The Complex Structure of Ferri-ferribactins

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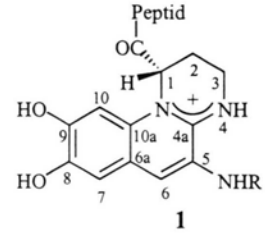
By comparison of the NMR data of the ferribactins from *Pseudomonas chlororaphis* ATCC 9446 and of *P. fluorescens* 18.1 with those of their Ga^{3+}-complexes as models for the Fe^{3+}-complexes it will be shown that only two bidentate ligands are provided for complexation, both located in the peptide chain. The two remainig free sites of the octahedral metal ion are probably occupied by solvent molecules.

Introduction

Fluorescent members of the rRNA homology group I of the genus *Pseudomonas* are characterized by the production of so-called pyoverdins, i.e., siderophores consisting of a dihydroxyquinoline chromophore (**1**) bound amidically to the N-terminus of a peptide chain comprising 6 to 12 amino acids, L as well as D and partially modified (Budzikiewicz, 1997). They are frequently accompanied by ferribactins. Ferribactins have the same peptide chain as the pyoverdins, but the quinoline nucleus is replaced by a condensation product of L-Dab with D-Tyr giving a tetrahydropyrimidine ring. The NH_2-group of the Tyr moiety is substituted by the γ-carboxyl group of L-Glu (**2**, **3**)



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(Taraz *et al.*, 1991). Ferribactins are considered to be the biogenetic precursors of the pyoverdins (Böckmann *et al.*, 1997). They form Fe^{3+}-complexes whose color changes from red at pH 3 to yellow at pH 10 (Budzikiewicz *et al.*, 1992). As in the case of the pyoverdins the peptide chain provides two bidentate ligands. Whether other parts of the ferribactin molecule (e.g., the hydroxyl group of Tyr or the Glu side chain) replace the catecholate unit of the pyoverdins, has been an open question. Clearly, the Fe^{3+}-ferribactin complexes are less stable than the corresponding pyoverdin complexes. The complex constants for the pair from *Pseudomonas chlororaphis* ATCC 9446 (pyoverdin: **1** with the peptide chain of **2**, ferribactin: **2**) (Hohlneicher *et al.*, 1995) assuming a 1:1 stoichiometry for the ferribactin complex are 8.0×10^{25} as compared with 3.1×10^{21} at pH 7.0 and 1.8×10^{19} vs. 5.0×10^{16} at pH 5.0 (Hohlneicher, 1993).

Materials and Methods

Mass spectrometry: Finnigan-MAT 900 ST (ESI). Ferri-**2** was dissolved in $CH_3OH/H_2O/CH_3COOH$ 250:250:1 (v/v) (c = 50 nmol/ml, pH ca. 3). For a second measurement the pH was adjusted to ca 8.5 by addition of a concentrated solution of ammonia. For the complexation experiment 0.1 ml of a solution of 1,10-phenanthroline in CH_3OH (c = 600 nmol/ml) were added to 0.4 ml of the original solution.

NMR: DRX 500 (^1H 500, ^{13}C 125 MHz) (Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ(TMS) = δ(DSS) for ^1H, δ(DSS) = −1.61 ppm for ^{13}C.

The ferribactins from *Pseudomonas chlororaphis* ATCC 9446 (**2**) and from *P. fluorescens* 18.1 (**3**, peptide chain D-Ser-L-Lys-Gly-FoOH-L-

Abbreviations: Common amino acids, 3-letter code; Dab, 2,4-diaminobutyric acid; Ac/FoOHOrn, N^5-acetyl/formyl-N^5-hydroxy-Orn; cOHOrn, *cyclo*-N^5-hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); DSS, [d_6]-2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane.

* Part XCIII of the series "Bacterial Constituents". For Part XCII see Amann *et al.* (2000).

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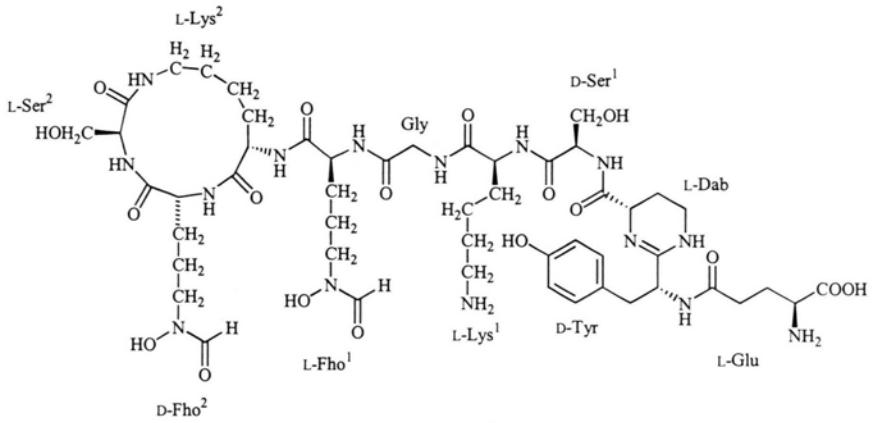

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Orn-L-Ser-D-Ser-Gly-[L-Lys-L-Ser-FoOH-D-Orn]) were isolated and characterized as described earlier (Hohlneicher *et al.*, 1992; Amann *et al.*, 2000).

While Ga^{3+}-complexes of pyoverdins can readily be purified by chromatography (e.g., Amann *et al.*, 2000), those of ferribactins decompose when a purification is attempted. Even when an excess of $Ga(NO_3)_3$ is added to a phosphate buffered solution of the ferribactin the complex formation is by far not complete as can be seen from the ^{1}H-NMR spectra. The phosphate ions seem to compete as complexing agents. For NMR analyses the following procedure proved to be satisfactory: To 15 mg **2** dissolved in 3 ml H_2O 1.1 equivalents of $Ga(NO_3)_3$ dissolved in 1 ml H_2O were added drop by drop under stirring. A pH of 4.2 was determined potentiometrically after complete addition of the Ga salt. After 1 hr the sample was brought to dryness i.v. 15 mg of Ga-**2** were dissolved in 0.9 ml H_2O and the pH was adjusted to 4.0 (potentiometric control); 0.1 ml D_2O were added for the lock signal.

Results and Discussion

Ga^{3+}-complexes have been used in several instances as models for Fe^{3+}-complexes which are not amenable to NMR spectroscopy, primarily to get information from the ^{1}H-data about the three-dimensional structures in solution (e.g., Mohn *et al.*, 1994). Both metal ions form octahedral complexes and the ion radius of Ga^{3+} (62 pm) is very close to that of Fe^{3+} (65 pm). Ga^{3+} causes changes

Table I. Differences in the ^{13}C chemical shifts $\Delta = \delta$(Ga-ligand) – δ(ligand).

Pyoverdin	Chromophore **1**					OHOrn		Lit.
	C-7	C-8	C-9	C-10	Fo	Ac	cyclo	
Pa 27853	−3.7	8.1	10.2	−4.1	−5.2		−6.7	[1]
Pf 51W	−5.1	9.2	11.0	−0.5			−4.5	[2]
Pf 18.1	−3.6	7.2	9.7	−3.9	−6.1			[3]
					−6.8			
Pf PL7	−3.6	8.0	10.5	−4.3		−10.2	−6.9	[4]
Pf PL8	−3.6	7.1	10.4	−4.3		−10.5	−7.0	[4]
Ferribactin	Dab/Tyr (**2**, **3**)							
	C-3	C-4	C-5	Glu				
2	0.1	0.0	0.1	0.1	−6.1			[5]
3	−0.1	0.0	−0.1	0.2	−6.3			[5]

[1] Tappe (1995); [2] Amann *et al.* (2000); [3] Voss *et al.* (1999); [4] Barelmann (1998); [5] present publication.

in the electron density at its binding sites resulting in chemical shift differences with reference to the free siderophore. This effect is especially notable for ^{13}C-resonances. In the ^{1}H-spectra influences due to conformational changes prevail, which bring certain structural units into different shielding or deshielding regions of the molecule. In Table I the shift differences for the binding sites of free pyoverdins and their Ga-complexes are compiled and compared with those of the two ferribactins. Note especially the effect on the C-atoms carrying the OH-groups of the catecholate system (C-8 and C-9) extending even to the neighboring C-atoms.

The shift differences observed for the formyl-CO of the ferribactins **2** and **3** (-6 ppm) agree with the values observed with pyoverdins. Hence, two ligands are provided by the two FoOHOrn units of **2** and **3**. Clearly, the hydroxyl group of Tyr does not occupy one of the free complexation sites of Ga^{3+}: There are no shift differences observed for the 4-hydroxyphenyl ring of Tyr. Another candidate would have been the carboxyl group of the side chain Glu. However, the Δ-values are negligible. This excludes a participation in the complex formation.

Structures and stoichiometries of dihydroxamate siderophore Fe^{3+}-complexes have been investigated in detail. Essentially two possibilities are under discussion, viz. the formation of 3:2-complexes with bridging ligands (e.g., Barclay *et al.*, 1984) and of 1:1-complexes (monomeric or dimeric with the two ligands as bridges) where the free sites of the octahedral metal are occupied by solvent molecules (Caudle *et al.*, 1994b). Equilibria may exist. In acidic media 1:1 complexes seem to prevail.

In the Ga complex of **2** several amino acids (Ser, Lys, Tyr and Glu) show doubled signals (shift differences <0.2 ppm) which might be interpreted as belonging to ferribactin ligands in a differing arrangement as in a 3:2-complex where two ligands are bound to one Ga^{3+} each and the third ligand acts as a bridge between the to metal ions. However, different conformations within an 1:1-complex had been observed also for pyoverdins and they resulted in an analogous doubling of signals. In favor of an 1:1-complex are the electrospray ionization mass spectral data. Between ca. pH 3 and 8 $[\mathbf{2}+^{56}Fe^{3+}-2H^{+}]^{+}$ (*m/z* 1231.5 and $[\mathbf{2}+^{56}Fe^{3+}-H^{+}]^{2+}$ (*m/z* 616.3) are formed. The isotope pattern of *m/z* 1231 shows that it is a singly charged species and not a doubly charged dimer (Caudle *et al.*, 1994b). Solvent molecules occupying the remaining two ligand sites are lost readily in the electrospray process (Caudle *et al.*, 1994a). However, after addition of 1,10-phenanthroline (phen) to the solution of Fe^{3+}-complex of **2** the ions $[\mathbf{2}+^{56}Fe^{3+}+phen]^{3+}$ (*m/z* 471.4) and $[\mathbf{2}+^{56}Fe^{3+}+phen-H^{+}]^{2+}$ (*m/z* 706.6) emerged. Phenanthroline is a more strongly bound ligand than H_2O or CH_3OH. The formation of the 1:1:1-complex confirms the assumption that only four ligand sites of Fe^{3+} are occupied by the two bidentate FoOHOrn groups of **2**, the remaining two being free for solvent molecules etc.

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