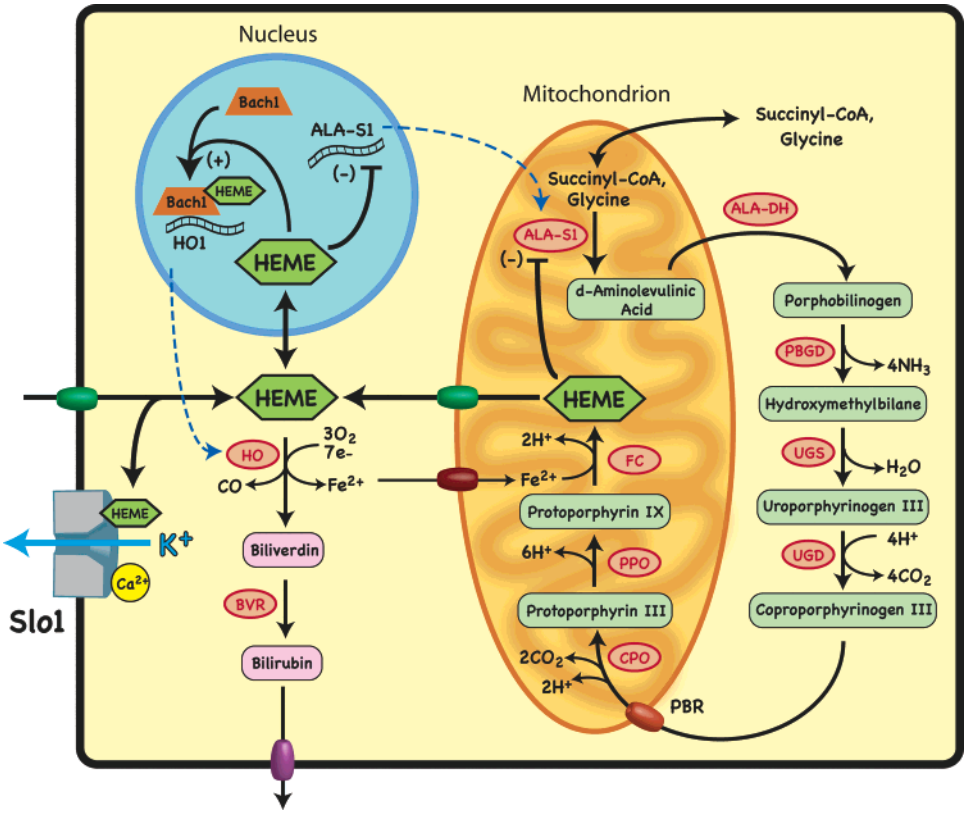

FIGURE 1. Intracellular heme: synthesis, signal transduction, and catabolism. Heme synthesis and catabolism pathways are schematically illustrated. Heme may be transported across the plasma membrane and modulate Slo1 BK channels and other proteins. Abbreviations: ALA-DH, minolevulinic acid, delta-, dehydratase; PBGD, porphobilinogen deaminase; UGS, uroporphyrinogen synthase; UGD, uroporphyrinogen decarboxylase; CPO, coproporphyrinogen oxidase; PPO, protoporphyrin oxidase; PBR, peripheral-type benzodiazepine receptor. Others are defined in the text. In plants, there is another pathway involving glutamyl transfer RNA.[48]

Intracellular Heme Homeostasis

While heme is essential for life, excess intracellular heme is highly toxic to cells in part because of its potent oxidizing propensity.[8] Accordingly, the concentration of intracellular free heme ($[heme]_i$) is tightly controlled at low levels. To maintain a low $[heme]_i$, multiple cellular mechanisms exist. A large fraction of heme, once synthesized, is incorporated into apo-hemoproteins to form hemoproteins. Heme also acts as a feedback inhibitor of its own synthesis and also as an inducer of HO1.[2] Degradation of heme is enzymatically catalyzed by HO. Despite these mechanisms in place, $[heme]_i$ may dramatically increase under certain conditions. For instance, hemorrhagic stroke and brain trauma lead to lysis of red blood cells and an increase in the extracellular heme concentration, which in turn could raise $[heme]_i$.[9] The mechanism of heme entry into cells, however, is not yet clear. The lipophilic nature of heme may allow it to passively transverse the membrane. Heme may be also transported by protein transporters/carriers.[10−12]

Heme as a Stable Prosthetic Group in Heme-Based Gas Sensors

The role of heme as a stable prosthetic group of hemoproteins is well-known; heme cofactors are present in numerous proteins, including hemoglobin, myoglobin, cytochromes, oxidases, and catalases, where they play critical structural and functional roles. In addition, the heme iron often confers an exquisite sensitivity to gases, such as O_2, NO, and CO, to selected hemoproteins. These gas-sensor proteins typically contain two distinct domains, a heme-containing regulatory domain and a catalytic domain, and gas binding to the heme-containing regulatory domain regulates the catalytic domain function.[13] Well-known examples of heme-containing regulatory domains are the Per-Arnt-Sim (PAS) domain, the globin-coupled sensor (GCS), the CooA heme binding domain, and the heme-NO-binding proteins (H-NOX).[1] The regulatory domains are coupled to a variety of catalytic domains including histidine protein kinases, cyclic nucleotide phosphodiesterases, and transcription factors. For example, the catalytic activity of soluble guanylate cyclase (sGC) is significantly enhanced by NO binding to the distal side of the heme iron.[14] The gas selectivity of H-NOX has been extensively studied.[15] Spectroscopic measurements suggest that NO binding to the heme iron in mammalian sGC displaces the proximal histidine ligand.[16] Although CO can also bind to the heme iron in sGC, the enzyme is not activated as efficiently.[17]

Heme as a Cellular Messenger

In the aforementioned hemoproteins, heme exists as a stable and essential prosthetic group. Removal of the heme cofactor from the apoproteins renders the proteins

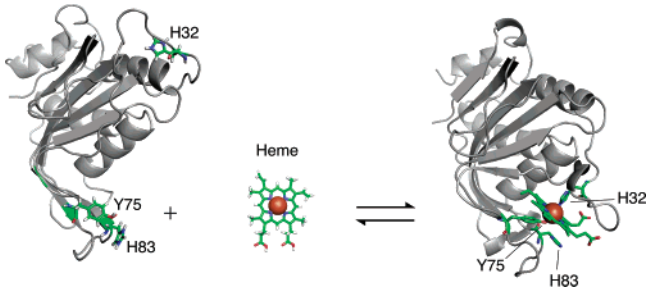

FIGURE 2. Reversible binding of heme to HasA. Structures of apoHasA characterized with NMR spectroscopy (1YBJ) (left)[21] and the heme-bound HasA determined with X-ray crystallography (1B2V) (right).[20] The numbering is according to 1B2V.

nonfunctional and structurally less stable. Recent results, however, show that the functions of selected proteins are acutely modulated by reversible binding of heme; thus heme acts as a cellular signaling messenger. This emerging role of heme is illustrated in selected gene transcriptional factors and ion channel proteins.

Heme Transporters and Carriers. Transport of heme from the extracellular medium to the cytoplasm across the plasma membrane is in part mediated by proteins.[10−12] The results obtained using these heme transport proteins may facilitate our understanding of the mechanisms of reversible binding of heme observed in transcription factors and ion channels and are briefly summarized below.

Several pathogenic bacteria take up iron by capturing heme from their hosts using a heme-binding protein called HasA, which captures a Fe^{3+} *b*-type heme from the host's hemoglobin or free heme with a high affinity ($K_a = 5.3 \times 10^{10}$ M^{-1}).[18] The holoprotein HasA binds to another protein located on the outer membrane, HasR, and transfers the heme to HasR, which then transports the heme to the cell interior.[19] The heme site environment of HasA (Figure 2) is noticeably different from those of other hemoproteins that contain heme as a stable prosthetic group.[20] In the fully coordinated state, the HasA heme is six-coordinate with His32 and Tyr75 bound to the heme iron, and the bound heme is solvent exposed. An initial step in heme unbinding is postulated to involve disruption of the axial ligation by Tyr75 by protonation of His83, placing the heme in the five-coordinate state.[21] The binding of heme in HasA may provide important structural clues about how heme binds to selected transcription factors and ion channel proteins.

Heme-Mediated Regulation of Gene Transcription. Heme-mediated gene transcription has been investigated extensively in yeast, where gene expression is finely regulated by oxygen tension.[22] In this model organism, intracellular free heme plays a pivotal role in up- and down-regulation of gene expression by reversibly binding to transcription factor proteins such as heme activator protein A (HAP1). Although the information is limited, similar transcriptional regulation by heme probably exists in mammals. For example, heme regulates expression of HO1 by binding to the transcription factor Bach1 to control its own degradation.[23]

Heme and Gene Expression in Yeast: HAP1. The yeast protein HAP1, a member of the GAL4 transcriptional regulator family, contains three functional domains: an N-terminal DNA binding domain with a zinc cluster motif, a heme-binding domain, and a C-terminal transcriptional activation domain.[24] In the absence of heme, HAP1 is found with other proteins,[25] forming a high molecular weight complex (HMC). The HAP1 proteins bound in HMCs are ineffective in binding to DNA but binding of heme to the heme-binding domain releases HAP1 from the large protein complex. HAP1 then forms dimers, which in turn bind to DNA with high affinity and activate transcription of several genes involved in cellular respiration and oxidative damage control.[24] The transcriptional activity of HAP1 reaches its maximal level at micromolar $[heme]_i$.[24] The unbinding of heme terminates the transcriptional activity by allowing HMC formation.

The heme-binding domain of HAP1 contains six heme-responsive motifs (HRMs), each of which is represented by the amino acid sequence K/R-C-P-V/I-D-H. The reversible interaction between this motif and heme was studied in a short synthetic peptide using spectrophotometric and gel filtration methods.[26] The peptide shifts the Soret absorption peak of heme from 388 to 362 nm, and the interaction with heme requires the cysteine residue in the sequence, which probably acts as an electron donor to chelate the heme iron.[24]

Heme and Gene Expression in Mammals: Bach1. HO1 is an inducible enzyme that breaks down heme, and the enzyme expression itself is stimulated by the substrate heme, constituting a physiological regulatory cycle. This heme-induced expression of HO1 is mediated by the transcription factor Bach1, to which heme binds directly.[27] UV−vis spectroscopy shows that heme binds to purified Bach1 with a K_d value of 100−200 nM.[27] While the Scatchard plot analysis shows that Bach1 functionally possesses only one heme-binding site, the protein contains six cysteine−proline (CP) motifs potentially capable of binding heme. Experiments using Bach1 protein fragments indicate that four of the six CP motifs in Bach1 are individually capable of accommodating heme, by noncovalently interacting with the heme-iron center. The multiple CP motifs may act to increase the local heme concentration.[27]

Heme also down-regulates its own synthesis by binding to Δ-aminolevulinate synthase 1 (ALAS1), the rate-limiting enzyme in heme synthesis. Binding of heme to the HRMs of the enzyme may occur in a cysteine-dependent manner and inhibits its import into mitochondria.[28]

Acute Modulation of Ion Channels by Heme. Recent experiments demonstrate that heme acutely modulates a selected type of ion channel involved in synaptic transmission and muscle contraction, further supporting the concept that heme may function as an intracellular messenger. Large conductance Ca^{2+}- and voltage-activated Slo1 K^+ channels are transmembrane ion channel proteins and provide gated pathways for transmembrane movement of K^+, thereby controlling the transmembrane potential (V_m).[29] Opening of Slo1 channels is facilitated

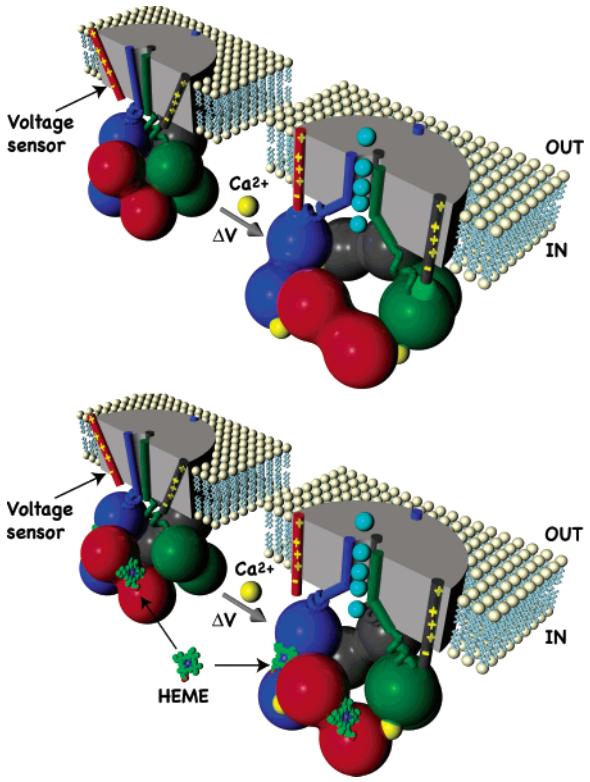

FIGURE 3. Reversible modulation of Slo1 BK channels by heme. The top row illustrates opening of a Slo1 channel by depolarization (ΔV), intracellular Ca^{2+}, or both. Only a portion of transmembrane domain of the channel is shown for clarity, but all four cytoplasmic RCK1−RCK2 dimers (spheres) are shown. Ca^{2+} binds to the sites in the RCK1−RCK2 domains and depolarization changes the conformation of the voltage sensors (cylinders with charges). These events are coupled to the central pore gate via mechanical spring-like connectors. When the gating ring formed by the RCK1−RCK2 domains expands (compare top left and top right), the central gate opens to allow a flux of K^+ ions (light blue balls). The bottom row depicts opening of the channel in the presence of heme. Note that heme binding slightly expands the gating ring even when the central pore gate is closed (compare top left and bottom left) and that when the gate is open, the gating ring expansion is not as complete as that without heme (compare top right and bottom right).

by depolarization of V_m and Ca^{2+} (Figure 3), and when open, the conductance of the pore of a Slo1 channel can be as high as 250−300 pS in symmetrical 150 mM K^+.[30] Because of this large single-channel conductance, Slo1 channels are also referred to as BK (big potassium) or MaxiK channels.

A BK channel complex contains four Slo1 subunits, each of which possesses several putative transmembrane segments, S0−S6.[30] Changes in V_m are detected by the charged voltage-sensor modules whose conformational changes in the membrane electric field are coupled to the channel's pore gate located at the inner mouth of the pore. Slo1 also possesses a large C-terminal cytoplasmic segment, which is considered to contain two homologous domains termed "regulators of conductance for K^+" (RCK1 and RCK2) based on partial sequence similarity to the bacterial channel MthK.[31] The cytoplasmic RCK domains most likely mediate the Ca^{2+} sensitivity of the Slo1 channel; binding of Ca^{2+} to multiple sites in the RCK domains alters the conformation of the cytoplasmic segment, which in turn mechanically opens the gate.[30] The channel's gate for K^+ is thus regulated by both V_m via the voltage-sensor domain and Ca^{2+} via the cytoplasmic RCK domains.[32,33]

Amino-acid sequence inspection of Slo1 shows that the linker segment between RCK1 and RCK2 contains the sequence CKACH, which conforms to the heme-binding motif CXXCH where X is any amino acid. The presence of the potential heme-binding motif in this cytoplasmic segment prompted Tang et al. to examine whether heme could modulate Slo1 from the intracellular side[34] using the patch-clamp method, which monitors conformational changes of ion channels in real time. Intracellular application of heme in the form of hemin (iron protoporphyrin IX chloride) acutely decreased K^+ currents through Slo1 channels. The inhibitory effect was specific to heme/hemin; heme analogues or derivatives failed to inhibit the channel at comparable concentrations. For example, Zn protoporphyrin IX and Co protoporphyrin IX were less effective, and MP-11, a short peptide with heme bound, did not inhibit the channel. Furthermore, the oxidation state of the heme-iron center did not alter the inhibitory effect. The inhibitory effect took a few minutes to fully develop, and heme was quite potent; the IC_{50} value was ~70 nM in the absence of cytoplasmic Ca^{2+}. While how many heme molecules are required to exert this apparent inhibitory action on each tetrameric Slo channel complex is not known, the concentration dependence suggests that the Slo1 channel is more sensitive to heme than the transcription factors HAP1 and Bach1.[24,27] The inhibitory effect of heme on the Slo1 channel was slowly reversible upon removal of heme. The study did note, however, that the time course of recovery was quite variable.

Tang et al. addressed the mechanism of heme binding to the Slo1 channel protein in multiple ways. First, pretreatment with diethylpyrocarbonate, which modifies the side chain of histidine, decreased the channel's sensitivity to heme, consistent with the idea that histidine is an axial ligand. Second, heme did not alter the currents recorded from the channels mutated in the potential heme-binding site (C615S or H616R) when examined using the same protocols as used for the wild-type channel. A separate study by Jaggar et al.[35] showed that the double mutation C615S:H616R also rendered the channel insensitive to heme. The chemical modification and the mutagenesis results therefore showed that the cysteine and histidine residues in the CXXCH motif in the RCK1−RCK2 linker segment are important in the overall heme sensitivity. But does heme really bind to this segment? Studies using short synthetic peptides corresponding to the RCK1−RCK2 linker segment support this notion.[34,35] Addition of heme to this peptide increases the absorbance at 420 and 550 nm, suggesting that heme can bind to this region. Electron paramagnetic resonance (EPR) measurements and simulations showed that the complex formed by hemin and the synthesized peptide exhibited a distinctive rhombic signal typical of low-spin ferric heme, indicative of two strong axial ligands bound

to the heme iron.[34] The study by Jaggar et al.[35] provided further evidence for the idea that the RCK1−RCK2 linker segment coordinates heme. Thin-layer chromatography assays showed that heme binds to the peptide containing the sequence CKACH but not to the mutant peptide with the sequence CKASR. That heme binds to the CKACH-containing peptide was also corroborated by electrospray ionization mass spectroscopy. The results from the model peptides thus suggest that the cytoplasmic RCK1−RCK2 linker segment containing the sequence CKACH is capable of coordinating heme. Whether Slo1 BK channels do in fact coordinate heme in this manner *in vivo* remains to be established.

How does binding of heme to the Slo1 channel protein affect its function? One possibility is that heme is a channel blocker that occludes the ion conduction pathway. This idea was largely excluded by the finding that, even in the presence of heme, the channels do open to allow ion flux with depolarization, Ca^{2+}, or a synthetic agonist.[34,36] These observations leave the idea that binding of heme somehow alters the gating of the Slo1 channel protein: how the protein conformation changes from the resting, closed nonconducting conformation to the open ion conducting conformation. Gating of the Slo1 channel is allosterically regulated by V_m and intracellular Ca^{2+} [30,32] and a complete description of the channel gating can require numerous kinetic states. Such a complexity can be circumvented in part by using extreme conditions to isolate subsets of the channel gating. For example, in the absence of intracellular divalent cations, the Slo1 channel essentially acts as a pure voltage-activated channel and its gating properties can be described by the model of Horrigan, Cui, and Aldrich (HCA model),[37] in which the intrinsic opening/closing of the channel pore is allosterically regulated by the movement of the four voltage sensors, one in each of the four subunits. Using the HCA model as the framework, Horrigan et al. carried out a series of biophysical measurements to elucidate the mechanism of the heme action.[36] In the absence of Ca^{2+}, Horrigan et al. found that depolarization increased the probability that the heme-bound Slo1 channel opened but not as efficiently as without heme. This finding immediately suggested two possible mechanisms. First, heme may hinder the voltage-sensor movement so that the channel responds less efficiently to depolarization. Second, the voltage-sensor movement may be unaltered, but its functional influence on the channel's gate is much less efficient with heme bound. The measurements of Slo1 gating currents, which track the movement of the charged voltage sensors in the membrane electric field, excluded the former possibility. The electrophysiological experiments of Horrigan et al. collectively showed that binding of heme to the channel drastically weakens the coupling between the voltage-sensor movement and the channel's pore gate, and that heme actually enhances the intrinsic opening of the channel gate.

An atomic-scale structure of the Slo1 channel is not yet available. However, the primary sequence of the cytoplasmic segment of Slo1 is relatively similar to that of the bacterial channel MthK whose high-resolution structure is known.[38] Based on the MthK structure, it is speculated that the putative cytoplasmic RCK1 and RCK2 domains of Slo1 form a dimeric structure, and four of them in turn form a structure termed "the gating ring"[38] (Figure 3). Expansion of the gating ring, for example, by binding of Ca^{2+}, may open the channel's pore gate using a passive mechanical spring-like mechanism.[39] In contrast, the structural arrangement of the transmembrane segments of the Slo1 channel probably resembles that of a voltage-gated K^+ channel, $K_v1.2$.[40] It is not clear how the voltage sensors "move" in response to changes in V_m, but the movement is likely coupled to the pore gate via the S4−S5 linker segment.[40] Once activated by depolarization, the voltage sensors may be stabilized by interactions with the cytoplasmic gating ring.[41] With this structural backdrop, Horrigan et al. proposed a speculative model of how heme binding may decrease the efficiency of coupling between the voltage sensors and the pore and favor the open conformation of the pore at the same time (Figure 3). Binding of heme to the RCK1−RCK2 linker segment expands the gating ring structure, thereby favoring the open state of the channel in the absence of any influence from the voltage sensors. The model explains the less efficient coupling between the voltage sensors and the pore by suggesting that heme binding *destabilizes* the interaction between the *activated* voltage sensors and the gating ring structure or alternatively heme *stabilizes* the interaction between the *resting* voltage sensors and the gating ring structure.

Simulations, however, show that the two key results obtained without Ca^{2+}, (i) decreased allosteric coupling between the voltage sensors and the pore and (ii) increased opening in the absence of voltage-sensor activation, are not entirely adequate to explain the results obtained at a saturating concentration of divalent cations.[41] Heme remains effective at high divalent cation concentrations, and to explain such results, Horrigan et al. postulated that heme interferes with the allosteric interactions among the channel's gate, voltage sensors, and Ca^{2+} binding segments.[32] The functional effects of heme can be reconciled with the putative structural organization of Slo1 based on MthK and $K_v1.2$ if the heme/heme-binding segment positions itself in the vicinity of the gating ring structure where the influences of the voltage sensors and Ca^{2+}-binding domains may converge onto the channel's gate (Figure 3).

The physiological relevance of the acute regulation of the Slo1 BK channel by heme is suggested by the recent results of Jaggar et al.,[35] which indicate that heme may confer CO sensitivity to the channel. CO is regarded by many as a potential gaseous messenger akin to NO.[4] For example, exogenous application of CO often dilates blood vessels.[42] In some systems, CO exerts its effect through enzymatic cascades involving such enzymes as guanylate cyclase.[43] In contrast, the activity of Slo1 BK channels appears to be enhanced directly by those experimental treatments designed to increase CO availability.[44−46] Jaggar et al. tested whether CO alters the efficacy of heme in

regulation of the Slo1 channel. The multidisciplinary experiments show that CO modulates the heme action in a redox-sensitive manner. Both hemin (with Fe^{3+}) and heme in the presence of the reducing agent dithionite (with Fe^{2+}) inhibited the Slo1 channel activity. However, CO increased the channel activity only when heme (Fe^{2+}) but not hemin (Fe^{3+}) was present, which is consistent with the results obtained from other hemoproteins in which CO preferentially interacts with heme (Fe^{2+}). Interestingly, CO applied in the presence of heme increases the channel activity beyond the level prior to application of heme. One idea consistent with this observation is that Slo1 channels may have some hemin bound and that, when treated with CO and dithionite, the activity is restored beyond the control level. This putative mechanism may contribute to the vessel dilation caused by CO. The double mutant C615S:H616R did not exhibit similar modulation by CO, and heme did not bind to a synthetic peptide containing the mutations, suggesting that C615 and H616 in the RCK1−RCK2 linker segment may represent the primary heme binding site responsible for the heme action.

Summary

The traditional role of heme as a stable prosthetic group has been augmented with its emerging role as an intracellular messenger capable of reversibly binding to several proteins. Binding of heme to selected transcription factors such as HAP1 and Bach1 markedly alters gene expression patterns, orchestrating cell-wide multicomponent responses to different stimuli. The type of protein ligands bound to the heme and the structure of the heme-binding pocket are critical in heme's role as a stable cofactor as well as a reversible modulator.

The finding that heme acutely modulates the Slo1 BK channel at nanomolar concentrations[34−36] suggests that heme functions as a rapid cellular signaling molecule, potentially acting as a CO sensor for the channel.[35] Additional functional and structural experiments are, however, required to thoroughly understand how heme modulates the Slo1 channel function. For example, it will be important to understand the structural changes that heme binding induces to alter the behavior of the channel's gate. It is also reasonable to speculate that the Slo1 channel may not be the only protein acutely modulated by heme. Other channels and enzymes may be similarly regulated by heme in a reversible manner.

The physiological significance of heme regulation of the Slo1 channel needs to be elucidated. If heme is to function as a physiological regulator, there must be mechanisms to increase and decrease its cytoplasmic concentration. A newly described mammalian heme transporter represents one mechanism to turn on the heme signaling mechanism,[11] and various HOs facilitate termination of the heme signal. The acute heme signaling mechanism involving Slo1 BK channels may also be relevant in certain pathological conditions.[47] One interesting aspect of the heme signaling mechanism is that heme and all of its catabolic byproducts, such as CO, bilirubin, Fe^{2+}, and biliverdin, appear to affect cell functions. How these molecules together modulate protein functions, such as those of Slo1 BK channels, is not clear. This complexity is daunting but may render heme a versatile and multifaceted modulator important in a multitude of physiological functions.

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