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Molecular recognition and signaling

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Abstract: Molecular recognition and signaling are at the heart of biological processes as they govern intercellular communication and information transfer. Here we probe the recognition phenomena of siderophore-mediated microbial iron(III) uptake, which involves two recognition processes: recognition of iron(III) by microbial siderophores (iron(III) carriers), and recognition and binding of siderophore-iron(III) complexes by microbial membrane receptors. In addition, we introduce fluorescent siderophore analogs that respond to iron binding with changes of their fluorescence and thereby provide artificial binders that combine iron recognition with signaling.

Introduction

Almost no living cell could exist without iron, as iron is involved in several fundamental enzymatic reactions such as oxygen metabolism, electron transfer processes, DNA and RNA synthesis. In order to guarantee adequate iron(III) (ferric ion) uptake, microorganisms have developed ingenious tools, such as iron(III) carriers or siderophores[1-7].

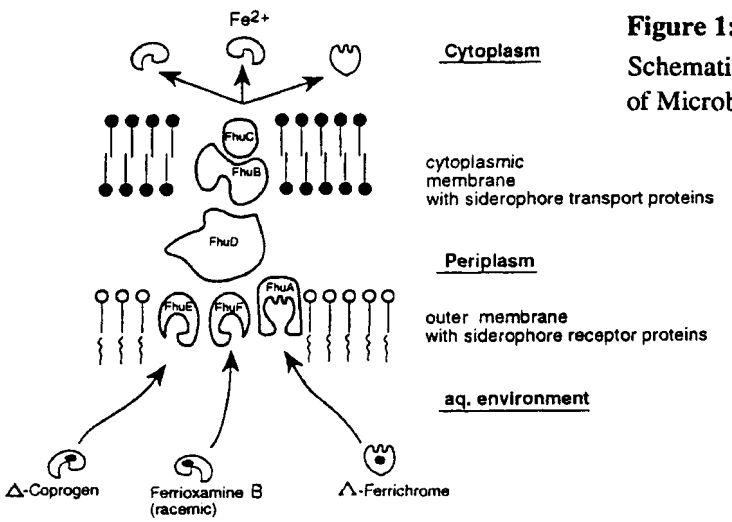

Figure 1: Schematic Representation of Microbial Membranes

Although siderophores may vary widely in chemical composition and geometry, they all function by the same three essential elements: high iron(III) binding selectivity, high iron(III) binding efficiency and high membrane permeability as iron(III) complexes. Most remarkably, siderophore mediated cellular iron(III) uptake is not diffusion controlled, but an energy dependent process which necessitates binding of the siderophore-iron(III) complexes to specific membrane receptors and transport proteins[8-12] (Figure 1). This process guarantees meticulous control of the intracellular iron(III) level within a narrow window and allows its accumulation against unfavorable concentration gradients. The iron(III) carrier properties and biological activity of siderophores are thus dictated by their three-dimensional structure and chirality, and the extent to which their shape fits specific membrane proteins.

The importance of iron for the proper function of almost all living systems, and the high specificity of natural siderophores in terms of their iron binding capacity and receptor recognition capability, prompted us to examine microbial recognition phenomena with all-artificial siderophore-analogs as structural probes. In the following

we wish to outline the principles of design and synthesis we pursued, describe the *in vitro* and *in vivo* properties of the compounds prepared and shortly indicate the potential of fluorescent siderophore conjugates as diagnostic tools.

Ferrichrome and Ferrichrome Analogs

As one of our first siderophore targets we selected the Ferrichrome molecule because of its broad range of activity.[1-3] Although produced only by fungi, it is taken up by a large variety of other microorganisms that have developed Ferrichrome-sensitive iron(III) uptake systems. We thus have a versatile setup to examine the biological activity of synthetic Ferrichrome analogs, and simultaneously to probe the structural characteristics of membrane receptors of different microbial origin.

In order to obtain biomimetic Ferrichrome analogs the non-symmetric hexapeptide ring of the natural Ferrichrome was replaced by C3-symmetric triscarboxylates as anchors. C3- symmetric extension of these anchors with amino acid residues allowed for systematic modifications by varying the nature of the amino acid residue, and enabled control of the complexes' preferred chirality. Termination with hydroxamate groups provided the ion binding sites (Figure 2)[13;14].

A unique element in this design is the use of amino acids as structural elements. The amino acids fulfill two major functions: They enable systematic modifications of the molecules' envelopes by varying the sidechains of the amino acid residues and impart chiral preference of choice by using either L or D amino acids. The latter feature is of paramount importance in view of the documented chiral discrimination of siderophore receptors.[1;11;12]

Following this principal design two families of analogs were prepared , Type 1 (m = 1) and Type 2 (m = 2), that are based on two homologous triscarboxylates as anchor (Figure 2)[13;14;15] Spectroscopic examination of the two types of analogs paired with molecular modeling showed, that both types are quite similar in the free state and adopt propeller-like conformations via intra-molecular H-bond networks[13]. On the other hand, the iron(III) complexes of the two types differ significantly. Although both types of compounds form complexes of L-cis configuration when built from L-amino acids, the complexes' envelopes differ in two major aspects: Type 1 complexes have their amide groups tangentially positioned and the pending side chains upward oriented, thus partially screening the iron binding domain. Type 2 complexes have their amides radially positioned with the amide NH groups H-bonded to the complexes' ether oxygens, and the side chains similarly radially oriented, leaving the iron binding domain exposed (Figure 3).

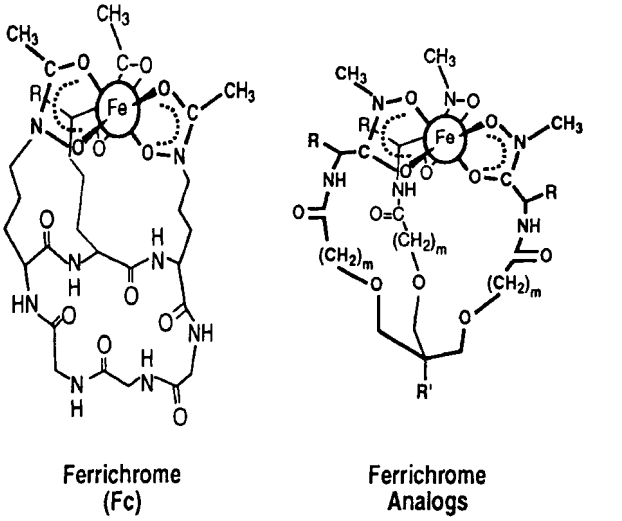

Figure 2: Principal Design of Ferrichrome Analogs

Figure 3

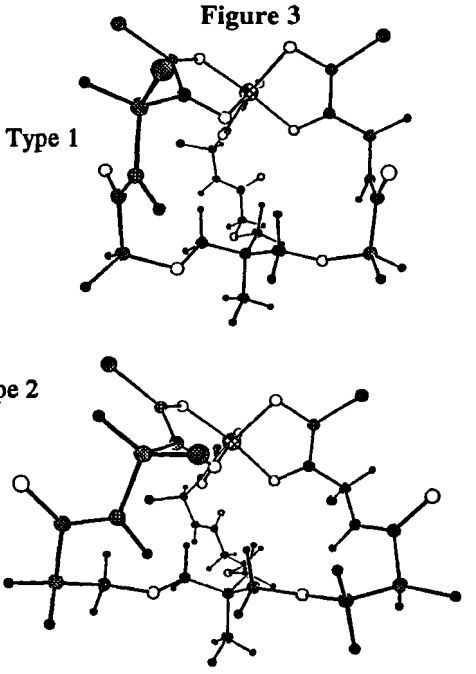

Figure 3: Calculated lowest energy conformations of Ferrichrome Analogs (Type 1, top; Type 2, bottom).

In light of these structural differences, we sought to compare both types' microbial activity. Towards this end we examined the analogs' effects on whole microorganisms in comparison with that of the natural siderophores[16-20]. For these tests we selected mutants that possess receptors for specific siderophores, but do not produce siderophores. These mutants cannot absorb iron(III) and can not grow, unless supplied with siderophores or siderophore analogs. We examined microbial growth as well as the rate of carrier mediated microbial iron uptake of radioactive iron(III) (^{55}Fe). The growth experiments gave the potency of the synthetic compounds, as compared to the natural siderophores. The radioactive iron(III) uptake experiments provided quantitative information on the compounds' activity as iron(III) carriers. In order to establish whether the observed iron(III) uptake involves the intervention of receptors and transport proteins, we then measured their dependence on NaN_3 and related agents, which inhibit energy driven processes. Finally we identified the nature of the specific siderophore receptor involved by competition experiments between the synthetic and the natural siderophores. Alternatively, the activity of the synthetic compounds was compared on a set of related mutants that possess only a single type of receptors.

When examined on mutants of *Pseudomonas putida*, representatives of Type 2 proved active,[18] while none of the Type 1 analogs showed any activity (Figure 4). For a siderophore or a siderophore analog to act as microbial iron(III) carrier, it has to first bind to the respective receptor of the outer membrane and then to the transport proteins of the cytoplasmic membrane. Most remarkably, the Type 2 analog derived from glycine (R = H) fully reproduced the action of Ferrichrome by promoting microbial iron(III) uptake and growth with almost the same efficiency, thereby functioning as agonist. Type 2 analog derived from L-alanine (R = Me) inhibited Ferrichrome mediated iron(III) uptake, thereby functioning as antagonist. The enantiomer derived from D-alanine had no effect. These results suggest that the glycine derivative of Type 2 is recognized by the microbial membrane receptor and activates the transport system, while the L-alanine derivative of Type 2 merely binds to the membrane receptor such as to inhibit the action of Ferrichrome.

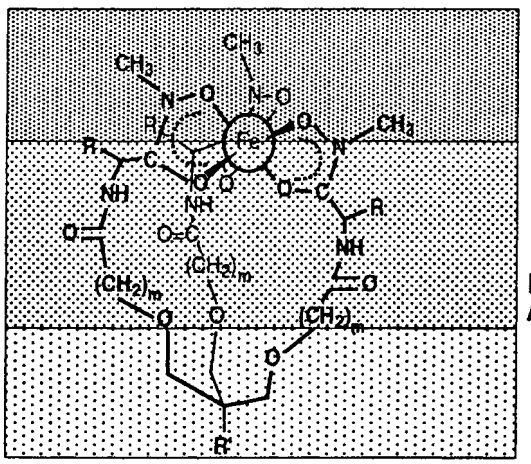

Figure 4: Microbial Activity of Ferrichrome Analogs.

Other organisms possessing Ferrichrome receptors responded similarly, but not identical, to the synthetic ferrichrome analogs. When examined on *Arthrobacter flavescens*, the L-alanine derivative of Type 2 proved not to act as inhibitor, but as full substitute of Ferrichrome[16]

At this stage it occurred to us, that it might be advantageous to attach a fluorescent marker to the siderophore analogs in order to couple iron recognition with signaling. Towards this end we linked aminoquinoline to the bioactive derivatives (Type 2, R = H, R = Me) and examined the binders' behavior.

UV/Fluorescence titration with iron(III) established quenching of the label's fluorescence upon iron loading (Figure 5). Conversely, release of the bound iron by treatment with EDTA restored the label's fluorescence. These experiments demonstrate,

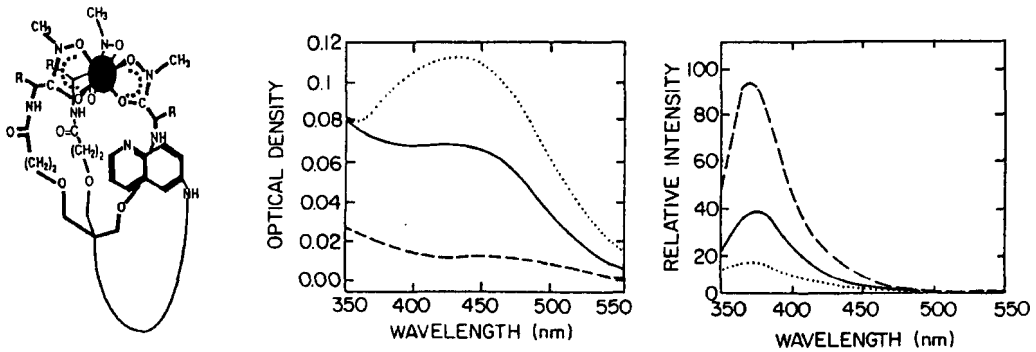

Figure 5: UV/Fluorescence Titration of Fluorescent Ferrichrome Analog

that the fluorescence of the marker is quenched by intramolecularly bound iron, and thereby encourage the use of such carriers as tools to monitor iron exchange processes *in vivo*.

Conclusions

The implications of the results described above are manifold. They illustrate the usefulness of synthetic binders as structural probes and demonstrate that it is indeed possible to match with all-synthetic iron(III) binders microbial iron uptake systems. The above data also indicate that the structural characteristics of microbial iron(III) uptake systems differ from species to species, and thereby pave the road towards the development of species- specific iron(III) carriers. In addition, the fluorescent derivatives of some of these carriers are hoped to provide new diagnostic tools for the identification of specific microorganisms provided, they will be recognized by the microbial iron uptake systems.

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References

[1] G. Winkelmann,D. van der Helm,J. B. Neilands In , Vol. VCH Verlagsgesellschaft mbH, D- 6940, Weinheim, Germany, **1987**
[2] J. B. Neilands Structure and Bonding **1984**, 58, 1-24.
[3] T. Emery Metal Ions in Biological Systems **1978**, 7, 77-126.
[4] K. N. Raymond,C. J. Carrano Acc. Chem. Res. **1979**, 12, 183.
[5] K. N. Raymond,G. Mueller,B. F. Matzanke Top. Cur. Chem. **1984**, 123, 49-102.
[6] B. F. Matzanke,G. Muller-Matzanke,K. N. Raymond In Physical Bioinorganic Chemistry, Vol. 5, VCH Publishers, NY, **1989**.
[7] R. C. Hider Structure and Bonding **1984**, 58, 25-87.
[8] A. Bagg,J. B. Neilands Microbiology Rev. **1987**, 51, 509.
[9] T. Emery,L. Emery,R. K. Olsen Biochem. Biophys. Res. Commun. **1984**, 119, 1191.
[10] G. Winkelmann FEBS Letters **1979**, 97, 43-46.
[11] G. Winkelmann,V. Braun FEMS Microbiol Letters **1981**, 11, 237-241.
[12] J. B. Neilands,T. J. Erickson,W. H. Rastetter J. Biol. Chem. **1981**, 256, 3831.
[13] Y. Tor,J. Libman,A. Shanzer J. Amer. Chem. Soc. **1987**, 109, 6518-6519.
[14] I. Dayan,J. Libman,Y. Agi,A. Shanzer Inorg. Chem. **1993**, 32, 1467-1475.
[15] The Role of Energy Calculations in the Design, Synthesis and Study of Biologically Active Iron(III) Carriers.; S. Lifson, C.E. Felder, J. Libman and A. Shanzer. NATO ASI Series, 426, (G. Wipff, Editor), Kluwer Academic Publishers, 349-376 (**1994**).
[16] A. Shanzer,J. Libman,R. Lazar,Y. Tor,T. Emery Biochem. Biophys. Res. Commun. **1988**, 157, 389-394.
[17] I. Berner,P. Yakirevitch,J. Libman,A. Shanzer,G. Winkelmann Biology of Metals **1991**, 4, 186-191.
[18] E. Jurkevitch,Y. Hadar,Y. Chen,J. Libman,A. Shanzer J. Bacteriol. **1992**, 174, 78-83.
[19] A. Shanzer,J. Libman In CRC-Handbook of Microbial Iron Chelates, Vol. (Eds.: G. Winkelmann), CRC Press, Inc., Boca Raton Ann Arbor Boston London, **1991**, p. 309.
[20] A. Shanzer,J. Libman,P. Yakirevitch,Y. Hadar,Y. Chen,E. Jurkevitch Chirality **1993**, 5, 359-365.