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Specificity and mechanism of rhizoferrin-mediated metal ion uptake

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Rhizoferrin-mediated iron uptake was studied in two different classes of organisms: a rhizoferrin producing fungus, *Absidia spinosa* (Zygomycetes), and a ferric rhizoferrin utilizing bacterium, *Morganella morganii* (Enterobacteriaceae). The uptake of iron rhizoferrin and some of its metal analogs (chromium, rhodium, gallium), was followed and kinetic parameters measured in *A. spinosa*. These metal ion complexes were taken up in a concentration- and energy-dependent manner indicative of an active transport system. The uptake of the kinetically inert chromium and rhodium and reductively inert gallium complexes suggests a variation of the so called 'shuttle' mechanism may be operative. The recognition of one geometrical isomer of chromium–rhizoferrin but not another argues for a degree of stereospecificity in the uptake process. A growth promotion plate assay was used to examine metal–rhizoferrin uptake in *M. morganii*. The results indicate that a number of factors including the nature of the chelating agent (e.g. bipyridyl or EDDHA) used to induce iron deficiency need to be considered before these simple plate assays can be reliably used to indicate the presence or absence of a particular siderophore uptake system.

Keywords: rhizoferrin, metal complexes, transport, siderophores, iron

Introduction

Rhizoferrin (Rf) is the archetype of a new class of recently discovered fungal siderophores which use hydroxy acids as donor groups rather than the hydroxamates common to this class of organism (Drechsel *et al.* 1991, 1992, Winkelmann 1992). It has been shown that rhizoferrin functions to deliver iron in a specific fashion not only to the producing organism but to other microbes as well (Drechsel *et al.* 1993). A rhizoferrin receptor from *Morganella morganii* has recently been cloned (Kühn 1995). Nevertheless the exact nature of the rhizoferrin-mediated iron transport mechanism in any organism has not yet been elucidated. One approach to obtaining information about the basic uptake mechanisms which may be operative in siderophore-mediated iron transport is to employ alternative metal ion complexes. In work originally pioneered by Raymond *et al.*, kinetically inert metal ions such as Cr^{3+} or Rh^{3+} have proven to be particularly informative in this regard since they are not readily exchanged or reduced (Chung *et al.* 1986, Ecker *et al.* 1988). We have recently synthesized and characterized such complexes for rhizoferrin (Carrano *et al.* 1996) and describe here their use in elucidating the mechanisms of iron transport in *A. spinosa*. In the present investigation the following metal ions complexed to rhizoferrin were studied: iron, aluminum, gallium, rhodium, chromium, vanadium, copper. Their transport and inhibition properties were analyzed using the producing fungal strain, *Absidia spinosa*, and a bacterial strain, *M. morganii*, possessing a transport system for iron–rhizoferrin.

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Materials and methods

Microbial strains, media and growth conditions

A. spinosa (Mucorales, Zygomycetes), a rhizoferrin-producing organism, was from the stock at the Institute. The strain was maintained on yeast–malt extract. The spores were harvested after 3 weeks and used to inoculate asparagine salts medium as described in Thieken & Winkelmann (1992). Young mycelia (12–24 h) were used for transport assays. *M. morganii* 13 (Proteae, Entobacteriaceae) was kindly provided by R. Reissbrodt. This strain has been shown previously to take up iron via a rhizoferrin-dependent pathway (Drechsel *et al.* 1993).

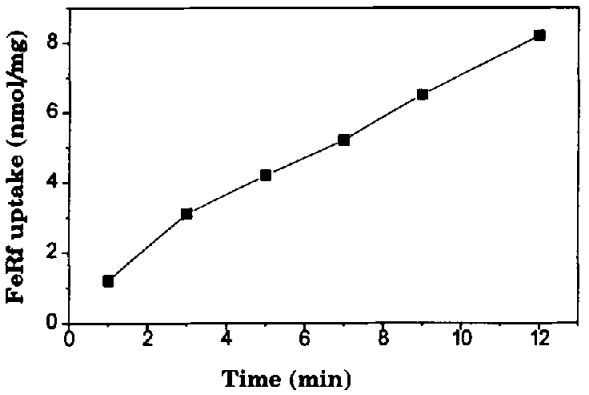

Figure 1. Time-dependent uptake of ferric rhizoferrin (20 μM) in mycelia of *A. spinosa*.

Transport and bioassays

Uptake protocols for the fungus *A. spinosa* were according to Thieken & Winkelmann (1992). Bioassays using *M. morganii* 13 were performed as described in Thieken and Winkelmann (1993) on agar plates containing either 2,2′-bipyridyl (300 μM) or ethylenediamine-*N*,*N*′-bis(2-hydroxy phenylacetic acid (EDDHA) (500 μM) to bind residual iron in the growth medium. Siderophores and metal analogs were placed on filter disks (10 μl of a 10 mM aqueous solution), dried in a microwave oven and placed on the agar plates. Alternatively the solution of siderophores (2–10 μl) was pipetted directly on to the agar surface.

Metal complexes

The Fe^{3+}, Ga^{3+}, Al^{3+}, Cr^{3+}, Rh^{3+}, VO^{2+} and Cu^{2+} complexes of rhizoferrin were prepared as described (Carrano *et al.* 1996) and are designated as FeRf, GaRf, AlRf, CrRf, RhRf, VRf and CuRf, respectively. Two geometrical isomers of CrRf (designated I and II) were purified and studied for uptake, while a single pure isomer, RhRf I, and an impure mixture of isomers, RhRf II, were used for RhRf uptake. For a spectroscopic characterization of these complexes, see Carrano *et al.* (1996). Uptake of the gallium, chromium and rhodium complexes was followed by atomic absorbtion on a PE 400 spectrophotometer equiped with a graphite furnace and autosampler following digestion of the filters and mycelia in concentrated nitric acid at 65°C overnight. We thank Professor U. Weser and Dr H. J. Hartmann of the Physiological Chemistry Institute, University of Tübingen for making this equipment available and assisting with its use. Iron–rhizoferrin uptake was monitored via scintillation counting of ^{55}Fe

Results

The time-dependent uptake of FeRf (20 μM) by *A. spinosa* is shown in Figure 1. The rate of uptake was high when the mycelia had been grown from conidiospores for at least 20 h under iron limitation in an asparagine salts glucose medium. Concentration-dependent uptake of FeRf revealed the expected saturation curves giving $K_m = 20\ \mu$M and $V_{max} =$ 1.0 nmol mg $^{-1}$ suggesting a mediated transport. To demonstrate that this uptake is indeed an active transport process, which should be sensitive to both temperature and the presence of metabolic inhibitors, we have investigated the effects of these parameters. A plot of uptake rate versus temperature revealed a maximum near 30°C with little uptake found at either 4 or 46°C (Figure 2). Metabolic inhibitors of various types, i.e. 1 mM 2,4-dinitrophenol (DNP; an uncoupler of oxidative phosphorylation), diethylstilbestrol (DES; ATPase inhibitor) and sodium azide (a cytochrome oxidase respiratory inhibitor), all effectively block rhizoferrin-mediated iron uptake, demonstrating that cellular energy and an intact membrane potential are required (Table 1). The FeRf uptake was also found to be pH dependent with a pH optimum for transport near pH 5 (Figure 3). Thus iron uptake via rhizoferrin in the producing fungus is clearly an active transport process which is temperature dependent and sensitive to metabolic inhibitors.

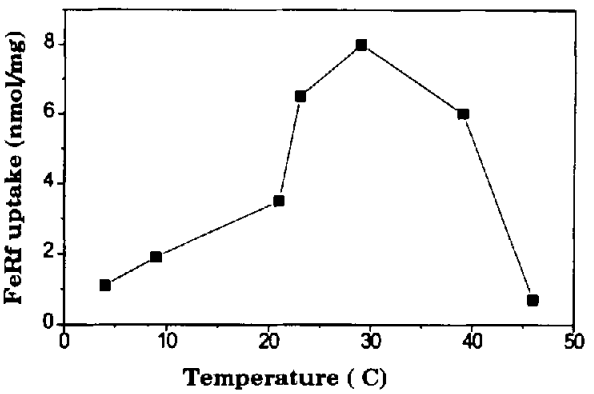

Figure 2. Temperature-dependent uptake of ^{55}FeRf. Mycelial suspension was preincubated in transport media for 30 min at the indicated temperature after which ^{55}FeRf was added to a final concentration of 40 μM. After incubation for an additional 10 min the cells were filtered and counted as previously described.

Table 1. Effect of inhibitors on ^{55}FeRf uptake in *A. spinosa*

Inhibitor	Concentration (mM)	Inhibition (% of control)
DNP	0.1	62
	0.5	88
	1.0	92
DES	0.1	26
	1.0	85
Sodium azide	1.0	95

Inhibitors were added together with the FeRf (40 μM) and uptake was measured after 8 min of incubation at 27°C.

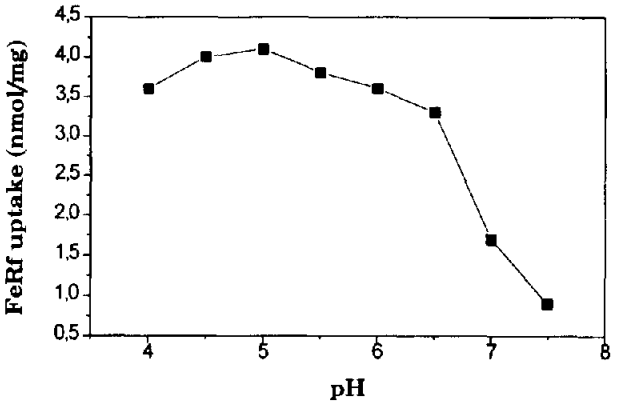

Figure 3. pH-dependent uptake of ^{55}FeRf. Cells were preincubated in 20 mM MES buffered media at the indicated pH for 30 min. ^{55}FeRf was then added to a final concentration of 40 μM and uptake measured after 8 min at 30°C.

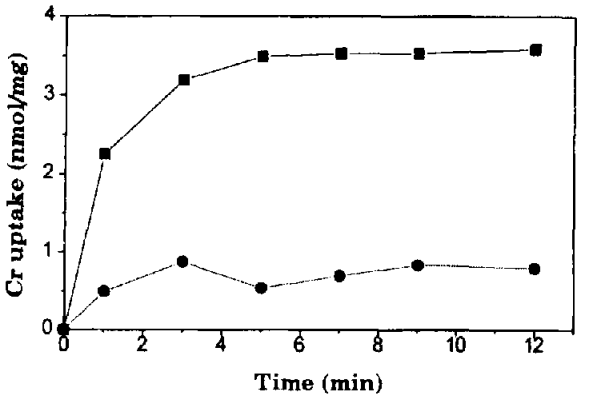

Figure 4. Time-dependent uptake of two isomers of CrRf, I (■) and II (●), at 26 and 33 μM, respectively.

Time-dependent uptake of the two isolated geometric isomers of CrRf by young mycelia of *A. spinosa* was followed by atomic absorbtion spectrophotometry. Isomer I was taken up in a time-dependent manner (Figure 4) with a rate that was similar to that of the natural iron–rhizoferrin while isomer II was apparently not recognized or transported. For RhRf both isomers I and II appeared to be transported (Figure 5). Similar results are seen for GaRf and uptake of gallium was abolished in the presence of 1 mM DNP (Figure 6).

Concentration-dependent uptake measurements were made on CrRf I and RhRf II in order to determine approximate kinetic parameters. Linewever–Burk plots indicated that the transport of both of these alternative metal complexes was saturable, as expected for a receptor-mediated uptake process. Kinetic parameters are given in Table 2 which show that the maximal rate of uptake of all the complexes were very similar although the affinity of the receptor for the chromium and rhodium complexes was clearly less than that for the iron complex.

The chromium and rhodium complexes were also tested for their ability to inhibit the uptake of radioactive iron by rhizoferrin. The results are shown in Figure 7, which indicate that both complexes inhibit iron uptake in a competitive manner. Inhibition constants derived from these plots are again given in Table 2. It is evident from these data that although the alternative metal ion complexes of rhizoferrin behaved kinetically as competitive inhibitors of iron uptake in *A. spinosa* they did so only weakly.

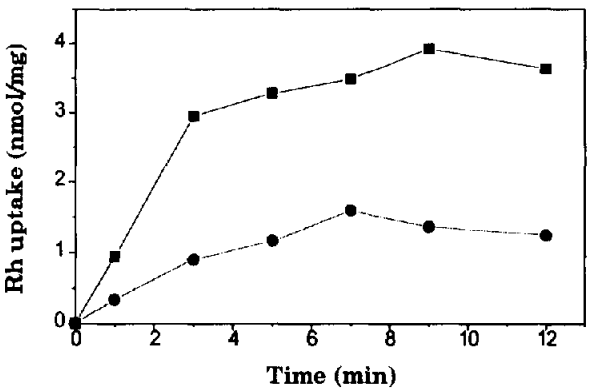

Figure 5. Time-dependent uptake of pure RhRf isomer I (■) and a mixture of isomers II (●) at 76 and 16 μM, respectively.

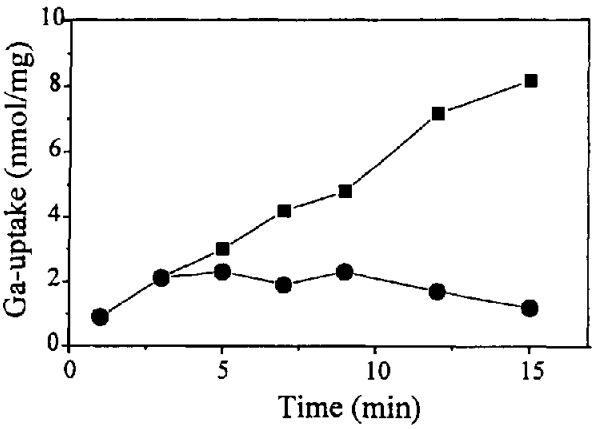

Figure 6. Time-dependent uptake of 20 μM GaRf in the absence (■) and presence (●) of 1 mM DNP.

Table 2. Kinetic parameters of transport and inhibition for metal comlexes of rhizoferrin

Metal	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	K_i (μM)
Fe	23	1.0	—
Cr	68	0.7	255
Rh	129	1.4	320

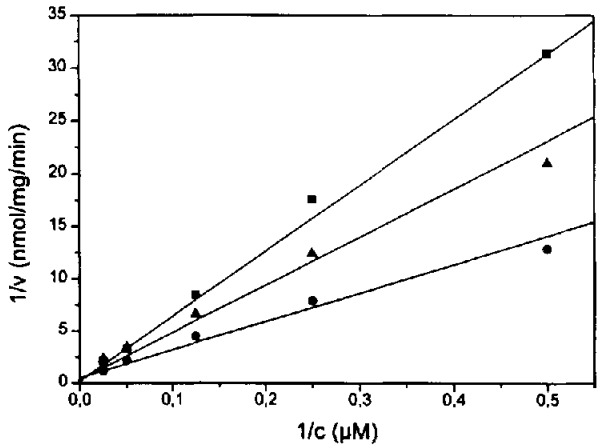

Figure 7. Dixon plot of concentration-dependent uptake of ^{55}FeRf in the absence of inhibitor (●) and in the presence of RhRf, isomer II (▲), or CrRf, isomer I (■), both at 200 μM.

Bioassays with M. morganii *13*

The metal analogs of iron–rhizoferrin were also studied in bioassay plates with the bacterium *M. morganii* 13. When plates employing bipyridyl to reduce the bioavailable iron were used we observed growth halos with both free rhizoferrin as well as its iron complex (20 mm diameter). However, the growth zone was smaller when the free ligand was utilized due to the need to complex iron from the media in competition with the bipyridyl. As expected, ferrioxamine E was ineffective at delivering iron to this organism due to the lack of a ferrioxamine receptor in *Morganella* (Drechsel *et al.* 1993). The kinetically inert complexes of rhizoferrin with chromium and rhodium, when combined with FeRf, showed the expected concentration-dependent reduction in the diameter of the growth halos with CrRf being the more effective inhibitor.

In contrast, the AlRf and GaRf complexes in the presence of FeRf showed a concentration-dependent clear zone surrounded by a ring of growth. Compared to the slight background growth of organisms that occurs even in the absence of added siderophore, the inner ring was completely clear, indicating a strong toxic effect. This clear zone of toxicity is also observed in the absence of FeRf. The kinetically labile CuRf and VRf complexes were not inhibitors of FeRf-mediated growth, instead we found an unexpected growth stimulatory effect when they were utilized alone or in combination with FeRf. The possibility that trace metals other than iron might be growth limiting lead us to experiments where we supplemented the bioassay plates with 1 μM $CuSO_4$. Strong background growth was observed indicating alleviation of iron deficiency.

When EDDHA was used for iron limitation in bioassay plates with *M. morganii* 13, free rhizoferrin became ineffective in supporting growth as compared with the iron complex. Due to the greater iron deficiency induced by EDDHA in the bioassay, background growth of organisms was absent and therefore the toxic effects of GaRf and AlRf found in the bipyridyl plates could not be observed. When we examined the CuRf and VRf complexes on EDDHA plates, CuRf now showed no growth stimulation while that displayed by VRf was still present.

Discussion

There are three basic mechanisms that have been described for siderophore-mediated iron transport in fungi (Van der Helm & Winkelmann 1994). These include the classical so called, 'shuttle' mechanism, where the intact metal siderophore complex is internalized and the metal removed either via a reductive or hydrolytic process; the 'taxi' mechanism, where the metal–siderophore complex carries metal to the cell surface where it is transferred to another carrier for internalization; and a surface reduction mechanism where the metal–siderophore complex is not internalized at all but reduced at the cell surface. There are almost certainly a number of variations on these basic mechanisms.

It is clear from the data presented here that at least some of the alternative metal complexes of rhizoferrin are recognized and taken up in an energy- and concentration-dependent manner indicative of an active transport system. The simple fact that both the kinetically (chromium and rhodium) and reductively (gallium) inert complexes are taken up suggests that neither exchange nor reduction of the metal ion can be necessary steps for transport and internalization. This effectively eliminates the taxi and surface reduction mechanisms from consideration. There is also compelling Mössbauer evidence that FeRf is internalized in *A. spinosa* in intact form (Matzanke 1994). It is also clear that there must be some form of stereospecificity for the coordination geometry around the metal, since at least one geometrical isomer of CrRf is not recognized.

One of the unique features of the chromium and rhodium complexes of rhizoferrin is that they have the opposite chirality at the metal center as compared to that of the iron complex (Carrano *et al.* 1996). The degree of chiral preference based on extinction coefficients varies from less than 5% for the chromium isomers to greater than 50% for the rhodium. However, the role that chirality about the metal center plays in the uptake process is difficult to assess in a quantitative way since none of the isomers existed in optically pure form. However, the trend in progressively lower transport affinity as the percentage of the lambda or anticlockwise isomer decreases could be interpreted to suggest some chiral specificity of transport, assuming that all three metal complexes utilize the same receptor/transport system.

Although the kinetic parameters for CrRf and RhRf in the fungal system are similar to those of FeRf, i.e. similar V_{max} although somewhat reduced affinity, this does not prove that they are all transported by the same system. To provide more conclusive data that the alternative metal ion complexes of rhizoferrin are transported by the same system as the iron we looked to inhibition studies. The data clearly showed competitive inhibition of iron transport by both the chromium and rhodium complexes. However, the surprisingly

weak inhibition observed, and the fact that the K_m and K_i values are not the same, which would be expected if both the iron and alternative metal complexes competed for a single receptor, suggest a system more complex than the simple shuttle mechanism must be involved in this fungus. One such a mechanism, previously described for another fungal system (*Neurospora crassa*), can also account for all the data presented here (Huschka *et al.* 1985). In this system there are two (or more) distinct receptor molecules with potentially independent affinities for their respective 'substrates'. These different receptors, however, share a common membrane carrier protein. In this way we can account for the recognition of alternative metal ion complexes despite the fact that they have opposite chiralities with respect to FeRf by postulating that they are recognized by different receptors. At the same time, if the rate determining step is transport of the receptor–metal–rhizoferin ternary complex by the carrier protein, we can expect similar V_{max}'s. It also accounts for the observation of competitive inhibition in the inhibition experiments and the fact that the K_m and K_i values for RhRf and CrRf differ since they are in fact measuring different parameters: in one case it is the affinity of the metal rhizoferrin for its receptor and in the other it is the affinity of the metal–rhizoferrin– receptor complex for the common carrier protein.

The differential effects of the various metal complexes seen between the two types of bioassay plates (bipyridyl and EDDHA) used in the bioassays can be explained if we consider both the stabilities of the complexes (thermodynamic and kinetic) as well as their redox properties. For example, the free ligand, rhizoferrin, is active in bipyridyl plates due to its ability to remove the iron from ferrous bipyridyl but it is ineffective on EDDHA plates due to the greater stability of the iron complex of the latter. The redox chemistry of copper and vanadium also explains their diverse effects on the two types of plates. Thus when divalent copper is incorporated directly into the media it completely alleviates iron limited growth due to its oxidation of the ferrous bipyridyl complex in the medium producing monovalent Cu^+ and Fe^{3+}. The latter can in turn be taken up by the relatively weak endogeous siderophores (keto acids) of *Morganella* (Drechsel *et al.* 1993) leading to high background growth. Both the CuRf and VRf complexes are also apparently capable of oxidizing ferrous bipyridyl leading to growth halos due to FeRf formation. On EDDHA plates CuRf had no effect since the iron of the medium is already bound in its oxidized state to EDDHA and cannot be mobilized. However, VRf was still effective in growth promotion probably due to the fact that vanadium can exist in three stable oxidation states, i.e. +3, +4 and +5, and hence may reduce Fe-EDDHA leading to V(V)Rf and ferrous ion. The resulting 'free' ferrous ion, being at most weakly, if at all bound to EDDHA, is then available for uptake by *Morganella* via a still unknown ferrous iron uptake system. The presence or absence of a particular type of receptor therefore should not be inferred from the presence or absence of growth halos without first considering the stability and redox properties of the metal complexes employed.

Acknowledgements

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