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Mechanism of Iron Transport in Mycelia Sterilia EP-76

Jonathan P. Adjimani
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MECHANISM OF IRON TRANSPORT IN \textit{MYCELIA STERILIA} EP-76

by

Jonathan P. Adjimani

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

UTAH STATE UNIVERSITY
Logan, Utah

1987
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Jonathan P. Adjimani
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<td>TAFC</td>
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<td>desFOB</td>
<td>Deferriferrioxamine B</td>
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ABSTRACT

Mechanism of Iron Transport in *Mycelia sterilia* EP-76

by

Jonathan P. Adjimani, Doctor of Philosophy

Utah State University, 1987

Major Professor: Dr. Thomas Emery
Department: Chemistry and Biochemistry

The cyclic trihydroxamic acid, N,N',N''-triacetyl-
fusarinine C, produced by *Mycelia sterilia* EP-76, is shown
to be a ferric ionophore for this organism. The association constant for ferric-N,N',N''-triacetylfusarinine C
complex was determined to be log $K=32.5$. Other iron
chelating agents, such as rhodotorulic acid, citric acid,
or the monomeric subunit of triacetylfusarinine C, N-acetyl-
fusarinine, delivered iron to the cells by an indirect
mechanism involving iron exchange into triacetylfusarinine
C. In vitro ferric ion exchange was found to be rapid with
triacetylfusarinine C. Gallium uptake rates comparable to
those of iron were observed with the chelating agents that
transport iron into the cell. Ferrichrome, but not
ferrichrome A, was also capable of delivering iron and
gallium to this organism, but not by an exchange mechanism.
Unlike triacetylfusarinine C, the $^{14}$C-ligand of ferrichrome
was retained by the cell. A mid-point potential of -690 mV
versus the saturated silver chloride electrode was obtained
for the ferric-N,N',N''-triacetylfusarinine C complex,
indicating that an unfavorable reduction potential was not
the reason for utilizing a hydrolytic mechanism of intra­
cellular iron release from the ferric triacetylfusarinine
C chelate. The iron transport system recognizes only the
$\Lambda$-cis coordination isomer of ferric-N,N',N''-triacetyl­
fusarinine C metal ligand complex even though the $\Delta$-cis
configuration predominates in solution. Ferrichrome and
ferric-N,N',N''-triacetylfusarinine C are both transported
by the same receptor.
INTRODUCTION

Microorganisms, like other forms of life, have an absolute requirement for iron and have evolved low molecular weight (500-1000 daltons) iron chelating agents called "siderophores", from the Greek "iron carriers", for sequestering the metal (Lankford, 1973). Siderophores are used by the cell to solubilize the highly insoluble ferric ion and enhance its mobilization into cells. These iron chelating agents have been useful in studying iron metabolism in microorganisms (Neilands, 1974; Emery, 1978), and for the development of drugs in the treatment of iron overload patients (Anderson, 1980; Weatherall, 1981). The fusarinines (fusigens) are a class of amino acid ester siderophores of the hydroxamate type produced by Fusarium roseum, Fusarium cubense and other fungi (Diekmann & Zahner, 1967; Sayer & Emery, 1968; Moore & Emery, 1976). The monomeric unit of fusaridine is \( \text{N}^\text{O}-(\text{cis}-5\text{-hydroxy}-3\text{-methyl-pent-2-enoyl})-\text{N}^\text{O}\text{-hydroxy-L-ornithine} \), which undergoes a head to tail cyclic polymerization via the formation of ester bonds instead of the peptide linkages found in many other hydroxamate type siderophores, such as ferrichrome. Investigation of the iron transport properties of the fusarinines has been
limited by the very labile ester bonds. More stable N-acetylated derivatives of the fusarinines, have been isolated in large quantities from iron-deficient cultures of *Mycelia sterilia* EP-76, previously incorrectly identified as a *Penicillium* species (Moore & Emery, 1976), and *Aspergillus* species. The structure of cyclic N,N',N''-triacetylfusarinine C, abbreviated TAFC, was elucidated by Moore & Emery (1976); Hossain et al., (1980). Culture fluids of both *Mycelia sterilia* EP-76 and *Aspergillus* species have been shown to contain in addition to the cyclic trimer, large amounts of the monomeric hydroxamic acid, the linear dimer, and the linear trimer, all of which are believed to be formed as the result of esterase activity within the cell (Emery, 1976a). The structure of TAFC and the products of its hydrolysis are shown in Figure 1. Paper electrophoretic pattern of *Mycelia sterilia* EP-76 hydroxamates is shown in Figure 2. These products have high iron coordinating capabilities typical of the hydroxamic acid functional group. Cyclic triacetylfusarinine C is thought to be the ferric ionophore for the fungal species that produce it (Winkelmann & Zahner, 1973). A mechanism by which TAFC might transport iron to cells was suggested by Emery (1976a), who isolated an enzyme from *Mycelia sterilia* EP-76 that could hydrolyze the ester bonds of the siderophore. It was proposed that the release of iron
FIGURE 1: Chemical structures of hydroxamates of Mycelia sterilia EP-76.
FIGURE 2: Paper electrophoretic pattern of Mycelia sterilia EP-76 hydroxamates. Electrophoresis was at pH 5.2 and 30V/cm for 45 min., using pyridine-acetic acid-water (31:15:1454 vol/vol). The number on each band represents the net charge on the molecule (Moore & Emery, 1976). Cyclic trimer (CT), linear trimer (LT), dimer (D), monomer (M).
from the cyclic trimer involves a hydrolytic mechanism similar to that put forward to explain the mechanism of iron release from enterobactin a cyclic trimer of 2,3-dihydroxybenzoylserine, a siderophore produced by enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Pollack & Neilands, 1970; O'Brien et al., 1971). Though esterase activity has been suggested in the release of iron from TAFC, virtually nothing is known about the precise mechanism by which TAFC producing species acquire and transport iron into the cell. Furthermore, the role of the products of hydrolysis of the siderophore is not known. Despite detailed work that has been carried out on the structure of TAFC, no work has been reported on the uptake of ferric-TAFC complex in any of the organisms that produce this stable siderophore. The present study provides data on the mechanism of TAFC mediated iron uptake in *Mycelia sterilia* EP-76.
Iron, the fourth most abundant element on the earth, is required for a large number of metabolic processes in virtually all organisms except some lactobacilli species. The element exists in the ferrous(II) and ferric(III) oxidation states, the former being rapidly converted to the ferric state in the presence of oxygen. Despite the abundance of iron in the environment, all forms of life have difficulty in assimilating the metal due to the very poor solubility of ferric ion at physiological pH. The free Fe(III) equilibrium concentration is about $10^{-18}$ M (Latimer, 1952), which is many orders of magnitude lower than the minimum amount of iron required for growth of organisms. Iron deficiency in plants is manifested by the yellowing of leaves, a condition called chlorosis. The mechanism by which plants acquire iron to meet their metabolic demands is not well understood. There is evidence that water-cultured plants excrete several hexadentate low molecular weight iron chelating organic acids from their roots (Fushiya et al., 1980; Sugiura & Tanaka, 1981). Whether these compounds are involved in transporting iron to plants has yet to be determined. Iron deficiency
anemia, in which too little iron is absorbed into the bloodstream, is common among humans. Transferrin, a plasma protein, functions to transport iron from the intestine to other tissues in humans (Huebers et al., 1978). Microorganisms use siderophores to solubilize and assimilate iron. There are presently more than 80 naturally characterized siderophores. In addition to their function in iron solubilization and transport, siderophores have been identified with certain physiological and pathological functions in higher organisms. For example, they have been shown to be important for germination and sporulation in some organisms (Horowitz et al., 1976). When microorganisms find their way into the bloodstream during infection, the siderophore that they produce compete for iron with the human iron transport protein, transferrin, with pathological consequences. It is also known that non-pathogenic root colonizing microorganisms can prevent invasion by pathogenic species by producing siderophores which deprive the invading organism of iron (Olsen et al., 1981; Schroth & Hancock, 1982).

Although there is a great deal of structural diversity among siderophores, two main types of ligating groups
prevail. These are the catecholate (a), and the hydroxamate (b) groups.

These bidentate ligating groups form six coordinate octahedral complexes with ferric ion. Typical trihydroxamate type siderophores include, coprogen, the ferrichromes, the fusarinines and the ferrioxamines (Figure 3). Enterobactin and parabactin are catecholate type siderophores. The former is produced by enteric bacteria such as *Escherichia coli* or *Salmonella typhimurium*, and the latter by *Paracoccus denitrificans*. The hydroxamate type siderophores are made up of an N5-hydroxyornithine subunit, which is the building block of the diketopiperazine containing siderophores, such as coprogen, rhodotorulic acid and dimerum acid (Atkins & Neilands, 1968; Diekmann, 1970; Keller-Schierlein & Diekmann, 1970), and the cyclic hexapeptides of the ferrichromes and the fusarinines (Emery, 1965; Diekmann, 1968; Sayer & Emery, 1968; Neilands, 1973). Some siderophores have mixed iron binding functions. For example, in addition to two hydroxamate groups, aerobactin,
FIGURE 3: Structures of representative hydroxamic acid and catechol-containing siderophores.
schizokinen and arthrobactin contain an α-hydroxy-carboxylate unit for binding the metal (Harris et al., 1979). All the siderophores described above, except rhodotorulic acid, form 1:1 complexes with ferric ion involving three bidentate ligating groups. Rhodotorulic acid (RA), a dihydroxamate siderophore elaborated by Rhodotorula pilimanae, forms a dimeric complex of stoichiometry Fe₂(RA)₃ at neutral pH (Carrano et al., 1979). The hydroxamate siderophores bind iron with each hydroxamate group contributing two oxygen atoms with the proton of the hydroxamic acid group (pKa=8-9) displaced by the metal:

\[
\begin{align*}
&\text{HO} \quad \text{O} \\
&\text{N} \quad \text{C}
\end{align*}
\] + \text{Fe}^{3+} \rightarrow \begin{align*}
&\text{O} \quad \text{O} \\
&\text{N} \quad \text{C}
\end{align*} + \text{H}^+

Iron binding to catecholate groups results in the displacement of two protons, pK₁=8.4; pK₂=12.1, (Raymond et al., 1980). The high pKa values of the ring hydroxyl groups lead to drastic reduction in affinity for Fe(III) as the pH is lowered below 7, giving rise to the greater acid stability of the iron hydroxamates over the catecholates at low pH (Hider, 1984). The strength of iron binding to siderophore may determine the mechanism by which iron is removed by the cell for metabolic needs. A comparison of
the stability constants of siderophores has shown differences of several orders of magnitude between them. Enterobactin, the strongest known siderophore, has a binding constant of approximately $10^{5.2}$ (Harris et al., 1979). Ferrioxamine E, the most stable hydroxamate siderophore, has a stability constant of $10^{3.5}$ (Anderegg et al., 1963). Because of differences in the ligand protonation constants, which describe the amount of free ligand in aqueous solution, a binding constant by itself does not provide enough information about the relative strength of a siderophore to compete with another for iron at a given pH. Apart from ferric ion, siderophores form stable complexes with other trivalent metals such as Al(III), Cr(III) and Ga(III) (Stover & Brunger, 1968; Clausen et al., 1974). These ions have radii, charge, and coordination geometry for oxygen ligands similar to that of Fe(III). Ferric ion has a high-spin $d^5$ electronic configuration as shown by EPR (Wickmann et al., 1965) and magnetic susceptibility measurements (Ehrenberg, 1956), that does not provide for crystal field stabilization energy. These properties make complexes between siderophores and iron susceptible to rapid isomerization and ligand exchange in solution. The iron chelate complexes are, therefore, not suitable for stereochemical investigation. This disadvantage inherent in iron has been circumvented by the use of chromium ion
which has a $d^3$ electronic configuration and significant crystal-field stabilization energy for kinetic inertness to ligand substitution (Snow, 1969). Individual coordination isomers of chromic complexes can, therefore, be isolated and their specific role in microbial iron transport studied (Leong et al., 1974; Leong & Raymond, 1975). The kinetic inertness of these isomers has allowed their use as biological probes in microbial siderophore transport systems to determine if receptors discriminate between different isomers of a given siderophore metal complex. Chromium siderophore complexes have also been used to determine if metal-ligand complexes are taken up by cells intact, or whether complex dissociation by reduction of metal ion at the cell surface preceeds uptake. Unlike chromium complexes, gallium siderophore complexes are kinetically labile, but they cannot be reduced. They have, therefore, been used to obtain evidence for reductive and non-reductive uptake mechanisms during transport (Ecker & Emery, 1983). Gallium and chromium analogs of ferrichrome and other siderophores have been prepared, and their rates of uptake are indistinguishable from those of the iron complexes (Leong et al., 1974; Emery & Hoffer, 1980; Muller & Raymond, 1984). It has been suggested that the accumulation of gallium in tumor and microbial cells could be the result of increased iron requirement during rapid cell
growth, with gallium imitating iron metabolism (Emery, 1986).

Iron transport studies in microorganisms for the past two decades have been directed towards answering questions about:

1. the nature of membrane receptors (Klebba et al., 1982),
2. the mechanisms of uptake and intracellular iron removal, (Leong et al., 1974; Leong & Neilands, 1976; Straka & Emery, 1979; Arceneaux & Byers, 1980; Ecker & Emery, 1983; Muller et al., 1985),
3. iron storage (Bauminger et al., 1980), and
4. genetic regulation of these processes (McIntosh & Earhart, 1976).

The large diversity of siderophores among microorganisms has raised the question of whether different siderophore structures require specific transport systems. Our present understanding of microbial iron transport shows that each organism produces its own siderophore for which it has a specific recognition receptor at the cell surface. An enterobactin outer membrane receptor of E. coli has been isolated by genetic techniques (Braun et al., 1976; Fiss et al., 1982). The isolation of a coprogen binding protein from the fungus Neurospora crassa (Ernst & Winkelmann, 1977), and the investigation of binding sites
on the cytoplasmic membrane have shown that a receptor mediates ferric siderophore transport in this species (Muller & Winkelmann, 1981). There is also evidence that some organisms have secondary recognition systems by which they can utilize siderophores produced by other organisms. For example, Ustilago sphaerogena can use iron of ferrioxamine B (Emery, 1971), and Neurospora crassa can obtain iron from ferrichrome (Winkelmann, 1974). It has become clear that siderophore recognition and transport depends on the different structural features in each siderophore molecule, such as the chirality and coordination geometry about the metal center, the ligand backbone, certain peripheral groups, and the molecule as a whole. Analysis of the specificity of receptors has revealed differences between fungal and bacterial recognition systems. It has been suggested that bacterial receptors recognize only the overall structure of the complex, whereas fungal receptors are specific in recognizing the coordination center and its surrounding residues (Winkelmann & Braun, 1981).

Siderophores form different stereochemical configurations about the metal center (Huschka et al., 1985). The hydroxamate siderophores are mainly hexadentate ligands with three unsymmetrical bidentate functional units (-CO-NO⁻⁻) attached to an asymmetric backbone. Jensen
(1977) has proposed a nomenclature in which ligand arrangement around the metal is designated Δ or Λ. Two geometric isomers cis and trans, are possible for an octahedral complex formed by coordination of ferric ion by three optically active hydroxamate groups. Each of the geometric isomers can exist as the Δ and Λ optical isomers. The Greek letters Δ and Λ indicate the absolute configuration of the metal-ligand complex. The Δ designation shows that the three hydroxamate groups form a right handed spiral configuration around the metal center, and the Λ isomer has the left handed configuration. Figure 4 shows such geometrical isomers of ferric hydroxamic acid complexes. The "cis" label indicates that the three identical oxygens each attached to a nitrogen occur on the same triangular face of the octahedron, and "trans" means the oxygens are on different faces. Because of the complex stereochemistry of siderophores, simple chromium complexes of the hydroxamates and catecholates, for which only five isomers are possible, have been used as models to correlate the absolute stereochemical configuration with UV-visible and CD spectra. Absolute configuration assignments have been made based on chromatographic properties of the isomers and by comparing their visible and CD spectra with reported data, such as those of octahedral complexes of various transition metals with asymmetric ligands (Leong & Raymond, 1974).
FIGURE 4: Geometrical and optical isomers of a ferric-trihydroxamate complex. It involves three unsymmetrical bidentate ligands, -CO-NOH-, attached to an asymmetric backbone (e.g. N,N',N''-triacetylfusarinine C). The isomers are designated $\Lambda$-cis and $\Lambda$-trans and their mirror images as $\Delta$-cis and $\Delta$-trans. Taken from Emery (1982).
Examination of molecular models indicates that the trans isomers of cyclic siderophores are improbable for steric reasons, although both geometric isomers of kinetically inert Cr(III) chelates of the linear trihydroxamates deferri-ferrioxamine B and deferriferrioxamine D have been prepared and resolved chromatographically (Leong & Raymond, 1975). Both $\Delta$ and $\Lambda$ isomers of ferric-$N,N',N''$-triacetylfusarinine C have been crystallized depending on conditions (Hossain et al., 1980). A correlation between coordination geometry and membrane recognition has been established by recent studies using synthetic siderophores (Winkelmann & Braun, 1981). Such studies have shown that the rate of uptake of $\Delta$-cis enantioferrichrome is reduced to 50% of the natural $\Lambda$-cis isomer. It has also been demonstrated that siderophore auxotroph mutants of E. coli show a definite discrimination between the natural $\Delta$-cis enterobactin and the synthetic $\Lambda$-cis enantioenterobactin (Neilands et al., 1981). These observations demonstrate the high degree of specificity in the iron transport system and show that cells contain a protein(s) in their membrane for the specific recognition of the natural siderophore.

Although microorganisms can accumulate iron in their cells complexed to siderophores, details of the mechanism by which these very strong chelators release the metal to the cell is not completely understood. Double-labelling
experiments in which either the iron or the ligand is isotopically labelled have revealed three different mechanisms by which the metal can be removed from siderophores by microorganisms (Figure 5). These mechanisms have been assigned based on the point at which the iron is released and on the fate of the ligand. Ferrichrome uptake in \textit{U. sphaerogena} is known to occur by a mechanism called the "iron shuttle mechanism" (Emery, 1971). In this mechanism, the complex is transported intact across the cell wall and the iron released by a process which does not result in ligand destruction. The ligand is excreted by the organism and can be used for another round of iron transport. In a second mechanism, called the "the iron taxi mechanism", or exchange mechanism, the ferric-siderophore complex binds to the cell surface and delivers the metal to the cell. The ligand remains extracellular and can be utilized for further iron transport. Iron uptake from ferric-rhodotorulic acid in \textit{Rhodotorula pilimanae} (Carrano & Raymond, 1978) and from ferrichrome A in \textit{U. sphaerogena} (Emery, 1971) occurs by this mechanism. The third mechanism involves transport of the iron siderophore complex across the cell wall and iron release by a process which results in ligand destruction by hydrolysis. Enterobactin uptake in \textit{E. coli} occurs by this mechanism (O'Brien et al., 1971; Bryce & Brot, 1972). Two processes are known whereby microorganisms
FIGURE 5: Three mechanisms proposed for microbial iron transport (Raymond & Carrano, 1979).
1. Iron shuttle mechanism,
2. Iron exchange mechanism, and
3. ligand destruction mechanism.
can overcome the thermodynamic barrier for removing iron from siderophores without ligand destruction: Ligand exchange, or reductive iron removal. Iron removal by exchange has been proposed for uptake in *Mycobacterium smegmatis* where the metal is exchanged from the extracellular exochelin into membrane bound mycobactin (Ratledge et al., 1982). The exchange of iron into another ligand can be achieved either by reductive removal of the metal, or by the involvement of a stronger chelating agent. However, in vitro studies on the mechanism and kinetics of iron exchange between siderophores (Tufano & Raymond, 1981; Ecker et al., 1982; Tufano, 1982) have shown that the metal is not easily exchanged between siderophores at physiological pH. Hence, the kinetics of ligand exchange can be rate limiting during uptake of iron. Thermodynamic association constants for siderophore metal complexes only determine the equilibrium distribution of the metal between chelators and do not provide information about the rate of ligand exchange which may be more relevant to in vivo transport. The reductive removal of ferric ion from siderophores was established with the discovery of enzymes called siderophore reductases (Lodge et al., 1982) which use cellular reducing agents like NADH to reduce ferric ion to the ferrous state for which siderophores have much lower affinity. Redox potential measurements for ferric
siderophore complexes obtained by cyclic voltammetry show that the hydroxamates have potentials which are within the range of biological reductants. The measurement of redox potentials for the tricatecholate siderophores has been limited to high pH, since direct determinations at neutral pH involve proton transfer associated with the reduction process giving rise to irreversible cyclic voltammogram waves from which the redox potential cannot be determined. Redox potentials have been estimated for the catecholates at neutral pH based on the observed dependence of half wave potentials on pH. Such estimates give the potentials for enterobactin and parabactin as -750 mV vs the normal hydrogen electrode (NHE) and -673 mV vs NHE, respectively. These unphysiologically low potentials indicate that reductive iron removal from these ligands can be achieved only by somehow changing the non-reducible form of the complex to one that can be reduced by the microorganism. It has been shown that siderophore redox potentials are highly dependent on pH. For example, the potential measured for ferric enterobactin at pH 7 (-750 mV vs the normal hydrogen electrode, NHE) increases to +170 mV at pH 4 (Pecoraro et al., 1983). An esterase has been found in E. coli extracts which hydrolyses the ester bonds of ferric enterobactin (Langmann et al., 1972). The ferric complexes of the 2,3-dihydroxybenzoylserine derived from enterobactin
hydrolysis and parabactin A obtained from parabactin hydrolysis have potentials that are within the range of biological reductants (Cooper et al., 1978). It has been suggested from such findings that ester bond cleavage may be part of the iron removal mechanism in \textit{E. coli} and \textit{P. denitrificans}. However, \textit{E. coli} can grow in the presence of synthetic analogs of enterobactin which lack ester linkages and cannot, therefore, be hydrolyzed (Heidinger et al., 1983). This observation is inconsistent with an esterase-catalyzed mechanism of iron removal from siderophores. It has been suggested that internal reduction of ferric ion by catecholate groups of enterobactin in non-aqueous environment, as in the membrane lipid bilayer, can facilitate the release of the metal by ligand exchange. An alternative is that an increase in redox potential due to locally low pH regions may bring the reduction potential of the siderophore metal complex within the physiological range. The latter suggestion is supported by a recent study in which the reductive removal of iron from enterobactin by glutathione at a pH below 6 has been observed (Hamed et al., 1982). Mossbauer spectra show that ferric-enterobactin accumulates as the complex in \textit{E. coli} which does not support an internal reduction mechanism. It has yet to be explained how \textit{E. coli} grows on non-hydrolyzable synthetic analogs of enterobactin.
In summary, there are many recent significant developments in iron transport research. An increased understanding of microbial iron transport mechanism may have important consequences in the treatment of microbial infections in humans as well as plants.
MATERIALS AND METHODS

Materials. Analytical and reagent grade chemicals were used in the entire study. Citric acid was obtained from Fisher Scientific Co., Fair Lawn, N.J., Deferri-ferrioxamine B (Desferal) was obtained from Ciba-Geigy Corp., Summit, N.J., \(^{59}\text{FeCl}_3, {^{67}\text{GaCl}_3, {^{51}\text{CrCl}_3, {^{14}\text{C-citric acid, and}^{14}\text{C-L-ornithine were obtained from New England Nuclear Corp.}}}}\)

Organism and Growth Conditions. The organism, \textit{Mycelia sterilia} EP-76, previously incorrectly identified as a \textit{Penicillium} species, was isolated from a garden variety of eggplant and cultured originally on Saubaroud-dextrose medium by Moore & Emery (1976). Cultures of the fungus were routinely maintained at room temperature (approx. 25 °C in a 250 mL flask with 40 mL iron-deficient medium at pH 6.8 (Emery, 1966). The medium contained per liter of deionized water 1 g \(\text{K}_2\text{SO}_4\), 4 g \(\text{K}_2\text{HPO}_4 . 3\text{H}_2\text{O}\), 3 g ammonium acetate, 1.2 g citric acid monohydrate, and 2 g sucrose. Metals were added as \(\text{MgSO}_4 . 7\text{H}_2\text{O}\) (0.8 g), \(\text{CuSO}_4 . 5\text{H}_2\text{O}\) (19.7 ug), \(\text{ZnSO}_4 . 7\text{H}_2\text{O}\) (8.83 mg) and \(\text{MnSO}_4 . \text{H}_2\text{O}\) (108 mg) for medium enrichment. The cultures were incubated on an Eberbach reciprocal shaker at 66 oscillations per min.
Isolation of Hydroxamates. Mycelial clumps from the stock cultures were inoculated into 400 mL of the iron-deficient medium described above in 2 L Fernbach flasks. The cells were grown for 5 to 7 days on an Eberbach reciprocal shaker and harvested by suction filtration. The filtrate was concentrated to a tenth of the original volume by evaporation on a Rotavapor and the hydroxamates extracted into phenol/chloroform (1:1) and back into water following the addition of five volumes of ether. The metal free ligands were purified by paper electrophoresis using pyridine acetate buffer pH 5.2 (Emery, 1965). The electrophoretic bands were localized after spraying a thin edge of the paper with 10% FeCl₃. The bands were eluted from the paper with deionized water. Siderophore concentration was determined by titration with FeCl₃ using a molar extinction coefficient of 3000 M⁻¹ cm⁻¹ (Moore, 1975). The purity of ferric-TAFC was further checked by thin-layer chromatography using 80% methanol as solvent. Ferrichrome and ferrichrome A were extracted from culture supernatants of U. sphaerogena as described by Emery (1966) Rhodotorulic acid was obtained from cultures of Rhodotorula pilimanae by the method of Atkins & Neilands (1968).

Preparation of Siderophore Metal Complexes. TAFC labelled with ⁵⁹Fe(III) was prepared by adding isotopic iron at 0 °C to give 80% saturation as determined by
spectrophotometric titration at 440 nm. The iron complex of rhodotorulic acid was prepared by the same procedure, based on an Fe(III):rhodotorulic acid ratio of 2:3 (Carrano & Raymond, 1978). Ferrichrome and ferrichrome A were labelled with $^{59}$Fe(III) as previously described by Emery (1971). Iron was chelated to a 20-fold excess of citrate under similar conditions. Gallium(III) complexes of the ligands were similarly prepared by addition of gallium nitrate (Emery & Hoffer, 1980). The formation of gallium complexes was confirmed by paper electrophoresis using pyridine acetate buffer or thin-layer chromatography using 80% methanol, and spraying the paper or plate with ferric chloride to displace the gallium (Emery, 1986). The presence of $^{67}$Ga radioactivity at siderophore positive regions on the plate or paper demonstrates co-migration of the gallium-siderophore complex. Chromium(III)-TAFC was prepared by freeze-drying a known quantity of previously purified TAFC and taking up the dried siderophore in methanol. An equal molar amount of CrCl$_3$ was added and the solution gently stirred at room temperature overnight. The methanol was removed by evaporation and the blue-green residue was dissolved in water. The product did not migrate on pyridine acetate paper electrophoresis at pH 5.2, but separated into two major bands with Rfs of 0.55 and 0.60 by silica gel thin-layer chromatography (chloroform:}
methanol: water, 35:12:2 vol/vol). The labelled siderophore was prepared by including \(^{51}\text{CrCl}_3\) in the methanol solution. Chromic-deferriferrichrome was prepared by a similar procedure except that anhydrous sodium acetate was added to the methanol solution of deferriferrichrome containing \(\text{CrCl}_3\) (Muller et al., 1985) and the mixture refluxed under dry air (CaCl\(_2\) drying tube) for 18 h. The resulting blue-green solution was concentrated to dryness by evaporation and resuspended in a small volume of deionized water. The product ran as a single spot on silica gel thin-layer chromatography (chloroform: methanol: water, 35:12:2), with an Rf of 0.42. The solution concentrations of chromium(III) were determined spectrophotometrically as \(\text{CrO}_4^{2-}\) \((\varepsilon_{372} = 4815 \text{ L M}^{-1} \text{ cm}^{-1})\) as described previously (Haupt, 1952). An an aliquot of each chromium containing solution was oxidized with alkaline hydrogen peroxide as follows: A 100 \(\mu\text{L}\) aliquot of the chromium solution was placed in a 2 \(\text{mL}\) volumetric tube, 5 drops of 0.5 N NaOH was added followed by 5 drops of 30\% hydrogen peroxide. Excess hydrogen peroxide was removed by placing the tube in a boiling water bath for 1 h. After cooling, the volume was made up to 2.0 \(\text{mL}\) and the absorbance at 372 nm was determined. Siderophores labelled with \(^{14}\text{C}\) were obtained by growing cells in the presence of \(^{14}\text{C}-\text{L-ornithine}\) as described by Emery (1966).
Uptake Experiments. Cells for uptake were grown under iron-deficient conditions for 24 h in 400 mL medium as previously described by Emery (1966), except that the culture was maintained in a teflon chemostat chamber with gentle stirring to maximize the production of relatively short, metabolically active mycelial tips. Uptake experiments were done using 10 mL of cell suspension (approx. 1 mg cellular protein/mL) in 50 mM K2HPO4 buffer, pH 6.8, containing 20 mM glucose, 2.5 mM ammonium acetate, and 1 mM citric acid. Cell protein concentration was determined by a modified Lowry method (Schacterle & Pollack, 1973) adapted for use in whole cells (Gordon & Moore, 1981). The cells were pre-incubated for 20 min at 30 °C in 125 mL Erlenmeyer flasks on a New Brunswick Rotary shaker at 180 rpm. Chelates were added to a final concentration of 40 uM unless stated otherwise. One mL aliquots of cell suspension were pipetted at timed intervals into chilled centrifuge tubes to stop uptake. The suspension was then centrifuged on an Eppendorf microfuge (Model 5412) to pellet the cells. One-half mL portions of the supernatant were counted for radioactivity in Aquasol 2 in a Packard Minaxi Tri-Carb 4000 scintillation counter. The percent of 59Fe uptake was calculated by taking the initial amount of 59Fe added per mL of cell suspension as 100%. A 100% uptake corresponds to 40 nmol of complex per mg of cellular protein (or
per 3.6 mg dry cell weight) unless stated otherwise. Because of the variability in iron uptake rates between different cell batches $^{59}$Fe uptake from the various ligands was compared to that from TAFC in all experiments.

**Iron Exchange.** Iron exchange experiments were carried out by mixing equal concentrations of ligands in the phosphate buffered uptake medium with one ligand chelated to $^{59}$Fe. The mixture was equilibrated at 30 °C and the distribution of $^{59}$Fe between the siderophores was determined by separating the component species by thin-layer chromatography using 80% methanol or by pyridine acetate electrophoresis (Emery, 1965), followed by scintillation counting of the eluted compounds.

**Electrochemistry of Ferric-TAFC.** Cyclic voltammetry is a very effective and versatile electroanalytical technique used for the mechanistic study of redox systems. It allows the electrode potentials to be rapidly scanned in search of redox couples. The ease of ferric-TAFC reduction was measured by cyclic voltammetry using a procedure similar to that described by Cooper et al., (1978). Solutions of iron siderophore complexes were prepared in 1 M KCl, 0.05 M sodium borate/0.05 M sodium phosphate buffer, pH 8.0, and deoxygenated by purging with nitrogen gas for 5 min. Electrochemical measurements were done on a hanging mercury drop electrode with a saturated silver chloride reference
and a platinum wire auxiliary electrode. Measurements were done at 26 °C. No correction for liquid junction potentials was applied. A Princeton Applied Research (PAR) Universal Programmer (Model 175) and a PAR potentiostat were used to generate triangular waves. Voltage current waves were recorded on a Houston Omnigraphics 2000 X-Y recorder.

Spectroscopic Measurements. Ultraviolet-visible spectra were measured on a Cary 17D spectrophotometer. Circular dichroism spectra were obtained with a Jasco J-500C recording spectropolarimeter. Calculations of values were made from the standard expression: \[ \theta = \frac{M \theta_A d c}{10} \] in which \( \theta_A \) is the observed ellipticity in degrees, \( M \) is the molecular weight, \( d \) is the path length in centimeters, and \( c \) is the concentration in grams per milliliter (Bunnenberg et al., 1962). \( \theta \) is related to \( \Delta \varepsilon \) by the equation \( \theta = 3300 \Delta \varepsilon \).

Hydrogenation of TAFC. Hexahydrotriacetylfusarinine C (HsTAFC) was prepared by hydrogenation of TAFC at 30 psi of hydrogen over platinum oxide until the absorbance at 260 nm reached a minimum value as described by Emery & Emery (1973). The ferric complex of the hydrogenated TAFC did not move on pyridine acetate buffer paper electrophoresis, but it resolved into four distinct bands with Rfs of 0.67, 0.70, 0.72 and 0.74, on thin-layer chromatography
using a freshly prepared chloroform:methanol:water, 35:12:2, solvent system.

**Preparation of Mycelia sterilia EP-76 Esterase.** The fungus was grown in a 400 mL culture medium as described previously on page 24. After 2 days the mycelia were harvested by filtration. The procedure used for the isolation of the enzyme was similar to that described by Emery (1976b). The mycelia were washed twice with 0.05 M potassium phosphate buffer, pH 7.5. A heavy suspension of the mycelia was then put into a 50 mL glass container and glass beads (0.45-0.50 mm in diameter) added. The mycelia were disrupted for 60 seconds at 4000 rpm in a Braun MSK homogenizer cooled with liquid carbon dioxide. The homogenate was centrifuged at 18,000 rpm for 1 h. To the supernatant was added (NH₄)₂SO₄ to 75% saturation at 4 °C. The precipitated proteins were resuspended in 0.05 M potassium phosphate buffer and dialyzed against a 1:5 dilution of the same buffer. The non-dialyzable fraction was used immediately for esterase assay with no further treatment.

**Esterase Assay.** The assay procedure used was the same as that described by Emery (1976b). The standard incubation mixture contained in a total of 3.0 mL, 150 umol of phosphate buffer pH 7.5, 6 umol EDTA (pH 7.5), 1.0 umol of ferric-TAFC or ferric-H₆TAFC, deionized water and enzyme
preparation. Controls were run with heat-inactivated enzyme. Esterase activity was determined by the rate of change in absorbance at 440 nm for ferric-TAFC or 430 nm for ferric-hydrogenated TAFC.
RESULTS

Iron Transport by Mycelia sterilis EP-76. $^{59}$Fe(III) transport kinetics using different iron chelating agents showed that, as in other fungal systems, Mycelia sterilis EP-76 is not fastidious with respect to its iron source. The ferric complex of TAFC is very efficient at donating iron to the cells (Figure 6). Uptake from TAFC is rapid and almost quantitative within 20 min. Initial transport rates as high as 770 pmol per min per mg dry weight of cells were achieved with this ligand. This rate is greater than those obtained in other siderophore mediated iron transport systems. No other source of iron was more efficient than the endogenously produced TAFC in delivering iron to the cell. Rhodotorulic acid and the monomer of TAFC were almost equally effective at donating iron. The rate of iron uptake from citric acid was much lower compared to TAFC, and reached only 80% completion after 60 min. Other monohydroxamates, such as acetohydroxamate and benzohydroxamate, were also capable of donating iron to the cell at rates comparable to that of the TAFC monomer (results not shown). Ferrioxamine B was a poor substrate (Figure 7), showing only 60% uptake after 3 hr.
FIGURE 6: Uptake of $^{59}$Fe-labelled TAFC, rhodotorulic acid, citrate and monomer. Conditions for uptake were as described in Materials and Methods. The flasks contained 3.6 mg/ml (dry weight) of cells, 40 uM ferric complexes in a 10 ml. total volume. Iron was complexed to 20 fold excess of citrate.
FIGURE 7: Comparison of time-dependent $^{59}$Fe uptake from ferric-TAFC and ferrioxamine B. The concentrations in the medium were 40 µM for both siderophores. Conditions were as described in Materials and Methods.
Ferrichrome Uptake. Ferrichrome and ferrichrome A are trihydroxamate type siderophores and were also used as sources of iron for *Mycelium sterilia* EP-76. The cells took up iron supplied as ferrichrome but not as ferrichrome A (Figure 8). The rate of ferrichrome uptake was about 50% that of ferric-TAFC during the initial 10 min of uptake.

Energy Dependence of Uptake. The dependence of iron uptake on energy was determined by incubating the cells with 1 mM NaN₃ for 5 min before initiating uptake. This treatment resulted in a complete inhibition of ⁵⁹Fe uptake from all ligands investigated.

¹⁴C-labelled Ligand Uptake. Microbial iron transport studies have shown that metal accumulation does not necessarily demonstrate an uptake of the intact ferric complex. To check whether the ligands that mediated iron uptake in *Mycelium sterilia* EP-76 were taken up during metal transport, the uptake of ¹⁴C-label of the ligands was examined in parallel experiments. As indicated in Figure 9, TAFC ligand was taken up initially by the cells but at a rate slower than the rate of ⁵⁹Fe uptake (Figure 6). The uptake of labeled TAFC ligand reached a maximum after 45% uptake, thereafter the label was excreted back into the medium. The uptake of ligand and its subsequent excretion has also been observed in ferrichrome uptake in *Ustilago sphaerogena* (Emery, 1971). There was no uptake of the
FIGURE 8: $^{59}$Fe uptake of ferrichrome and ferrichrome A. Conditions were as described in Materials and Methods. The flasks contained 4 mg/ml (dry weight) of cells. $^{59}$Fe-TAFC uptake is presented for comparison. The medium concentrations of all three siderophores were 40 uM.
FIGURE 9: $^{14}$C label uptake of iron complexes of TAFC, monomer, citrate, and RA. All ferric complexes were added at 40 uM concentration. Conditions were as described in Materials and Methods. Flasks contained 4.0 mg/ml (dry weight) of cells in a 10 ml volume.
$^{14}$C-label of TAFC when it is not complexed to iron (Figure 9). The $^{14}$C-label of ferrichrome was taken up at a rate similar to that of $^{59}$Fe-labelled ferrichrome (Figure 10). Unlike TAFC however, the label of ferrichrome was retained by the cells. Contrary to the observation that the ligands were also taken up during TAFC or ferrichrome mediated iron transport, no uptake of $^{14}$C-label was observed during iron transport mediated by rhodotorulic acid, citric acid or the monomer of TAFC (Figure 9). The fact that these ligands did not gain access to the cell suggests a reductive mechanism as has been observed in ferrichrome A uptake by U. sphaerogena (Ecker & Emery, 1983). Alternatively, the ligands may exchange their iron into another carrier in the cell membrane or its environment.

Electrophoresis of Cell Suspension Medium. A ferric-TAFC esterase has been isolated from Mycelia sterilia EP-76 and shown to hydrolyze the metal ligand complex to its monohydroxamic acid subunits (Emery, 1976a). It was suggested that the aminoacyl esterase might function in the cell to hydrolyze the ferric chelate of TAFC to facilitate the release of the metal. A similar hydrolytic cleavage of ligand following iron uptake has been observed in E. coli, where the ester linked cyclic enterobactin is hydrolyzed by a specific esterase and the monomeric subunit, dihydroxybenzoylserine, is released from the cell (O'Brien
FIGURE 10: Uptake of $^{59}$Fe- and $^{14}$C- ferrichrome. Conditions for uptake were as described in Materials and Methods. The uptake of $^{59}$Fe-TAFC is shown for comparison. Ferrichrome and ferric TAFC were added to 40 uM final concentration.
et al., 1971). It was important in this study, therefore, to determine the form in which the $^{14}$C-label of TAFC was excreted from the cell. Electrophoretic examination of the medium in which the cells were suspended during uptake of $^{14}$C-TAFC-Fe revealed that the label of TAFC was excreted mainly as the monomer units of TAFC, and smaller amounts of the dimer and linear trimer units. The production of labelled hydrolysis products demonstrates that the ligand was structurally altered by the cells, probably through the activity of the esterase. Incubation of TAFC or ferric TAFC with heated or azide treated cells did not produce any detectable hydrolysis of the ligand. The retention of $^{14}$C-label of ferrichrome by the cell could be due to inability of the cells to hydrolyze the amide linkages of ferrichrome.

**Gallium Uptake.** Since gallium cannot be reduced to the divalent state, substitution of Fe(III) by Ga(III) is an excellent probe for investigating mechanisms of iron uptake. The ability of cells to take up gallium provides evidence for the absence of an obligatory extracellular reductive mechanism for siderophore mediated metal uptake. Figures 11, 12, 13, 14, show that $^{67}$Ga was taken up at rates similar to iron when added to the cells complexed to TAFC, rhodotorulic acid, monomer or citrate. Gallium-deferriferrichrome was also taken up at rates similar to
FIGURE 11: Comparison of $^{59}$Fe and $^{67}$Ga uptake mediated by TAFC. Uptake of $^{14}$C-label of TAFC complexed to iron and gallium is also shown. Conditions for uptake were as described in Materials and Methods. Metal complexes of TAFC were added to give 40 uM concentration.
FIGURE 12: Comparison of $^{59}$Fe and $^{67}$Ga uptake mediated by RA. Uptake of $^{14}$C-label of rhodotorulic acid complexed to iron and gallium is also shown. Conditions were as described in Materials and Methods. The cell suspension contained 4.2 mg/ml (dry weight) of cells, 40 uM metal chelates in a 10 ml volume.
FIGURE 13: Comparison of $^{59}$Fe and $^{67}$Ga uptake mediated by the monomer of TAFC. Uptake of $^{14}$C-label of monomer complexed to iron and gallium is also shown. Conditions were as described in Materials and Methods. The flasks contained 4.0 mg/ml (dry weight) of cells, 40 uM of ferric and gallium complexes of monomer.
FIGURE 14: Uptake of $^{59}$Fe and $^{67}$Ga mediated by citrate. Uptake of $^{14}$C-label of citrate complexes of iron and gallium were also measured under the same conditions. The flasks contained 3.5 mg/ml (dry weight) of cells, 40 uM metal chelated to 20 fold excess of citrate.
ferrichrome (Figure 15). Gallium associated $^{14}$C-labelled ligand uptake patterns for TAFC, rhodotorulic acid, monomer and citrate were similar to those observed for their iron complexes. This observation suggests that reduction of metal was not rate limiting during metal uptake from these ligands. A reductive dissociation of the metal ion from the ligand prior to entering the cell, as has been found in ferrichrome A uptake by U. sphaerogena, is therefore unlikely.

Iron Exchange into TAFC. Rather than an extracellular reduction of ferric ion for release of the metal for transport into the cell, an alternative mechanism exist whereby cells are able to take up iron without the ligand. This method involves extracellular exchange of ferric ion into another chelating agent, which then acts as a true siderophore to transport the metal into the cell as an intact complex. Therefore, the possibility of iron exchange into another chelator was examined. Advantage was taken of the observation that uncomplexed TAFC does not enter the cell under conditions where the ferric complex of TAFC is rapidly transported (Figure 16). The fact that the ligand must be complexed to the metal before transport offers a means of determining if metal exchange is a physiologically important mechanism by which Mycelia sterilia EP-76 transports iron. In these experiments the cells were
FIGURE 15: Comparison of $^{59}$Fe and $^{67}$Ga uptake mediated by ferrichrome and deferriferrichrome. Conditions were as described in Materials and Methods. Metal complexes of both siderophores were added to a final concentration of 40 μM.
FIGURE 16: Uptake of $^{14}$C-labelled TAFC in the presence of non-isotopic ferric complexes of citrate, monomer and RA. Cells were incubated with 40 μM of iron-free $^{14}$C-TAFC for 5 min, followed by the addition of non-isotopic complexes. The concentration of all ferric complexes was 40 μM in a 10 ml volume of cell suspension. Uptake conditions were as described under Materials and Methods.
incubated with $^{14}$C-labelled TAFC for 5 min followed by the addition of non-isotopic ferric complexes of citrate, rhodotorulic acid or the monomer of TAFC. Uptake of the $^{14}$C-label was observed (Figure 16), demonstrating an extracellular exchange of the metal into TAFC.

In vitro Exchange of Iron into TAFC. Earlier exchange studies by Ecker et al., 1982, using ferrichrome A and ferrichrome and a similar investigation by Tufano & Raymond (1981) with ferrioxamine B and ferrichrome A showed that chemical exchange is too slow at physiological pH to account for observed iron uptake rates. To determine if the rate of exchange of iron into TAFC is great enough to be physiologically significant, the rate of iron exchange between TAFC and other ligands was examined under conditions identical to those used in the uptake experiments. This was done by mixing equimolar concentrations of a $^{59}$Fe-labelled complex and a competing ligand in a phosphate buffered medium, in the absence of cells. The ferric complexes were separated after 30 min and 12 h by thin-layer chromatography or paper electrophoresis, and the distribution of $^{59}$Fe-label between the two ligands was determined by elution of the bands with water and counting in a liquid scintillation counter. As indicated in Table I, TAFC removed more than 50% of the iron from rhodotorulic acid, citric acid and the monomer within 30 min of incubation.
TABLE I: In vitro Iron Exchange Between Siderophore ligands.

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<th>Ligandsa</th>
<th>Percent exchange</th>
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<tr>
<td></td>
<td>0.5h</td>
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<tr>
<td>$^{59}$Fe-ferrichrome + TAFC</td>
<td>42</td>
</tr>
<tr>
<td>$^{59}$Fe-TAFC +</td>
<td></td>
</tr>
<tr>
<td>deferriferrichrome</td>
<td>4</td>
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<tr>
<td>$^{59}$Fe-ferrioxamine B + TAFC</td>
<td>8</td>
</tr>
<tr>
<td>$^{59}$Fe-TAFC +</td>
<td></td>
</tr>
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<td>92</td>
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<tr>
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<td>derriferrichrome</td>
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<tr>
<td>$^{59}$Fe-rhodotorulic acid + TAFC</td>
<td>82</td>
</tr>
<tr>
<td>$^{59}$Fe-citric acid + TAFC</td>
<td>63</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Chelates labelled with $^{59}$Fe(III) were mixed with competing ligands in 0.4 mM phosphate buffered medium at pH 6.8, 30 °C. Percent exchange was measured by separation on TLC and scintillation counting.

\textsuperscript{b} Determined at 16h rather than 12h.
Exchange of iron into TAFC was almost quantitative within 12 h. The thermodynamic stability of ferric-TAFC is evident from the fact that after 12 h, TAFC removed about 74, 64 and 90% of the metal from such stable ferric siderophore complexes as ferrioxamine B (K=10^{30.5}), ferrichrome A (K=10^{32}) and ferrichrome (K=10^{29}), respectively (Table 1), with only 19, 38 and 6% of metal exchanged from the respective complexes into TAFC. In spite of the fact that more than 40% the of iron exchanged into TAFC from ferrichrome within 30 min, uptake of ^{14}C-label was not detected when cells were incubated with uncomplexed ^{14}C-TAFC and non-isotopic ferrichrome. This shows that, unlike rhodotorulic acid, citrate or the monomer, ferrichrome did not exchange iron into TAFC outside the cell.

**Stability Constant of Ferric-TAFC.** The stability constant of ferric-TAFC was estimated from equilibrium distribution of iron between ferrioxamine B (FOB) and TAFC using labelling techniques based on the following equilibria:

\[
\text{Fe}^{3+} + \text{TAFC} \rightleftharpoons \text{Fe-TAFC} + 3H^+ \quad k_{\text{Fe-TAFC}} = \frac{[\text{Fe-TAFC}][H^+]^3}{[\text{Fe}^{3+}][\text{TAFC}]}
\]

\[
\text{Fe}^{3+} + \text{desFOB} \rightleftharpoons \text{FOB} + 3H^+ \quad k_{\text{FOB}} = \frac{[\text{FOB}][H^+]^3}{[\text{Fe}^{3+}][\text{desFOB}]}
\]
The exchange process involves the transfer of ferric ion which is common to both reactions, thus the overall process can be summarized as:

$$\text{FOB} + \text{TAFC} \rightleftharpoons \text{desFOB} + \text{Fe-TAFC}$$

The ratio of the binding constants, $K_R$, is given by:

$$K_R = \frac{k_{\text{Fe-TAFC}}}{k_{\text{FOB}}} = \frac{[\text{Fe-TAFC}][\text{desFOB}]}{[\text{FOB}][\text{TAFC}]}$$

Figure 17 shows the reaction profile of iron exchange between $^{59}\text{Fe}$-labelled ferrioxamine B and TAFC. As indicated in this Figure, 89% of $^{59}\text{Fe}$-label exchanged into TAFC from ferrioxamine B at equilibrium. The corresponding amount of iron exchanged in the reverse direction after 72 h was found to be 9% when $^{59}\text{Fe}$-labelled TAFC was equilibrated with deferriferrioxamine B (desFOB), demonstrating approach to equilibrium from both directions. Given the initial concentrations of TAFC and ferrioxamine B as 0.4 mM, the fraction of label exchanged from ferrioxamine B into TAFC is equivalent to a ferric-TAFC concentration of 0.36 mM and a non-complexed TAFC concentration of 0.04 mM at equilibrium. By the same reasoning the amounts of ferrioxamine B and deferriferrioxamine B at equilibrium are 0.04 mM and 0.36 mM, respectively. Substituting the equilibrium
FIGURE 17: Reaction profile for iron exchange between $^{59}$Fe-labelled ferrioxamine B and TAFC. Equimolar concentrations of $^{59}$Fe-labelled ferrioxamine B and non-isotopic TAFC were mixed in a test tube. The mixture was incubated at 30°C. Samples were withdrawn at timed intervals and separated by thin-layer chromatography using a chloroform/methanol/water (35:12:2 vol/vol) solvent system. Bands corresponding to Fe-TAFC and ferrioxamine B were eluted and counted in a liquid scintillation counter.
concentration data into the above expression, gives a value of 81 for $K_R$. The value of $k_{FOB}$ is $10^{3.0.6}$ (Anderegg et al., 1963). Using the value of $K_R$ obtained from this study and the calculated $k$ for FOB, the binding constant for ferric-TAFC was calculated to be $10^{32.5}$. This value is the same as that reported for the most stable hydroxamate complex so far known, ferrioxamine E ($K=10^{32.5}$).

**Electrochemistry of Ferric-TAFC.** It is generally accepted that enzymatic reduction of ferric ion to the ferrous state is an intracellular mechanism by which the cell releases the metal from siderophore complexes. Hydrolysis of ferric-enterobactin by enteric bacteria to release the metal is believed necessary because of the very low redox potential of the complex, $-750$ mV versus the normal hydrogen electrode (NHE) at pH 7 (Cooper et al., 1978). To determine if TAFC hydrolysis during iron transport in *Mycelia sterilia* EP-76 is obligatory due to an unusually low redox potential for this complex, the potential for ferric-TAFC was determined by cyclic voltammetry and compared to that of ferrichrome A. As shown in Figure 18, a mid-point potential of $-690$ mV (versus the saturated silver chloride electrode) was obtained for ferric-TAFC compared to $-655$ mV for ferrichrome A. These values, which correspond to $-468$ mV and $-433$ mV versus the normal hydrogen electrode, are within the range of known biological
FIGURE 18: Cyclic voltammograms of ferrichrome A and ferric-N,N-'N''-triacetylfusarinine C (at pH 8). Both siderophores were in 1 M KCl, 0.05 M sodium borate/0.05 M sodium phosphate buffer. Cyclic voltammograms were at a hanging mercury drop electrode with a saturated silver chloride reference electrode. Scanning was at a rate of 100 mV/sec. Mid-point potential = (Epa + Epc)/2. Epc and Epa are potentials corresponding to the cathodic and anodic peak currents, respectively.
reductants. An unfavorable redox potential cannot, therefore, be the primary reason for TAFC hydrolysis by the cell during iron transport. A cyclic voltammetric reduction wave at -530 mV (pH 8) versus the saturated silver chloride electrode was measured for the ferric-monomer complex (Figure 19). The absence of an associated oxidation wave does not allow an estimation of the mid-point potential for the iron monomer complex. The reduction wave at -530 mV provides the lower boundary for the mid-point potential.

Stereochemistry of Chromium Complexes. It is well documented that siderophores undergo significant changes in conformation upon ferric ion coordination (Emery, 1967). Stereochemical specificity for the ferric isomer that is transported has been demonstrated (Naegeli & Keller-Schierlein, 1978). Such observations have led to considerable interest in the conformational and stereospecific requirements of membrane siderophore receptors. The high crystal-field stabilization for chromium(III) compared to iron(III) gives kinetic inertness to chromium ligand complexes. The well characterized d-d transitions with distinct UV-Visible and circular dichroism spectra of chromium complexes allow their use as chemical probes to elucidate the octahedral coordination geometry of siderophores. Chromic TAFC was prepared as described in Materials and Methods. Two distinct bands with Rfs of 0.55 and 0.60
FIGURE 19: Cyclic voltammograms of ferric-monomer and ferrichrome A. Measurements were under the same conditions as described in Figure 18.
were obtained. Solution CD spectra of these two chromic-
TAFC complexes and a Cr(III)-deferriferrichrome band
obtained by the same procedure are shown in Figure 20.
The slower moving sample was identified as Δ-cis and the
faster moving band as Λ-cis, based on the spectra for model
chromium(III) ligand complexes (Raymond et al., 1984).
Similarly, the spectrum for Cr(III)-deferriferrichrome was
identified as Λ-cis. The Λ configuration in the model
compounds has a positive CD band for the low energy
transition region at 500-600 nm, whereas the Δ isomer has
a negative CD band in this region. A comparison of CD
spectra of chromium complexes of siderophores with iron
complexes has shown that the same rule applied to the CD
spectra of chromium complexes can be used to identify the
iron complexes (Raymond et al., 1984). Ferric siderophore
complexes have a Λ configuration in solution if the CD
band in the region of the absorption maximum at 400-500 nm
has a positive sign. By comparing the crystal structure
and the solution CD spectra, Hossain et al.,(1980) and van
der Helm et al.,(1980), have shown that the configuration
of ferric-TAFC and ferrichrome in solution is Λ-cis and
Λ-cis, respectively. They also found that crystals of
ferric-TAFC obtained from ethanol/benzene assume the
Λ-cis configuration whereas Δ-cis is the predominant
form in crystals obtained from chloroform. The aqueous CD
FIGURE 20: Solution circular dichroism spectra of isomers of Cr-TAFC and Cr-deferriferrichrome. The negative band with maximum at 575 nm indicates a Δ configuration of the metal chelate octahedron, and the positive band at the same wavelength indicates the opposite configuration. The CD spectrum of Λ-cis Cr-TAFC has been multiplied by two.
spectra of ferrichrome and ferric-TAFC measured in this study (Figure 21) confirm the observation made in the laboratory of van der Helm.

**Chromium Uptake.** Figure 22 shows a clear discrimination between the $\Lambda$-cis and $\Delta$-cis isomers of Cr-TAFC. The $\Lambda$-cis isomer was taken up whereas the $\Delta$-cis isomer was completely inactive. The uptake of $\Lambda$-cis chromic TAFC was rapid and reached a maximum after about 50% uptake within 20 min. The $^{51}$Cr-label was thereafter excreted back into the medium (Figure 22). Electrophoretic examination of the medium showed that the metal was excreted complexed to the monomer of TAFC. This observation shows that reduction is not rate limiting during hydrolysis of metal ligand complex. Hydrolysis of $\Lambda$-cis Cr-TAFC isomer was also observed with the *Mycelia sterilia* EP-76 esterase in vitro. The excretion of chromium from the cell as a complex to the monomer suggests that, unlike gallium or iron, the cells are not able to remove chromium from the hydrolyzed units of TAFC. Like $\Lambda$-cis Cr-TAFC, $^{51}$Cr-deferri-ferrichrome was taken up by the cell at a rate not significantly different from that of ferrichrome (Figure 23). Unlike Cr-TAFC, however, there was no excretion of chromium following uptake of Cr-deferri-ferrichrome.

**Inhibition of Iron Uptake by Chromium Complexes.** Since ferrichrome is not synthesized by *Mycelia sterilia*
FIGURE 21: Circular dichroism spectra of ferrichrome and ferric-TAFC in aqueous solution.
FIGURE 22: Uptake of $^{59}$Fe and $^{51}$Cr mediated by TAFC. Conditions for uptake were the same as described under Materials and Methods. Metal siderophores complexes were added to a final concentration of 35 uM.
FIGURE 23: Uptake of $^{59}$Fe-ferrichrome and $^{51}$Cr-deferriferrichrome. Complexes were added to a final concentration of 40 uM. Conditions for uptake were the same as described in Materials and Methods.
EP-76, the question arises as to which transport system is used by this siderophore. Inhibition studies using siderophore metal complex analogs can provide evidence concerning interaction of different substrates with one uptake system. The kinetically inert chromic complexes of TAFC and deferriferrichrome were examined as inhibitors of iron uptake. The cells were incubated with the inhibitor 5 min before initiating iron uptake. As shown in Figure 24, the initial uptake rate of $^{59}$Fe-TAFC was reduced 30% by Cr-deferriferrichrome at an inhibitor concentration of 15 uM. The $\Lambda$-cis isomer of Cr-TAFC (15 uM) inhibited the initial uptake of ferric-TAFC by 70%. There was no inhibition of $^{59}$Fe-TAFC uptake by the $\Delta$-cis isomer when added to the cells at a final concentration of 50 uM (results not shown). The uptake of ferrichrome was also inhibited by $\Lambda$-cis Cr-TAFC and Cr-deferriferrichrome (results not shown). The inhibition of ferrichrome uptake by $\Lambda$-cis Cr-TAFC, and a similar inhibition by Cr-deferriferrichrome on ferric TAFC uptake, indicates that the two siderophore have a common receptor or share an overlapping iron transport pathway in Mycelia sterilia EP-76.

**Effect of Hydrogenation of TAFC on Uptake.** It has been demonstrated by Emery (1971), that fungal membrane receptors are able to recognize minor changes in structure and conformation of the hexapeptide ring of siderophores.
FIGURE 24: Inhibition of TAFC mediated $^{59}$Fe uptake by $\Lambda$-cis Cr-TAFC and Cr-deferriferrichrome. Both inhibitors were present at 15 μM final concentration. Ferric TAFC was added to give 40 μM concentration. Cells were preincubated with the inhibitors for 5 min before $^{59}$Fe uptake was initiated.
In order to probe the sensitivity of TAFC mediated iron transport to small changes in TAFC structure, the ligand was hydrogenated as described under Materials and Methods to reduce the carbon-carbon double bonds conjugated to the hydroxamate groups. Four bands of ferric complexes of the hydrogenated product were resolved by thin-layer chromatography (Figure 25). The UV-Visible absorption ratio (260 nm:430 nm) for the four samples were the same within the limits of experimental error, demonstrating that hydrogenation completely reduced the double bonds of TAFC. It is also known that platinum catalysts are completely inactive in the reduction of esters (Hudlicky, 1984), so it can be assumed that the three ester linkages of TAFC remained intact during the hydrogenation procedure. The four ferric-hydrogenated TAFC bands must, therefore, represent diastereoisomers that differ in the face of the molecule attacked by hydrogen. Eight such isomers are possible by inspection of TAFC structure. The introduction of additional chiral centers upon hydrogenation, further complicates the interpretation. Time-dependent $^{59}$Fe uptake showed that all the four isomers delivered iron to the cell at rates lower than TAFC (Figure 26).

**Esterase Activity on Metal TAFC Complexes.** In vitro Mycelia sterilia EP-76 esterase activity on gallium and iron complexes of TAFC was investigated. Using
FIGURE 25: Thin layer chromatography of ferric complexes of hexahydrogenated TAFC. Chromatographic separation was carried out as described in Materials and Methods. I, II, III and IV indicate bands with Rfs of 0.67, 0.70, 0.72 and 0.74 respectively.
FIGURE 26: Comparison of $^{58}$Fe uptake mediated by four isomers of hexahydrogenated TAFC and TAFC. All ferric complexes were added to give a final concentration of 35 uM. Conditions of uptake were the same as described under Materials and Methods. Uptake of, $^{58}$Fe-TAFC (■); HsTAFC with Rf, 0.67 (□); Rf, 0.70 (●); Rf, 0.72 (○); Rf, 0.74 (△).
14C-labelled gallium complexes of TAFC, it was found that incubation of 20 μM complex with 1 mg of enzyme for 1 h resulted in ligand hydrolysis, mainly to the monomer units. This was determined by electrophoretic separation of the reaction mixture and counting the 14C-radioactivity that moved with a ferric-monomer standard. The fact that gallium cannot be reduced demonstrates that metal reduction is not obligatory for ligand hydrolysis. Both ferric-TAFC complex and the fastest moving (Rf 0.74) ferric hexahydrogenated TAFC complex were also found to be good substrates for the enzyme (Figure 27). The activity of the esterase on ferric-TAFC was first demonstrated by Emery (1976a).
FIGURE 27: Enzymatic hydrolysis of ferric-TAFC and ferric-hexahydrogenated TAFC. Incubation mixture contained 0.02 M phosphate buffer, pH 7.5, 0.02 M EDTA, pH 7.5, 0.02 mM Fe-TAFC or Fe-H$_2$TAFC. Absorbance changes were measured at 430 nm for Fe-H$_2$TAFC and 440 nm for Fe-TAFC after addition of 1 mg of enzyme. Figure shows esterase activity on the fastest moving Fe-H$_2$TAFC sample, Rf=0.74.
DISCUSSION

The mechanism by which iron uptake is facilitated by triacetylfusarinine C (TAFC) and other ligands such as citrate, rhodotorulic acid and the monomer of TAFC in a *Mycelia sterilia* EP-76 was investigated using radiolabelled iron, gallium and chromium complexes of these ligands. The evidence obtained supports a model in which TAFC transports iron into the cell as the intact metal chelate complex. The ligand is hydrolyzed to release the metal and the hydrolyzed ligand units excreted as the monomer. The excretion of monomer is consistent with hydrolysis of the ligand during iron transport. TAFC is made up of three monomeric hydroxamic acids N-acetylfusarinine, linked together through ester bonds (Figure 1). It was earlier suggested that an ornithine esterase found in this species may be involved in the intracellular release of iron (Emery, 1976a). The present study lends support to this hypothesis. The data presented in this dissertation clearly show that *Mycelia sterilia* EP-76, like other fungi, can sequester iron from a variety of complexes that differ widely in chemistry. Among the siderophores tested, the organism's own siderophore, TAFC, was found to be the most efficient
iron donor to the cell. Inhibition of transport by azide showed that the iron uptake process is energy dependent. Though several microbial iron transport systems have been shown to be both receptor mediated and energy dependent processes, there is presently very little data available to determine if inhibition of transport is directly on the receptor or is a general inhibition of cellular energy production.

Microbial uptake of siderophores has usually been followed by monitoring either labelled ligand or metal, seldom both together. Synchronous uptake of both the ligand and the metal has been demonstrated with ferric schizokenin in *Bacillus megaterium* and ferric aerobactin in *Aerobacter aerogenes* (Arceneaux et al., 1973), and ferrichrome in *U. spherogena* (Emery, 1971). However, high spin ferric complexes readily undergo isomerization and ligand exchange reactions in aqueous solution, and monitoring ligand or metal uptake alone is not sufficient to determine the mechanism of iron transport. Using $^{14}$C-labelled ligands, I have shown that ferric-TAFC and ferrichrome enter the cell intact. Other chelating agents, such as RA, the monomer of TAFC, and citric acid, mediate iron transport in *Mycelia sterilia* EP-76 indirectly by exchange of the metal into TAFC which in turn acts as the true iron carrier for the cell. This observation is
significant since data from our laboratory (Ecker et al., 1982), as well as work in the laboratory of Raymond (Tufano & Raymond, 1981) led to the conclusion that extracellular exchange is too slow at physiological pH to be of any significance in microbial iron transport. I confirmed through in vitro studies that exchange rates into TAFC occurred at rapid enough rates to account for the observed uptake of iron from these ligands. The rapid rates of iron exchange into TAFC from other ligands shows that iron extraction from weaker chelators may be a very important mechanism for iron transport in this organism. It is reasonable to assume that some TAFC is still produced and excreted even after washing the cells, accounting for the uptake of iron from rhodotorulic acid, citrate and the TAFC monomer through exchange.

TAFC appears to be different from other trihydroxamate siderophores such as ferrichrome in its ability to participate in rapid exchange of ferric ion at neutral pH. The chemical explanation for this difference is not apparent. Work by Monzyk & Crumbliss (1983) and Brink & Crumbliss (1984) has shown the importance of electron donating substituents on the hydroxamate groups in interligand iron exchange catalysis. However, ferrichrome A has substituents on the hydroxamate groups very similar to TAFC, but it does not participate in rapid metal exchange. The
flexibility of the TAFC macrocyclic ring system may be responsible for the ligand's ability to participate in a much more rapid exchange compared to the rigid coordination centers of the ferrichrome compounds. The production of siderophores closely related to TAFC by other fungal species, such as *Fusarium roseum*, suggests that this exchange process may not be unique to *Mycelia sterilia* EP-76. Extracellular iron exchange may also be involved in the ability of *E. coli* to grow on non-hydrolyzable synthetic analogs of enterobactin (Heidinger et al., 1983). These analogs have stability constants several orders of magnitude lower than enterobactin produced by *E. coli* (Raymond et al., 1984).

In almost all fungal iron transport systems studied to date, ferrichrome has been found to be an effective donor of iron even when it is not endogenous to the organism. This was found true in the present study. Though the in vitro exchange of iron from ferrichrome into TAFC was significant, ferrichrome does not exchange iron to TAFC for subsequent transport into *Mycelia sterilia* EP-76. Rather, ferrichrome enters the cell as the intact chelate as shown by $^{14}$C-labelling experiments. It is possible that binding of the complex to the cell stabilizes it towards ligand exchange. In contrast to TAFC, the labelled ligand of ferrichrome does not exit from the cell. The monomeric
hydroxamic acid units of ferrichrome are joined by amide bonds rather than the ester bonds found in TAFC, and it is therefore not a substrate for the esterase. The mechanism by which iron is removed from ferrichrome inside the cell is not known. I speculate that decomplexation of ferrichrome may occur either by intracellular exchange of its iron into TAFC, as demonstrated by in vitro exchange rates, Table I, or by reduction of the ferric ion to the low affinity ferrous oxidation state. Ferrichrome A does not appear to share the ferrichrome pathway of iron transport since it does not donate iron to the cells. Ferrichrome contains three glycine units whereas the sequence seryl-seryl-glycine is found in ferrichrome A. In the latter siderophore the methyl groups adjacent to the hydroxamate carbonyl are substituted by (Z)-3-methyl-3-butenoic acid. This substitution introduces three negative charges and very bulky residues at the metal center portion of the molecule. A possible explanation for the lack of biological activity of ferrichrome A is that the negative charges on the molecule prevents transport of the complex across the cell membrane.

Gallium cannot be reduced to the divalent state and the ability of cells to transport this element complexed to hydroxamate or citrate has been used as evidence that the transport mechanism does not require a mandatory
extracellular reduction step. The present finding shows that regardless of ligand the rates of gallium uptake by *Mycelia sterilia* EP-76 are identical to iron uptake rates, demonstrating that decomplexation of metal from ligand by reduction is not a prerequisite for its uptake.

Whereas the mechanism of iron release has not been conclusively established for most microorganisms, the present investigation confirms the participation of an esterase in rendering available iron of ferric-TAFC. This mechanism is analogous to that of enterobactin mediated iron transport in *E. coli*. From cyclic voltammetric measurements, it was observed that ferric-TAFC has a mid-point potential of approximately -690 mV versus the saturated silver chloride electrode. This value, which corresponds to -468 mV versus the normal hydrogen electrode, is similar to those reported for other ferric trihydroxamate siderophores and much higher than the value of -750 mV estimated for ferric-enterobactin at pH 7 (Cooper et al., 1978). It therefore appears that a hydrolytic destruction of TAFC is not necessitated by an unfavorable reduction potential of the metal ligand complex as has been suggested for enterobactin. Results of the chromium uptake studies strikingly show that the siderophore metal transport system of *Mycelia sterilia* EP-76 has a recognition capacity which distinguishes between the left handed (Λ) and right handed (Δ) propeller configurations.
at the metal center. The cell transports only the $\Lambda$-cis isomer of the TAFC metal complex. The complete lack of activity of the $\Delta$-cis isomer demonstrates that this configuration of the hydroxamate complex has the wrong propeller metal center chirality. Figure 28 shows the difference between $\Delta$-cis and $\Lambda$-cis propeller configurations of enterobactin. Evidence for stereospecific recognition of a siderophore iron complex has been shown with a synthetic enantiomer of ferrichrome, which has the $\Delta$-cis configuration (Winkelmann & Braun, 1981). This compound showed a 50% decrease in uptake rate relative to the naturally occurring $\Lambda$-cis isomer. It is interesting that in *Mycelia sterilia* EP-76 the $\Delta$-cis enantiomer is completely inactive. Inability of the cell to take up the $\Delta$-cis chromium TAFC configuration suggests that transport of the labile ferric complex must rely on rapid isomerization to the $\Lambda$-cis isomer. This rapid equilibration is made possible by the $d^5$ electronic configuration of iron which makes the complex kinetically labile with respect to isomerization and ligand exchange in solution. Evidence from enantiomorphous CD spectra obtained upon dissolution of a KBr disk containing crystals of ferric-$N,N',N''$-triacetylfusarinine showed that such an isomeric equilibrium is established in solution in less than 1 min (Hossain et al., 1980). The chromium containing analog of
FIGURE 28: Natural ferric enterobactin and synthetic enantioenterobactin (Neilands et al., 1981). The Δ-cis compound is a right handed coordination propeller and the Λ-cis enantiomer has a left handed propeller configuration.
ferrichrome, which is locked in the $\Lambda$-cis configuration is, transported at a rate similar to ferrichrome. This provides further evidence that the iron transport receptor only recognizes the $\Lambda$-cis isomer. The kinetically inert d$^3$ chromium complexes preclude any isomerization of ligand during transport (Raymond & Carrano, 1979) and enable their use as inhibitors of siderophore mediated iron transport. Inhibition of ferric-TAFC uptake by chromium deferriferri­chrome and the inhibition of ferrichrome uptake by chromium TAFC suggest competition between these siderophores for the receptor. The use of chromium ligand complexes to probe the specificity of Mycelia sterilia EP-76 iron transport system has allowed the direct examination of the high sensitivity of the siderophore receptor to a change in just the metal coordination geometry. The finding from the present study that the less common $\Lambda$-cis isomer is the active form of the metal chelate complex represents the first demonstration of an organism exhibiting preference for a less dominant stereochemical isomer. The fact that the metal center recognized is the $\Lambda$-cis configuration supports the trend found among the other optically active hydroxamate siderophores which are also transported as the $\Lambda$-cis isomer.

From inspection of models of ferrichrome and TAFC, it is obvious that few similarities exist between the ligand
structures. The difference in transport activity between ferrichrome and ferric-TAFC in *Mycelia sterilia* EP-76 may reflect differences in receptor affinity for the two siderophores resulting from conformational changes introduced by differences in the amino acid skeleton. Fungal membrane receptors can recognize small changes in structure and conformation of the hexapeptide ring of siderophores (van der Helm et al., 1980). Although the exact mechanism of iron transport remains elusive, very important features of the transport process have been established from this study which shows that three different mechanisms for siderophore iron uptake are operative in *Mycelia sterilia* EP-76.

1. A true siderophore system utilizing TAFC that is excreted into the environment under conditions of low iron stress and involves uptake of the intact metal chelate followed by cellular hydrolysis of the complex to remove iron;

2. An exchange of iron from other weaker complexes into extracellular TAFC, which then functions as the true ferric ionophore;

3. A ferrichrome transport system which may involve either a reductive or an exchange mechanism of iron removal within the cell.

A model for iron uptake involving these three mechanisms is illustrated in Figure 29. The ligand destruction
FIGURE 29: Model proposed for uptake from ferric TAFC, ferrichrome and other iron chelators by Mycelia sterilia EP-76 Ligand (X), indicates chelators that donate iron to TAFC.
process which accompanies intracellular iron removal has often been considered wasteful. However, the present study shows that hydrolysis of the ligand may serve as the signal for the excretion of the monomer units and any metal that remains bound to them, as observed in the excretion of chromium following Cr-TAFC uptake (Figure 22). This finding provides the first evidence for siderophore mediated metal excretion by a microorganism and could be a useful mechanism by which the cell gets rid of metals such as aluminum and chromium whose siderophore complexes are extremely similar to those of iron but which have no metabolic role and might even be toxic to the cell if accumulated.

An explanation for the observed differences in the rates of iron uptake from the four diastereoisomers obtained by TAFC hydrogenation is not apparent. It is possible that hydrogenation introduces changes in the ligand backbone that affect receptor affinity for the siderophore. The hydrogenation of TAFC could also result in lowering the binding constant of the siderophore, thus allowing ferric bound hydrogenated isomers to exchange their iron into TAFC. The introduction of a second chiral center on hydrogenation further complicates the nature of possible stereo-chemical products of TAFC hydrogenation. An investigation of the binding constant of ferric hydrogenated TAFC by ligand exchange experiments should also shed some light on
the mechanism of hydrogenated TAFC mediated iron uptake. It would be interesting to obtain information about the CD spectra of chromic complexes of the different hydrogenated isomers to determine how hydrogenation affects the chirality at the metal center.

Evidence obtained from the transport of gallium mediated by TAFC shows that although gallium is not reducible, there is hydrolysis of the complex during transport followed by excretion of the hydrolysed units of TAFC. The fact that gallium is released from TAFC as efficiently as iron (Figure 11), suggests that iron removal from TAFC inside the cell may be achieved solely through hydrolysis of the ligand without the need for reductive dissociation of the ferric monomer complex. Ferric and gallium complexes of different chelates have very close binding constants (Raymond et al., 1984). The question of whether ferric-monomer dissociation in the cell does involve reduction would require EPR analysis of TAFC mediated iron uptake. If reduction is required to remove the metal from the monomer units, then a disappearance of the EPR signal should accompany the TAFC mediated iron transport process.
SUMMARY OF MAJOR CONCLUSIONS

1. Under conditions of low iron stress, *Mycelia sterilia* EP-76 excretes cyclic triacetylfusarinine C which binds and transports iron directly into the cell by an energy dependent transport system. Iron removal from the ligand involves hydrolytic destruction of the ligand. Iron uptake from citrate, rhodotorulic acid and TAFC monomer is not direct but occur via metal exchange into TAFC, which in turn acts as the true ferric ionophore.

2. TAFC differs from other trihydroxamate siderophores such as ferrichrome in its ability to participate in rapid exchange of ferric ion at neutral pH.

3. The cell can transport chromic and gallium complexes of TAFC at rates identical to that of the ferric-TAFC. Since neither gallium nor chromium can be reduced to a lower oxidation state, the mechanism of metal transport does not include a mandatory reduction step.

4. *Mycelia sterilia* EP-76 can recognize and transport ferrichrome. Iron removal from ferrichrome may involve reduction of ferric ion by an endogenous reducing agent such as NADH, or by ligand exchange.
5. Identification of the metal center configuration of chromic complexes of TAFC and chromium deferriferrichrome revealed that only the $\Lambda$-cis isomer is transported by the cell although the $\Delta$-cis isomer predominates in solution. The kinetic lability of iron with respect to isomerization and ligand exchange in solution results in a rapid re-equilibration of the $\Delta$ to the $\Lambda$ isomer as the latter is taken up.

6. Cyclic voltammetric measurements show that ferric TAFC has a mid-point potential that is similar to that reported for other trihydroxamate siderophores. The hydrolytic mechanism for iron removal from TAFC is thus not neccessitated by an unfavorable reduction potential of the metal chelate.
REFERENCES


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