Molecular Recognition of Siderophores in Fungi: Role of Iron-Surrounding N-Acyl Residues and the Peptide Backbone during Membrane Transport in Neurospora crassa

HANS-GEORG HUSCHKA,1 MAHBUBUL A. F. JALAL,2 DICK VAN DER HELM,2 AND GÜNTHER WINKELMANN1*

Institut für Biologie I, Mikrobiologie I, Universität Tübingen, Tübingen, Federal Republic of Germany,1 and Department of Chemistry, University of Oklahoma, Norman, Oklahoma 730192

Received 26 December 1985/Accepted 9 June 1986

Recognition of ferric siderophores in Neurospora crassa was found to depend on the number and kind of N-acyl residues that surrounded the iron coordination center. In the coprogen series, uptake decreased in the order of coprogen, neocoprogen I, and neocoprogen II, indicating that gradual replacement of the N-trans-anhydroacetyl groups had an adverse effect on uptake. The reverse effect was observed in the ferrichrome series, where uptake decreased in the order of ferrichromes, asperchrome D1, asperchrome B1, and ferrirubin. Configuration of the anhydroacetylvalon group (cis or trans) in ferrichromes was also an important determinant in the recognition process. On the basis of uptake and inhibition studies, it is proposed that in ferrichromes part of the molecule (iron configuration and the N-acyl groups) is responsible for binding, whereas another (cyclic peptide ring) is involved in the subsequent process of transport.

Siderophores are natural compounds designed for chelation and transport of iron (III) in microorganisms. Fungi synthesize a variety of siderophores with different structural backbones such as ferrichromes, coprogens, and fusigens (fusarinins); these vary depending on the strain and the medium and cultivation conditions used. A number of reviews of the structures and functions of siderophores have been published recently (5, 11, 14). A characteristic feature of the ferrichrome-type siderophores is their cyclic peptide backbone, which forces the metal center to adopt a Δ-cis absolute configuration (12, 15, 16). On the other hand, coprogens adopt a Δ-trans configuration (2, 15; M. B. Hos-sain, M. A. F. Jalal, B. Benson, C. L. Barnes, and D. van der Helm, Abstr. 23rd Int. Conf. Coordination Chem., p. 440, 1984). Despite the different structures and absolute configurations about the metal center, coprogens and ferrichromes have revealed competition during uptake in Neurospora crassa, confirming a shared transport system (6). However, uptake of coprogen and competition of coprogen with ferrichromes have not been found in Penicil-lium parvum, indicating that this fungus does not recognize coprogen-type siderophores. From these results it was inferred that recognition and transport are distinct events.

Recent findings point to the importance of the iron-surrounding N-acyl residues for recognition and transport to trihydroxamate siderophores (3, 4). The predominant hydroxamic N-acyl residue of ferrichromes is acetic acid (AC). Another commonly found acid is 5-hydroxy-3-methylpent-2-enoic acid, known as anhydroacetylenic acid (AM). Its configuration may be trans as in coprogen (three trans-AM) and ferrirubin (three trans-AM) or cis as in fusigen (three cis-AM) and ferrirubin (three cis-AM).

A common feature of all these siderophores is the homogeneity of the N-acyl residues. By use of improved chromatographic methods it was possible to isolate a variety of minor siderophores containing heterogeneous iron-surrounding (outer) N-acyl residues (see Fig. 1). Thus, neocoprogen I (one AC, one trans-AM) and neocoprogen II (two AC) were isolated from Curvularia subulata (9; Hos-sain et al., Abstr. 23rd Int. Conf. Coordination Chem.); these differ from coprogen (two trans-AM) isolated from N. crassa (10). Aspergillus ochraceus produces, in addition to ferrichrysin and ferrirubin, a collection of asperchromes possessing heterogeneous N-acyl residues (8). Asperchromes D1 (two AC, one trans-AM) and B1 (one AC, two trans-AM) are of particular value for investigating the role of N-acyl residues during recognition and transport of siderophores in N. crassa and other fungi. Des(iserylgly-cyl)ferrirhodin (DDF) isolated from A. ochraceus (7) presents a different case, in which the iron coordination center (A-cis) and the N-acyl groups (three cis-AM) are similar to those of ferrirhodin but the characteristic peptide ring of ferrichrome is absent. The natural occurrence of siderophores possessing heterogeneous N-acyl residues and the absence of the peptide ring in DDF enabled us to study in more detail the process of recognition between coprogens and their receptor and ferrichromes and their receptor. The present investigation is aimed at answering the following questions. How specific is the recognition of the N-acyl residues, and what is the role of the peptide part of the ferrichrome-type siderophore molecule?

MATERIALS AND METHODS

Cultures and maintenance. N. crassa strains were obtained from the Fungal Genetic Stock Center, Arcata, Calif. The ornithine-free mutant N. crassa (arg-5 ota aga) was a gift from R. H. Davis, Irvine, Calif. Penicillium variabile Sopp CBS 385.48 and Fusarium dimerum Penz var. pusillum CBS 254.50 were from the Centraalbureau voor Schimmel cultures. A. melleus Tü 142 was from the stock of the Institut für Biologie, Mikrobiologie I, Universität Tübingen, Tübingen, Federal Republic of Germany; Neurospora indica was kindly provided by Günther Deml, Universität Tübingen; and A. ochraceus and C. subulata were from the stock of the Department of Chemistry, University of Oklahoma, Norman.

* Corresponding author.
VOL. 167, glucose, containing harvested and used for (6).

earlier cultures of dride. adding a B Coprogen obtained from cultures Ferrichrysin was described previously and purified from (6).

scribed (CDDF) DDF filtrate of established and separated (7). Neocoprogens previously magnetic proton 23rd and resonance magnetic Storage-Metab., 1985), respectively.

FIG. 1. Structures of siderophores used in kinetic studies: a, ferrichromes; b, coprogens; c, DDF and CDDF.

All fungal strains were maintained on YMG agar slants containing 4 g of yeast extract, 10 g of malt extract, 4 g of glucose, and 15 g of agar per liter. The incubation temperature was 27°C. Conidia of N. crassa (arg-5 ota aga) were harvested and used for kinetic measurements as described earlier (6).

Siderophores. Ferrichrome was prepared from low-iron cultures of Neovossia indica as previously described (3). Ferrichrysin was isolated from A. melius. Ferrirubin was obtained from cultures of P. variabile. Coprogen was isolated from N. crassa wild type 74A as described earlier (15). Coprogen B was obtained from cultures of F. dimerum and acetylated with [1-14C]acetic anhydride as previously described (6). The procedure was altered to some extent by adding a bicarbonate solution for removing unreacted anhydride. The asperchromes B1, B2, C1, D1, D2, and DDF were isolated and purified from culture filtrates of A. ochraceus as previously described (7, 9). Ferrirhodin was kindly provided by W. Keller-Schierlein, Eidgenössische Technische Hochschule Zürich, Zürich, Switzerland.

N-Acetyl DDF and N-acetyl DDF methyl ester or covered DDF (CDDF) were prepared from DDF as described earlier (7). Neocoprogens I and II were extracted from the culture filtrate of C. subulata by the chloroform-phenol method (8) and separated by the chromatographic procedure described previously (9). The structures of neocoprogens I and II were established by X-ray crystallography (Hossain et al., Abstr. 23rd Int. Conf. Coordination Chem.) and high-resolution proton magnetic resonance spectroscopy or 13C nuclear magnetic resonance spectroscopy (M. A. F. Jalal, M. B. Hossain, and D. van der Helm, 1st Int. Symp. Iron Transp.-Storage-Metab., Tübingen, Federal Republic of Germany, 1985), respectively.

Analytical methods. Siderophores were quantitated spectrophotometrically by using known extinction coefficients (5, 8, 11, 15). Thin-layer chromatography was performed on Silica Gel 60 plates (E. Merck AG, Darmstadt, Federal Republic of Germany) with chloroform-methanol-water (65:25:4). Mycelia were filtered on cellulose nitrate membrane filters (8.0-µm pore size) (Sartorius, Göttingen, Federal Republic of Germany). Desferrisiderophores were prepared by the hydroxyquinoline method. 55Fe-labeled siderophores were prepared with 55FeCl3 in 0.1 M HCl (carrier free) purchased from Radiochemical Centre, Amersham, England. The procedures used for preparation of radiolabeled siderophores and uptake measurements have been described earlier (6).

RESULTS

When 55Fe-labeled coprogen or neocoprogen I or II (Fig. 1b) was added to a culture of young mycelia of N. crassa (arg-5 ota aga) grown under iron limitation and in the absence of ornithine to suppress siderophore biosynthesis, decreased uptake was observed in the order of coprogen, neocoprogen I, and neocoprogen II (Fig. 2a), indicating that the iron-surrounding N-acetyl (AC) residues possessed lower biological activity than the trans-anhydromevalonyl (AM) residues. That the N-acetyl residues were not devoid of biological activity was confirmed by the finding that neocoprogen II, possessing N-acetyl residues at both ends of the molecule, retained at least 50% of the transport activity found with coprogen. Neocoprogen I, possessing one N-acetyl and one N-trans-AM residue in these positions behaved as an intermediate between coprogen and neocoprogen II, retaining about 75% of the coprogen transport activity. Coprogen-mediated 55Fe transport reached saturation levels earlier than that mediated by either neocoprogen I or II (Fig. 2a).
Inhibition of [14C]coprogen uptake by unlabeled coprogen corresponded well with the theoretical value of 50% at equimolar concentrations (10 μM) (Fig. 2b). Neocoprogen I was less effective as a competitive inhibitor of coprogen uptake, resulting in 37% inhibition at a 10-μM concentration. Substitution of both of the terminal N-trans-anhydromevalonyl residues by N-acetyl groups (neocoprogen II) resulted in only 21% inhibition of coprogen uptake at an equimolar concentration (Fig. 2b). In general, competitive inhibition of [14C]coprogen uptake by the neocoprogen corresponded well with the above-described uptake kinetics determined by using 55Fe-labeled siderophores. Transport experiments with 55Fe-labeled ferrichrome-type siderophores (Fig. 1a) possessing identical cyclic peptide backbones (-Ser-Ser-Gly-Orn-Orn-Orn-) and different N-acetyl residues, e.g., ferrichrysin (three AC), asperchrome D1 (two AC, one AM), asperchrome B1 (one AC, two AM), and ferrirubin (three AM) revealed the following order of transport rates in N. crassa: ferrichrysin > asperchrome D1 > asperchrome B1 > ferrirubin (Table 1). The successive substitution of three acetyl groups by trans-AM groups led to a corresponding successive decrease in transport rates. However, in contrast to the neocoprogen, in which trans-AM residues enhanced transport, complete substitution of N-acetyl groups by N-trans-AM groups in ferrichrysin yielded a nearly inactive siderophore known as ferrirubin. Besides being the siderophore possessing the lowest transport activity in N. crassa, ferrirubin also inhibits uptake of all other siderophores in this fungus.

Inhibition experiments performed with [14C]coprogen in the presence of ferrichrysin, asperchrome D1, asperchrome B1, and ferrirubin (Table 1) revealed that in this case transport of coprogen was more strongly affected by ferrichromes possessing N-trans-AM residues than by ferrichromes possessing N-acetyl residues. Furthermore, inhibition increased with increasing degree of substitution by trans-AM. At an equimolar concentration, inhibition of coprogen uptake was 60% by ferrichrysin, 92% by asperchrome D1, 96% by asperchrome B1, and 97% by ferrirubin.

To investigate whether the presence of an N-acetyl chain in a particular position in the molecule is important, the inhibitory activities of asperchromes B1 and B2 were compared. Both asperchromes B1 and B2 possess one AC and two trans-AM groups, but in asperchrome B1 the AC group is on ornithine 1, which is in the vicinity of the peptide backbone, whereas in asperchrome B2 this group is on either ornithine 2 or ornithine 3, which are positioned at the outside (Fig. 1). In uptake experiments, asperchromes B1 and B2 behaved quite similarly (data not shown). The same applied to asperchromes D1 and D2, each of which contains two AC groups and only one trans-AM group but at different positions. Acetylation of one trans-AM group of ferrirubin, as found in asperchrome C, reduced inhibition slightly compared with ferrirubin (data not shown).

Ferrirhodin contains three cis-AM groups instead of the trans-AM groups found in ferrirubin. Uptake (and also inhibition) rates by these compounds were quite different (Table 1). Ferrirhodin was taken up quite well, similar to ferrichrysin, but its inhibition of coprogen uptake (74%) was considerably less than that of ferrirubin (97%).

DDF (Fig. 1c), in contrast to ferrirhodin, was not taken up by the fungus (Table 1). Its inhibition of coprogen uptake (17%) was less effective than that by ferrirubin (74%). N-Acetyl DDF and CDDF were also not taken up; they inhibited coprogen uptake 50 and 72%, respectively.

**DISCUSSION**

Our previous results (2, 6) indicated quite clearly that two different receptors are operative in N. crassa, one for Δ-trans coprogen and the other for Δ-cis ferrichromes. In addition, a common transport system was postulated for both types of siderophores, although a strong allosteric influence of both receptors remains an alternative possibility. Stereospecificity of the uptake phenomena was very clear in that enantiomeroferricrocin (Δ-cis) was not taken up and did not inhibit coprogen uptake (6, 13). The present experiments elaborate on these results and further investigate the influence of the N-acyl groups of both the coprogen and ferrichromes, as well as in the latter case, the possible function of the peptide portion of the molecule.

The gradual decrease in uptake observed for neocoprogen I and II clearly indicated the importance for the

---

**TABLE 1. Uptake of siderophores and their inhibition of [14C]coprogen uptake in N. crassa**

<table>
<thead>
<tr>
<th>Siderophore</th>
<th>Uptake (nmol/mg of dry wt after 15 min)</th>
<th>Inhibition of coprogen uptake (% of [14C]coprogen uptake at equimolar concn = 10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrichrysin</td>
<td>1.06</td>
<td>59</td>
</tr>
<tr>
<td>Asperchrome D1</td>
<td>0.59</td>
<td>92</td>
</tr>
<tr>
<td>Asperchrome B1</td>
<td>0.10</td>
<td>96</td>
</tr>
<tr>
<td>Ferrirubin</td>
<td>0.07</td>
<td>97</td>
</tr>
<tr>
<td>Ferrirhodin</td>
<td>1.02</td>
<td>74</td>
</tr>
<tr>
<td>DDF</td>
<td>0.037</td>
<td>17</td>
</tr>
<tr>
<td>N-Acetyl DDF</td>
<td>0.040</td>
<td>50</td>
</tr>
<tr>
<td>CDDF</td>
<td>0.041</td>
<td>72</td>
</tr>
<tr>
<td>Coprogen</td>
<td>1.20</td>
<td>50</td>
</tr>
<tr>
<td>Neocoprogen I</td>
<td>0.95</td>
<td>37</td>
</tr>
<tr>
<td>Neocoprogen II</td>
<td>0.46</td>
<td>21</td>
</tr>
</tbody>
</table>

---

**FIG. 2.** (a) Rate of 55Fe-siderophore uptake and (b) inhibition of [14C]coprogen uptake in N. crassa. Symbols: ●, coprogen; ●, neocoprogen I; ■, neocoprogen II.
coprogen system of the N-acetyl groups in either the initial receptor process or subsequent transport. One explanation for their reduced uptake (and also reduced inhibition of coprogen uptake) is the replacement of AM groups by AC groups renders the iron coordination surrounding of the siderophore less amenable to interaction with the receptor system. The coprogens and ferrichromes have different structures. In the coprogens the iron is in the center of the disk-shaped molecule, whereas in the ferrichromes the iron is at the side of the molecule, separated from the cyclic peptide. The influence of the N-acetyl groups is quite different in the ferrichromes than in the coprogens. In addition, there seems to be a definite function of the peptide backbone in the ferrichromes.

Replacing one (B1 and B2) or two (D1 and D2) of the trans-AM groups with AC groups had a significant influence on uptake. However, the change had remarkably little influence on inhibition of coprogen uptake, which was 97% for ferrirubin and 92% for asperchrome D1 possessing only one AM residue. Increasing the size of one AM group (asperchrome C) had little influence at all. It indicates that one and at the most two trans-AM groups are sufficient to inactivate the postulated common transport system.

The distinct difference between ferrirubin and ferrirubin in both uptake and inhibition indicates that the difference in the configuration (Fig. 3) of the N-acetyl groups (cis and trans-AM) rather than their bulk is important. In addition, it is quite likely that the AM groups occur in a distinct conformation. Crystallographic data (1) showed that the torsional angle $\phi$ of the O=C-C=C bond (Fig. 3) is always close to $0^\circ$. For the trans-AM group this was observed 18 different times in three independent ferrirubin molecules (9 times), asperchrome A (3 times), asperchrome B1 (2 times), asperchrome C (3 times) and asperchrome D1 (1 time), whereas the same observation was made for the cis-AM group in the structure of ferrirubin. Furthermore, X-ray crystal structure analyses of a large number of ferrichromes have proven that the conformation of the peptide backbone is stable and independent of the N-acetyl groups. The distinct conformations of the AM groups give quite different appearances to the iron-surrounding residues of ferrirubin and ferrirubin. This is therefore the most obvious feature responsible for the difference in transport between ferrirubin and ferrirubin.

The function of the cyclic peptide portion of the ferrichromes and asperchromes can be most directly investi-

gated from the results on ferrirubin and DDF. In DDF three ornithyl residues (with three cis-AM groups) form a tripeptide which is identical to the ornithyl part of ferrirubin. The iron coordination geometry of DDF, determined by circular dichroism spectroscopy (data not shown), was found to be $\Lambda$ as seen in all ferrichromes. However, it lacks the cyclic peptide ring present in ferrirubin and other ferrichromes. To eliminate the effect of the dipolar ion in DDF, N-acetyl DDF and CDDF were prepared and found to be similarly inactive in uptake but increasingly effective as inhibitors of coprogen uptake. The fact that CDDF was not taken up and ferrirubin was, whereas their inhibitory properties were quite similar, confirmed that the peptide portion is important for the function.

Although we have shown in a previous paper (6) that bulky residues in the peptide portion do not seriously impair the transport properties of ferrichromes, the present investigation clearly indicates that the cyclic nature of the peptide portion is a necessary requirement for the transport process. It appears that the peptide is not important for initial recognition but is essential for further transport across the membrane. The suggestion that part of the molecule (iron configuration and N-acetyl groups) is responsible for binding, whereas another part (the peptide) is involved in the process of subsequent transport is in agreement with the kinetic aspects of a specific transport mechanism.

The strong (more than 90%) inhibition of coprogen uptake found for ferrirubin and asperchromes B1, B2, D1, D2, and C is probably caused by one or more of the trans-AM acyl groups occurring in a preferred conformation. However, the variation of the number of trans-AM acyl groups in these compounds indicates that the binding causing inhibition is not a perfect hand-to-glove type of binding.

The overall picture that part of the molecule being transported is responsible for binding, whereas another part is responsible for response, i.e., further transport, is probably a general feature of biological phenomena involving the processing of small molecules by macromolecules.

ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft, Wi 628/3-1 (G.W.), and by Public Health Service grant GM 21822 (D.v.d.H.) from the National Institutes of Health.

LITERATURE CITED


