Mammalian Siderophores, Siderophore-binding Lipocalins, and the Labile Iron Pool*

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Bacteria use tight-binding, ferric-specific chelators called siderophores to acquire iron from the environment and from the host during infection; animals use proteins such as transferrin and ferritin to transport and store iron. Recently, candidate compounds that could serve endogenously as mammalian siderophore equivalents have been identified and characterized through associations with siderocalin, the only mammalian siderophore-binding protein currently known. Siderocalin, an antibacterial protein, acts by sequestering iron away from infecting bacteria as siderophore complexes. Candidate endogenous siderophores include compounds that only effectively transport iron as ternary complexes with siderocalin, explaining pleiotropic activities in normal cellular processes and specific disease states.

Host Utilization of Iron

Iron is required by virtually all living things (1). The biological versatility of iron, which participates in a variety of chemical reactions and as a structural component in proteins, is partly due to its tunable redox states. Despite its abundance in the biosphere, free ferric iron (Fe(III)), the form of iron present under aerobic conditions, is relatively inaccessible to biological systems, as it forms hydroxides, which are insoluble at physiological pH, limiting Fe(III) concentrations to \(~10^{-18}\) M in the absence of solubilizing agents (2, 3). Within the body, where the majority of iron is present as heme, free iron concentrations are maintained at even lower levels, partly because free iron is cytotoxic through its facility to catalyze Fenton chemistry, reacting with oxidants to generate damaging hydroxyl radical species (4–6). Consequently, animals have evolved highly specialized networks of proteins that maintain normal iron homeostasis and prevent deleterious side reactions during storage (e.g. ferritin) and transport (e.g. transferrin (Tf)) of iron; the translation of these proteins and their receptors is regulated in response to intracellular iron levels (7–9).

Whereas Fe(III) predominates in aerobic environments, ferrous iron (Fe(II)) is more bioavailable (soluble and transportable) and predominates under the reducing conditions inside cells, constraints that combine to require a constant cycling between ferric and ferrous forms during import and export in vivo, with many organisms coupling enzymatic reduction of iron with transport (10–12). In plasma, iron is found almost exclusively bound to Tf, which is present at concentrations as high as 50 \(\mu\)M (13) and is normally 30–40% iron-saturated (14); consequently, plasma concentrations of free Fe(III) average \(~10^{-24}\) M (15). Tf binds two atoms of Fe(III) with extremely high affinity (\(K_d = 10^{-23}\) M\(^{-1}\)) (16) and delivers bound iron to cells through a specific cell-surface Tf receptor (TfR1) (17, 18).

The Tf-TfR1 complex is endocytosed and recycled through low pH endosomes, where iron is released from Tf and concomitantly reduced. Released Fe(II) is transported into the cytoplasm by DMT1 (divalent metal transporter 1), where it becomes available for utilization or storage (19). Under normal physiological conditions, excess iron is stored within ferritin, a spherical cage consisting of 24 protein protomers arranged with cubic (432) symmetry as a hydrous ferric oxide core (20). The heavy chain of ferritin is responsible for oxidizing cytoplasmic ferrous iron to facilitate its storage within the ferritin cage (21). The continuous demand of iron for cellular processes requires a constant flux from the extracellular milieu to the cytoplasm; however, how iron is transported to sites of storage and utilization within the cell is still poorly understood. Because of its reactivity, transitory iron is presumably bound to and solubilized by small molecule (<1 kDa) ligands, making up what is known as the chelatable or labile iron pool (LIP), proposed to be a transitory source or sink of extracellular and intracellular Fe(III) and Fe(II), although its exact composition remains unclear (22–24). Characterization of the LIP has been difficult due to its transient nature and the complexity of chemically identifying specific iron complexes within the cell or following cell disruption (22). Proposed ferric ligands include ionic compounds such as citrate and phosphate, polypeptides, and phospholipids, but the physiological relevance of these compounds and their roles in iron homeostasis have not been determined (25).

Interplay of Host and Pathogen Iron Requirements

Normal iron homeostasis can be altered in a variety of disease states, including infections and cancer, where the tightly controlled levels of available iron in the body are further reduced to slow or stop the growth of pathogens and tumors through depletion of this necessary resource. In patients with microbial infections, iron supplementation often significantly worsens their condition, demonstrating the importance of the tight control of iron in disease (26–28). An example of a component of this defense is the iron-binding protein lactoferrin (first discovered in milk), which is a bacteriostatic agent that is released from neutrophil granules at sites of inflammation, inhibiting the growth of infecting pathogens by directly sequestering iron (13, 29, 30). To acquire iron in the face of the tight controls imposed through normal homeostasis and by antibacterial defenses, colonizing or infecting microbes use several approaches: dispensing with any need for iron (a rare example is Borrelia burgdorferi, the causative agent of Lyme disease (31));

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2 The abbreviations used are: Tf, transferrin; TfR1, Tf receptor 1; LIP, labile iron pool; Ent, enterobactin; DHBA, dihydroxybenzoate; Cat, catechol.
Siderophores are secreted, low molecular mass (≤1 kDa) compounds with remarkable iron complex formation constants; pFe₇.₄ is 23.5 for the siderophore aerobactin and 35.5 for the siderophore enterobactin (Ent; also enterochelin). Siderophore iron affinities are sufficient to solubilize iron exogenously in marine and terrestrial environments and to strip iron from direct iron-binding proteins endogenously, such as lactoferrin and Tf (36–38).

Siderophores form kinetically stable complexes with iron that are entropically favored by encapsulating ferric ions and displacing hexacoordinated water molecules; siderophores are often synthesized from amino acid precursors through non-ribosomal peptide synthesis (34). Siderophore chelating functionalities include catecholates, hydroxamates, and α-hydroxycarboxylates (although other chemistries are known) that contain hard base oxygen atoms to satisfy the preferred hexadentate coordination of ferric iron (Fig. 1). Siderophores display only weak affinities for Fe(II), allowing microbes to couple enzymatic reduction, leading to iron release, with siderophore import (39). Ent, a triscatecholate siderophore secreted by many enteric bacteria, displays essentially ideal hexadentate iron coordination, making it among the most potent siderophores known (40, 41). Ent consists of three 2,3-dihydroxybenzoate (2,3-DHBA) groups, a bidentate iron chelator on its own, organized into a single hexadentate compound through amide linkages to a backbone consisting of three serine residues coupled through lactone linkages (42). Siderophores that do not contain six ligating groups, such as bidentate and tetradeutate compounds, fully satisfy iron coordination by forming higher order iron complexes, as is seen with bidentate 2,3-DHBA (forming FeL₃ complexes) and tetradeutate siderophores like alcaligin and amonabactin, which form Fe₂L₃ complexes with Fe(III) (43–45). Many siderophores combine multiple ligation chemistries, such as carboxymycolactin, which contains phenolate-oxazoline and hydroxamate groups, and citrate-based siderophores like aerobactin and schizokinen, which utilize
α-hydroxycarboxylate and hydroxamate-chelating groups (Fig. 1) (36, 46–48). Virulence is often associated with the utilization of distinct or multiple siderophores with varying chelation chemistries and backbone structures, as is seen in pathogenic *Escherichia coli*, which secretes both Ent and aerobactin, although the explanation for this association was not initially apparent (47, 49–51).

**Siderocalins and Anti-siderophore Host Defenses**

In response to the use of siderophores by bacteria to steal iron from host iron-sequestering proteins, the armamentarium of the immune system includes proteins that sequester ferric siderophore complexes away from bacterial siderophore receptors (52). All of the known or hypothesized members of this functional group of proteins belong to the lipocalin family of binding proteins and so are known as “siderocalins” for “sidero-phore-binding lipocalins.” The lipocalin family of binding proteins displays a conserved eight-stranded β-barrel fold, which encompasses a highly sculpted binding site known as a calyx. Siderocalins include the mammalian lipocalins Lcn1 (lipocalin 1; also tear lipocalin or von Ebner’s gland protein (53)) and the archetype of the family, Scn (also know as NGAL (neutrophil gelatinsae-associated lipocalin), Lcn2, or 24p3 (54)), and the close pair of avian orthologs Q83 and Ex-FABP (55, 56). Siderocalins often bind siderophores with subnanomolar affinities (54–60), using, in the cases of Scn and the avian siderocalins, calyces lined with positively charged lysine and arginine side chains to interact, through cation–π and Coulombic interactions, with negatively charged siderophores with aromatic catecholate groups. To broaden the range of this antibacterial defense, Scn, the best characterized member of the family, uses a highly polyspecific recognition mechanism to sequester both a wide range of related Ent-like siderophores and the chemically distinct carboxymycobactins (54–60). Scn knock-out mice are significantly more susceptible to infections with bacteria that rely on these siderophores for iron acquisition in the absence of any other gross phenotype (61–63). Pathogenic bacteria have evolved responses to these defenses by using multiple siderophores that include examples that do not bind to Scn or by modifying siderophores in ways to block Scn binding, allowing iron to be acquired even in the presence of Scn and explaining much of the previously mysterious association of variant siderophores with virulence (54, 58, 59, 62, 64). For instance, Scn does not bind aerobactin, a weaker siderophore than Ent, the primary siderophore of many enteric bacteria, but aerobactin is associated with virulence by evading Scn sequestration.

**Mammalian Siderophores**

The idea that animals may utilize siderophores, much like bacteria, fungi, and even plants (monocots use phytosiderophores like mugineic acids to acquire iron (65)), has intrigued researchers for decades (66, 67), but until recently, no candidate animal siderophores have been identified, characterized, and validated. Despite the fact that animals primarily acquire iron through diet and transport iron using specialized proteins, it is reasonable to speculate that animals may also take advantage of the beneficial properties of siderophores in managing the endogenous iron budget. Endogenous iron transport systems alternative to Tf have been hypothesized based on the phenotype of hypotransferrinemic mice and atransferrinemic humans, who display severe hematopoietic and neurodevelopmental defects even though epithelial organogenesis is normal, and mice lacking TfR1, which display a more severe phenotype than hypotransferrinemic mice but successfully initiate organogenesis (68, 69). In these examples, iron availability is sufficient in the absence of Tf to initiate or complete organogenesis, a later stage of development than might be expected on first principles.

In this context, a mammalian siderophore would be defined as a compound that solubilizes iron and keeps it accessible for transport and systemic utilization. Even though the LIP only constitutes a small fraction of cellular iron (3–5%), a mammalian siderophore would also, ideally, keep iron in a non-reactive state and have affinities for iron compatible with Tf-mediated transport. Consideration of the latter point is important because, unlike microbial interactions with the environment, an endogenous siderophore needs to interact with extracellular iron predominantly bound to Tf. If the affinity of a siderophore for iron is considerably less than that of Tf, then the iron-bound fraction will remain small; if the affinity greatly exceeds that of Tf, iron will stripped away from the Tf delivery pathway. Bidentate chelators generally do not have affinities for iron sufficient to outcompete Tf, applicable to the wide array of endogenous catecholate and catecholamine compounds (e.g. dopamine, epinephrine, and norepinephrine) that might otherwise disrupt iron transport. The affinity of Ent and many microbial siderophores is sufficient to outcompete Tf, allowing iron acquisition during infection (38). The breakthrough in identifying such compounds was the discovery of host siderophore-binding proteins, the siderocalins, enabling the co-purification and identification of candidate endogenous siderophores.

**Mammalian Siderophore/Siderocalin Interactions**

Scn has been implicated in diverse cellular processes seemingly unrelated to iron transport in general or its primary function as an antibacterial specifically, including apoptosis and cellular differentiation (70, 71), with contradictory effects observed in different systems. Scn has also been associated with tumorigenesis and metastasis (70, 72–75), although, again, with considerable disagreement in the field as to the mechanisms involved. These observations also have to be reconciled with the lack of any apparent phenotypes in Scn knock-out mice in the absence of disease. The avian siderocalins like Scn also participate in a variety of endogenous cellular processes, including differentiation, development, and apoptosis for Ex-FABP (76, 77) and tumorigenesis for Q83 (78), that are also not obviously linked to bacteriostatic effects. Recent studies focusing on the pleiotropic functions of Scn have revealed that the protein can function as an iron shuttle, either delivering iron to or stealing iron from specific cell types. In two contrasting studies, small molecule siderophore-like compounds capable of solubilizing iron were found bound to Scn, confirming the existence of endogenous siderophores. Two different stories have developed from these observations, leading to the identification of two different endogenous siderophore candidates operating in distinct contexts.
The differentiation of non-epithelial mesenchymal cells into epithelial tubules is a key step in kidney development (79). The process is controlled by factors secreted from the ureteric bud, including Scn, which acts as an epithelial inducer. In this first context, it was discovered that Scn delivers iron to the cytoplasm, activating or repressing iron-responsive genes involved in the differentiation program. Iron delivery depends on endocytosis, likely mediated by the multi-ligand receptor megalin (80), and subsequent passage of Scn through acidic endosomes (Fig. 2, left). Interestingly, it was shown that the pH inducing iron release and the subcellular targeting of Scn were both distinct from Tf, and although it was clear that Scn associated with iron, the nature of the interaction was never characterized (79). However, Scn does not bind iron directly, requiring the equivalent of a siderophore to mediate any interaction with iron (54), suggesting the presence of an endogenous siderophore. Bacterial catecholate siderophores like Ent and its substituent, 2,3-DHBA, present in vivo from the colonizing microbiota, are unlikely to function as part of an endogenous iron delivery pathway because iron is not released from these ternary Scn-siderophore-iron complexes in the absence of specific reductases until acidification below pH 4.0 (60), which is not readily achieved in most cellular compartments such as endocytic vesicles (Fig. 3A). Because Scn is abundant throughout the urinary system, aseptic urine was used as a source to search for candidate compounds that could enable iron binding by Scn in a variety of qualitative and quantitative binding assays (81). A subset of simple catechols, including catechol itself, 3-methylcatechol, and pyrogallol, were shown to form ternary Scn-catechol-iron complexes at physiological pH with nanomolar dissociation constants. The complexes were directly visualizable by crystallography and supported complete iron release at pH values below 6 (Fig. 3B). Catechol was also shown to effectively solubilize iron at neutral pH and support iron delivery to cells through endosomes in vivo through interactions with Scn. Bidentate chelators such as these simple catechols do not bind iron with affinities approaching hexadentate chelators, but their efficiency is increased through Scn, which acts as an organizing scaffold, stabilizing FeL₃ complexes. The proposed mechanism of Scn-mediated iron transport using the siderophore catechol involves the sequential binding of Fe(Cat)₂, followed by recruitment of the third catechol moiety through the scaffolding effect, increasing the stability of iron complexation from that seen with bidentate FeL complexes to that of higher order complexes (Fig. 3C). Catechols derive from bacterial and mammalian metabolism of dietary compounds, representing a novel host/microbiome interaction and mimicking the role of Scn in innate immunity but instead serving to traffic iron in aseptic tissues. However, it is not clear if catechols serve systematically to maintain the LIP in the absence of Scn or enable iron transport outside of the urinary tract. Although the scaffolding effect improves iron chelation sufficiently to function efficiently in the urinary tract, it is unlikely to generate affinities sufficient to outcompete Tf and so may not function in the presence of serum components. Catechol alone can release iron from ferritin by direct chelation, potentially mobilizing stored iron and increasing the possibility that this Scn-mediated transport pathway may be more broadly applicable (82).

Certain murine hematopoietic cell lines, when deprived of IL-3, undergo apoptosis, a process that involves transcriptional up-regulation and secretion of Scn. In a second set of studies (83–85), it was observed that conditioned medium from apo-
ptotic cells contained Scn and was sufficient to induce apoptosis in susceptible cells (hematopoietic cell lines and leukocytes) even in the presence of IL-3. Furthermore, the simple addition of recombinant Scn to the culture medium (up to 0.5 μM) had a similar pro-apoptotic effect, suggesting that the protein alone was responsible for providing the apoptotic signal. In a series of subsequent studies encompassing dramatic experimental scope, it was shown that Scn could induce apoptosis in a variety of cells expressing a specific cell-surface receptor: BOCT1 (brain-type organic cation transporter 1; SLC22A17). Ectopic expression of SLC22A17 conferred on cells the ability to import or export iron, in the latter case driving apoptosis through the induction of the pro-apoptotic protein Bim in response to decreased intracellular iron levels. Interestingly, SLC22A17 was observed to be down-regulated on tumor cells with concomitant up-regulation of Scn, suggesting an unexpected role for iron in BCR-ABL-induced tumorigenesis. These results argued for a model in which empty, ligand-free Scn (apo-Scn), secreted in response to cytokine withdrawal or tumorigenesis, is internalized in a receptor-mediated process to sequester and export intracellular iron, driving apoptosis through autocrine, paracrine, or exocrine mechanisms (Fig. 2, right). This mechanism potentially explains the association of Scn with certain hematopoietic cancers, with tumor cells up-regulating Scn secretion and down-regulating its receptor to outcompete normal cells in the compartment by driving their apoptosis. The compound serving as the siderophore in this context was not fully characterized but was proposed to have 2,5-DHBA substituents, an isomer of 2,3-DHBA, synthesized endogenously by a cytosolic type 2 R-β-hydroxybutyrate dehydrogenase, DHRS6, also known as 3-hydroxybutyrate dehydrogenase type 2. On its own, 2,5-DHBA (also known as gentisic acid) was shown to bind to Scn with a reported dissociation constant of 12 nM, although these studies were not repeated with the ferric complex.

Although compelling, both of these stories raise a series of questions about the nature of this unique iron transport mechanism and its role in normal cellular physiology. Catechols

FIGURE 3. pH-dependent iron-recycling mechanisms mediated by Scn and catechol. A and B, fluorescence titrations as a function of pH of Scn with FeEnt and of Scn with Fe(Cat)3, respectively. In the case of FeEnt and 2,3-DHBA (not shown), siderophore release is not observed until pH 2.0, due in part to the structural stability of Scn, which sterically prevents the salicylate transition required in the absence of a reductase to release iron (60). In the case of Fe(Cat)3, siderophore is released at pH 6.0, which complements its ability to deliver iron physiologically. C, proposed mechanism of Fe(Cat)3 binding and release based on the co-crystal structure of Scn and FeCat at pH 4.5. Only one catechol was seen in the crystal structure at this pH, suggesting that release is correlated with protonation of the catechols.
readily undergo oxidation to form reactive radicals that can be deleterious to cells. Furthermore, although ferric catechol complexes may constitute some fraction of the LIP, it is unclear if they represent a practical source of iron for developing kidney cells. Although iron delivery to kidney cells does not require siderophores with iron affinities sufficient to outcompete Tf, which is filtered out of the urine, iron withdrawal in the hematopoietic compartment does, or else the endogenous Tf-bound iron pool would not be depleted and would be available to replenish cellular demands. Bidentate chelators like 2,5-DHBA are unlikely to outcompete Tf and therefore would not significantly affect the endogenous iron budget, even when boosted by scaffolding by binding to Scn. Additionally, the initial Scn knock-out mouse studies found no effect on apotransferin in the hematopoietic compartment (61, 62), as would be expected if this hypothesis were valid, although a third, independent study did find profound effects linked to the genetic background of the knock-out (86). Finally, 2,5-DHBA and other related compounds such as salicylic acid have been shown not to support the knock-out mouse studies found no effect on apoptosis in the hematopoietic compartment (61, 62), as would be expected if this hypothesis were valid, although a third, independent study did find profound effects linked to the genetic background of the knock-out (86). Finally, 2,5-DHBA and other related compounds such as salicylic acid have been shown not to support iron binding by Scn on its own (81), and it is unclear how DHRS6, a highly substrate-specific enzyme catalyzing the conversion of β-hydroxybutyrate into acetoacetate (87), would also efficiently generate the unrelated compound 2,5-DHBA.

The identification of candidate endogenous mammalian siderophores through two different lines of inquiry has potentially revealed unique Scn-mediated, siderophore-dependent iron transport pathways that do not involve Tf, with physiological implications ranging from normal cellular differentiation and apoptosis (in both immune system homeostasis and cancer progression) to tumorigenesis. Further studies will be needed to fully define (i) the range of endogenous compounds acting as siderophores in concert with Scn; (ii) the precise mechanisms involved; and (iii) whether additional mammalian siderophore-binding proteins, with potentially distinct specificities, exist and affect similar endogenous processes.

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