The Mechanism and Specificity of Iron Transport in *Rhodotorula pilimanae* Probed by Synthetic Analogs of Rhodotorulic Acid*

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The yeast *Rhodotorula pilimanae* produces the dihydroxamate siderophore rhodotorulic acid (RA) in prodigious amounts when starved for iron. Synthetic dihydroxamate analogs of RA have been prepared in which the diketopiperazine ring of RA is replaced by a simple chain of \( n \) methylene groups. It is found that *R. pilimanae* is able to accumulate iron using these achiral complexes, as well as from simple monohydroxamate analogs, at rates comparable to those of RA. While the Fe\(_3\)RA\(_3\) complex does not enter the cell, there is a receptor system whose geometric requirements for siderophore recognition have been probed using analogs. In contrast to mono- or dihydroxamate ligands, the trihydroxamate siderophores such as ferroxamine B are completely ineffective at delivering iron to *R. pilimanae*. This is ascribed to the greater stability of these complexes, which blocks release of the Fe(III) in a ligand exchange process that is required for uptake. To explore whether this ligand exchange involves re- dox catalysis, Ga(III) was substituted for Fe(III). The gallium was taken up at rates near those of iron and were also energy-dependent, as determined by metabolic inhibition with KCN.

Since all life forms appear to have an absolute requirement for iron, the introduction of this element into a wide range of biochemical processes appears to have taken place very early in the evolution of life. For aerobic organisms the concentration of free, aqueous ferric ion is limited to \( 10^{-16} \) M at neutral pH due to the insolubility of Fe(OH)\(_3\). This is the driving force for the excretion by microbes of strong iron-chelating agents (siderophores) and the expression of high-affinity transport systems which provide a reliable cellular iron supply. Investigators have done much to determine the trihydroxamate siderophores such as ferroxamine B in systems of this type. For a receptor/uptake system several features of the ferric RA complexes may potentially be important for recognition: 1) the diketopiperazine rings, 2) the geometrical configuration about the metal centers and the direct surroundings (e.g. methyl groups), 3) one or both iron centers, 4) the distance between the iron centers, and 5) the entire molecular structure. In order to characterize those molecular features of RA which are critical to the iron acquisition process of the yeast, we have synthesized analogs of RA with selectively modified sites. In these model ligands the hydroxamate groups are separated by varying numbers (\( n = 3 \) to 6 and 8) of methylene groups. These molecules are designed to probe the importance of the diketopiperazine rings and the iron-iron distance in the uptake process. These dihydroxamate ligands form complexes in aqueous solution with Fe\(^{II}\) that are analogous to those of RA. The formation constants per ferric ion are \( 10^7 \) identical within experimental error to that determined for Fe\(_3\)RA\(_3\) (15).

To test the influence of steric hindrance around the hydroxamate metal centers, dimeric acid (Fig. 1) was also used as a probe. Furthermore, in order to test whether one or two metal centers are necessary for iron transport, the uptake characteristics of synthetic monomeric trihydroxamate complexes such as acetylhydroxamate and 1-hydroxy-2-(1H)-pyridinones (Fig. 1) were also investigated.

**MATERIALS AND METHODS**

1. The abbreviation used is: RA, rhodotorulic acid.

2. Portions of this paper (including "Materials and Methods" and Figs. 4 and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry.

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Iron Uptake in *R. pilimanae* Mediated by Dimeric Iron Complexes—Fig. 2 shows the comparison of Fe uptake mediated by dimeric hydroxamates and trihydroxamate siderophores. All complexes were added to cell cultures at 10 µM concentrations. Iron uptake only occurred in the presence of dimeric complexes; no uptake occurred with trihydroxamate siderophores such as ferrioxamine B, coprogen, and ferricrocin. The species own siderophore, RA, exhibited the highest uptake rate: the ferric complex of dimerum acid gave the lowest rate (28% of the ferric RA uptake, measured after 10 min). Iron was supplied to the cells by the synthetic analogs n = 6 iso, n = 6 me, and n = 8 me at rates between these extrema, even though these analogs lack the diketopiperazine ring.

Effect of Chain Length on 55Fe Uptake—In order to probe the effect of the iron-iron distance in the dimeric hydroxamate complexes on the 55Fe uptake in *R. pilimanae*, the series of N-isopropyl analogs in which the number of bridging methylene groups varies from 3 to 6 was investigated. Although the n = 6 compound is still shorter than the chain length of RA, the higher chain length analogs are too insoluble for these experiments. Time-dependent uptake measurements with the ferric complexes of the synthetic isopropyl analogs n = 3 to 6 (Fig. 1) showed that n = 6 and n = 8 supply iron at 80 and 74% of the rate of 55Fe, respectively. The n = 4 iso and n = 3 iso compounds mediated the iron transport at rates similar to monomeric ferric tris(acetohydroxamate), which was between 50 and 60% of ferric RA. All experiments were performed at 10 µM substrate concentrations, where diffusion into the cell membrane contributes less than 1.5% to the overall iron accumulation after 10 min, as measured after addition of 1 mM KCN, a respiratory poison (data not shown).

Fig. 3 illustrates that the concentration dependence of uptake of the RA analogs is quite different from that for ferric RA. A maximum in uptake rate for 55Fe, RA, was reached at 10 to 20 µM. However, the analogs did not show saturation kinetics in supplying iron to *R. pilimanae*. At substrate concentrations less than 10 to 20 µM the uptake rates were lower

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**RESULTS**

Iron Uptake in *R. pilimanae* Mediated by Dimeric Iron Complexes—Fig. 2 shows the comparison of 55Fe uptake mediated by dimeric hydroxamates and trihydroxamate siderophores. All complexes were added to cell cultures at 10 µM concentrations. Iron uptake only occurred in the presence of dimeric complexes; no uptake occurred with trihydroxamate siderophores such as ferrioxamine B, coprogen, and ferricrocin. The species own siderophore, RA, exhibited the highest uptake rate: the ferric complex of dimerum acid gave the lowest rate (28% of the ferric RA uptake, measured after 10 min). Iron was supplied to the cells by the synthetic analogs n = 6 iso, n = 6 me, and n = 8 me at rates between these extrema, even though these analogs lack the diketopiperazine ring.

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**FIG. 1.** Structures of rhodotoreric acid, the synthetic dihydroxamates, and the 1,2-dihydroxyhydroxamidones used in this study. n = 6 me, N,N′-dihydroxy-N,N′-dimethyldecanamide; n = 8 me, N,N′-dihydroxy-N,N′-dimethyldecanamide; n = 3 iso, N,N′-dihydroxy-N,N′-dissopropylpentanamidamide; n = 4 iso, N,N′-dihydroxy-N,N′-dissopropylhexanamide; n = 5 iso, N,N′-dihydroxy-N,N′-dissopropylheptanamide; n = 6 iso, N,N′-dihydroxy-N,N′-dissopropyloctanamide; 1,2-HOPO-6-X, 1-hydroxy-2-(1H)-pyridinone-6-carboxylic acid; 1,2-HOPO-6-Y, N,N-dimethyl-1-hydroxy-2-(1H)-pyridinone-6-carboxamide.

**FIG. 2.** Time-dependent uptake of 55Fe mediated by RA, RA analogs, and trihydroxamate siderophores. The same abbreviations for ligand names are used as in the legend to Fig. 1. **Iron Transport in R. pilimanae**, the series of N-isopropyl analogs in which the number of bridging methylene groups varies from 3 to 6 was investigated. Although the n = 6 compound is still shorter than the chain length of RA, the higher chain length analogs are too insoluble for these experiments. Time-dependent uptake measurements with the ferric complexes of the synthetic isopropyl analogs n = 3 to 6 (Fig. 1) showed that n = 6 and n = 8 supply iron at 80 and 74% of the rate of 55Fe, RA, respectively. The n = 4 iso and n = 3 iso compounds mediated the iron transport at rates similar to monomeric ferric tris(acetohydroxamate), which was between 50 and 60% of ferric RA. All experiments were performed at 10 µM substrate concentrations, where diffusion into the cell membrane contributes less than 1.5% to the overall iron accumulation after 10 min, as measured after addition of 1 mM KCN, a respiratory poison (data not shown).

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process; the uptake curve of Fig. 3. The difference curve for the concentration-dependent kinetic experiments with the analog and probed using the respiratory pbison KCN. The same concentration of ferric RA accumulation. The active portion of this was RA complexes revealed that, in contrast to FezR&, iron transport rates mediated by the analogs exceeded the values that of RA. The active transport portions of the ferric analogs analog is similar, but reaches a saturation rate that is 70% of 1 mM indicate the uptake mediated of iron. The concentration-dependent uptake kinetics were showed subs~tia~ly the same kinetics as the isopropyl derivatives (data not shown).

In order to show the portion of uptake that is due to whether these compounds might serve as iron transport agents in R. pilimanae. The solution chemistry of the iron complexes of these powerful chelating agents has been well characterized (20). At neutral pH and excess ligand (L) a 3:1 complex is formed (FeL3) with ferric ion. All complexes were assayed at 10 µM concentrations. The uptake of labeled iron from these compounds, RA, and acetohydroxamate (a simple monohydroxamate) is linear for the first 10 min. Regardless of differences in the structure of these compounds, the uptake rates were about 50% of the iron accumulation from dimeric RA complexes. After 10 min about 2 nmol of Fe/mg of cells were absorbed, compared to 4 for FezRA3. Incubation of the cells with 10 mM NaN3, an inhibitor of oxidative phosphorylation, resulted in total inhibition of iron uptake (less than 1% after 10 min, data not shown). This indicates that iron from the monomeric compounds, as well as free RA, is transported by a process requiring energy. This is the first time it has been shown that hydroxypyridinones can act as siderophores.

Inhibition of Iron Uptake—The inhibition of iron hydroxamate uptake by increasing concentrations of chromic RA complexes should test whether RA analogs deliver iron to the cells by the same uptake system as RA. The chromic complexes, which are kinetically inert to ligand exchange, were separated into geometrical and optical isomers as described elsewhere (17). Fig. 4 (Miniprint Section) shows the inhibition of 55Fe uptake mediated by RA, dimerum acid, and the n = 6 me and n = 6 iso analogs (2.5 µM) in the presence of increasing concentrations of the Δ-trans-CrzRA3 preparation. At that concentration inhibition of uptake occurred for all complexes except for n = 5 iso; it was only inhibited if added at 0.5 µM to the assay, in which case iron accumulation decreased to 80% at 30 µM CrzRA3 concentration.

Incorporation of iron from complexes of tris(acetohydroxamate) (Ac) and tris 1-hydroxy-2-(1H)-pyridinone (HOPO) was also impeded in the presence of Δ-cis-CrzRA3. 40 µM CrzRA3 diminished Fe(HOPO)3 uptake to 75% and Fe(Ad3) uptake to 68% (data not shown).

In summary, CrzRA3 inhibits uptake of iron from synthetic monomeric and dimeric hydroxamate in the same way as it does from RA. This indicates that the same receptor system is used in the iron acquisition mediated by these ligands.

**DISCUSSION**

In this study it has been shown that R. pilimanae is able to accumulate iron at good rates from analogs of the siderophore rhodotorulic acid which include achiral synthetic dimeric complexes as well as from synthetic monomeric iron complexes. As with RA, iron uptake mediated by RA analogs involves an active transport system. Diffusion or passive accumulation only becomes significant at very high substrate concentrations and accounts for less than 1.5% of the overall transport at 10 µM complex concentration in the assay. In transport of both RA and the analogs, reduction is not re-
quired, as shown with the $^{67}$Ga complexes. Of particular importance is the inhibitory effect of chromic RA on the accumulation of analog-bound ferric ion. This indicates that the synthetic analogs and dimerum acid provide iron by the same transport system as RA. In addition, all synthetic ligands lack the diketopiperazine ring. Therefore, we conclude that the diketopiperazine ring does not play a major role in the recognition and uptake process.

The influence of the residues of the hydroxamate carboxyl group is demonstrated by dimerum acid (Fig. 2). The dimerum acid is identical to RA except that the terminal methyl groups are substituted by isopentalen residues. Compared to RA, the uptake of ferric dimerum acid is reduced by 70%, indicating a steric hindrance of the isopentalen groups for recognition and uptake. A further strong argument for the hypothesis that it is the terminal metal center which is of primary importance in recognition and uptake is the comparison of dimerum acid and the synthetic analogs $n = 6$ me and $n = 6$ iso. Iron from complexes of the analogs is taken up at higher rates than from dimerum acid. As shown in another investigation, there exists a strong discrimination in iron transport between RA and its enantiomer (17). Since the diketopiperazine ring is not of primary importance for recognition/uptake, the mirror image hydroxamate "propellors" and the opposite twist of the methylene chains must account for the reduced uptake of enantio-RA. These results again illustrate the importance of the terminal region in the accumulation process.

Unexpectedly, the $n = 6$ iso analog shows even higher uptake than $n = 5$ me (Fig. 2). The latter possesses methyl residues, like RA, whereas the isopropyl groups of $n = 6$ iso are bulkier. Competition experiments with these analogs and chromic RA at low and high substrate concentration indicate that $n = 6$ iso has a different interaction with the RA transport system than $n = 6$ me. Since $n = 8$ me is taken up at almost the same rate as $n = 6$ me, the deviant behavior of the $n = 6$ iso iron complexes cannot be due to changes in the length of the molecule. It must be ascribed to the increase of hydrophobic portions around the metal centers. Emery and Emery (23) have observed a similar effect with semisynthetic analogs of ferrichrome, in which substitution of the hydroxamate-linked methyl groups by butyl functionalities resulted in increased iron supply to *U. sphaerogena* (23) compared to the native ferrichrome. Thus, lipophilic groups seem to be one important part of the recognition/transport process.

In order to measure the possible influence of the iron-iron distances on uptake rates, homologous analogs ($n = 3$ iso to $n = 6$ iso) have been compared. With decreasing chain length a decrease in uptake rates is observed. Accumulation of iron from analogs $n = 3$ iso and $n = 4$ iso are in the range of acetohydroxamate, whereas analogs $n = 5$ iso and $n = 6$ iso display 74 and 80% of the ferric RA uptake. Since the geometry of the metal center is the same for all of these dimeric compounds, the decrease of uptake rates is due to the reduced molecular length.

All hydroxamic analogs show lower uptake rates compared to RA. Since the diketopiperazine ring plays no major role in accumulation, this must be due to additional structural features. In contrast to RA, the analogs have the hydroxamate carbonyl and $N$-hydroxyl groups interchanged, and they are achiral. For the racemic mixtures of the analogs the concentration of receptor active species might be as low as 50% compared to RA complexes which are predominantly $\Delta$. In summary, we suggest three features to account for the observed differences in uptake rates: 1) the terminal residues, 2) the iron-iron distances, and 3) the chirality at the metal center and its effect on the position of the terminal groups.

It has been shown in an earlier paper, using $^{3}$H-labeled ligand and chromic RA that the ligand is not taken up in *R. pilimanae* (16). However, it remained unclear whether the iron removal involves reduction of the metal or merely ligand exchange by a superior substrate. The $^{67}$Ga experiments in this study eliminate the reduction mechanism for metal incorporation.

In *Rhodotorula gracilis* and another fungus, *N. crassa*, it was shown that the potential of the cytoplasmic membrane is caused by a proton gradient. Low pH regions at the membrane surface, produced by the proton motive force, could catalyze a shift in species distribution of ferric RA and analogs and promote the ligand exchange process which must accompany uptake. In fact, the results with hydroxamate siderophores, such as coprogen, ferricrocin, and ferrichrome B (all of which are not taken up), strongly suggest that the higher complex formation constants and/or the stability of these complexes over a wide pH range (2 to 8) are crucial for the lack of any ferric ion removal. The absence of metal accumulation from these complexes cannot be due to different stereochemistry. Ferricrocin, for example, displays the same structural features at the metal center part of the molecule as enantio-RA (21). The latter compound exhibits uptake rates which are half as high as those for ferric RA, whereas ferricrocin-bound iron is not taken up at all. Similarly, dimerum acid shows low accumulation rates and coprogen does not release iron to the membrane-associated ferric ion acceptor.

From uptake measurements with monomeric ferric complexes of hydroxamate and 1-hydroxy-2-(1H)-pyridinones it has been demonstrated that two iron center substrate molecules are not absolutely required for transport by the RA uptake system. However, it cannot be completely excluded that these molecules might be small enough to occupy simultaneously two iron binding centers at the same recognition site. There is only poor resemblance of ferric 1-hydroxy-2-(1H)-pyridinone to ferric RA. The two systems have in common a hydrophobic portion adjacent to the hydroxamate group of the ring and the solution chemistry is comparable. Lowering the pH leads to shifts in the species distribution favoring $\text{FeL}^{2+}$ and $\text{FeL}^{3+}$ species. Finally, it should be mentioned that hydroxypyridinones are similar to the piperdine isolated by Akers and Neilands (22) from *R. pilimanae* low-iron cultures. This compound may serve as a second siderophore or as part of a membrane-bound system which functions as a ferric ion acceptor.

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**Materials and Methods**

**Materials.** In all experiments reagent grade chemicals were used. Desferrioxamine B (Deferal®) was obtained from Ciba-Geigy Pharmaceutical Company, New Jersey. Coprogen was a generous gift of S. F. Matzanke. Rhodotorulic acid was isolated as described previously and recrystallized three times from hot methanol (18). The dihydropyrazine analogs were synthesized from the acid chloride and 1-aminopyrazoline or 1-aminopyrazol ylamine. Their properties and characterization are described elsewhere (18, 19). The syntheses of the 1-hydroxy-2-(10)-pyridazines (HPO) derivatives have been reported elsewhere (20). Solutions of 55FeCl₃ and ⁶⁷GaCl₃ were purchased from New England Nuclear. Complexes of rhodotorulic acid were prepared from C₆H₅NO₃ and Cl⁻ as described (17). Tritiated RA was prepared by microwave discharge activation of tritium gas at the Lawrence Berkeley Laboratory. In inhibitor. BA-RA was purified by eight recrystallizations from water until all labile tritium had been removed.

Specific activity was 0.09 mCi/μmol.

**Cyanides and growth conditions.** Stock cultures of *Rhodotorula pilimanae* (ATCC 26422) were kept on agar slants (2.1% Difco TM broth; 2.1%) incubated at 27°C. From this preculture 1 mL of cell suspension was transferred to an iron-deficient salt medium, containing per 1 liter: 1 g KNO₃, 2 g HPO₄, 0.25% NaCl and 5 g MgSO₄. Solutions of these salts were passed over Chelex-100 (Na⁺ form) to remove the iron. Thiamine (2 mg), 24 glucose, and solutions of trace salts (including 0.005% CaCl₂·2H₂O, 0.015% MgSO₄·7H₂O, 2 mg KH₂PO₄, 7H₂O, 80 mg MgSO₄ and 100 mg CaCl₂) were added separately. The water was deionized twice and then distilled in a glass still. All glassware was washed with nitric acid. After inoculation of the cells in iron deficient medium, the cells were centrifuged at 5000 ×g/10 min and washed twice with medium, incubated for an additional two hours and then put on ice. The cell suspension was adjusted to an optical density of 0.5 at 660 nm (1:5 dilution) which corresponds to a cell dry weight of 5.6 mg/mL culture.

**Transport assay.** For time-dependent measurements, radiolabeled ferric complexes of RA or the analogs were added to 20 mL of a cell culture. Some aliquots were removed at regular intervals and then added to 10 mL of ice-cold 0.9% saline. The cell suspension (lopahtic growth phase) was filtered through nitrocellulose filters (3 μm pore size; Schleicher & Schuell) and washed with cold saline. The filters with the cells were counted in a liquid scintillation counter (Searle Mark III) after equilibration in Aquasol 2 liquid scintillation fluid. For kinetic measurements 0.9% aliquots of cell suspension were added to test-tubes containing increasing amounts of labeled ferric complexes in 0.1 mL volumes and these were vigorously shaken at 27°C. After either two or ten minute uptake periods, assays were diluted with 10 mL of ice-cold saline (0.9% NaCl solution), filtered, washed again twice with ice-cold saline and counted as described above. The uptake rates were determined from the interval between the second and the 15th minute of uptake (which was already shown by the time dependent experiments to be linear).

In order to compare more closely the specific receptor-mediated ⁵⁵Fe-uptake rate with the non-specific ⁵⁵Fe-uptake rate, all time dependent uptake curves were corrected for non-specific adsorption of the labeled complex onto the cell surface and filters. This was done by subtracting the amount of label adsorbed in the first ten seconds from each of the curves.

**Labeled complexes.** The ⁵⁵Fe or ⁶⁷Ga-labeled complexes of the dimeric hydroxamates were prepared by adding ⁵⁵FeCl₃ or ⁶⁷GaCl₃ in 0.1 mL H₂O to an approximately 10% excess of ligand (based on Fe₃(PO₄)₅) being the form of the complexes and the pH was adjusted to 7. The monomeric ⁵⁵Fe-complexes of 1,2-HPOs and acetyldihydroxamate were similarly synthesized using a tenfold excess of ligand. Comparison of the viable spores with results of spectrophotometric titrations showed that the tri-complexes were formed under these conditions (20). Isotopic ferric complexes of coprogen, ferricrocin B and ferricrocin were prepared as described for the dimeric ligands. Desferriferrocyan and desferriferroxalate were obtained using hydroquinone (21). The concentrations of the iron complex solutions were determined spectrophotometrically to be 0.5 mM. Their specific activities were 10 to 20 mCi/μmol.

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**Figure 4.** Inhibition of ⁵⁵Fe-uptake iron dimeric hydroxamates by ⁴⁻-trans chelating complexes of RA. All ferric complexes were added at 2.5 μM concentration. The 10% uptake was calculated from the ⁵⁵Fe-uptake rate without inhibitor, n = 6, iso (●); dimeroxalate (ட); BA (△); n = 6, m (●).

**Figure 5.** Comparison of the time-dependent ⁵⁵Fe- and ⁶⁷Ga-uptake mediated by RA and analogs. Upper curves: ⁵⁵Fe-uptake in presence of KCN (1 mM) (mediated by n = 6, m (●); n = 6, iso (○); RA (△)).