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Perspective

Understanding the Logistics for the Distribution of Heme in Cells

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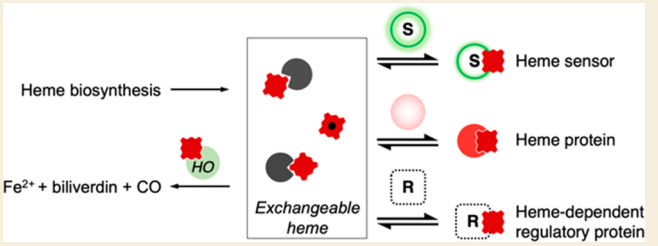
ABSTRACT: Heme is essential for the survival of virtually all living systems—from bacteria, fungi, and yeast, through plants to animals. No eukaryote has been identified that can survive without heme. There are thousands of different proteins that require heme in order to function properly, and these are responsible for processes such as oxygen transport, electron transfer, oxidative stress response, respiration, and catalysis. Further to this, in the past few years, heme has been shown to have an important regulatory role in cells, in processes such as transcription, regulation of the circadian clock, and the gating of ion channels. To act in a regulatory capacity, heme needs to move from its place of synthesis (in mitochondria) to other locations in cells. But while there is detailed information on how the heme lifecycle begins (heme synthesis), and how it ends (heme degradation), what happens in between is largely a mystery. Here we summarize recent information on the quantification of heme in cells, and we present a discussion of a mechanistic framework that could meet the logistical challenge of heme distribution.



KEYWORDS: *heme, heme quantification, heme trafficking, heme homeostasis, heme sensors*

INTRODUCTION

Heme is a macrocyclic complex of iron, with the metal ion coordinated equatorially to the four nitrogen atoms of an electronically delocalized protoporphyrin IX ring (Figure 1A) and to one or two axial ligands. Broadly following an IUPAC convention that defines heme as a complex of iron coordinated to a porphyrin and to one or two axial ligands,[1] the term heme is used widely, and often interchangeably, to refer to different types of iron protoporphyrin IX where the precise heme structure (e.g., heme *a*, heme *b*, heme *c*, heme *d*, etc.) is not always elaborated. When one of these heme structures is bound to a protein, the two axial ligation positions are normally occupied by donor ligands provided by protein amino acids. Heme ligation to the side chains of His, Cys, Met, Tyr, Lys, Glu residues and even the N-terminus is known.[2,3] In binding to a protein in this way, the heme becomes either 5- or 6-coordinated, Figure 1B. By convention, the proximal position, which is most commonly a histidine, is assigned as the fifth ligand and is normally shown underneath the heme when visualizing heme structures. The sixth ligand, if there is one, is then drawn above the heme on the so-called distal side. This proximal/distal terminology dates back at least as far as the crystallography studies of Kendrew and Perutz.[4,5] The distal position can be occupied by a protein amino acid; by a diatomic gas (e.g., O_2, NO, CO); by water, hydroxide, or another small molecule ligand (e.g., H_2S, CN^-); or it can be vacant giving a 5-coordinate heme species. The reactivity of the heme is controlled in part by the number and identity of these

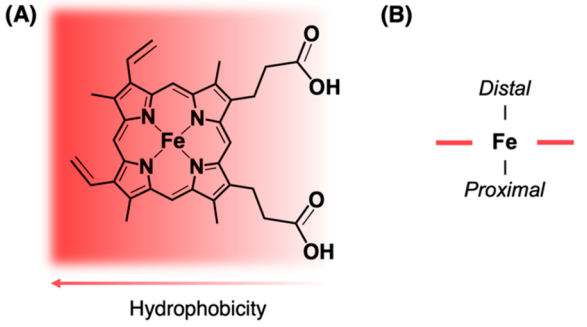


Figure 1. (A) The structure of iron protoporphyrin IX, also known as heme *b*. While heme is mostly hydrophobic, the carboxylate groups enable hydrogen-bonding interactions between the heme group and other molecules, including assisting the binding of heme to a protein. (B) The heme group is classified as containing distal and proximal sides, which are conventionally drawn above and below the plane of the ring, respectively. In heme proteins, the proximal side is usually bound to an amino acid residue provided by the protein; this helps to control its reactivity as a redox center[190,191] and its properties as a gas-binding molecule for storage and signaling.[11]

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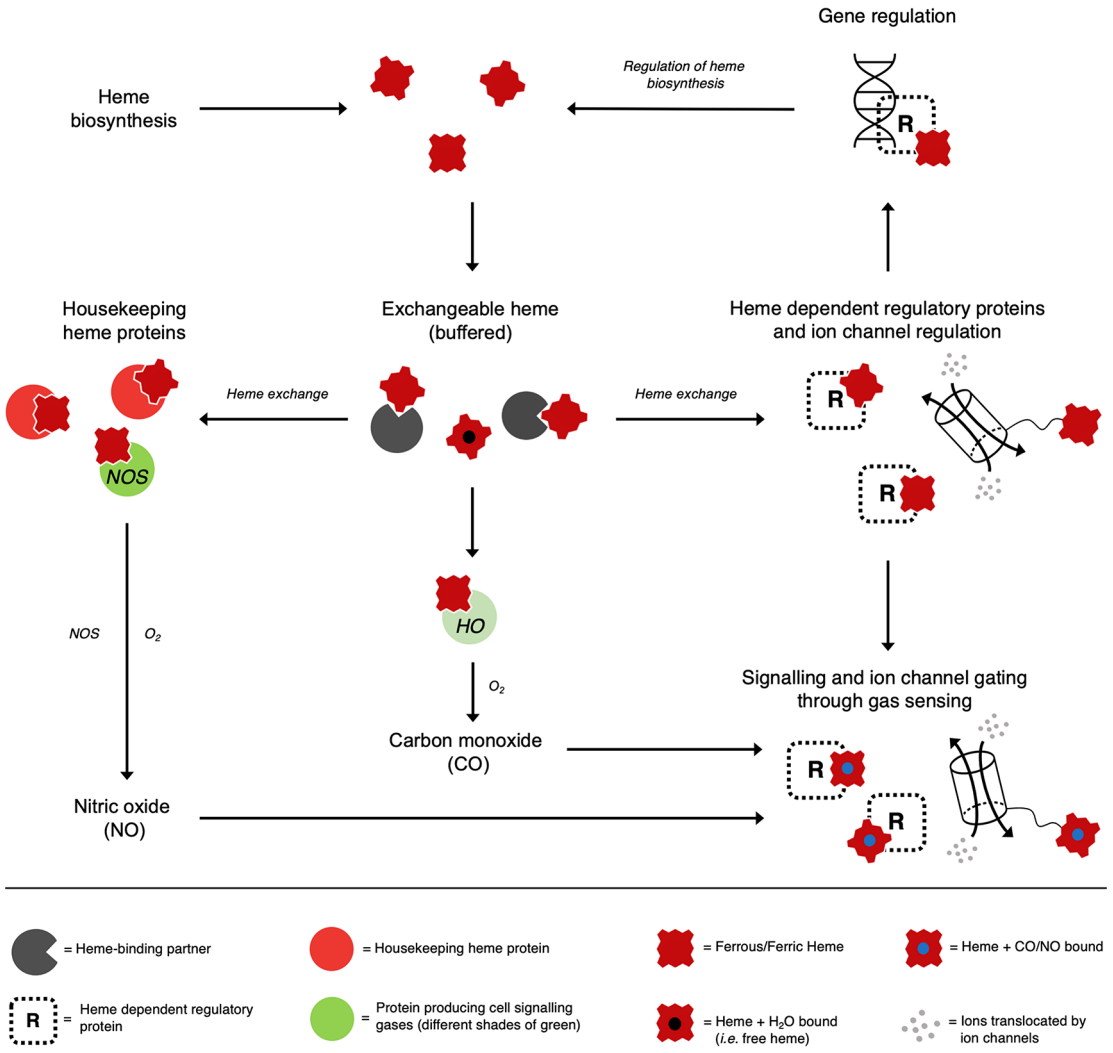

Figure 2. The possible interconnected pathways for the movements of heme in cells, and the links to signaling gases such as CO and NO. From the total heme synthesized in the cell (top), a proportion is bound irreversibly to heme-binding housekeeping proteins (red circles) that are essential for cell survival. A body of exchangeable heme is envisaged as being mostly weakly bound, soon after heme biosynthesis, to heme-binding partners (dark gray pacmans in this and other figures, which could be heme proteins or non-heme proteins) and available for exchange to heme dependent regulatory proteins (R, right). These heme-binding partners constitute an exchangeable, buffered, reservoir that can provide a flexible supply of heme and protect against changes in heme concentration. Once formed, these heme-bound proteins can serve in regulatory roles by, for example, binding to DNA for transcriptional control (top right; including the regulation of heme biosynthesis[17,21,28,37,192−194]) or to ion channels (middle and bottom right[54,195]). In green circles are shown the proteins that produce cell signaling gases—nitric oxide synthase (NOS, left) and heme oxygenase (NOS, middle). The synthesis of NO by NOS, and the production of CO by the heme degrading HO enzyme, adds multiple layers of complexity by coupling the formation of cell signaling gases to the heme-binding process. This would allow both CO and NO to bind to any heme protein with a regulatory function (bottom right) but could equally well occur for other heme dependent regulatory processes. It is worth noting that, while the binding of π-acid ligands like CO is traditionally associated exclusively with heme in its ferrous form, ferric heme has also been shown capable of binding CO/NO.[41] For the purposes of this review, movement of ferric/ferrous heme is presumed to mean heme *b*.

ligands at the proximal and distal positions, while stabilization of the heme molecule is further controlled by the heme-binding pocket via hydrophobic interactions with the porphyrin ring and vinyl groups, and hydrogen bonds between the propionate groups and the solvent.[6]

The role of the protein can thus be envisaged as solubilizing the hydrophobic and thus poorly soluble heme molecule, and in doing so controlling the reactivity of the heme group for biological purposes. Over the past several decades, most of what has been learned about the role of heme in biology relates to binding of heme to individual proteins such as those involved in oxygen transport (e.g., myoglobin, hemoglobin) and sensing (e.g., cytoglobin, neuroglobin),[7] bioenergetics (e.g., cytochrome *c*, cytochrome *c* oxidase),[6] metabolism and catalysis driven by the insertion of oxygen (e.g., cytochrome P450, indoleamine 2,3-dioxygenase),[6] peroxidase catalysis,[8] formation of nitric oxide,[9] and lipid metabolism (e.g., COX-2).[10]

In addition to these widely known roles for heme in biology, there is now a substantial body of evidence that identifies heme as a regulator of various cellular activities.[11−16] The first report, to our knowledge, of heme as a regulator was as far back as 1975, when heme was observed as regulating the synthesis of δ-aminolevulinic acid synthase which catalyzes the first step of the 8-step heme biosynthesis pathway.[17] We see chemical logic in the concept of regulatory mechanisms in cells that are linked to heme binding, but the idea of heme as a regulator laid dormant in the literature for many years. Despite its toxicity

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and lack of solubility, heme has a number of redeeming features that make it easier to understand why it could regulate cellular functions. The first is that heme is redox active and can respond to the redox status of the cell by modulating its oxidation state. The concentration of heme in cells is controlled by the balance of heme biosynthesis (to increase heme concentrations) and heme oxygenase activity (which degrades heme and produces CO as a byproduct). Cell signaling gases, such as O_2, NO, and CO, can bind rapidly and sometimes reversibly to heme—this could be exploited alongside the association of heme with a protein to create a second layer of regulatory control. CO is itself produced via degradation of heme by the O_2-dependent heme oxygenase enzyme,[18−20] and NO is produced by the heme- and O_2-dependent NO synthase enzyme;[9] i.e., the production of cell signaling gases also depends on the presence of heme. We envisage that the redox state of the cell, the oxidation state of the heme, the balance between heme synthesis and degradation, and the relative concentrations of O_2, NO, and CO may all be interconnected as outlined in Figure 2. This could be used as a versatile mechanism for cell signaling. Examples of heme-dependent regulation are beyond the scope of this review but have been summarized recently.[11,12] They include, among others, transcriptional regulation and gas sensing,[11,12,15] degradation of the p53 protein,[21] regulation of the circadian clock,[22−42] immune response,[43,44] aging,[45] and the gating of numerous ion channels.[46−69]

THE BIOLOGICAL NEED FOR HEME DISTRIBUTION

The fundamental requirement for heme across virtually all organisms—both in catalytic and regulatory roles as outlined above—means that cells need mechanisms to manage heme supply and distribution. Supply of heme is regulated by the well-studied heme biosynthesis pathway.[70] At the other end of the heme lifecycle, surplus heme (when it exists) is degraded by the O_2-dependent heme oxygenase enzyme.[18,19] But while the enzymatic machinery for making heme−heme synthesis—and removing heme−heme degradation—is well established, what happens in between is almost completely unknown. It has been recognized as far back as the 1970s[71,72] that cells need a supply of heme to respond to cellular demands, but until quite recently tools to examine this question have been lacking. In this review, we outline recent advances in the development of methods for measuring heme in cells, and we present ideas on, and possible mechanisms for, heme mobilization consistent with quantitative determinations of heme concentration.

TERMINOLOGY

To begin the discussion, we describe and define the terminology. For the purposes of this review, we take heme (in the context of heme transport) to mean heme *b*. Literature on the role and distributions of heme in cells has used a bewildering, and at times imprecise, range of terminologies. Phrases such as "labile heme", "regulatory heme", "free heme", and "heme pool" are all used in the literature, often interchangeably.[73] While the need to discriminate between these types of heme in cells is obvious, it is challenging to provide a precise description of each. What does "labile heme" mean? How can "free heme" exist in the cell, given that heme is cytotoxic and poorly soluble? Does the phrase "regulatory heme" describe a supply of heme dedicated *exclusively* for regulatory purposes, or might it be used for other purposes? Given its insolubility and cytotoxicity, does it actually make sense to talk about "pools" of heme when current estimates for heme concentration are in the nM to μM range?[74−79] The lack of precise descriptions for the nature of different types of heme can lead to confusing or contradictory interpretations. Below we provide a summary and offer some suggestions, which we hope will be helpful in future discussions for both specialists and newcomers to the field.

Regulatory Heme

In disciplines that vary from blood disorder (e.g., porphyria),[80,81] gene expression,[71,72] and heme biosynthesis,[82,83] a small portion of the total heme content of the cell has been referred to over many years as being present in a regulatory or intracellular heme pool. These terms originate from early papers[71,72,83−86] where it was also suggested that the nature of heme was a free molecule (see below). The existence of a form of regulatory heme is conceptually useful, and because of this the nature of regulatory heme has been debated as long ago as 1975 when new roles for heme—above and beyond the traditional roles in oxygen binding, electron transfer, and catalysis—were identified by Granick.[83] In Granick's work, the regulation of heme biosynthesis was proposed as being controlled by fluctuations in the concentrations of heme in a dedicated heme pool where heme was envisaged as being weakly associated with an ensemble of cytosolic proteins.[83,87] These weak binding interactions were considered important for heme to be readily exchangeable.[83,86] The terms were then later adopted by scientists in multiple fields.[46,80,88−98] Hence, since Granick's initial studies, the phrases "regulatory heme" and "free heme" have been widely used although not precisely defined.

Free Heme

To the best of our knowledge, the term free heme was introduced in the 1970s[72,85,99] and was interpreted as an intracellular population of free heme molecules either on their own (free) or all together (in a pool). These early papers noted the difficulties associated with the idea of a pool of free heme, but, nevertheless, the concept and the terms "free heme" and "pool of free heme" became ingrained in the literature.[85,92,94,95,100,101] The term "free heme" has never been precisely defined—broadly speaking it has been taken to mean a heme molecule that is not bound to anything else. There are difficulties with that idea, however, and the concept of large amounts of free heme, because heme is cytotoxic through Fenton chemistry and radical formation.[92,94,96,102−104] So if heme is free in the cells, it would cause problems. Heme is also a hydrophobic molecule and poorly soluble, so presumably cannot exist in cells without being solubilized by binding to a protein, cellular membranes,[105] or even nucleic acids.[45,106] Moreover, heme needs to be ligated at the fifth and sixth ligation position, otherwise, it will stack to form dimers, or higher multimers, in solution.[107,108] This is problematic for cellular handing because, in a dimeric or multimeric form, heme could not be delivered to proteins that require only one molecule per binding site.

We envisage that a "free" molecule of heme can therefore only exist transiently in cells. In this review, we will use the term "free heme" to indicate molecules of iron protoporphyrin IX, in the ferrous or ferric oxidation state, existing in the absence of any protein binding partner but weakly associated with water molecules as ligands at the fifth and sixth coordination sites.

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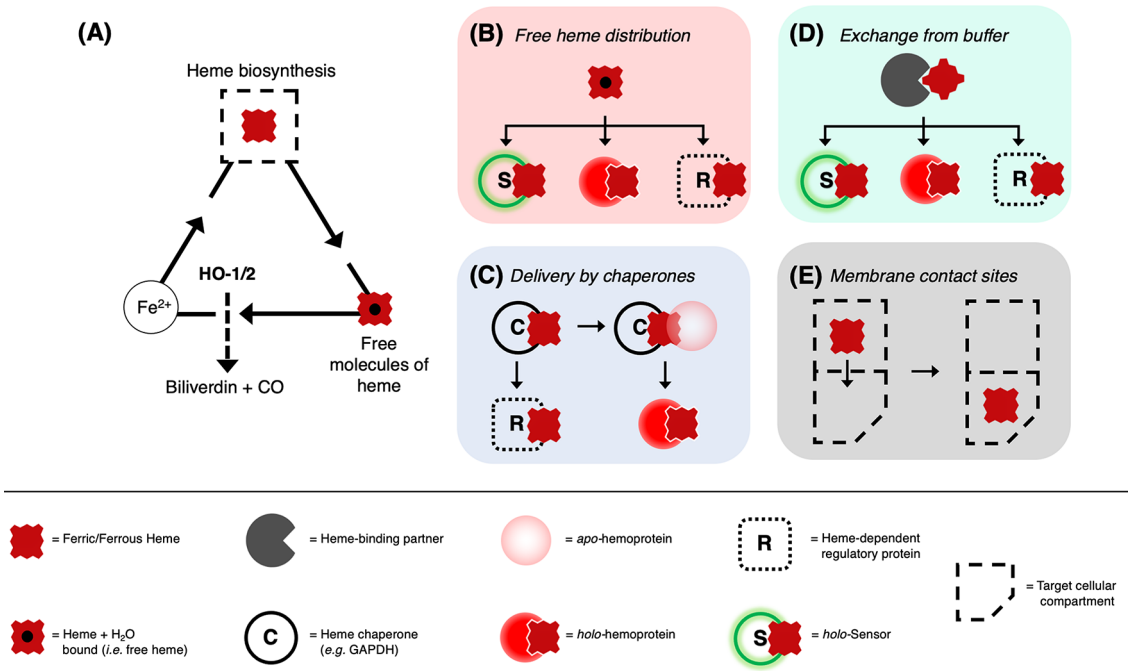

Figure 3. Representation of possible mechanisms for distribution and delivery of heme across the cell. (A) The life cycle of heme in cells starts with its biosynthesis in the mitochondria (full dashed square) and ends with its degradation by heme oxygenase (HO-1/2). Heme oxygenase generates Fe^{2+} which can be recycled for the synthesis of new heme molecules. A balance between synthesis and degradation contributes to the controlling heme concentrations in cells. (B–E) The supply of heme to the locations where it is in demand is suggested as occurring via four possible mechanisms (colored panels). In (B), direct distribution of free heme into a heme protein (red circle), a heme-dependent regulatory function (R, white box), or a genetically encoded/synthetic heme sensor for quantification studies (S, green circle). Free heme (either ferrous or ferric) is envisaged as being present in minuscule concentrations but will still represent a mechanism for heme to be made available in cells.[77] In (C), chaperone-mediated heme delivery to an *apo*-heme protein (pale red circle), for example by GAPDH.[113−115] In (D), heme bound to heme-binding partners (dark gray pacman) constitutes a body of exchangeable heme readily available for downstream applications, in the same way as in (B). Possible candidates for heme-binding proteins are IDO, HBP22/23, SOUL, and albumin.[111,163,196] In (E), distribution of heme via membrane contact sites between mitochondria—where heme is synthesized—to target cellular compartments which bypasses the need for transporters to mediate the delivery of heme.[171]

Labile Heme

In recent years the term "labile heme" has appeared.[44,78,96,105,109−120] The use of this term allows a distinction to be made between the proportion of the total heme content that is available for mobilization, and the proportion that is unavailable (or, more precisely, inert for exchange) because it is bound with a high affinity, and therefore irreversibly, to proteins. Ideas on what the concepts of labile heme actually means mimic the early ideas on free heme.[83,87] Labile heme is envisaged as being continuously engaged in transient binding to intracellular proteins that exist to actively buffer heme concentrations in the cell. Since some heme is unavailable for distribution in cells, labile heme is thus envisaged as a intermediary through which heme can move and be distributed through the cell.[105,110−112] The term labile heme probably finds its origins as an adaptation of "labile iron pool" that predated it.[121]

Exchangeable Heme

The use of the term lability to distinguish a more mobile fraction of the total heme from that which is permanently (irreversibly) embedded into heme proteins is similar in concept to the kinetic lability of ligands in inorganic metal complexes.[122−124] Labile ligands exchange very fast, and inert ligands exchange slowly. But the availability and distribution of heme will be defined not just by the kinetic lability of the ligands bound to the heme, but also by thermodynamics. This is because cascades of heme exchange events down a thermodynamic gradient (dictated by heme binding affinity) will control the overall distribution of heme. Hence, our opinion is that "exchangeable heme"—which has been adopted in a limited number of examples[77,86,97,125]—is a more precise term to convey the concept that both kinetic and thermodynamic processes are relevant when considering the bioavailability of heme.

Definitions Used in This Review

We propose here a working set of definitions.

Total Heme (H_t). The total heme content of a cell. This includes the fraction of heme that is bound irreversibly to proteins and thus not available for other purposes (defined here as H_h), and the fraction of exchangeable heme (which includes small quantities of free heme, see below).

Exchangeable Heme (H_e). A fraction of the total heme that is reversibly bound to proteins or small ligand molecules (e.g., free amino acids, H_2O). Exchangeable heme can be considered as a reservoir that provides an accessible supply of heme to the cell.

Free Heme (H_f). Molecules of heme that are not bound to a protein but weakly coordinated with water molecules. We expect free heme to exist in vanishingly small quantities. Long and co-workers[126] have recently offered a definition for free heme which is similar to that proposed here.

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In principle, $H_t = H_h + H_e + H_f$, where H_h is the amount of heme that is bound irreversibly to heme proteins (including membrane and cytosolic proteins).

THE LOGISTICS OF HEME SUPPLY AND DEMAND

Given the need to move heme from its place of synthesis (in the mitochondria) to other regions of the cell, how can cells control the balance of supply and demand and what mechanisms are in place to do this?

Controlling Supply and Demand

The balance between heme biosynthesis and degradation is part of the regulatory control that the cell needs to maintain heme availability below the cytotoxic threshold (Figure 3A).[94,102,127] Heme oxygenase, whose mechanism of degradation of heme into biliverdin and CO is well-known,[18,19] plays a crucial role in maintaining this balance. Heme oxygenase-1 is transcriptionally regulated and induced by infection, inflammation, oxidative stress or increasing concentrations of heme.[128,129] Interestingly, the post-transcriptional regulation of heme oxygenase-2 (abundant in the brain, testes, and neural tissue) has been shown to be controlled by heme occupancy in the active site.[130] Specifically, heme oxygenase-2 appears to be destabilized and more likely to be degraded when heme is not bound, which is the likely situation under conditions of heme deficiency and when high heme oxygenase activity is undesirable. This regulation would help to keep heme concentrations within a suitable physiological range under conditions of cellular heme deficiency.[130]

The Involvement of Heme Chaperones

The existence of biological chaperone systems is documented for other redox active transition metal ions (e.g., iron, copper, molybdenum, cobalt, nickel).[131−134] Cells adopt strategies to tightly regulate the concentrations of different metals ions, ensuring suitable levels of bioavailability, effective physiological responses, and prevention of cytotoxicity.[133−149] The concentration of transition metal ions in cells is kept at very low levels to avoid inappropriate activation of signaling pathways. For instance, the intracellular free copper concentration in *S. cerevisiae* is limited to less than one molecule per cell.[133,137] Similarly, free zinc is estimated in the low femtomolar range in *E. coli*.[133,136] Instead of being free to circulate around the cell, metal ions are stored, locked in an inaccessible state, and released to a network of chaperones or chelators that control their delivery, repurposing, and ensure fidelity in metalation when two competitive metals can bind to the same site.[134,138−144] By comparison, it is reasonable to presume that cells use similar principles for the movement and distribution of free heme (Figure 3C,D).

The identification of heme chaperones with the specific role of transporting and selectively delivering heme to downstream proteins would thus clarify the picture considerably. But very few heme chaperones have been identified over the years. A notable example is the CcmE chaperone, part of the multi component Ccm membrane heme maturation machinery for heme translocation to cytochrome *c* in most proteobacteria, archaea, deinococcales, and plant mitochondria.[150−153] However, this is a special case as it speaks only to the need for formation of covalent links to heme in the specialized *c*-type cytochromes. GAPDH is perhaps the most compelling example of a heme chaperone so far identified.[113−115,154] The interaction between GAPDH and heme was initially thought to occur to protect heme from degradation during its transport (which at the same time reduces its cytotoxicity).[155] But GAPDH has since been found to be responsible for the delivery of heme to the nuclear transcription factor Hap1[156] in *S. cerevisiae*, to cytosolic iNOS in mouse macrophage cells,[113] and to soluble guanylate cyclase in HEK293 cells.[114]

The involvement of GAPDH in heme delivery in both yeast and mammalian systems is indicative of a broadly conserved strategy for heme transport.[113] GAPDH is primarily a glycolytic enzyme, and, therefore, its function as a heme chaperone looks to be opportunistic as far as the cell is concerned. This begs the question as to whether there are other as-yet unidentified heme chaperones lurking about the cell in disguise. Certainly, if heme chaperones are used at all then more than one could be required to create an effective supply chain. Arguably, chaperones of chaperones might even exist.[115] But if heme binding can be opportunistic and does not need a specific heme-binding motif—as GAPDH seems to indicate—then identification of heme chaperones becomes an incredibly demanding task which would need to take into account noncanonical heme-binding motifs in addition to the large number of proteins with known heme-binding sites.[3,157]

All of this leads us to the conclusion that existing, well-known heme-binding proteins could be repurposed for the task of cellular heme delivery under certain conditions. For example, indoleamine 2,3-dioxygenase, whose mechanism and structure is well-documented,[158−162] has recently been shown to lose its heme under certain conditions,[163] which suggests the possibility that it could be used as a source of heme in specific regions of cells. In all likelihood, there may be other cellular proteins that can similarly masquerade as heme chaperones.

Possible Limitations on the Utility of Chaperones for the Distribution of Heme

The distribution of heme via chaperones can be used to explain how heme moves from the mitochondria to other locations, and then subsequently to the endoplasmic reticulum for degradation by heme oxygenase. But the chaperone mechanism is ill-equipped to deal with rapid changes in heme availability. If increases or decreases in heme concentration are required, then the rate-limiting steps are heme synthesis and degradation. Upregulation of heme synthesis, by expression of the ALA synthase enzyme, in response to low heme availability could take at least 30 min,[164] while induction of heme oxygenase-1 for heme degradation could take hours.[165] Both of these mechanisms are too slow by far if rapid control of heme concentration is required, for example in the case of ion channel regulation which is necessary for appropriate cardiac function.[54] Hence, if the cell is solely reliant on heme chaperones, then it runs the risk of spikes in heme availability should there be a mismatch in timings between the delivery and the removal of heme. Taken together, and bearing in mind the failure (so far) to identify large numbers of heme chaperones, we suggest that complementary mechanisms will be required to regulate heme distribution. We elaborate on this below.

Buffering of Free Heme Concentration as a Mechanism of Control

An ability to store heme in cells would decrease the reliance of cells on heme synthesis and degradation and could be coupled to mechanisms to make heme more or less available, on-demand and at speed (Figure 3D). To date, there have been no proteins identified that have the dedicated function of

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Table 1. Summary of Reported Cellular Heme Concentrations from Known Heme Reporters or Fluorescent Heme Sensors

reporter	heme probe	fluorescent tag(s)	$K_{d,heme}$ (nM)[a,b]	measured heme concentration (nM)	quantitation method	tested in	ref.
CYSDY-9	IsdX1, IsdC	ECFP, EYFP	63.5 ± 14.3	Cytosol 25.6 ± 5.5 Mitochondria 23.3 ± 4.9 Nucleus 31.0 ± 7.0 ER 5.4 ± 1.4[c]	FRET	HeLa	76
HS1	Cytochrome b_{562}	EGFP, mKATE2	3 (ferric), <1 (ferrous)	Cytosol 20−40 Nucleus <2.5 Mitochondria <2.5	FRET	*S. cerevisiae*	79
CHY	*P. falciparum* Histidine rich protein	ECFP, EYFP	∼250	Cytosol 1600	FRET	*P. falciparum*	78
apo-HRP	Horseradish peroxidase	–	–	600	peroxidase activity[d]	Human lung fibroblasts (IRM90)	73,97
apo-HRP	Horseradish peroxidase	–	–	2100 ± 2	Reconstitution of catalytic activity[d]	Human erythrocytes	74
apo-HRP	Horseradish peroxidase	–	270 ± 40	433 ± 125	Reconstitution of catalytic activity[d]	HEK293	75
mAPXmEGFP[e]	Ascorbate peroxidase	mEGFP	22	Cytosol 4	RET	HEK293	77

[a]The heme affinity, or conversely the dissociation constant $K_{d,heme}$, is a key parameter in heme quantitation. $K_{d,heme}$ values should be close to the physiological concentrations of heme (in the nM to μM range), to ensure prompt response of the sensor to changes in [heme] but also avoiding the sensor becoming either fully saturated or fully unsaturated (which is essential to study dynamic cellular processes). $K_{d,heme}$ defines the fraction of total cellular heme which is available for donor−acceptor heme exchange (acceptor = *apo*-heme sensor). Heme sensors with the highest heme affinities will accept heme from a larger ensemble of heme donors, and vice versa. Caution is needed when comparing heme concentrations obtained with different sensors, as the sensors with lower $K_{d,heme}$ will draw heme from a larger number of donors. [b]For ferric heme unless otherwise stated. [c]A relatively low concentration of heme was measured in the endoplasmic reticulum of HeLa cells compared to other cell lines (mouse melanoma B16, human HCT116 colon cancer cell, human PANC-1, hamster kidney fibroblast BHK-21, and CHO), which was interpreted as due to the high expression levels of heme oxygenase-1 in the endoplasmic reticulum surface of HeLa cells. [d]*holo*-HRP was formed by mixing *apo*-HRP with HEK293 lysates. The reconstitution of the catalytic activity of the HRP reporter by its binding to cellular heme was then used to measure heme concentration. [e]mAPX = monomeric APX; mEGFP = monomeric EGFP.

storing heme, but there is evidence, from recent work,[77] of a buffering capacity within cells that can accommodate changes in either the total concentration of heme (i.e., $[H_t]$), or the concentration of exchangeable heme (i.e., $[H_e]$), while maintain the capability to mobilize heme as and when necessary. As indicated above, it is entirely possible that known heme proteins (such as indoleamine 2,3-dioxygenase, and GAPDH) participate in the buffering of free heme concentration ($[H_f]$) and protect the cell against increases or decreases in total, or exchangeable, heme concentration. In this scenario for heme buffering, the ability of any given protein to acquire heme is based on how well it competes for heme (i.e., the heme affinity) against other proteins. Hence the availability of heme in the cell would be dependent entirely on the K_d, and abundance, of these heme-binding partners. In this way, the concentration of free heme is buffered—or, in other words, the availability of heme is kept within a limited range—by the many heme-binding proteins in the buffer and would be distributed among those proteins according to their relative heme affinities.

Crucially, in this buffering model the hierarchy of heme-binding affinity would be responsive to changes in the composition of the buffering proteins. As an example, upregulation of proteins in the buffer with high heme affinity (e.g., a globin, or a cytochrome) would deplete the capacity of the exchangeable heme reservoir to release heme, as heme was dragged to thermodynamically inaccessible sites. As well as providing a reservoir of exchangeable heme able to meet the localized and instantaneous demands of the cell, such a buffering system would, along with the heme degradation pathways, prevent heme-induced cytotoxicity by limiting the concentrations of free heme within the cell. The interplay between heme biosynthesis, degradation, transcriptional and post-transcriptional regulation of heme buffering proteins, are all envisaged as jointly contributing to the buffering of heme in cells.

Trafficking through Membranes

Evidence is emerging for the involvement of biological membranes as important factors in the trafficking of cellular heme. Heme has been shown to be capable of diffusing or even being retained by cellular lipid bilayers[166−168] and examples of putative membrane heme transporters have been identified, most notably the mitochondrial exporter Flvcr1b, which have been reviewed elsewhere.[94,169] But while heme may or may not necessitate membrane transporters for its movement across compartments, the contribution of lipid bilayers to heme trafficking is still not completely clarified. A recent study has proposed that the trafficking of heme through membranes does not rely on transporters and is, in contrast, a chaperone-less pathway made possible by interorganellar contact sites. This provides an elegant solution for the exchange of heme between compartments (Figure 3E).[170,171]

QUANTIFYING HEME CONCENTRATIONS IN CELLS

Early Estimation of Heme Concentration

Granick speculated in the 1970s[83,87] that the concentration of exchangeable heme in the cytosol was in the range 10−100 nM. This value is, in fact, remarkably close to recent measurements (Table 1). In the past, the cellular heme concentration has had to be inferred from the amount of heme measured in soluble cell lysis extracts determined using either fluorometric approaches[172] or enzymatic reconstitution

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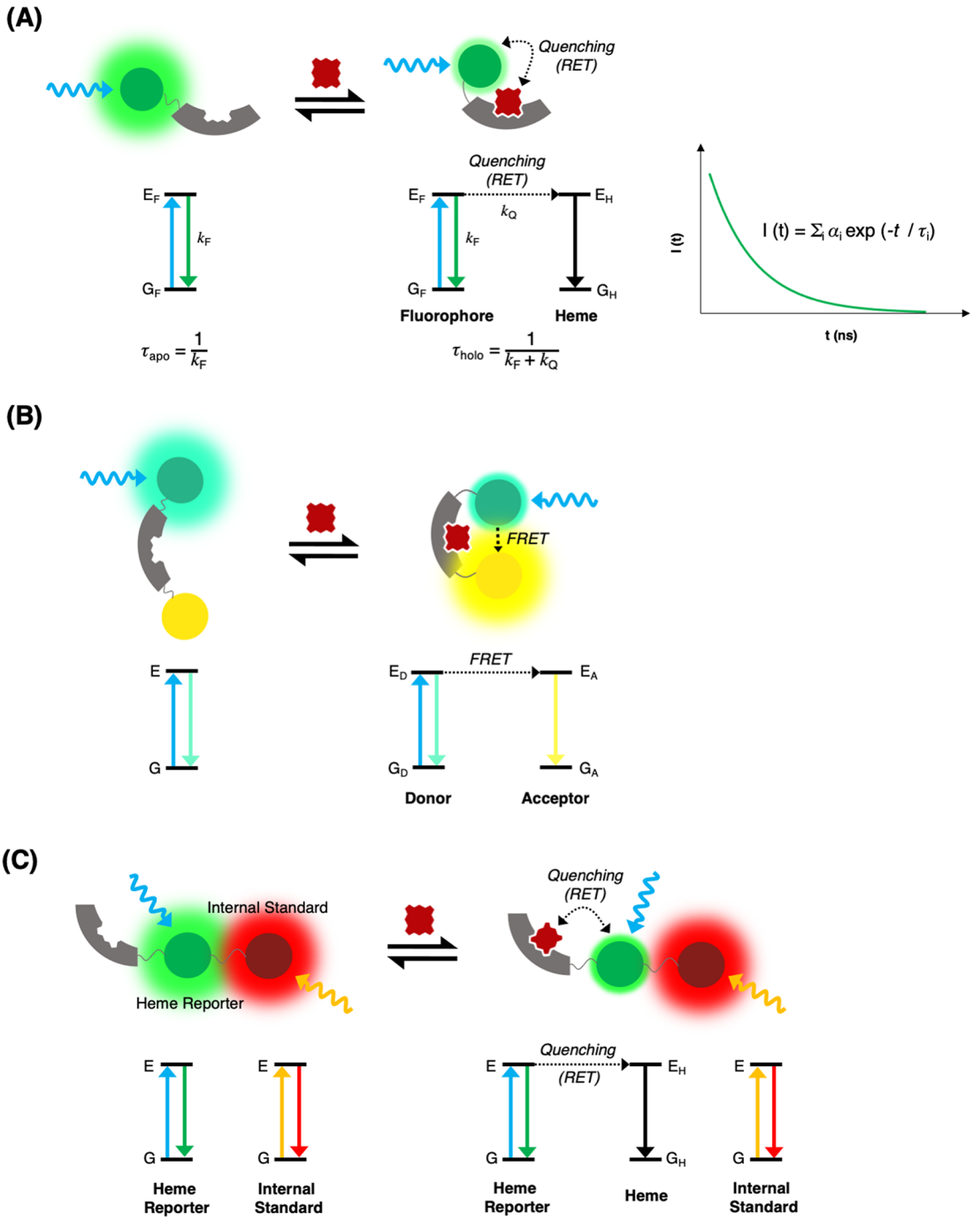

Figure 4. Basic principles of heme sensor designs. (A) RET sensors: Heme-binding to this type of sensor introduces an additional relaxation pathway for the electronic excited state of the fluorophore. The mean fluorescence lifetime of the probe changes between the limiting values of τ_{apo} to τ_{holo} for the pure *apo* and *holo* forms of the sensor dependent on heme concentration.[77] (B) FRET sensors: The heme-binding domain of the sensor undergoes a conformational change that brings two fluorophores into close proximity to one another. In this example, Förster energy transfer results in a decrease in the emission of a green fluorophore and an increase in emission of a yellow fluorophore. Hence changes in the relative emission intensities of the two fluorophores can be used to determine heme concentration.[76] Multiple heme-binding sites may be present in the heme-binding domain.[78] (C) A RET sensor (similar to that shown in (A)) that incorporates an additional fluorophore in order to measure a ratiometric intensity. The sensor is composed of two fluorophores, but heme-binding triggers the selective quenching of the fluorescence of only one of them. The unperturbed tag can then be used as an internal reference to monitor the changes in the intensity of emission for the quenched fluorophore, providing a method for precise heme quantitation.[79,112]

techniques.[74,89,97,173] These methods are likely to report a concentration that lies somewhere between that for exchangeable heme ($[H_e]$) and the total amount of heme present in the cell ($[H_t]$) because the denaturing methods lead to release of heme from many heme proteins. In order to selectively measure concentrations of exchangeable heme, it is necessary to use a method that is compatible with in vivo fluorescence imaging or spectroscopy of live cells.[174] Such an approach is possible by the design and application of genetically encoded

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heme sensors which can be expressed recombinantly in different cell lines.

The Development of Cellular Heme Sensors

Genetically encoded sensors comprise a heme-binding protein conjugated to one or more fluorescent proteins. For the sensor to report on the presence and quantify the concentration of heme, the fluorescence output must be modulated by heme binding. There are different mechanisms that can lead to suitable modulation of the fluorescence output.

a. Förster Resonance-Energy Transfer between the Fluorescent Protein and a Bound Molecule of Heme. The heme-binding pocket of the sensor must be located in close proximity to the chromophore-forming amino acids in the fluorescent protein, and the emission of the fluorescent protein must have spectral overlap with either the Soret or Q absorption bands of heme.[175] In this case, resonance-energy transfer provides a nonradiative decay pathway for the photoexcited state of the chromophore, via the heme moiety, that competes with the radiative-decay pathway responsible for the fluorescence signal (Figure 4A,C).

b. Förster Resonance-Energy Transfer between a Pair of Fluorescent Proteins. Unlike in (a), the heme-binding pocket must be distant from both fluorescent proteins, and, instead, the binding of heme to the sensor must lead to a conformational change that brings the chromophore-forming amino acids on each of the fluorescent proteins (i.e., the donor and acceptor sites) into close proximity; see Figure 4B. In this case, resonance-energy transfer between the donor and acceptor leads to subsequent radiative decay from the acceptor. Hence the binding of a molecule of heme is detected by the attenuation of the short wavelength emission band of one the fluorescent proteins (the donor), and the appearance of the long wavelength emission band of the other fluorescent protein (the acceptor).

In order to distinguish between the mode of operation of these two different designs for heme sensors, we will refer to the mechanism described in (a) as just resonance-energy transfer (RET) and the mechanism described in (b) as fluorescence resonance-energy transfer (FRET).

Further consideration is given here to the heme sensor design comprising a heme-binding domain and a single fluorescent protein (i.e., that shown in Figure 4A). If the distance between the chromophore-forming amino acids on the fluorescent protein and the heme-binding pocket, r, is much less than the Förster distance, R_0 (calculated from the spectral overlap integral[175]), then the efficiency of RET in the *holo*-sensor will be near to 100%. The fluorescence quantum yield of the *holo*-sensor (i.e., the sensor with heme bound to it) will be near to 0%, and the presence of heme can be inferred from the quenching of the intensity of the fluorescence emission. Alternatively, if the distance r is close to the R_0, then there will be an intermediate efficiency for RET in the *holo*-sensor (given by $100\% \times 1/(1 + (r/R_0)^6)$. The fluorescence quantum yield of the *holo*-sensor will be reduced (but will not equal zero), and the presence of heme can be inferred from either the reduction in the emission intensity or from a reduction in the fluorescence lifetime.

A change in the fluorescence lifetime can be observed by time-correlated single photon counting, where the proportions of the *apo*- and *holo*-forms of the sensor can also be deduced by determining the relative amplitudes of functions in the fitting of a multiexponential decay model, Figure 4A. For a genetically encoded heme sensor, a measurement of a fluorescence decay profile has significant advantages over a measurement of fluorescence intensity: (i) The fluorescence decay can be used to determine the ratio of *holo*- to *apo*-forms of the sensor, but the intensity can only be used to detect the *apo*-form of the sensor (because the *holo*-form of the sensor is quenched). Sensor designs that rely on measurements of fluorescence quenching require an additional tag (distant from the heme-binding pocket and the other chromophore site) to normalize the signal intensity. (ii) The quantitative accuracy of fluorescence lifetime imaging or spectroscopy is independent of the inner filter effect or partial photobleaching of the fluorescent protein.

Another property of the emission of a fluorescent protein is the anisotropy (polarization), which might be modulated by changes in the rotational diffusion between the *apo*- and *holo*-forms of the sensor. The sensitivity of a polarization measurement will be highly dependent on the local viscosity and, as yet, it has not been exploited in the context of heme sensing.

Maintaining Homeostasis in the Presence of Heme Sensors

The in vivo deployment of a heme sensor in cells introduces an additional heme-binding species into the cellular milieu. Hence the sensor will compete with other heme-binding proteins in the cell for molecules of exchangeable heme. While there would be no issue with the sensor competing for heme with either heme-binding proteins, or small molecules, whose roles are to buffer the concentration of free heme, it is critical that this competition does not extend to bona fide heme proteins that require heme either for their function or to adjust their properties. To accurately measure the availability of heme in cellular compartments, the expressed sensor must integrate into the network of buffering partners, and its presence must not result in a significant change either to the amount of exchangeable heme (H_e) associated with these heme-binding partners, or to the miniscule amount of free heme (H_f) in the cell. For a certain concentration of exchangeable heme, it is fairly straightforward to estimate an appropriate range of concentrations for the sensor that will preserve heme homeostasis in the cell.[77] On the basis of measurements in the cytosol of HEK293 cells,[77] a sensor concentration of up to 1 μM would not be expected to alter the availability of heme for exchangeable heme concentrations equal to, or in excess of, 3 μM. It is realistic to assume that, if a sensor is deployed in a localized area of the cell (e.g., directed specifically to the nucleus), then the sensor concentration may exceed 1 μM. Hence, we anticipate that, in future, controlling the concentration of expressed sensor will be increasingly important.

Summary of Known RET and FRET-Based Heme Sensors

There are a number of reports of different fluorescent heme sensors in the literature. We summarize them briefly below. Heme concentrations reported from the studies of these sensors are given in Table 1.

In the early 2000s, Takeda et al. provided the first proof-of-concept for a heme sensor. The sensor comprised an EGFP-cytochrome b_{562} fusion protein,[176] and it was subsequently optimized for enhanced RET.[177] Fluorescently labeled variants of heme oxygenase-1 (K18C[178] and D140H[179] variants) have also been used to detect heme in vitro. These sensors were a step forward in terms of heme quantification, but were not

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deployed in cells for real-time monitoring of heme concentrations.

The first genetically encoded heme sensor was CYSDY-9.[76] CISDY-9 was designed to exploit the natural dimerization of a pair of bacterial chaperones (IsdX1 and IsdC). Tagging each chaperone with a fluorescent probe and linking them with a short peptide tether produced an intracellular FRET reporter which was used to analyze heme population in HeLa cells, Table 1. With the exception of the endoplasmic reticulum, heme concentrations were found to be evenly distributed across the cytosol, the mitochondria and the nucleus, which is different from the observations in yeast (see above and ref 79). It may be the case that results from different sensor systems cannot be directly compared, see Table 1.

Cytochrome b_{562} was later engineered with EGFP and mKATE2 fluorescent tags. This created a sensor that could be deployed in cells[79] in which the fluorescence intensity of EGFP was quenched (via RET) on heme-binding; the intensity of mKATE2 was unchanged providing a means of assessing heme concentration by ratiometric analysis. A somewhat heterogeneous distribution of heme throughout yeast cells was reported, Table 1, and the behavior of the sensor in cells[112] supported the concept of a (buffered) reservoir of exchangeable heme that is readily available for regulatory roles and crucial in the overall physiology of yeast. The same sensor was then utilized to demonstrate that heme delivery from mitochondria—the place of heme biosynthesis—to the nucleus was 25% faster than to the cytosol and the mitochondrial inner matrix. The authors suggested direct membrane contacts between mitochondria and the ER as a possible pathway for heme delivery to the nucleus.[170,171]

A genetically encoded (FRET-based) heme sensor based on histidine rich protein 2 has been used to quantify heme concentrations in the parasite *P. falciparum*.[78] In this case, heme concentrations are higher (ca. 1.6 μM), but this is not completely unexpected considering that 80% of the amount of heme contained in a human body is synthesized by red blood cells.[73]

Peroxidase-based fluorescent sensors, based on horseradish peroxidase and ascorbate peroxidase, were first used to measure dynamic heme fluxes in *C. elegans*.[75] Ascorbate peroxidase has also been used recently to monitor heme concentrations and distributions in live cells[77] using fluorescence lifetime imaging microscopy. In this work, miniscule concentrations (a few nM) of free heme were identified—corresponding to one molecule or less per cellular compartment. These observations are consistent with a system that sequesters free heme.

The Use of Heme Sensors to Study Heme Protein Maturation

Applications of heme sensors can extend beyond the determination of heme concentrations. A GFP-labeled version of cytochrome *c* peroxidase has been recently developed and used to study heme protein maturation by heme insertion.[119] In this design, the percentage of the heme-bound sensor in yeast cells was revealed by the relative amplitudes of exponential-decay components in the emission measured by fluorescence lifetime imaging. This provides a forward-looking approach to establish in real-time the dynamic processes involved in the folding process that accompanies the formation of heme proteins.

Alternative Approaches

There are other technological approaches to heme quantification, including the use of the antimalarial 4-aminoquinoline probe[180] and peptide-based fluorescent probes.[181] The use of heme catabolites—biliverdin and bilirubin—as a way of tracking and studying heme biochemistry is also promising and is starting to provide interesting insights into the interplay between the biochemistry of heme and other biological pathways.[182−189]

POSSIBLE MECHANISMS OF HEME TRANSFER

Detailed information on the mechanism to transfer heme from one place to another—for example between protein partners—is as yet poorly defined. It has been suggested[77] that an exchange mechanism between protein partners might play a role, as shown in Figure 5. This is envisaged as heme exchange between a donor, which could be a buffering molecule or a chaperone, and an acceptor such as a heme protein or a heme sensor. This heme exchange could take place by a range of different pathways (corresponding to dissociative or associative mechanisms) for delivery of heme to specific acceptors. The existence of such a mechanism, as outlined in Figure 5, would account for the buffering of the concentration of free heme, changes in heme availability, and how cells are able to hoard supplies of heme despite the undesirable effects of heme. It

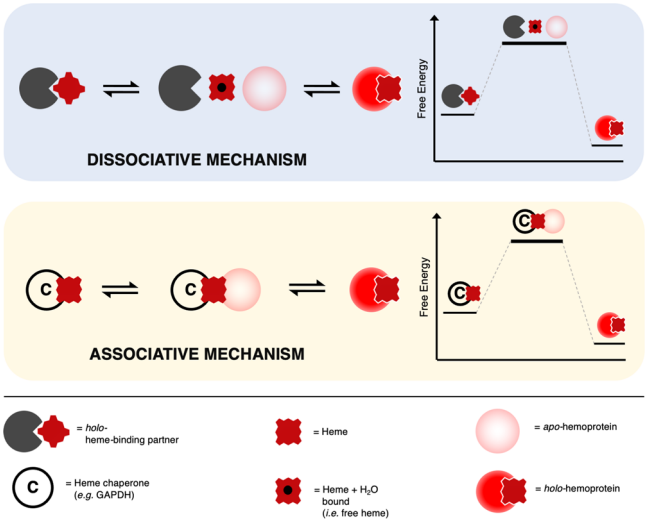

Figure 5. Possible mechanisms for exchange of heme. Heme-binding partners (dark gray pacmans) are envisaged as transferring heme to heme proteins (red circles) by dissociative (pale gray box) or associative (pale yellow box) pathways, resembling classical ligand exchange mechanisms in coordination chemistry. In a dissociative pathway, a free molecule of heme (assumed to be coordinated by a water molecule) is formed transiently following dissociation from a heme-binding partner, and is intercepted by an *apo*-heme protein (faded red circles). Alternatively, an associative exchange of heme is possible and is shown here for the example of heme delivery by chaperones (C, circles). This latter mechanism may provide better selectivity toward the target heme protein. However, we do not envisage this as being exclusive to chaperones, but a mechanism which, in principle, is available to be used by heme-binding partners as well in delivering heme to apo heme proteins. The different mechanisms of heme exchange may help to fine-tune the delivery of heme to specific acceptors.

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would also provide a responsive capability for mobilization of heme when and where needed.

SUMMARY AND FUTURE OUTLOOK

The logistics for the distribution of heme in cells have yet to be fully discovered. In practice, the dynamic requirements of cellular supply and demand may be complex, and perhaps there is no single mechanism for movement of heme within cells that dominates. Chaperones, heme-binding partners, membranes, and transporters may well work together in a concerted way and could provide contingencies for heme supply under conditions where the cell needs to react quickly to changes in heme demand. We see the role of a buffered reservoir of heme-binding proteins, potentially in large numbers, as being important. This could allow heme to travel from one place to another, where molecules of heme hitchhike across the cell by binding to whichever protein(s) they can find en route to their final destination.

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Notes

The authors declare no competing financial interest.

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