Stereospecificity of Siderophore-mediated Iron Uptake in *Rhodotorula pilimanae* as Probed by Enantiorhodotorulic Acid and Isomers of Chromic Rhodotorulate*

(Received for publication, November 9, 1984)

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Rhodotorulic acid (RA), a dihydroxamate siderophore produced by *Rhodotorula pilimanae*, forms 3:2 complexes with ferric and chromic ions (M₂RA₃) at pH 7. Kinetically inert chromic complexes of RA have been separated into geometrical isomers and for the first time partially resolved into optical isomers. The three isomers Δ-cis, Δ-trans, and Λ-trans were characterized by their visible and circular dichroism spectra. Inhibition by both Δ-isomers of radiolabeled ferric RA uptake in *R. pilimanae* was equally effective. However the Δ-cis isomer was significantly less effective as an inhibitor. Concentration-dependent uptake kinetics were performed with ferric RA and the ferric complex of synthetic enantiomeric RA, which form predominantly Δ and Λ complexes, respectively. The Δ-enantiomeric Fe₃RA₃ was 50% less effective in supplying iron to *R. pilimanae* than was Fe₃RA₂. An additional synthetic analog of RA, which lacks a carbonyl group at the diketopiperazine ring, exhibited the same uptake rates as ferric RA. We conclude that stereoselective recognition of optical isomers takes place during iron uptake mediated by RA and that this recognition primarily involves the right-handed Δ coordination "propeller" of the metal center and its adjacent functionalities.

As described in the previous paper (1), the absolute requirement of iron for microbial growth and the great insolubility of ferric hydroxide require chelating agents to solubilize Fe(III) and facilitate its mobilization into microbial cells. As a class these low-molecular-weight complexing agents are called siderophores. We are concerned with the mechanisms by which these agents transport iron into the cell. All of these compounds, which typically incorporate hydroxamate or catecholate groups, form high-spin complexes of Fe(III) in which the ferric ion is transported into the cell by receptor-mediated processes that consume metabolic energy (2–4). In the preceding paper synthetic analogs of rhodotorulic acid (RA)

* This research was supported by National Institutes of Health Grant AI 11744. This is Paper 32 in the series "Coordination Chemistry of Microbial Iron Transport Compounds." The preceding paper (Ref. 1) is the previous paper in this series. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§§ The abbreviations used are: RA, rhodotorulic acid; HPC, high-performance liquid chromatography.

*Fig. 1, Miniprint Section* were used to probe the mechanism by which this siderophore mediates iron uptake in the yeast *Rhodotorula pilimanae*, which produces RA in prodigious amounts when grown in iron-deficient culture media. This and other transport studies with synthetic siderophores have revealed important correlations between the coordination geometry of the ferric siderophore complex and its recognition and uptake by microbial cells. For example, natural ferrichrome, which has a preferred Δ geometry at the metal center (2) is produced by various species of fungi. Both *Neurospora crassa* and *Escherichia coli* have receptor systems which recognize this siderophore and clearly discriminate between it and the mirror image Δ-enantioferrichrome (5, 6). Similarly, *E. coli* produces enterobactin, in which the ferric ion has a preferred Δ configuration, and a clear discrimination is made between ferric enterobactin and the synthetic mirror image ferric enantiomerobactin, which does not support growth of *E. coli* (7).

For siderophores such as enterobactin and ferrichrome, synthesis of the enantiomeric ligand requires that the preferred metal ion chirality will also be enantiomeric. Thus, the microbial discrimination between natural Δ ferric enterobactin and the synthetic Δ ferric enantiomerobactin really involves a response to two changes of chirality: 1) the ligand structure and 2) the metal ion coordination site. In principle, the response to these two changes cannot be separated. However, if metal complexes of the same ligand can be isolated as different isomers, then only the effect of the metal ion coordination geometry change can be probed. This is not feasible for high-spin ferric complexes in aqueous solution because of the relative lability of these complexes to both ligand exchange and isomerization processes. However, the substitution of Cr(III) for Fe(III) in the hydroxamate siderophores produces kinetically inert complexes which are essentially unchanged in geometry from the natural ferric complexes (8).

In this study chromic complexes of rhodotorulic acid, the dimeric cyclic diketopiperazine of δ-N-acetyl-L-(S)-δ-N-hydroxyornithine, have been synthesized and for the first time resolved into optical and geometrical isomers. With these isomers the stereoselectivity of recognition of the RA uptake system in *R. pilimanae* has been probed. These results have been confirmed by uptake studies with synthetic radiolabeled ferric Δ-enantiomeric RA complexes. A second synthetic analog of RA, which lacks one carbonyl group of the diketopiperazine ring, has been used to probe the role of this molecular region with respect to specificity of binding (Fig. 1, Miniprint Section).
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EXPERIMENTAL PROCEDURES

RESULTS

Iron Transport Mediated by RA, Enanto-RA, and the RA Analog—As required for enantiomeric complexes, the circular dichroism spectra of ferric RA and ferric enanto-RA at neutral pH are mirror images: Fe₂RA₃ has a negative CD band at 465 nm (Δε = -1.52) whereas enanto-Fe₂RA₃ has a positive CD band at 465 nm (Δε = +1.49) (data not shown). The Fe₂RA₃ exists predominantly as the Δ-complex (14). Apparently the chirality of the two L-hydroxynorhine groups of the diketopiperazine in RA favors the Δ helicity at the iron hydroxamate centers. The ferric enanto-RA, therefore, has a preferred optical absolute configuration at the iron centers. (For a detailed discussion of assignments of chirality of siderophore complexes see Refs. 2 and 3.) As expected, the CD spectrum of ferric enanto-RA is very similar to that of ferrichrome, in which the Fe(III) is also octahedrally coordinated by three hydroxamate groups in a Δ configuration (15).

The concentration-dependent iron uptake kinetics mediated by the two enantiomeric RA complexes probe whether stereoselective recognition takes place in this system during transport. Iron from both ferric complexes is accumulated by a saturable process with increasing substrate concentration. Fig. 2 shows a clear discrimination between the two optical isomers of ferric RA, which results in a 50% lower iron uptake rate for the Δ-enanto-RA complex compared to RA. The maximum velocity of iron uptake remains 50% less than for RA even at a 60 μM concentration. The naturally occurring RA complex is thus a more effective substrate for iron supply to the cells.

Iron uptake measurements with ferric complexes of the RA analog, which lacks one carboxyl group at the diketopiperazine ring, probe the sensitivity of the RA transport system to minor changes of the ligand structure (Fig. 1). In this case the uptake rates were the same as for Fe₂RA₃, indicating that this part of the diketopiperazine ring is not important for recognition and transport (data not shown).

Separation of Isomers of Chromatic Rhodotorulate—Rhodotorulic acid, a dihydroxamate siderophore, forms dimeric complexes with iron, aluminum, and chromium of the stoichiometry M₆(RA)₃ at neutral pH (14). Since no crystal structure of Fe₂RA₃ has been reported, it cannot be unambiguously stated that the binuclear complex has the structure shown in Fig. 1 (Miniprint Section). In an effort to lend support for the triply bridged dimeric structure proposed for ferric rhodotorulicate (Fig. 1, Miniprint Section), a heteroanalogue of RA, N₃,N₅-(1-hydroxy-2-(H)-pyridonecarboxyl)diaminopropane has been synthesized. The crystal structure of the ferric Fe₆L₈ complex reveals a structure such as that shown in Fig. 1 (Miniprint Section) (16). Assuming this same bridged structure for Fe₂RA₃ complexes, the combination of cis-trans, Δ, and Λ configurations of two iron centers connected by three RA molecules allows, in principle, 44 distinct isomers; each can be simulated by molecular models. Reverse-phase HPLC analysis of the chromic RA on a C₁₈ preparative column resulted in a six-component chromatogram (Fig. 3, Miniprint Section). The fractions were collected, re-injected, and eluted with the same gradient. Fractions 1, 2, and 3 were well resolved single peaks while fraction 4 contained a 1% contamination of fraction 3 and fractions 5, 6, and 7 contained increasing amounts of contamination from earlier components of up to 10%. The spectroscopic properties of the main HPLC fractions are summarized in Table I and Figs. 4 and 5 (Miniprint Section).

The CD spectra of these chromic siderophore complexes are composed of 3 bands (8). Their symmetry designations are based on a local point group symmetry C₉. The shortest wavelength doubly degenerate band is designated B and, except for the sign change that occurs for all 3 bands on going from a Δ to Λ metal chirality, is relatively insensitive to changes in geometry at the metal center. Similarly, the longest wavelength, nondegenerate A band is not greatly sensitive. However, the most intense, middle wavelength band, which is double degenerate, changes significantly as the geometry at the metal center changes. This, the E band, is slightly split in the trans isomers and shows changes in both relative maxima and wavelength compared to the cis isomers as first established in a series of optically active model hydroxamate complexes (18). Both main fractions (2 and 3 in Fig. 3, Miniprint Section) have visible absorption maxima very similar to those of the trans optical isomers of the model complex of tris(N-methyl-1-methoxyacetohydroxamate) chromium(III), which has bands at 416 (50) and 596 (70) nm (18). These fractions are even more similar to trans chromic desferriferroxamine B which has bands in the VIS-UV at 411 (50.7) and 589 (71.5) nm (17). We conclude that the two main fractions consist of one or more trans geometrical isomers. As the CD spectra of Fig. 5 show, the net chiralities for these two predominant isomers are opposite. Since the Δ maxima of the CD bands are the same for a wide variety of optically active hydroxamate chromic complexes, the magnitude of Δε for the Eₕ band is a measure of the optical purity of the complex. Since the Δ-trans isomer has a CD maximum at 572 nm (ε = +1.92) which is significantly smaller in absolute magnitude than the Δ-trans isomer (ε = -4.35), we must conclude that fraction 2, the Δ-trans preparation, is an unresolved mixture of Δ- and Δ-trans isomers in which the net

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2 Portions of this paper (including “Experimental Procedures,” Figs. 1 and 3–5 and Table I) 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, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. M4-3451, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
metal chirality is \( \Delta \). In any case, this is the first reported resolution of optical isomers of a siderophore complex. The principal significance is that, since the ligand remains unchanged in the resolved (or partially resolved) isomers, the sensitivity of the transport system to changes in just the metal coordination geometry can be probed.

**Stability of the Chromic Isomers of RA and Oxidation to Chromate**—In order to test the stability of the isolated chromic complexes, an aqueous pH 7.0 solution of the trans isomer of Cr\(_2\)RA\(_3\) was exposed to light at room temperature and the visible spectra recorded every 20 min. A slow increase and broadening of the high energy peak occurred. By subtraction of the original spectrum from the subsequent spectra the development of a species with a peak at 372 nm was observed; this band is typical of [CrO\(_4\)]\(^{2-}\). Although the chromate concentration after 1 h was only 0.88%, this becomes an obvious feature of the spectrum, due to the intense absorption at 372 nm. Chromate could be removed by DEAE-Sephadex (Cl\(^{-}\)) anion exchange chromatography. The process of formation of [CrO\(_4\)]\(^{2-}\) was much slower with the unseparated Cr\(_2\)RA\(_3\) complexes and did not occur in pure MeOH. After 50 h under the same conditions as described for the \( \Delta \)-trans isomers the concentration of [CrO\(_4\)]\(^{2-}\) was 1.35% (data not shown).

**Inhibition of \(^{55}\)Fe Transport by Isomers of Cr\(_2\)RA\(_3\)**—A previous investigation showed that kinetically inert chromic complexes of RA are not accumulated by *R. pilimanae* (19). However, this result does not preclude the possibility that the complexes bind to the membrane receptor but do not undergo subsequent transport. In order to probe the interaction of different separated chromic isomers of RA with the Fe\(_2\)RA\(_3\) transport system, inhibition measurements were performed. The effect of chromic isomers on the uptake of \(^{55}\)Fe mediated by RA or enantio-RA was measured. Fig. 6 illustrates that the \( \Delta \)-trans fraction of Cr\(_2\)RA\(_3\) is a stronger inhibitor of Fe\(_2\)RA\(_3\) uptake than the predominantly \( \Delta \)-trans fraction. Iron transport of \( \Delta \)-enantio-Fe\(_2\)RA\(_3\) is even more strongly inhibited by \( \Delta \)-trans-Cr\(_2\)RA\(_3\) than is \( \Delta \)-Fe\(_2\)RA\(_3\). However, no difference was observed in the inhibition caused by the geometrical isomers \( \Delta \)-cis- and \( \Delta \)-trans-Cr\(_2\)RA\(_3\).

**DISCUSSION**

In this study isomers of a metal siderophore complex have been separated for the first time. The three different isomeric fractions of chromic rhodotorulate, Cr\(_2\)RA\(_3\), have been separated by HPLC and cation exchange chromatography. This has allowed the first direct examination of the sensitivity of a siderophore receptor to change in just the metal coordination geometry. Although the chromic complexes are not transported into the cell, inhibition studies show that they do differentially interact with the receptor. The \( \Delta \)-cis and \( \Delta \)-trans preparations showed the same inhibition, which was greater than the \( \Delta \)-trans isomer. This is inferential evidence that the chirality at the metal center is more important in this receptor recognition than is the cis/trans rearrangement of ligand groups. For the ferric RA receptor a \( \Delta \) (right-handed spiral) arrangement is preferentially bound. This conclusion is supported by the observation that the chromic RA complex inhibition is more pronounced for ferric uptake mediated by enantiorthoroludoric acid, in which the ferric ion has a predominant \( \Delta \) chirality and hence binds more weakly to the receptor.

Since the maximum iron uptake rates achieved by ferric enanti-RA do not increase with metal complex concentration, the observed rate of iron uptake cannot be due to a small fraction of the (predominantly \( \Delta \)) ferric enanti-RA which is a \( \Delta \) isomer. Assume, for example, that 10% of the enanti-oRA complex were an isomeric species with a \( \Delta \) configuration and that this configuration had the same recognition and transport characteristics as the natural \( \Delta \) ferric RA. In that case a saturation in the uptake rate would not be reached until the concentration of Fe\(_3\)(enantio-RA) is 10 times that of natural Fe\(_3\)(RA) for the same saturation level. Hence the different \( V_{\text{max}} \) values for the \(^{55}\)Fe uptake of enanti-RA versus RA imply that both the \( \Delta \) and \( \Delta \) configurations at the metal center are recognized and transported, but the \( \Delta \) configuration is preferred.

A more quantitative assessment of the relative binding can be made if one assumes Michaelis-Menten kinetics (20) with the "enzyme" being the receptor sites of the microbe and the "substrate" being the ferric siderophore complex. For the ferric siderophore complex (FeL), the membrane-bound siderophore receptor (R), the siderophore bound to the receptor (FeL), and the iron transported into the cell (Fe), this kinetic model is:

\[
\text{FeL} + R \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \text{FeLR} \overset{k_2}{\longrightarrow} \text{Fe} + R + L
\]

and

\[
K_m = \frac{k_{-1} + k_2}{k_1}, \quad K_D = \frac{k_{-1}}{k_1}
\]

Double reciprocal plots of the uptake data shown in Fig. 2 are linear, with linear regression correlation coefficients (21) of 0.97 and 0.98 for RA and enantio-RA, respectively. The corresponding rate parameters for RA and enanti-oRA are: \( V_{\text{max}} \) (0.72 and 0.36 \( \mu \)mol mg\(^{-1}\) min\(^{-1}\)) and \( K_m \) (6.8 and 4.8 \( \mu \)M). Since the maximum velocity in this model is simply the rate constant \( k_1 \) times the total number of receptor sites (20), the differences in maximum uptake rates between enanti-RA and RA must be ascribed to a change in the rate constant.
$k_0$, since the total number of receptor sites is constant. Thus, within this model, the difference in recognition of RA and enantio-RA is due primarily to their differential rate of processing by the receptor rather than a difference in binding constant.

However, the chromic inhibition studies show that the above model is too simple to explain the observed behavior. If the chromic complexes which are the most effective inhibitors are assumed to have the same binding to the receptor as the ferric RA complex, then competitive inhibition should be observed. For either competitive or noncompetitive inhibition the rate of the reaction should decrease steadily with the inhibitor concentration (20). Clearly the inhibition data of Fig. 5 (Miniprint Section) are not consistent with this; for all three chromic isomers a limit in the inhibition is reached at about 20 $\mu$M complex concentration and the $^{56}$Fe uptake rate remains constant after this. Thus, either through additional transport pathways or the presence of additional steps in the transport process not included in a model such as that represented by Eq. 1, the mechanism of RA-mediated iron uptake is more complex than would seem to be implied by the simple transport kinetics.

Previous studies which have dealt with relative rates of iron uptake mediated by siderophores and their enantiomers have necessarily been unable to separate the change in recognition and transport due to the ligand chirality or the metal center, although they have confirmed a stereospecific interaction (6, 7). In the studies reported here and in the previous paper (1) and in two related studies of iron transport in *Streptomyces pilosus* (22, 23), it has been possible to demonstrate directly the important role the metal center plays in siderophore recognition and transport. In *R. pilimanae* the right-handed $\Delta$ coordination propeller of the metal center and its adjacent functionalities dominate the recognition process. The evidence is summarized as follows:

1. The RA uptake system discriminates between ferric RA and its enantiomer.
2. Inhibition of ferric RA uptake with $\Delta$-trans and $\Delta$-trans isomers of Cr$_2$RAs, which are formed from the identical ligand, is different.
3. Iron uptake mediated by an RA analog which is chemically modified in the diketopiperazine ring portion of the molecule occurs at the same rate as RA.
4. Synthetic RA analogs in which the diketopiperazine ring is replaced by $-\text{CH}_2-$ units supply iron at 50 to 80% the active transport rate of RA (the preceding paper (1)).

**Acknowledgments**—We thank Dr. David White for assistance with HPLC techniques.

**REFERENCES**

21. It is recognized that linear regression analysis of reciprocal plots involves a distortion of the weighting scheme for a true least-squares refinement, but at the confidence level we use these data the difference is insignificant.
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EXPERIMENTAL SECTION

**Materials.** Reagent grade chemicals were used for all experiments. The labile tetrahydrofuran (THF) complex Cr(III)TPP was prepared by literature methods (9). Ion exchange chromatography was performed on the anion-exchange resin CM-32 Sephadex, and anion exchange chromatography on DVB-Sephadex. Adsorption chromatography was performed on KAD-2 resin (200-250 mesh). The reagents were products of Serva; 55Fe as described previously (11). 55Fe(II) was purchased from New England Nuclear.

**Rhodotorulic Acid (RA).** Enantiomeric and an RA-analog. The RA was isolated from low iron cultures of *R. pilimanae* as described previously (10). The RA so obtained was crystallized three times from hot MeOH.

**Chromic Rhodotorulate.** The chromic complexes of RA were prepared under anaerobic conditions and all steps were carried out in the dark. Methanolic (MeOH) was distilled from Mg(OMe)2 and all chemicals were dried over P2O5. To a solution of 500 µg RA and 952.1 mg hydrogen sulphide in 50 mL MeOH was added 54.7 mg CrCl3-3H2O dissolved in 50 mL MeOH. The solution was heated at reflux under dry air (H2) drying) for 12 hrs. The resulting blue solution was concentrated to dryness in vacuo and resuspended in a small volume of glass distilled water. Salts were removed on a Sephadex resin bed and eluted with saline containing 50% MeOH. After washing several times with MeOH. The chromic RA complexes were eluted with 50% MeOH. Anions were removed by evaporation and the aqueous solution was dried. The chromic and ferric RA complexes had identical M-values (0.97) on reverse phase C18-HPLC. The solvent system was 90% MeOH containing 0.125 M formic acid.

**Preparation of Isomers.** The compound subsequently characterized as the α isomer was separated from a mixture of isomers by chromatography on CM-Sephadex (Na+-form) at 4°C. 50 µg Cr-RA was applied to the column (1.0, 0.5 cm; bed volume 20 mL). Over a period of 1.5 hrs two fractions were collected. The faster eluting band also contained CrO42- which was removed on Sephadex-G25 (II+-form). The fractions were then stored in liquid nitrogen. Chromic RA complexes were also separated by HPLC on a reversed phase C18 preparative column with an elution gradient ranging from pure MeOH to 20% MeOH in H2O over 18 min. The Shimadzu chromatograms were monitored at 254 and 578 nm and were essentially identical (although the sensitivity at 578 nm was drastically reduced). The isolated fractions were rechromatographed to insure purity. Methanol was removed by evaporation before redissolution and recording spectra of the aqueous solutions. The solution concentrations of Cr(III) were determined spectrophotometrically by oxidation to CrO42- (cmax = 4815 1·mol⁻¹·cm⁻¹) (11).

**Organisms and Growth Conditions.** Stock cultures of *R. pilimanae* and growth of cultures were the same as described previously (1).

**Transport Assays.** The 55Fe-labeled complexes of RA and its analogs were prepared by adding 55FeCl3 in 0.1 M HCl to an approximately 1% excess of ligand, and the pH was adjusted to 7. The specific activity of the 0.5 mL stock solution was 16 to 20 mCi/ml. Radiolabeled 55FeCl3 was adsorbed on aluminum backed cellulose TLC plates (Merck) which were cut into squares after development with propionatinbutane (5:95) and counted by liquid scintillation. For kinetic measurements, 10 µL aliquots of cell suspension were added to test-tubes containing increasing amounts of the 55Fe complexes in 0.1 M buffered solutions and these were vigorously shaken at 27°C. After 2 min or 10 min uptake period, the test-tubes were centrifuged with 10 mL of ice-cold 0.14 saline solution. The supernatants were applied to microcrystalline cellulose filters (3 cm pore size; Schleicher & Schuell) washed twice with saline and counted by liquid scintillation after precollimation in the liquid scintillation fluid Aquasol 2. The uptake rates were determined from the mean of the 2nd and 10th min of uptake. Equilibrium studies were performed in the same way except that the concentrations of the 55Fe complexes was 1.5 µCi in each test tube as increasingly concentrated of chromic RA complexes were added.

**Figure 1.** The structures of: a) rhodotorulate acid, b) a synthetic analog which lacks one carboxyl group, and the proposed geometry of the 3·α isomer of ferric RA.

**Figure 2.** The elution profile of trans isomers of Cr-RA on reversed phase C18-HPLC. The solvent system was a MeOH/H2O gradient which increased from 0 to 20% MeOH over 18 min. Numbers on the peaks correspond to the identified fractions of Figure 4.
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Figure 1. Absorption spectra of a cis and two trans (or mixture of trans) isomers of Cr₃⁺₄. (Fraction numbers 2 and 3 of Figure 3): α-cis chromic Cr (-); β-trans chromic Cr (- - -); γ-trans chromic Cr [- -].

Figure 2. Circular dichroism spectra of the predominant cis and trans fractions eluted by HPLC-chromatography.