Inhibitory Effect of the Partially Resolved Coordination Isomers of Chromic Desferricoprogen on Coprogen Uptake in *Neurospora crassa*[†]

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Two partially resolved chromatographic fractions of geometrical and optical isomers of the chromic complexes of desferricoprogen, a siderophore from *Neurospora crassa*, were obtained from high-pressure liquid chromatography on a reverse-phase matrix. The first fraction was identified as a *cis* complex with a 20% diastereomeric excess of the Λ isomer. The second fraction was identified as a mixture of several of the possible *trans* isomers with a net 20% diastereomeric excess of the Δ isomers. These fractions were used to evaluate the stereospecificity of the coprogen-mediated iron uptake system with respect to the metal coordination center. Fraction II competitively inhibited coprogen uptake, whereas fraction I showed only slight inhibition. *N. crassa* accumulated chromium from fraction II faster than the rate of chromium uptake from fraction I. Neither fraction had a significant effect on the uptake of ferricrocin, suggesting that coprogen and ferricrocin are taken up by different receptor systems.

The extreme insolubility of ferric ion at neutral to alkaline pH values ($K_{sp} = 10^{-38.7}$ M) (11) severely restricts the bioavailability of iron. Thus, aerobic microorganisms solubilized iron(III) by producing chelating agents called siderophores, whose metal complexes facilitate transport of iron into the cells by specific receptor-mediated uptake systems (2, 18, 20).

Investigations of the siderophore transport systems in *Escherichia coli* have been advanced through the use of available genetic mutants (2). Evidence has been provided for enantioselective recognition of Λ -*cis* ferrichrome (26), Δ -rhodotorulate (14), and Δ -*cis* enterobactin (19) (and their synthetic enantiomers) by the outer membrane receptors determined by *fhuA* (*tonA*), *fhuE*, and *fepA*, respectively. The enantiomers of ferrichrome and rhodotorulic acid display considerably decreased uptake rates (50 and 33%, respectively) compared with the natural siderophores, whereas synthetic Λ -*cis* enantio-enterobactin failed to promote growth. In addition, it has been shown that the transport process involves recognition of the metal chelate portions of the molecules, whereas the backbones have minor or no importance (1, 4, 5, 9, 10, 14, 19). Similar conclusions have been derived from experiments with *Streptomyces pilosus* and *Rhodotorula pilimanae* (16, 17; G. Müller, I. Isowa, and K. N. Raymond, J. Biol. Chem., in press; G. Müller, S. J. Barclay, D. L. White, and K. N. Raymond, J. Biol. Chem., in press).

The fungus *Neurospora crassa* excretes desferricrocin and desferricoprogen, which bind Fe(III) and are then transported intact as ferric ion complexes, as shown by Mössbauer studies and uptake kinetics (15, 24). Although it does not produce ferrichrome and ferrichrysin, *N. crassa* takes up these siderophores at rates similar to the ferricrocin uptake rate. However, synthetic Δ -*cis* enantioferrichrome is not

transported (10a, 25), thus providing evidence for strong enantioselectivity of the receptor system. Moreover, the coordination geometry of the metal ion may also influence recognition and uptake rates. Ferrichromes, of which ferricrocin is an example, are cyclic siderophores whose structures permit only *cis* geometrical isomers (Fig. 1). Linear siderophores, such as ferrioxamine B and coprogen (Fig. 1), have greater flexibility. A *cis* geometrical isomer and a *trans* isomer or a mixture of *trans* geometrical isomers of the substitution-inert chromic desferrioxamine B complexes (16) have been reported. The crystal structure of a coprogen homolog (neocoprogen) established that there is *trans* geometrical coordination at the metal center for the isolated Fe(III) complex (D. van der Helm et al., Abstr. XXII Int. Conf. Coordination Chem., 1984, p. 440). In contrast to the ferrichromes, the circular dichroism spectrum of ferric coprogen indicates that there is a predominantly Δ absolute configuration at the metal center (27).

In this study, for the first time geometrical and optical isomers of chromic desferricoprogen were partially separated by reverse-phase high-pressure liquid chromatography (HPLC). The spectrophotometric features were studied, and the interaction of the isomers with the coprogen and ferricrocin transport system was tested in a siderophore-negative mutant of *N. crassa*.

MATERIALS AND METHODS

Circular dichroism spectra were obtained with a Japan Spectroscopic Co. model J-500C recording spectropolarimeter by using a model DP-500N data-processing unit for spectral manipulations and base-line corrections. UV-visible absorption spectra were determined with a Hewlett-Packard model 8450A spectrophotometer. HPLC was performed with a Beckmann model 344 integrated HPLC system interfaced with a Gilson model 201 programmable fraction collector.

Chemicals. All chemicals were reagent grade. The tetrahydrofuran adduct of anhydrous CrCl_3 was prepared by

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FIG. 1. Structural formulas of coprogen (A) and ferricrocin (B).

using previously described procedures and was stored under dry nitrogen (3). Dimethyl sulfoxide, a nonhydrolytic solvent, was distilled under a vacuum at 45 to 50°C from CaH₂ and was stored over Linde type 3A molecular sieves under a dry nitrogen atmosphere (21). Triethylamine (Aldrich Chemical Co., Inc.) was purified by passage over a small alumina column (3 by 15 mm) immediately before use. The methods used to isolate coprogen and prepare desferricoprogen have been described previously (27). The ⁵⁵Fe complexes were prepared by adding ⁵⁵FeCl₃ in 0.1 M HCl to a 10% excess of free ligand and then adjusting the preparation to pH 7 and to a final iron concentration of 0.2 mM.

Synthesis of chromic desferricoprogen. Chromic desferricoprogen synthesis was carried out under anaerobic, anhydrous conditions by using standard a Schlenk apparatus and techniques (23) to prevent the hydrolysis of the tetrahydrofuran adduct of CrCl₃ before it reacted with desferricoprogen. Desferricoprogen · 1.5H₂O (175 mg; 220 μmol) was dissolved in 15 ml of dimethyl sulfoxide, 100 μl of triethylamine was added, and then the chromic reagent (136 mg; 362 μmol) was added to this ligand solution from an addition tube to yield a grayish purple solution, which became grayish green within 10 min. The solution was heated to 55 to 65°C for 3 h and then stirred overnight at room temperature. The solvent was removed in vacuo from the deep green solution at 55°C. The resulting dark green oil was taken up in dry methanol and evaporated to dryness in vacuo three times; this yielded a deep green film, which was dissolved in 1.5 ml of deionized distilled water. The product was chromatographed on a Bio-Rex 70 gel (Na⁺ form; 200/400 mesh; 1.3 by 15 cm; Bio-Rad Laboratories) that was equilibrated and eluted with water. The dark green eluant was concentrated to dryness in vacuo, dissolved in 1 ml of water, and chromatographed on a Bio-Gel P2 gel (400 mesh; 1.3 by 18 cm; Bio-Rad) that was equilibrated and eluted with water. The resulting greenish blue solution was lyophilized, dissolved in water, and stored in the dark at -196°C.

Partial separation of chromic desferricoprogen isomers. Partial separation of the isomers was achieved by preparative HPLC on an Altex Ultrasphere octadecylsilyl (C₁₈) reverse-phase column (1.0 by 25 cm; average particle diameter, 5 μm) in five 200-μl portions by using a flow rate of 6.0 ml/min (5,000 lb/in²) and a linear gradient of 15 to 45% methanol in water for 25 min. Peaks were collected (0.05 min per fraction) and placed at -20°C immediately after elution. Corresponding peaks from each sample were pooled and stored at -100°C until work-up. Methanol was removed

from the pooled samples in vacuo at room temperature, and the samples were frozen, lyophilized, dissolved in 250 μl of water, and kept at -196°C in the dark. Each peak was chromatographed under identical conditions and then assayed for purity on an analytical reverse-phase HPLC C₁₈ column (4.6 by 150 mm) by using the same gradient and a flow rate of 1.5 ml/min. Purified peaks were worked up as described above and stored at -196°C in the dark until they were used.

Physical measurements. Absorbance due to CrO₄²⁻ (probably from photooxidation) was removed by chromatography on DEAE-Sephadex A-25 gels (fine; 0.6 by 4 cm; Pharmacia Fine Chemicals) that were equilibrated and eluted with 0.1 M Tris hydrochloride (pH 7.4). Absorbance and ellipticity data were converted to molar extinction coefficients based on the chromium concentrations of samples, which were determined spectrophotometrically as CrO₄²⁻ (ε_{max} at 372 nm, 4,815 l mol⁻¹ cm⁻¹ [7]) alkaline oxidation by H₂O₂. Circular dichroism signal calibration was verified with (+)-Co(en)₃ · tartrate · Cl (8).

Strain, media, and growth conditions. The strain used was a *N. crassa* triple mutant (*arg-5 ota aga*) which in the absence of L-ornithine is not able to synthesize N-hydroxyornithine-containing siderophores (6). Spores were formed and harvested as described previously (6). Cultures were grown in chemically defined minimal salt medium (24) which was not supplemented with iron. After inoculation with approximately 10⁹ conidia (0.1 ml of densely packed wet cells), the cultures were grown at 27°C for 16 h. Incubation for longer times resulted in unpipettable mycelia. Typically, suspensions (250-ml) in 1-liter Erlenmeyer flasks were used for each set of experiments. After growth, the cells were washed, suspended in fresh medium, and kept on ice while being stirred. About 30 min before each experiment, 20 to 100 ml of culture was taken from the stock preparation and shaken at 120 rpm and 27°C in a water bath. Every experiment was performed twice by using the same culture.

Transport assays. The ⁵⁵Fe siderophore uptake measurements were done as described previously (24, 25). To follow time-dependent uptake of chromic coprogen isomers, 10-ml samples of a cell suspension were removed at regular intervals (15 min) from a 50-ml culture, diluted, and filtered as described previously (24, 25). Filters and mycelia were dissolved in boiling concentrated HNO₃, and their chromium contents were assayed by atomic absorption spectrophotometry.

For inhibition measurements, 0.8-ml suspensions were added at regular intervals to test tubes containing $5 \mu\text{M}$ ^{55}Fe -labeled siderophore and increasing concentrations of chromic inhibitor (5 to $50 \mu\text{M}$) adjusted to equal volumes (0.2 ml). At 2 and 8 min after uptake was initiated, 5 ml of ice-cold saline was added to each tube. Then the cells were filtered, washed, and counted as described previously (25). To prevent isomerization of the chromic complexes, isomers were kept in liquid nitrogen and added to the assay mixture shortly before the cells were added. Control experiments showed that uptake of coprogen and ferricrocin were linear for 10 min at both limiting concentrations of inhibitor (data not shown).

RESULTS AND DISCUSSION

Separation and characterization of chromic coprogen isomers. A representative preparative chromatogram of a crude mixture (Fig. 2) showed two major peaks, designated fractions I and II, which had retention times of about 12.5 and 13.7 min, respectively. Each fraction was chromatographed again, and the analytical HPLC chromatograms (Fig. 2) indicated that fraction I was relatively pure, whereas fraction II was a mixture of at least three isomers (although more may have been superimposed and not separated on this stationary phase). Chromatography on C_8 reverse-phase

FIG. 2. (A) Chromatography of crude chromic desferricoprogen mixture on a preparative high-pressure liquid reverse-phase C_{18} column (Altex Ultrasphere $5 \mu\text{m}$; 1.0 by 25 mm). The following conditions were used; sample size, $100 \mu\text{l}$ (13 mM solution); flow rate, 6.0 ml/h; gradient, 15 to 45% methanol in water; 25 min; ambient temperature. (B and C) Chromatography of fraction I (B) and fraction II (C) from the preparative column on an analytical C_{18} column (Altex Ultrasphere $5 \mu\text{m}$; 4.6 by 150 mm). The conditions were the same as those described above, except that a flow rate of 1.5 ml/min was used. The sample size was $5 \mu\text{l}$ of the collected preparative fraction collected.

FIG. 3. Absorption (A) and circular dichroism spectra of partially resolved isomers of chromic desferricoprogen in 0.1 M Tris hydrochloride (pH 7.4). The solid lines indicate the fraction I (Λ) isomer, and the dashed lines indicate the fraction II (Δ) isomer. See text for assignments.

columns gave the same pattern of peaks, but the peaks were more closely spaced. Chiral agents in the mobile phase did not cause any significant broadening of the peaks.

The UV-visible and circular dichroism spectra of fractions I and II are shown in Fig. 3, and the essential features of the fractions are summarized in Table 1. Fraction I had a diastereomeric excess of the Λ configuration about the metal, while fraction II had the opposite (Δ) configuration, as judged from the sign of the higher-energy E_d symmetry band at 560 nm for a trigonal d^3 complex (8). The circular dichroism intensity of these fractions was only 20% of the intensity of the previously prepared Tris-hydroxamate com-

TABLE 1. Absorption and circular dichroism spectra of the partially resolved isomers of chromic desferricoprogen^a

Fraction	Absorption spectrum ^a		Circular dichroism spectrum ^b	
	Maxima (nm)	ϵ ($\text{mol}^{-1} \text{cm}^{-1}$)	Maxima (nm)	$\Delta \epsilon$
I (<i>cis</i>) ^b	406	88.2	418	-0.73
	584	82.7	561	+1.61
			654	-1.01
II (<i>trans</i>) ^b	406	82.7	414	+0.50
	588	85.3	567	-1.77
			666	+0.47

^a Spectra were determined in 0.1 M Tris hydrochloride (pH 7.4) at room temperature by using a 1-cm path length.

^b See text for assignments.

FIG. 4. Lineweaver-Burk plot indicating the concentration dependence of the uptake of ^{55}Fe -labeled coprogen in the presence (●) and absence (○) of an unseparated mixture of chromic coprogen isomers (100 μM). Cells of *N. crassa* (10 ml) were incubated in a rotary shaker bath at 27°C with 10 μM ferric coprogen. At intervals 0.5 ml of the mycelium suspension was treated as described in Materials and Methods.

plexes (which had absolute intensities of 7.8 to 8.2 $\text{mol}^{-1} \text{l cm}^{-1}$ [12]). The UV-visible extinction coefficients and band positions for these fractions were of the correct magnitude compared with the model Tris-hydroxamate complexes containing conjugated systems (13).

Fraction I was tentatively identified as a diastereomeric mixture of one of the two possible *cis-cis* isomers, whose higher polarity was consistent with a shorter retention time on a reverse-phase matrix. The single peak observed on the analytical column and the reduced circular dichroism intensity was consistent with a diastereomeric mixture (60% Λ , 40% Δ) of one coordination isomer. The polarity and lipophilicity differences between diastereomers were expected to be subtle and were not distinguished on this

FIG. 5. Uptake of ^{55}Fe -labeled coprogen (●) and partially separated chromic desferricoprogen fractions I (▲) and II (■). Cr uptake was determined by atomic absorption (see text).

nonchiral matrix. The higher extinction coefficient of the high-energy visible band was consistent with the higher oscillator strength of the *cis* chromic complexes (12).

Fraction II was identified as a mixture of several of the *trans* isomers, as indicated by the multiple peaks in the analytical HPLC gels. The retention times for the *trans* isomers were expected to be similar, as we observed, and to be impossible to separate with the system which we used. The circular dichroism spectrum of fraction II exhibited some broadening of the higher-energy band, which indicated that there was some splitting of the $^4\text{A}_2 \rightarrow ^4\text{T}_1$ to an E-and-A band expected for *trans* complexes.

Uptake studies. An initial experiment showed that uptake of ^{55}Fe -labeled coprogen was markedly inhibited when an unseparated mixture of chromic desferricoprogen was added 4 min after the start of the assay (data not shown). Concentration-dependent uptake of ^{55}Fe -labeled coprogen in the presence of unseparated chromic isomers (25 μM) indicated that uptake was competitively inhibited by chromic coprogen (Fig. 4). Since in this experiment a mixture of isomers was tested, the competitive species could not be identified.

The time-dependent uptake of ^{55}Fe -labeled coprogen and the chromic coprogen fractions is shown in Fig. 5. The accumulation of the kinetically inert chromic complexes agreed well with a mechanism proposed previously, which excluded ligand exchange or reduction. (After 60 min, 5.5 nmol of ferric coprogen per mg [dry weight], 4.9 nmol of fraction II per mg [dry weight], and 1.8 nmol of fraction I per mg [dry weight] were assimilated.)

Figure 6 shows the inhibition of coprogen and ferricocin uptake in *N. crassa* by the isolated fractions. Fraction II strongly inhibited ferric coprogen uptake, even at low inhibitor concentrations. In contrast, increasing the concentration of fraction I decreased the level of ferric coprogen accumulation slowly; uptake was decreased by 25% when there was a fivefold excess of inhibitor (50 μM). At the same concen-

FIG. 6. Inhibition of ^{55}Fe -labeled coprogen uptake by increasing concentrations of fractions I (○) and II (●). Uptake of 5 μM ^{55}Fe -labeled coprogen without any inhibitor was used as a reference (100% = 56 pmol of Fe per mg per min). In addition, the inhibitory effects of fractions I (Δ) and II (▲) on the uptake of ^{55}Fe -labeled ferricocin were tested.

tration the inhibitory effect of fraction II was as high as 67% of the initial uptake rate without any inhibitor.

The low accumulation of fraction I may have been due to geometrical or optical isomerism. Unfortunately, as neither fraction was optically pure, no quantitative conclusions could be drawn regarding the effect of chirality about the metal center on the recognition process. However, our data indicate that a *cis-cis* geometrical isomer is not recognized or transported as efficiently as at least one of the *trans* isomers contained in fraction II. Taken together, the competitive inhibition and transport rates of the chromic mixtures strongly suggest that chromic desferricoprogen is recognized and transported by the same receptor used for coprogen. Furthermore, this receptor exhibits a preference for a *trans* isomer rather than the *cis-cis* isomer.

The level of inhibition of ferricrocin uptake by the isolated chromic coprogen isomers was small compared with the effect on ferric coprogen. Furthermore, no difference in the inhibitory effect of either set of isomers was observed. Therefore, chromic coprogen does not compete effectively for ferricrocin-mediated ferric ion accumulation. This finding strongly suggests that there are two independent uptake systems for the ferric complexes of ferricrocin and coprogen.

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