Siderophores: Structure and Function of Microbial Iron Transport Compounds*

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Siderophores (from the Greek: "iron carriers") are defined as relatively low molecular weight, ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress. The role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cell. Research in this field began about five decades ago, and interest in it has accrued with the realization that most aerobic and facultative anaerobic microorganisms synthesize at least one siderophore. Siderophores have been related to virulence mechanisms in microorganisms pathogenic to both animals and plants. In addition, they have clinical applications and are possibly important in agriculture. For an introduction to the field, the reader is referred to one of the comprehensive monographs on the subject (1, 2).

Iron in the Environment and in Microbiology

The aerobic atmosphere of the planet has caused the surface iron to become converted to oxyhydroxides polymers of very sparing solubility. The concentration of free ferric ion at neutral pH is dictated by the solubility product constant of ferric hydroxide. Depending on the value selected for this constant, the maximum amount of uncomplexed ferric ion in solution at biological pH is probably not greater than $10^{-18} \text{M}$ (3). Microorganisms growing under aerobic conditions need iron for a variety of functions including reduction of oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, for formation of heme, and for other essential purposes. A level of at least one micromolar iron is needed for optimum growth. These environmental restrictions and biological imperatives have required that microorganisms form specific molecules that can compete effectively with hydroxyl ion for the ferric state of iron, a nutrient which is abundant but essentially unavailable.

It must be stressed that not all microbes require iron, and siderophores can be dispensed with in these rare cases. Some lactic acid bacteria are not stimulated to greater growth with iron, they have no heme enzymes, and the crucial iron-containing ribotide reductase (4) has been replaced with an enzyme using adenosylcobalamin as the radical generator. Other microbes need iron but grow anaerobically on Fe(II). While nearly all fungi make siderophores, both budding and fission yeast appear to be exceptions (3).

Among the alternative means of assimilating iron are surface reduction to the more soluble ferrous species, lowering the pH, utilization of heme, or extraction of protein-complexed metal. Siderophores appear to be confined to microbes and are not products of the metabolism of plants or animals, which have their own pathways for uptake of iron.

Detection, Isolation, and Structure

Detection of siderophores is most readily achieved in iron-limited media, which generally means either a synthetic (minimal) recipe or introduction of a complexing agent that will render the iron selectively unavailable. Although most siderophores are either hydroxamates or catechols, earlier tests based on such functional groups proved unreliable since they are absent from a few siderophores. The chrome azurol sulfonate assay (5) has become widely used since it is comprehensive, exceptionally responsive, and more convenient than microbiological assays which, although sensitive, may be rigidly specific. The chrome azurol sulfonate assay may be applied on agar surfaces or in solution. It is based on the color change that accompanies transfer of the ferric ion from its intense (extinction coefficient of at least 100,000) blue complex to the siderophore. A detergent must be present in order to achieve the intense color; otherwise, only a charge-transfer extinction of a few thousand is realized. Gram-negative bacteria are impervious to detergents and hence the chrome azurol sulfonate reagent can be incorporated in the agar media where it has the potential for isolation of biosynthetic, regulatory, and transport mutants. For Gram-positive bacteria and fungi, the toxicity of the reagent must be mitigated in some way (6). These qualifications, however, do not compromise use of the dye in solution or as a spray on chromatograms (5).

Since siderophores differ substantially in structure, no uniform procedure is available for their isolation. A preliminary examination by paper electrophoresis should reveal the charge profile as a function of pH, following which appropriate exchange resins can be applied for retention and elution of the compound(s). Most are water-soluble, and it is thus usually expedient to drive the siderophore into an organic solvent, such as benzyl alcohol or phenol-chloroform, in order to eliminate salt.

The siderophore may be isolated per se or as its iron chelate. The latter has the advantage of visual color, but the iron must be removed before any natural product can be characterized. Vigorous hydrolysis in the presence of iron will destroy oxidizable moieties, and direct NMR analysis is ruled out by the paramagnetism of the ferric ion.

Structural characterization is best carried out by a combination of NMR and mass spectroscopy. Both of these techniques are sensitive and capable of providing absolute answers. Less than half of the known siderophores will crystallize, otherwise x-ray diffraction is the method of choice since it affords the configuration of those molecules containing a chiral center (7).

Coordination Characteristics

Siderophores form high-spin, kinetically labile chelates with ferric ion which are characterized by exceptional thermodynamic stability (8, 9). The formation constant for typical molecules containing three bidentate ligands is $10^{30}$ or greater. The affinity for gallium is also high, but the attraction for aluminum and for all divalent ions is substantially less. Thus, the siderophore ligand can be said to be "virtually specific" for Fe(III) among the naturally occurring metal ions of abundance. Synthetic man-made elements in the actinide series are also firmly bound.
The fact that the siderophore ligand shows strong affinity for only the higher oxidation state of iron sets this natural complexing agent apart from molecules such as heme, which serve effectively as electron shuttles. At the same time, the relatively weak complexing of Fe(II) affords an efficient means of release, via reduction, inside the cell. This large discrepancy in the binding constants for Fe(II) and Fe(III) drives down the oxidation-reduction potential, and there has been some discussion that the actual value may be beyond the range of natural reducing agents. This aspect of the problem requires clarification and elucidation at the enzyme level. Probably the significant feature is the oxidation-reduction potential of the enzyme-ferric siderophore complex rather than the potential of the free ferric chelate.

With few exceptions, the “hard” acid ion, Fe(III), is linked to hard base atoms, such as oxygen, which accounts for the preference for ferric ion. Chirality in the ligand means that the binding sites for the metal ion are disposed in space in a particular orientation, and, hence, optical isomers are possible. Thus, ferrichrome (Fig. 1), in which the binding sites for the metal ion are mounted on an L-ornithine derivative, forms a cis-propeller of the highest known binding constant for ferric ion (9). Enterobactin is produced generally by enteric bacteria.

**Biosynthesis**

The siderophore for which we have the greatest inventory of information with regard to its anabolism is aerobactin (Fig. 3), first isolated from Aerobacter aerogenes (12). Subsequently, it was detected as a product of pCovK30, a plasmid commonly borne by clinical isolates of Escherichia coli. The aerobactin determinants from the latter source have been cloned and shown to occur in an operon preceded by a regulatory element (reviewed in Ref. 11). Aerobactin, which consists of citrate substituted on the distal carboxyls with residues of N<sup>6</sup>-hydroxylsine (14). Lysine-N<sup>6</sup>-hydroxylase, which carries loosely bound FAD, oxidizes the substrate at the expense of NADPH and molecular oxygen (15).

**Transport**

In constructing a binding agent with requisite affinity/specificity for Fe(III), nature appears to have exceeded the free diffusion limit of the outer membrane of Gram-negative bacteria (16). Thus, enterobactin, the siderophore indigenous to E. coli, has a molecular weight of 669 while that of ferrichrome, produced by fungi and utilized avidly by E. coli and other bacteria, is 740. This exclusion-by-size has required the insertion in the outer membrane of specialized receptors. In the course of evolution, these receptors have become “parasitized” by lethal agents such as bacteriophage, bacteriocins (“killer proteins”), and antibiotics. One of the earliest genetic lesions studied in E. coli, tonA ("T-one"), now fhuA, was that specifying ability to attach phage T1. This pore turned out to be the receptor for ferrichrome (17), although it also enables transport of several phages, colicin M, and albomycin. Similarly, the receptor for ferric enterobactin, FepA, is the site of penetration of colicins B and D, and the bacteriocin cladin utilizes the
ferric aerobactin receptor. This is the general pattern with siderophore receptors; namely, they also act as receptors for a variety of lethal agents. Thus, the receptor for an uncharacterized siderophore of Yersinia enterocolitica has been shown to serve as receptor for pesticin (18).

A second classical gene in E. coli codes for the TonB protein, required for phage infection and for iron supply via the many siderophore and inorganic iron uptake systems of the bacterium (19). TonB, located in the cytoplasmic membrane, was viewed as providing some kind of link to the outer membrane, but clarification, in molecular terms, had to await sequencing of the genes for the receptors. In an elegant experiment, the deletion of a particular loop converted FepA into a nonspecific diffusion channel (20). A similar finding was reported for ferrichrome transport in FhuA (21). Apparently these bacteria have evolved a sophisticated mode of active iron transport in which the energy of the cytoplasmic membrane has been linked to the outer membrane siderophore receptors.

**Regulation**

It has been known for many years that all components of siderophore systems are derepressed at low levels of iron. The first report on the molecular genetics of the process came with work on Salmonella typhimurium. Chemical mutagenesis identified a gene, designated fur (ferric uptake regulation), which controlled expression of the siderophore, again enterobactin, and a brace of large outer membrane proteins, one of which is the equivalent of FepA of E. coli (22). In the latter organism, the gene was cloned and sequenced, and the product was isolated and shown to act as a classical negative repressor of transcription (reviewed in Ref. 23). Although any first row divalent transition element will "organize" Fur to bind the operator, Fe(II) is thought to be the natural activator because of the relative abundance of iron. The "iron box" or "fur box" consensus sequence in the operator is GATAAGATAACTCATC, an array which occurs with some variation in the regulatory DNA of iron-affected systems in many microbial species. Polymerization of Fur around the operator has been suggested as the mode of binding (24), and this is supported by observations with the electron microscope (25). On the other hand, both Fur and ArcA, the latter the repressor for the sodA gene coding for manganese-superoxide dismutase, bind at the same site. Footprinting experiments demonstrated polymerized binding in the −10 to −35 region of the promoter but suggested interaction with one face of the double helix (26). The interaction of metallo-Fur with DNA was reinvestigated, and it was concluded that the repressor, which lacks the classical helix-turn-helix motif, contacts one face of the DNA across almost three successive major grooves (27). Earlier it was established that the N-terminal region of Fur recognizes DNA while other domains of the repressor are involved in separate functions such as binding metal or polymerization (28).

A still baffling aspect is the fact that a number of genes seemingly unrelated to iron acquisition, in addition to that for superoxide dismutase, are also part of the Fur regulon. A Fur titration assay has been proposed as a means of identifying all genes regulated by the repressor (29).

In contrast to the straightforward regulatory mechanism of the aerobactin operon by ferrous-Fur, regulation of the fur gene itself seems considerably more baroque. As well as an iron box, sites for binding of CAP have been identified (30).

The negative regulation scheme with Fe(II) as co-repressor for a small, Fur-like protein appears valid in many other bacterial species such as in the iron-regulated formation of toxin by Corynebacterium diphtheriae (31). Some variation in the structure of the repressor and the operator can be anticipated. However, in pseudomonads, a positive mechanism may underlie the observed overproduction of the fluorescent siderophores variously known as pseudobactins and pyoverdines (32).

The fur mutants of E. coli grow poorly (23), possibly because of oxidative stress (33). The mutation appears to be lethal in Neisseria spp. (34).

**Siderophores and Virulence**

A role for iron in the virulence mechanism of several microbes attacking man and other animals is well established. An adequate iron supply for many pathogenic species is critical since transferrin has a very high affinity for the metal and the protein is normally only about one-third saturated with iron. Strains of E. coli causing disseminating infection were found to harbor Co1 plasmids carrying the aerobactin synthesis and transport genes (reviewed in Ref. 35). The siderophore system of Y. enterocolitica is correlated with the virulence of the organism (18). On this vast topic, we can only refer to a monograph on iron and infection (36) and to two excellent reviews documenting the elaborate host defense systems based on the principle of withholding of iron (37, 38).

Regarding phytopathogens, it should be recalled that the virulence-associated iron chrysobactin uptake apparatus of Erwinia chrysanthemi involves an operon encoding transport and biosynthetic functions (39).

**Clinical Applications**

As naturally occurring chelating agents for iron, siderophores might be expected to be somewhat less noxious for deferrization of patients suffering from transfusion-induced siderosis. A siderophore from Streptomyces pilosus, desferrioxamine B, is marketed as the mesylate salt under the trade name Desferal and is advocated for removal of excess iron resulting from the supportive therapy for thalassemia. The drug must be injected, however, and an oral replacement is needed (40).

The potency of common antibiotics has been elevated by building into the molecules the iron-binding functional groups of siderophores (41). The objective here is to take advantage of the high affinity, siderophore-mediated iron uptake system of the bacteria.

**Agricultural Interest**

Fluorescent pseudomonads form a line of siderophores comprised of a quinoline moiety, responsible for the fluorescence, and a peptid chain of variable length bearing hydroxamic acid and α-hydroxy acid functions. Capacity to form these pseudobactin or pyoverdine type siderophores has been associated with improved plant growth either through a direct effect on the plant, through control of noxious organisms in the soil, or via some other route. Nitrogenase can be said to be an iron-intensive enzyme complex and the symbiotic variety, as found in Rhizobium spp., may require an intact siderophore system for expression of this exclusively prokaryotic catalyst upon which all life depends. These topics are explored in a recent volume dealing with siderophores in the plant world (42).

**Summary**

Siderophores are common products of aerobic and facultative anaerobic bacteria and of fungi. Elucidation of the molecular genetics of siderophore synthesis, and the regulation of this process by iron, has been facilitated by the fact that E. coli uses its own siderophores as well as those derived from other species, including fungi. Overproduction of the siderophore and its transport system at low iron is in this species well established to be the result of negative transcriptional repression, but the detailed mechanism may be positive in other organisms. Siderophores are transported across the double membrane enve-
lope of E. coli via a gating mechanism linking the inner and outer membranes.

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