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Iron Uptake in *Mycelia sterilia* EP-76

JONATHAN P. ADJIMANI AND THOMAS EMERY*

Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322

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The cyclic trihydroxamic acid, *N,N',N''*-triacetylfusarinine C, produced by *Mycelia sterilia* EP-76, was shown to be a ferric ionophore for this organism. The logarithm of the association constant *k* for the ferric triacetylfusarinine C chelate was determined to be 31.8. Other iron-chelating agents, such as rhodotorulic acid, citric acid, and the monomeric subunit of triacetylfusarinine C, *N*-acetylfusarinine, 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 midpoint potential of −690 mV with respect to the saturated silver chloride electrode was obtained for the ferric triacetylfusarinine C complex, indicating that an unfavorable reduction potential was not the reason for the use of a hydrolytic mechanism of intracellular iron release from the ferric triacetylfusarinine C chelate.

The fusarinines are a class of amino acid ester siderophores of the hydroxamate type that have been isolated from various species of fungi, such as *Fusarium roseum* (1, 6, 9). The monomeric unit of the fusarinines is N^{δ}-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-N^{δ}-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 siderophores such as ferrichrome. The cyclic triester of fusarinine is named fusarinine C (23), or fusigen (6), and is believed to be the ferric ionophore for *F. roseum*. Investigation of the iron transport function of this siderophore has been limited because of its very labile ester bonds. More stable N-acetylated derivatives of the fusarinines have been isolated from cultures of *Mycelia sterilia* EP-76 (previously incorrectly identified as a *Penicillium* sp.) and *Aspergillus* species, and their structures have been elucidated (15, 18). The structure of *N,N',N''*-triacetylfusarinine C (TAFC) is shown in Fig. 1. The N-acetylation of the amino groups imparts stability to the fusarinines. Culture fluids of both *Mycelia sterilia* EP-76 and *Aspergillus* species have been shown to contain, in addition to this cyclic triester, large amounts of the monomeric hydroxamic acid, the linear dimer, and the linear trimer, all of which are believed to be products of esterase activity within the cell (12). These hydrolysis products have high iron-coordinating capabilities typical of hydroxamic acids. TAFC is thought to be the ferric ionophore for the fungal species that produce it (18, 26). This compound has also been reported to possess antibiotic activity against certain bacteria (1, 16, 26).

A proposed mechanism by which TAFC transports iron involves an enzyme from *M. sterilia* EP-76 that hydrolyzes the ester bonds of this iron chelate (12). It was suggested that the release of iron from TAFC involves a hydrolytic mechanism similar to that put forward to explain the release of iron from enterobactin (also called enterochelin), the cyclic triester of 2,3-dihydroxybenzyolserine, a catechol-type siderophore produced by *Escherichia coli* and other enteric bacteria (19–21).

* Corresponding author.

Although esterase activity has been implicated in the release of iron from TAFC, little is known about the mechanism by which TAFC-producing species sequester and transport iron into the cell. Despite detailed structural studies of TAFC, no work has been reported on the transport of the ferric-TAFC complex in organisms that produce this siderophore. This paper presents data on the mechanism of TAFC-mediated iron uptake in *M. sterilia* EP-76.

MATERIALS AND METHODS

Preparation of siderophore-metal complexes. The *N*-acetylfusarinine compounds were obtained from cultures of *M. sterilia* EP-76 as previously described (18), except that the organism was grown in a Teflon chemostat with gentle stirring to minimize clumping. The compounds were purified by paper electrophoresis with pyridine acetate buffer (9). Rhodotorulic acid was obtained from cultures of *Rhodotorula pilimanae* as described by Atkin and Neilands (2). Ferrichrome and ferrichrome A were crystallized from culture supernatants of *Ustilago sphaerogena* and labeled with ^{59}Fe as previously described (11). TAFC labeled with ^{59}Fe was prepared by addition of $^{59}Fe^{3+}$ at 0°C to about 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 iron-to-rhodotorulate ratio of 2:3 (4). Iron was chelated to a 20-fold excess of citrate. Gallium complexes were similarly prepared by addition of a solution of gallium nitrate (13). The purity of the gallium complexes was confirmed by thin-layer chromatography with 80% methanol and detection with a spray of 10% $FeCl_3$. Ligands labeled with ^{14}C were obtained by growing cells in the presence of L-[^{14}C]ornithine (10).

Uptake. Uptake studies were carried out as previously described with a cell concentration of 1 mg of protein per ml (11). The protein concentration in cells was determined by a modification of the Lowry et al. method (24) adapted for use with whole cells (14). Cells were preincubated 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 μM unless otherwise stated. Portions of the supernatant were counted in Aquasol 2 in a Packard Minaxi Tri-Carb 4000 scintillation counter. A 100% uptake

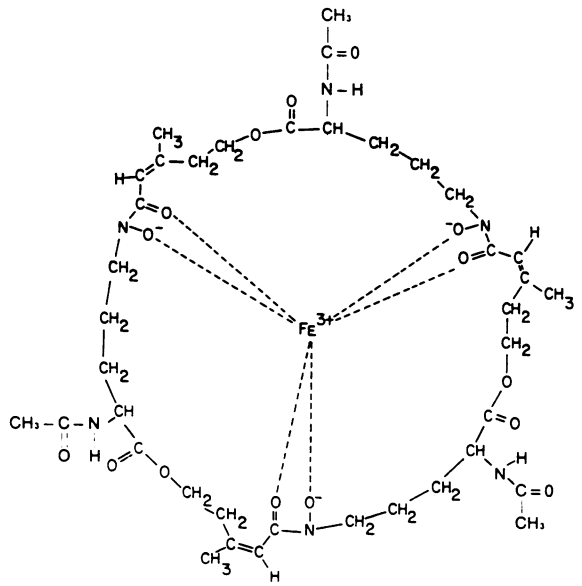

FIG. 1. Structure of the Fe^{3+}-TAFC complex.

corresponds to 40 nmol of metal-ligand complex per mg of cellular protein.

Iron exchange. Iron exchange studies were carried out by mixing equal concentrations of the corresponding ligands in the phosphate-buffered uptake medium with one ligand chelated to ^{59}Fe. The mixture was equilibrated at 30°C. The distribution of ^{59}Fe among the siderophores was determined by separating the component species by thin-layer chromatography with 80% methanol or by pyridine acetate electrophoresis (9) followed by scintillation counting of the eluted compounds.

Electrochemistry of ferric iron-TAFC. Reduction of ferric iron-TAFC was compared with that of its monomeric subunit iron complex and ferrichrome A by cyclic voltammetry by using a procedure similar to that described by Cooper et al. (5). Solutions of the 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 being purged with nitrogen 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 made at 26°C. No correction was applied for liquid junction potentials. A Princeton Applied Research Universal Programmer (model 175) and a Princeton Applied Research potentiostat were used to generate triangular waves.

RESULTS

Iron transport by *M. sterilia* EP-76. The ^{59}Fe-labeled chelate of TAFC is a very effective source of iron for *M. sterilia* EP-76 (Fig. 2). Within 20 min the metal was quantitatively taken up by the cell suspension, corresponding to a V_{max} of 770 pmol/min per mg (dry weight) of cells. This is greater than rates reported for most other fungal siderophore transport systems and is several times greater than ferrichrome uptake by *U. sphaerogena* (11). The iron complex of the TAFC monomeric subunit, *N*-acetylfusarinine, and ferric rhodotorulate (not shown) were found to be almost equally effective as donors of iron, with V_{max} values of 690 pmol/min per mg). Although the uptake rate of ferric citrate was significantly lower, greater than 80% uptake of the metal from this complex was observed in less than 60 min (V_{max} = 472 pmol/min per mg). Thus, as for other fungal systems, *M. sterilia* EP-76 is not fastidious with respect to its iron source. In every case, complete inhibition by 1 mM sodium azide was observed (Fig. 2). Substitution of ^{67}Ga for ^{59}Fe in each of the above complexes, including citrate, resulted in uptake of the label at a rate identical to that of the iron complex within the limits of experimental error (data not shown).

When we examined ^{14}C-labeled ferric iron-TAFC in a parallel experiment, we observed initial transport of the label followed by its release from the cell (Fig. 3). This phenomenon has been observed with other microbial uptake systems, such as the uptake of ferrichrome by *U. sphaerogena* (11). However, in the latter system the excreted deferriferrichrome ligand can complex additional extracellular iron and serve repeatedly as a ferric ionophore. This was not the case in the present study. When the extracellular medium was examined by paper electrophoresis, most of the label was found in the monomer, with smaller amounts in the linear dimer and trimer (data not shown), which demonstrates that cellular hydrolysis of the TAFC ligand had occurred with subsequent excretion of the hydrolytic fragments. Incubation of TAFC or ferric iron-TAFC with heated or azide-treated cells led to no detectable hydrolysis.

In contrast to the results with ferric iron-TAFC, when the ferric chelates of rhodotorulic acid, citric acid, or the TAFC monomer were tested by using the ^{14}C-labeled ligand under conditions of rapid iron uptake, no detectable amount of label entered the cell (data not shown). Although it is not possible to rule out an extremely rapid uptake followed by an equally rapid excretion, we believe that a more probable explanation is a dissociation of the metal-ligand complex prior to entry of the metal into the cell cytosol. A reductive dissociation has been observed for iron uptake from ferrichrome A by *U. sphaerogena* (7).

In vivo iron exchange into TAFC. It is possible that a

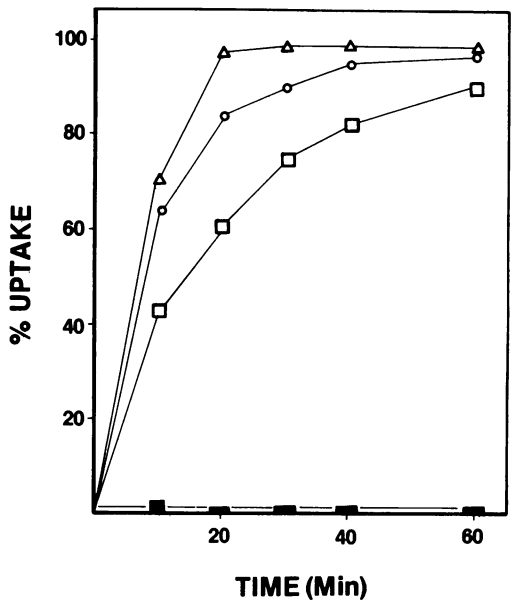

FIG. 2. Iron uptake in *M. sterilia* EP-76. Iron ligand complexes were added to cell suspensions to give a final concentration of 40 μM. Ferric iron was chelated to a 20-fold excess of citrate. ^{59}Fe-TAFC (△), ^{59}Fe-TAFC monomer (○), ^{59}Fe-citrate (□), ^{59}Fe-TAFC plus 1 mM azide or heated cells (■).

nonenzymatic, extracellular exchange of the ferric ion into the true siderophore ligand occurs. The siderophore could then carry the metal into the cell as the intact chelate. To examine this possibility, we took advantage of the fact that uncomplexed TAFC ligand remains extracellular. The data in Fig. 3 show that under conditions when the ^{59}Fe-TAFC chelate was quantitatively taken up within 20 min, no significant amount of uncomplexed [^{14}C]TAFC entered the cell even after a 60-min incubation. The fact that TAFC must be complexed to metal before transport into the cells can occur offers a method of determining whether metal exchange is physiologically important with *M. sterilia* EP-76. Cells were incubated with ^{14}C-labeled TAFC ligand, and then nonisotopic ferric citrate, ferric rhodotorulate, or the ferric complex of the monomeric subunit of TAFC was added (Fig. 3). In all three cases significant uptake of label, i.e., TAFC, was observed, conclusively demonstrating that iron exchange into extracellular TAFC had occurred.

In vitro iron exchange into TAFC. In vitro exchange of iron between various ligands was examined. Equimolar concentrations of an isotopically labeled iron complex and a competing ligand were incubated under the same conditions as in our uptake experiments. After 30 min or 12 h, the compounds were separated by thin-layer chromatography or paper electrophoresis and the extent of exchange was measured by scintillation counting. TAFC was very efficient at removing iron from the monomer, rhodotorulic acid, and citrate, with greater than 50% exchange occurring in 30 min (Table 1). Even for the stronger chelate, ferrichrome, 42% of the metal exchanged into TAFC in the same period, and the exchange was almost quantitative after 12 hr. These data support the conclusion that exchange of iron from the monomer and other ligands into TAFC may be an important factor in iron assimilation by this organism. The thermodynamic stability of the ferric iron-TAFC complex was demonstrated by the fact that after 12 h TAFC removed more than 50% of the metal from such stable siderophores as ferrioxamine B and ferrichrome A, with little iron exchanging in the reverse direction (Table 1). By allowing the exchange of the ferric ion to reach equilibrium between the TAFC and ferrioxamine B ligands, we were able to calculate a value of 31.8 for the logarithm of the association constant k of the ferric iron-TAFC complex.

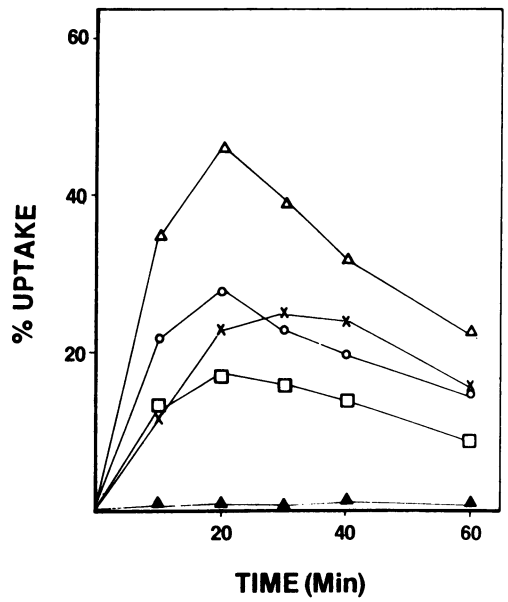

FIG. 3. Uptake of [^{14}C]TAFC. Cells were incubated for 5 min with [^{14}C]TAFC ligand, and nonisotopic ferric complexes were then added. Symbols: △, uptake of ferric iron-[^{14}C]TAFC; ▲, [^{14}C]TAFC; ○, [^{14}C]TAFC plus ferric monomer; □, [^{14}C]TAFC plus ferric citrate; ×, [^{14}C]TAFC plus ferric rhodotorulic acid. The concentration for all ligands and complexes was 40 μM.

TABLE 1. In vitro iron exchange between siderophore ligands

Ligands[a]	% Exchange after:	
	0.5 h	12 h
[^{59}Fe]ferrichrome + TAFC	42	90
[^{59}Fe]TAFC + deferriferrichrome	4	6
[^{59}Fe]ferrioxamine B + TAFC	8	74
[^{59}Fe]TAFC + deferriferrioxamine B	1	19
[^{59}Fe]ferrichrome A + TAFC	4	64
[^{59}Fe]TAFC + deferriferrichrome A	10	38
^{59}Fe-monomer + TAFC	92	99
^{59}Fe-monomer + derriferrichrome	8	86
[^{59}Fe]rhodotorulic acid + TAFC	82	85[b]
[^{59}Fe]citric acid + TAFC	63	90[b]

[a] Chelates labeled with $^{59}Fe^{3+}$ were mixed with competing ligands each at 0.4 mM in phosphate-buffered medium at pH 6.8 and 30°C. Percent exchange was measured by scintillation counting after separation by thin-layer chromatography.

[b] Determined at 16 h rather than 12 h.

Ferrichrome uptake by *M. sterilia* EP-76. The cyclic peptide trihydroxamates ferrichrome and ferrichrome A were examined as sources of iron for *M. sterilia* EP-76. The cells rapidly take up iron from ferrichrome, but not ferrichrome A (data not shown). By using a ^{14}C label, we found that the ferrichrome ligand was completely retained by the cells during the course of the uptake experiment. Although TAFC was hydrolyzed by a cellular esterase, no corresponding peptidase has been described, and the retention of the ferrichrome ligand may be due to the inability of the cells to hydrolyze peptide-based iron complexes. Gallium-deferriferrichrome was also taken up by cells at a rate similar to ferrichrome.

Electrochemistry of ferric iron-TAFC. It is widely accepted that enzymatic reduction of ferric ion to the ferrous state is an important mechanism in the intracellular release of iron from siderophores. To determine whether an unfavorably low redox potential of ferric iron-TAFC necessitates a hydrolytic mechanism for iron release, we examined the redox potential of ferric iron-TAFC (Fig. 4). A midpoint potential of −690 mV with respect to the saturated silver chloride electrode was estimated for ferric iron-TAFC compared with −655 mV for ferrichrome A, included for comparison. A cyclic voltammetric reduction wave at −530 mV was found for the ferric iron-monomer complex (data not shown), but the absence of an associated oxidation wave did not allow an estimation of the midpoint potential. The observed value, however, does establish a lower boundary for the midpoint potential.

DISCUSSION

M. sterilia EP-76 is similar to other fungi in its ability to sequester iron from a variety of complexes that differ greatly in their chemistry. Its own siderophore, TAFC, is nevertheless the most efficient donor of the metal as measured by initial uptake rates. TAFC consists of three monomeric hydroxamic acids, *N*-acetylfusarinine, linked together by ester bonds (Fig. 1). We have previously suggested that an

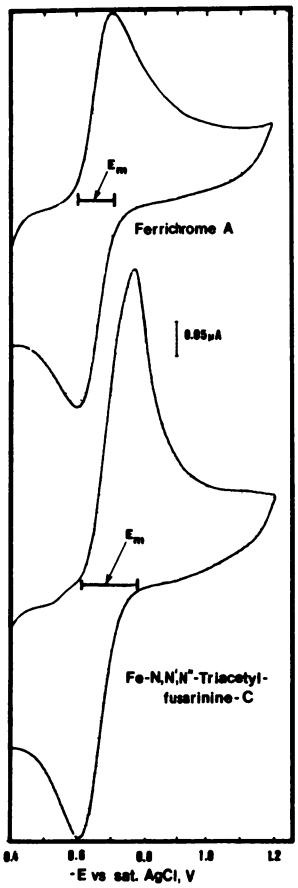

FIG. 4. Cyclic voltammograms of ferrichrome A and ferric iron-TAFC at pH 8. Siderophores were prepared in 1 M KCl–0.05 M sodium borate–0.05 M phosphate buffer (pH 8.0). Cyclic voltammograms were at a hanging mercury electrode with a saturated silver chloride reference electrode. Scanning was at a rate of 100 mV/s.

ornithine esterase found in this organism may be involved in the intracellular release of the ferric ion (12). Our present results support this hypothesis. After uptake of the ^{14}C-labeled ferric iron-TAFC complex, the metal was retained by the cells while the ^{14}C label was excreted mainly as the hydrolysis product, *N*-acetylfusarinine. Smaller amounts of the dimer and linear trimer were found, and these products were also observed during initial in vitro enzymatic hydrolysis of ferric iron-TAFC. Other ligand-labeled ferric iron complexes, such as ferric *N*-acetylfusarinine or ferric citrate, yielded the metal to the mycelia without concomitant uptake of the ligand, clearly indicating a unique role of the TAFC in the iron metabolism of this organism, analogous to the role of ferrichrome as an iron donor to *U. sphaerogena*.

Gallium cannot be reduced to the divalent state, and the ability of cells to transport gallium complexed to hydroxamates or citrate demonstrates that extracellular metal reduction is not obligatory for transport. Data from the laboratory of Tufano and Raymond (25), as well as our own work with *U. sphaerogena* (8), led to the conclusion that extracellular exchange is too slow at physiological pHs to be of significance in microbial iron transport. However, our present finding that (regardless of ligand) the rates of gallium uptake by *M. sterilia* EP-76 are identical to iron uptake rates prompted us to reexamine this assumption. Addition of unlabeled ferric citrate, ferric rhodotorulate, or ferric *N*-acetylfusarinine caused the rapid uptake of ^{14}C-labeled TAFC (Fig. 3). Since uncomplexed TAFC remained extracellular, we conclude that metal from these complexes entered the cells via exchange into TAFC. We also found in vitro exchange rates under our uptake conditions to be rapid enough to account for the observed uptake (Table 1). Thus TAFC appears to differ from many other trihydroxamate siderophores, such as the ferrichrome-type siderophores, in its ability to participate in rapid exchange of ferric ion at neutral pH. The chemical reason for this difference is not apparent. Work from the laboratory of Brink and co-workers (3, 17) has demonstrated the importance of electron donation by substituents on the hydroxamate groups in catalyzing interligand iron exchange. However, the substituents on the hydroxamate groups of TAFC are very similar to those of ferrichrome A, but ferrichrome A did not participate in rapid metal exchange, nor was it a functional siderophore for *M. sterilia* EP-76. It may be significant that although the stereochemistry of trihydroxamate chelates is typically Λ-*cis*, a rapid equilibrium is established between the Λ-*cis* and Δ-*cis* isomers for ferric iron-TAFC, with the latter isomer predominating (15). Such facile isomerization indicates kinetic lability of the hydroxamate groups. It may be that the greater flexibility of the macrocyclic ring system of TAFC is a more important factor than electronic inductive effects in its ability to engage in rapid exchange relative to the much more rigid chelation centers of the ferrichrome compounds. Other organisms, such as *F. roseum*, produce siderophores closely related to TAFC, and so this exchange phenomenon may not be unique to *M. sterilia* EP-76.

In every fungal system examined to date, ferrichrome has been found to be an effective donor of iron even when it is not synthesized by the organism. The same is true in the present study. Although the in vitro rate of exchange of iron from ferrichrome into TAFC was significant, ferrichrome did not engage in an exchange mechanism, but rather it entered the mycelia as the intact chelate, as shown by ^{14}C labeling. In contrast to TAFC, the label from the ferrichrome did not exit from the cells. The monomeric hydroxamate subunits of ferrichrome are linked by peptide bonds rather than ester bonds, and ferrichrome is therefore not a substrate for the esterase. Ferrichrome A does not share the ferrichrome pathway, since it does not yield iron to the cells, nor is it effective in exchanging its iron with TAFC.

One possible explanation for the wasteful hydrolytic mechanism of ferric iron-TAFC iron transport is that the reduction potential of the chelate is unfavorable for a reductive mechanism of iron removal. This idea was first put forward by O'Brien et al. (19) in their study of ferric iron-enterobactin (enterochelin) transport in *E. coli*. We found by cyclic voltammetry that ferric iron-TAFC has a midpoint potential of approximately −690 mV with respect to the saturated silver chloride electrode, corresponding to a value of −468 mV with respect to the normal hydrogen electrode. This value is very similar to that reported for other trihydroxamate siderophores and much higher than the value of −750 mV found for ferric enterobactin (22). It would thus appear that a hydrolytic mechanism for iron removal from TAFC is not necessitated by an unfavorable reduction potential of the metal in this chelate.

We conclude that *M. sterilia* EP-76 possesses at least three distinctive pathways of iron uptake: (i) a true siderophore system utilizing the ferric iron-TAFC complex and involving cellular hydrolysis of the chelate for iron release; (ii) an exchange mechanism of iron from other complexes

into extracellular TAFC, followed by uptake of ferric iron-TAFC; and (iii) a ferrichrome transport system.

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