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Current Opinion in Chemical Biology

Siderocalins: Siderophore binding proteins evolved for primary pathogen host defense

Allyson K Sia, Benjamin E Allred and Kenneth N Raymond

Bacterial pathogens use siderophores to obtain iron from the host in order to survive and grow. The host defends against siderophore-mediated iron acquisition by producing siderocalins. Siderocalins are a siderophore binding subset of the lipocalin family of proteins. The design of the siderophore binding pocket gives siderocalins the ability to bind a wide variety of siderophores and protect the host against several pathogens. Siderocalins have been identified in humans, chickens, and quail, among other animals. The differences in the respective siderocalins suggest that each was developed in response to the most serious pathogens encountered by that animal. Additionally, siderocalins have been observed in many roles unrelated to pathogen defense including differentiation, embryogenesis, inflammation, and cancer.

Address
Department of Chemistry, University of California, Berkeley, CA 94720-1460, USA

Corresponding author: Raymond, Kenneth N (raymond@socrates.berkeley.edu)

Current Opinion in Chemical Biology 2013, **17**:150–157

This review comes from a themed issue on **Bioinorganic Chemistry**

Edited by **Christopher J Chang** and **Chuan He**

For a complete overview see the Issue and the Editorial

Available online 22nd December 2012

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http://dx.doi.org/10.1016/j.cbpa.2012.11.014

Introduction

Both pathogens and hosts require iron for several vital processes including oxygen binding, catalysis, and gene expression [1]. Demand for the same iron resources pressures the host and pathogen to coevolve competing systems to acquire and withhold iron [2]. The host limits the free iron concentration by using the iron transport and storage proteins transferrin and ferritin. Many bacteria acquire iron from the host by secreting siderophores that remove iron from the host proteins and carry it to the pathogen [3].

In response to siderophore-mediated iron acquisition, many animal hosts produce siderocalins. Siderocalins are a siderophore-binding subset of the lipocalin family of proteins defined by the lipocalin fold, an eight-stranded anti-parallel beta-barrel that forms a calyx or binding pocket [4,5]. Siderocalins limit pathogen growth by intercepting siderophores and preventing the delivery of iron to the pathogen [6]. Siderocalins have recently been observed in several animals including humans, chickens, and quail. Subtle differences among the siderocalins suggest that each host has adapted to the most dangerous pathogens it encounters.

In addition to pathogen defense, siderocalins function in many other processes. Cell differentiation, embryogenesis, inflammation, cancer, and other diseases have been associated with the expression of siderocalins. The role of the siderocalins in these processes is largely unknown, but the characterization of the siderophore binding properties provides the first molecular insights. The structure, function and properties of human Siderocalin (Scn), human tear lipocalin (TL), galline extracellular fatty-acid binding protein (Ex-FABP), and quail siderocalin (Q83) will be discussed in this review.

Siderocalin

The antimicrobial human immunoprotein Siderocalin (Scn, also NGAL, Lcn2, HNL, 24p3) has garnered interest from a wide spectrum of scientists in recent years. Arguably the most significant function of Scn is that it acts as part of the first line of defense against microbial invaders by intercepting bacterial siderophores [6–8]. While it is also expressed in non-disease cases such as modulation of intracellular iron stores and iron delivery in early embryogenesis, Scn is also a biomarker that is upregulated in certain diseases such as acute kidney injury, psoriasis and ovarian and gastric cancers (Figure 3) [9–15]. Whether these functions are ligand-dependent or why Scn expression is upregulated in these cases is not fully understood [16–24].

A collection of crystallographic structures of Scn with bound ligands elucidated the role of the protein as an antimicrobial ligand scavenger. Scn has three rigid binding subpockets which are defined by the residue side chains that line them. The positively charged, polar residues Lys125, Lys134 and Arg81 are involved in cation-pi interactions necessary for molecular recognition by Scn [25] (Figure 1). Tyr106 is key in stabilizing bound ligands by hydrogen bond stabilization [7]. Several papers have shown the *tris*-catecholate siderophores, such as enterobactin from *Escherichia coli* and bacillibactin from *Bacillus anthracis* (Figure 2) are bound tightly by Scn *in vivo* and *in vitro* with subnanomolar dissociation constants [7,25,4]. Siderophores bound by Scn are not limited to *tris*-catecholates. The carboxymycobactins of

Mycobacterium tuberculosis (Figure 2) bind iron with two hydroxamates and one phenyloxazoline moiety. Several carboxymycobactins are secreted from *M. tuberculosis*, and differ from one another in fatty acid tail length [26]. The phenyloxazoline unit of all carboxymycobactins is the single aromatic unit of this siderophore that anchors the carboxymycobactin into Scn subpocket 1. Scn, however, binds only carboxymycobactins with $n = 6–8$ as well as corresponding ferric complexes. Hoette et al. attribute the selectivity of Scn for carboxymycobactins $n = 6–8$ to the ability of longer carboxylate tails to tuck into a recess behind subpocket 2 [26]. The shorter carboxylate tails of carboxymycobactins $n = 3–5$ protrude from subpocket 3 away from the calyx, thus diminishing Scn affinity for the siderophore [26]. The 'tail-out' configuration of carboxymycobactin carboxylate tails can be viewed as a sort of stealth mechanism in which the bacterium produces several siderophores of varying lipophilicity and varying abilities to evade Scn sequestration.

Other bacterial pathogens have capitalized on the strict Scn binding requirements to produce stealth siderophores, or iron chelators that are sterically or electronically incompatible with Scn. Salmochelins and aerobactin of *E. coli* are not Scn-bound due to sterics and lack of aromatic groups for cation-pi stabilization, respectively [27,28]. Petrobactin of *B. anthracis* and its ferric complex clash with the Scn walls and thus are not bound (Figure 2) [29,30].

In the medical sciences the role of Scn as a biomarker or a potential iron transport agent has been a topic of recent interest. Scn has been identified as an iron delivery agent in early embryogenesis, a relationship established from growth experiments with atransferrinemic mice (Figure 3) [14,31]. Scn has no measurable affinity for iron alone and requires an endogenous siderophore to traffic iron.

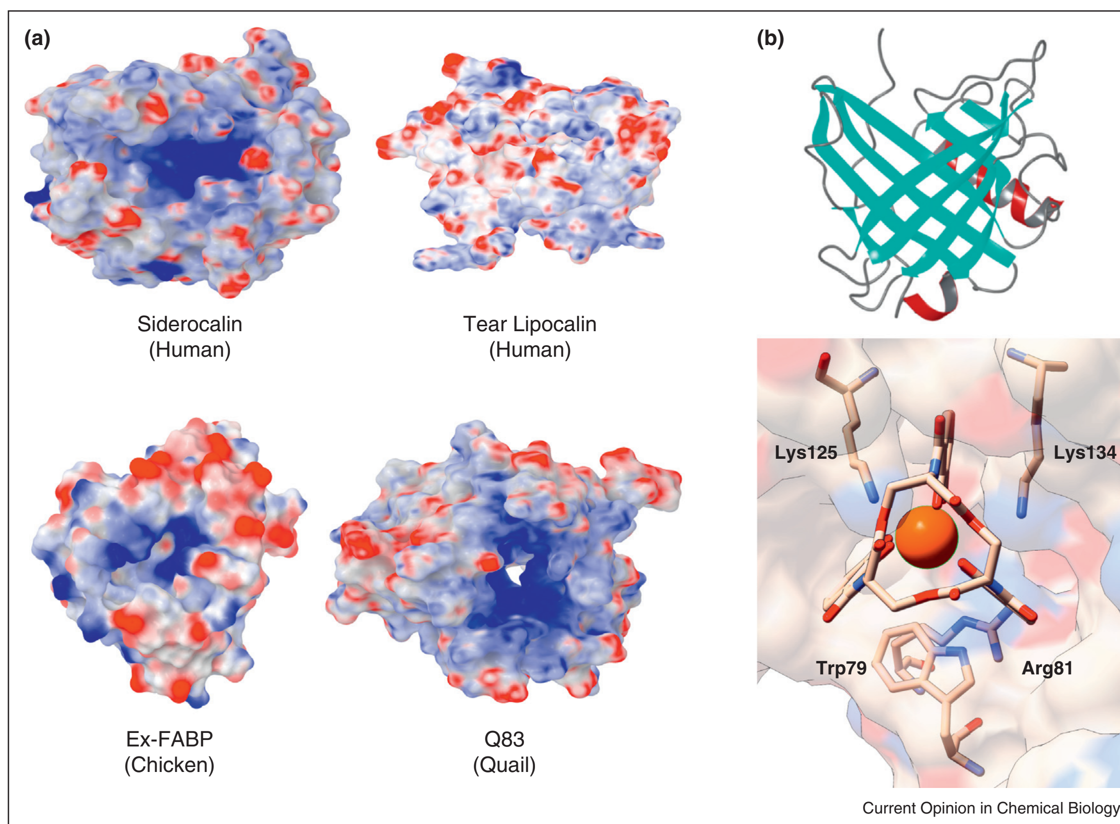
Bao and coworkers have shown that simple catechols can serve as the endogenous mammalian siderophore equivalent [13,32]. A scheme of this is depicted in Figure 3. Among the mammalian siderophore candidates isolated from mouse urine was 2,3-dihydroxybenzoic acid, the monomeric unit of enterobactin [13]. All cofactors which can be classified as mammalian siderophores must obey the binding requirements of Scn. Claims that 2,5-dihydroxybenzoic acid (25DHB), also known as gentisic acid and an isomer of 2,3-dihydroxybenzoic acid, is a mammalian siderophore have been made [33], but are inconsistent with the definition of mammalian siderophore. Catechols form high affinity iron complexes due to the bidentate chelation by deprotonated *ortho*-hydroxyl groups to form a 5-membered ring. 25DHB is not a catechol; it is a catechol isomer which binds iron via salicylate mode using one oxygen from the 2-hydroxyl group and one from the deprotonated carboxylic acid. Salicylate-siderophores are inherently weaker iron chelators. Correnti et al. have performed solution thermodynamic analyses detailing the distribution of $Fe(25DHB)_3$ complexes in solution under various conditions only to find that either the *bis*- or *tris*- 25DHB ferric complexes exist at negligible concentrations. The possibility that the $Fe(25DHB)_3$ complex form by ternary association in the Scn calyx is also implausible since the 5-hydroxyl groups sterically clash with the calyx walls. A hexadentate ligand based on 25DHB units was synthesized and tested in Scn binding assays to further illustrate the weak affinity Scn has for salicylate-mode chelators and/or for chelators with substituents on the 5 position of the aromatic ring [32].

Unfortunately, the misunderstanding about 25DHB as a mammalian siderophore has been the basis for other claims that the ternary Scn:Fe:25DHB complex can mediate apoptosis [33]. Repetitions of Scn:Fe:25DHB-mediated apoptosis experiments, followed by more direct and complementary apoptosis experiments, have shown that 25DHB-based siderophores do not chelate iron strongly enough to generate an apoptotic response in hematopoietic cell lines [32]. An important statement within the scope of this review is that Scn does not and cannot use 25DHB chelators as endogenous siderophores; 25DHB-based siderophores more likely resemble stealth siderophores due to the steric incompatibility of these molecules with Scn binding pockets. Cellular responses which are interpreted to be dependent on the Scn:Fe:25DHB interaction likely require alternative explanation or further study.

Tear lipocalin

Tear lipocalin (TL, also known as Lipocalin1, von Ebner's gland protein, or human tear prealbumin) is the second-most concentrated protein in tears and protects the ocular surface by scavenging harmful lipid peroxidation products. More than an extracellular ligand delivery protein, TL is a catalytic endonuclease in tears which is important for the breakdown of invading viral or microbial DNA [34]. TL also disrupts bacterial or viral colonization in human tears by inhibiting microbial cysteine proteinases and intercepting bacterial siderophores [35–38]. In the early 1990's, TL was found in several other types of human mucosae, such as trachobroncheal, lingual, prostate and pituitary glandular mucosae [39–42]. The various roles of TL are attributed, in part, to its ligands. TL accommodates ligands of strikingly different chemical structures. Fatty acids with chains up to 18 carbons long or with aromatic moieties tethered to the end of the chain are bound by TL with high affinity [41,45]. TL also binds bacterial and fungal siderophores that are structurally quite different from fatty acids, such as enterobactin, aerobactin, DFOB, ferricrocin, ferrichrome, rhodotorulic acid, coprogen and triacetylfusarinine C [37].

Figure 1



Structure of siderocalins. **(a)** Surface structure diagrams of the 4 lipocalins discussed in this review. Proteins are colored according to electrostatic potential (blue = positively charged regions, red = negatively charged regions). All calyces are oriented to face the reader. For TL, the positively charged residues that line the calyx are difficult to show in two dimensions. **(b)** Structural highlights of Scn. The ribbon diagram of Scn illustrates the typical beta-barrel. The binding pocket of Scn uses three positive residues (sticks) to bind ferric enterobactin (sticks and an orange sphere for iron).

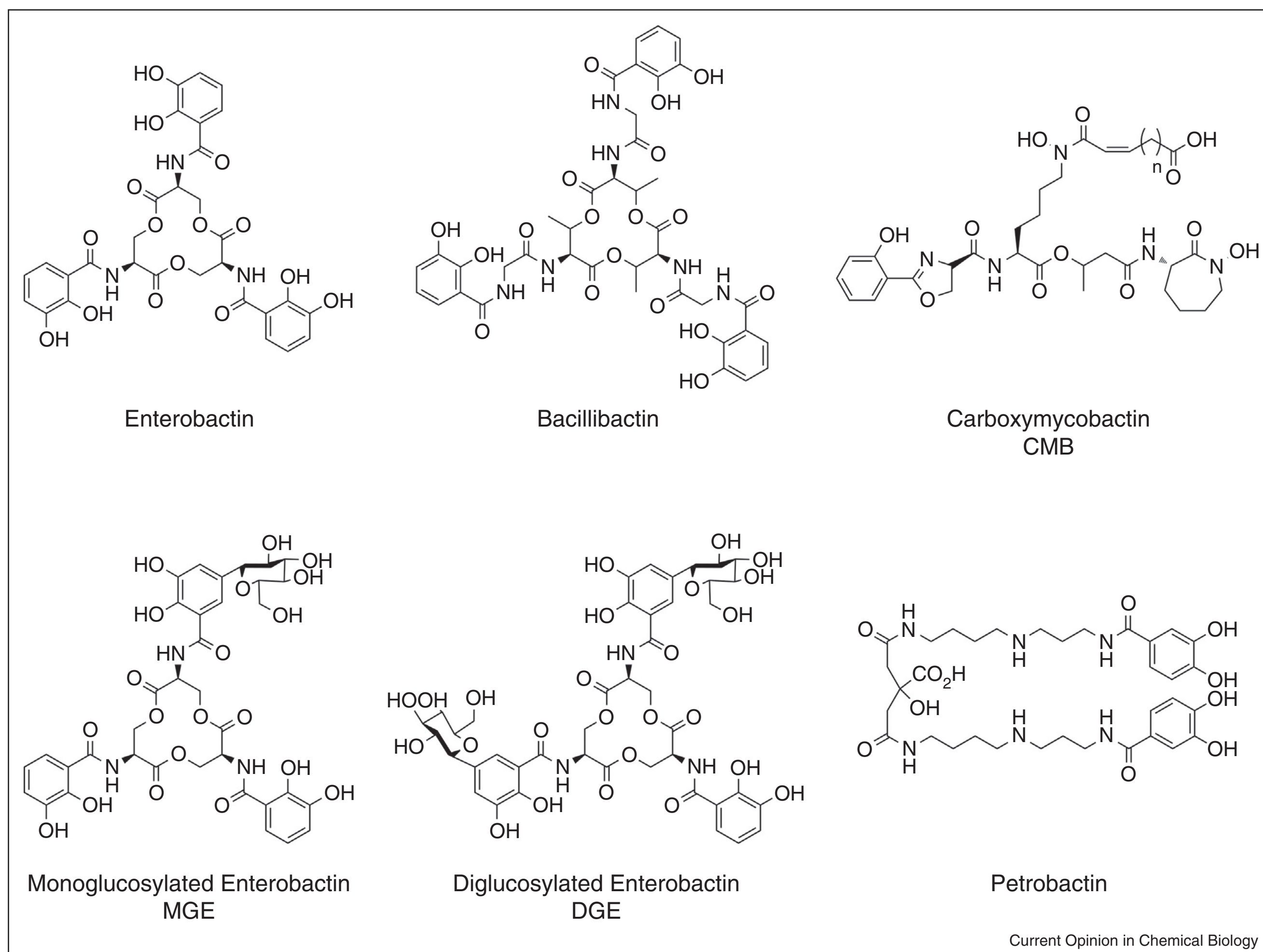
Unlike other lipocalins, TL has a much bigger calyx that can also change cavity sizes and rigidity by rearrangement of residue side chains within the calyx (Figure 1). A phenylalanine residue (Phe99) within the TL calyx can rearrange to reversibly form another beta-sheet that rigidifies the protein–ligand interaction [43]. A methionine residue (Met39) can rearrange upon ligand binding to expand the calyx volume [44]. Furthermore, TL has 4 flexible loops that aid in ligand recognition [45]. These flexibility features demonstrate the multipurpose nature of TL. TL accommodates chemically different ligands and the resulting conformational changes of the protein are dependent on the identity of a ligand. For example, TL binding of a lipid results in β-structure formation and a rigidification of TL [41]. At various epithelial surfaces where TL is a scavenger, enzyme or enzyme inhibitor, the ultimate fate of one TL protein is determined by the bound ligand and the resulting TL conformational change. The fact that TL binds a vast array of ligands and is expressed in several types of cells suggests that TL is part of a general protective response for the human body. Furthermore, other roles of TL such as its endonuclease role may be available in other physiological contexts beyond protection of ocular surfaces.

Ex-FABP

Ex-FABP is a 21 kDa lipocalin found in chickens [4,46]. The structural similarity to Scn, including a wide, positively charged calyx, prompted investigating the siderophore binding properties of Ex-FABP and the discovery that it is a siderocalin [47] (Figure 1).

The calyx interacts with siderophores in a manner similar to Scn. Three basic residues, Lys82, Arg101, and Arg112, give the calyx a positive charge and define three catechol-binding subpockets within the calyx. Arg101 and Arg112 provide electrostatic interactions with bound catechols and Lys82 hydrogen bonds with the meta hydroxyl of the catechols in two of the subpockets. Ex-FABP binds ferric enterobactin with a dissociation constant of 0.2 nM, comparable to the high affinity of Scn for ferric enterobactin. Ex-FABP also binds monoglucosylated enterobactin (MGE) with a subnanomolar dissociation constant, but it does not bind

Figure 2



Chemical structures of siderophores. MGE and DGE are also referred to as salmochelins.

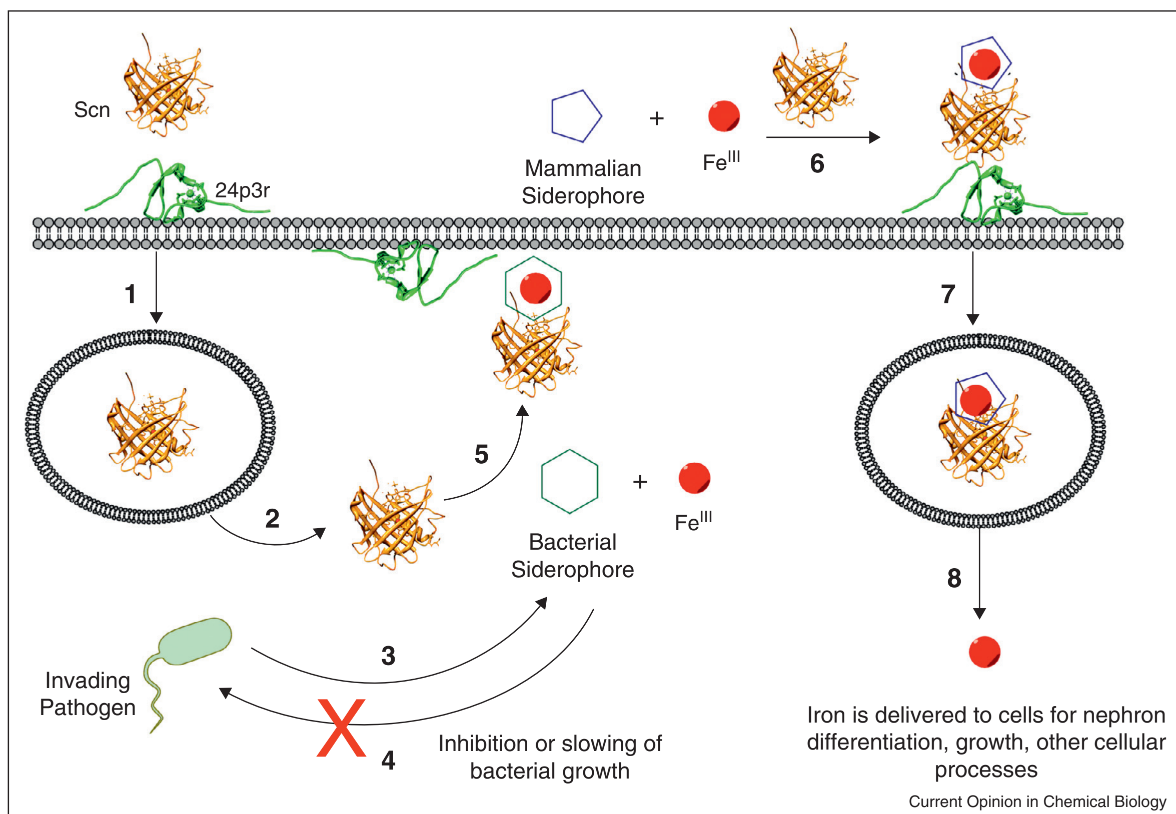
diglucosylated enterobactin (DGE) (Figure 2). This is because one subpocket of Ex-FABP is extended to allow space for the single glucose subunit branching from a catechol of MGE. The glucose would clash with the calyx wall of the corresponding subpocket in Scn [47].

The difference in ability to bind MGE between Scn and Ex-FABP strongly suggests that chickens have developed a siderocalin defense specific to chicken pathogens. MGE and DGE are members of a family of siderophores called salmochelins which also includes various hydrolysis products of MGE or DGE [28,48]. These siderophores are produced and transported by products of the *iro* gene locus found in *Salmonella enterica* and extra-intestinal pathogenic *E. coli*, including avian pathogenic *E. coli* (APEC) [48–50]. In APEC, the enterobactin system alone is insufficient for virulence [50] presumably because the enterobactin is intercepted by Ex-FABP. Since MGE is bound by Ex-FABP [47], it likely does not support virulence. Unsurprisingly, DGE is secreted in addition to MGE because it can circumvent the Ex-FABP defense [49,50]. The *iro* locus remains a virulence factor in APEC that is significantly associated with pathogenicity and lethality [50,51]. In addition to *E. coli*, pathogenic *S. enterica* isolated from chickens has the *iro* virulence factor [52].

The observed expression of Ex-FABP supports the siderocalin activity of the protein. It has been observed in the bacteriostatic hen egg [53]. Granulocytes express it [54]. Tracheal infection with infectious bronchitis coronavirus increases expression of the protein [55], and the inflammatory signals IL6 and LPS, Gram-negative endotoxin, induce expression [56,57].

The siderocalin activity of Ex-FABP is the most characterized function of the protein, but it plays a role in a variety of other processes. Ex-FABP has been observed in

Figure 3



Scheme depicting intracellular Scn-mediated processes. (1) Scn (shown in its apo form in step 1) binds its putative receptor, 24p3 or megalin. Scn is endocytosed and secreted intracellularly (2) thus available to intercept (5) bacterial siderophores secreted (3) by an invading pathogen. The result is inhibition of bacterial growth (4) since the bacteria are rendered growth-limited by Scn. The Scn:ferric siderophore complex is then trafficked to extracellular space. In a separate event, mediated by mammalian siderophores (6), a Scn:mammalian siderophore-iron complex can bind either 24p3 or megalin, and the ternary structure is endocytosed and trafficked to intracellular space (7) where the iron is released in acidic environments of the late endosome (8). This intracellular iron supply is used for growth, differentiation, or other cellular processes.

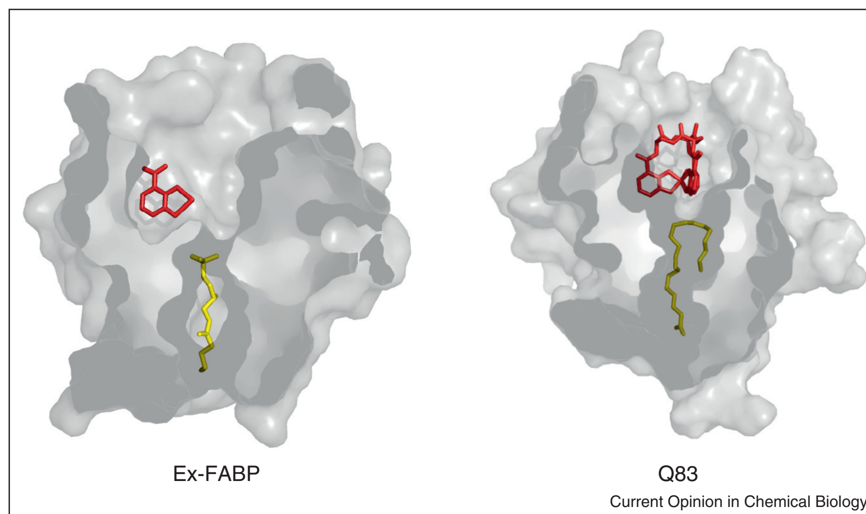
differentiating cartilage cells of chicken embryos as well as skin, brain, heart, and muscle tissues [46,54]. Expression increases during inflammation and acute phase response. Pathological cartilage of dyschondroplastic and osteoarthritic chickens has an elevated amount of Ex-FABP [56]. A review of the different processes that involve Ex-FABP, and Scn has proposed that these proteins, in addition to being siderocalins, are stress proteins that function in tissues experiencing active remodeling or acute phase response [58].

A breakthrough in the way Ex-FABP contributes to these different processes was uncovered while characterizing the structure of the Ex-FABP:siderophore complex. The beta-barrel forms a second binding site that extends from the siderophore binding pocket to the opposite side of the protein. Having two binding sites enables Ex-FABP to simultaneously bind two structurally, chemically, and physiologically distinct molecules. The crystal structure shows a molecule of lysophosphatidic acid (LPA) bound in the long, narrow pocket while ferric-enterobactin sits in the broad pocket near the surface (Figure 4). LPA is signal for a variety of cellular processes, and Ex-FABP may serve as a LPA sensor [47]. Two reported instances have linked Ex-FABP to binding lipophilic molecules. In adult chickens, polymorphisms of Ex-FABP are related to subcutaneous fat and skin thickness of cocks [59]. The protein also protects the heart during acute phase response, possibly by fatty acid scavenging [57]. As of now, it is unknown whether each binding site operates independently, cooperatively, or both, but it is likely that the many functions of Ex-FABP depend on the status of each binding site.

Q83

Q83 is the quail homolog of Ex-FABP. It was identified in avian fibroblasts transformed by the v-*myc* oncogene [60]. Q83 has 64% sequence similarity to Scn and 87%

Figure 4



Dual binding pockets of Ex-FABP and Q83. Ex-FABP contains a red ferric dihydroxybenzoic acid molecule in the siderophore binding pocket and a yellow lysophosphatidic acid in the fatty acid binding pocket. Q83 contains a red ferric enterobactin molecule in the siderophore binding pocket and a yellow arachidonic acid molecule in the fatty acid binding pocket.

sequence similarity to Ex-FABP [60,61]. The structural similarity between Q83 and Ex-FABP is confirmed in the NMR structure of Q83. The typical beta-barrel forms a calyx containing the three basic residues Lys83, Arg102, and Arg113 (Figure 1). The basic triad provides the interactions to tightly bind enterobactin with a dissociation constant of 0.5 nM [61], implying that it defends against pathogens that use enterobactin. It may have the same activity against avian pathogens as Ex-FABP by binding the salmochelin MGE, but the glucose subunit has not been modeled in the expanded subpocket 3 to verify this.

Similar to Ex-FABP, Q83 has a second binding pocket that extends through the core of the protein for binding arachidonic acid and other fatty acids with nanomolar dissociation constants (Figure 4). NMR studies showed that the binding status of each binding site has an allosteric effect on the dynamics of the other binding site [62,63]. The dual binding capability of Q83 potentially links iron transport, antimicrobial activity, and fatty acid pathways. The different roles of Q83 have not been investigated, but it is inferred to have pleiotropic functions similar to the other siderocalins. The characterization of the dual binding sites of Q83 sheds light on how the siderocalins may participate in the variety of processes that have been identified.

Concluding remarks

The bacterial pilfering of the host iron supply is accomplished by a number of siderophores and the respective iron-uptake systems. Siderocalins are immunoproteins that incapacitate some bacterial iron uptake systems by intercepting bacterial siderophores. The isolation and characterization of siderocalins from quail, chickens, and humans, suggests that siderocalins exist for all animals that suffer from microbial infections. It seems only a matter of time before siderocalins are identified for all forms of life that can be considered a host, thus enhancing our appreciation for the evolutionary pressure of host–pathogen interactions. The discovery of siderocalins will give an added perspective as to why certain species or individuals within a species are at higher risk to bacterial infections. Searching for new siderocalins may also reveal that a single host expresses a variety of location-specific siderocalins designed to manage the microbiome of the host by preventing an outbreak of pathogenic bacteria.

With regard to the multiple developmental and disease related functions of siderocalins, it is unknown whether these functions coevolved with the siderophore-binding functions or if the different functions developed independently. Were these proteins originally developed for fatty acid binding and eventually customized by human evolution to sequester bacterial siderophores, or did the development follow another sequence? By manipulating a protein already encoded in the genome to serve multiple purposes, hosts would increase the chances for survival at little extra cost. Furthermore, an evolutionary map of siderocalin development may indicate when a pathogen emerged since it appears that siderocalins evolved to bind siderophores secreted by the most threatening pathogens.

Acknowledgements

We are grateful for support from the National Institutes of Health (R01AI011744). The authors also thank Prof. Roland K. Strong and his laboratory members for providing important pdb files.

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