Siderophores

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Structural Basis and Stereochemistry of Triscatecholate Siderophore Binding by FeuA**

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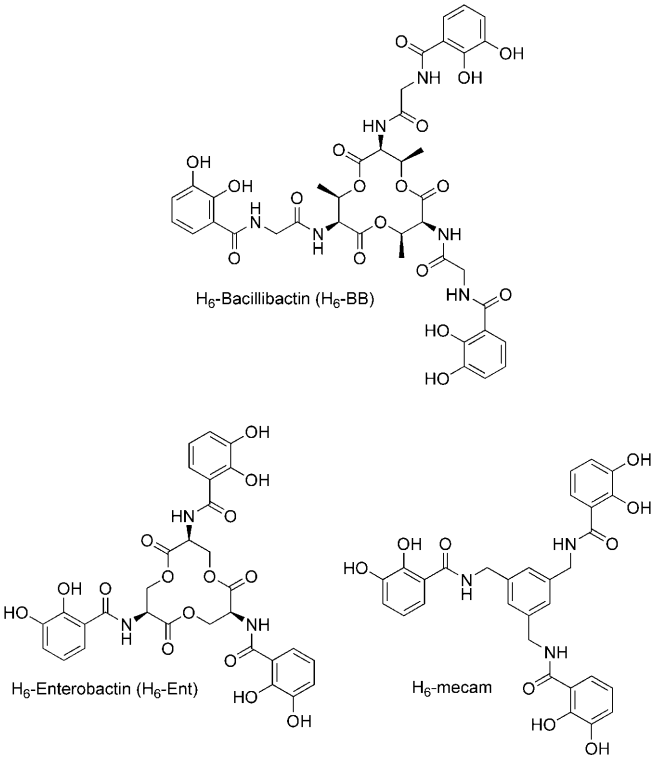
The acquisition of iron is a key feature of microbial growth, in particular for the development of virulence in animal and human hosts. The secretion of low-molecular-weight organic chelators called siderophores is one of the main iron-mobilizing strategies. The strict homeostasis of iron in mammalians led in particular to the development of chelators with extremely high affinity.[1,2] The two strongest chelators are represented by the triscatecholate-trilactone derivatives bacillibactin and enterobactin (H_6-BB and H_6-Ent, respectively; Scheme 1), which have formation constants of 10^{48} and $10^{49}\,M^{-1}$, respectively.[3,4] As a result of their great importance in pathogenicity, both siderophores are targets of the innate defense system that acts by siderocalin (NGAL, lipocalin 2) dependent sequestration.[5,6] *Bacillus anthracis*, *B. cereus*, and nonpathogenic relatives such as *B. subtilis* secrete bacillibactin, a cyclic trilactone depsipeptide, comprising three subunits of the 2,3-dihydroxybenzoate (2,3-DHB)-Gly-Thr, which are assembled by a nonribosomal peptide synthetase (NRPS).[7] Cellular uptake of ferribacillibactin ($[Fe^{III}(BB)]^{3-}$) relies on the ATP-binding cassette transporter FeuABC-YusV, which is also able to import $[Fe^{III}(Ent)]^{3-}$.[8,9]

A comprehension of the coevolution of hosts and pathogens in regard to siderophore scavenging requires a precise understanding of existing siderophore–protein interactions. These may help to create novel strategies for defense against pathogens, for example, by siderophore–drug design or by affinity engineering of available binding pockets. We report here the 1.7 Å crystal structure of the siderophore binding protein FeuA from *B. subtilis* in a complex with $[Fe^{III}(BB)]^{3-}$. Detailed analysis of the protein–ligand interactions at high structural resolution is complemented by fluorescence and CD spectroscopic studies on variants of the binding site and ligand configuration.



Scheme 1. Bacillibactin, its relative enterobactin, and the catecholate siderophore mimic mecam.

The siderophore binding protein FeuA (297 amino acids without a signal peptide) is attached to the cytoplasmic membrane by a lipid anchor tethered to the N-terminal cysteine residue of the mature protein. We obtained crystals in different space groups of FeuA without the lipid anchor (lacking the first 20 amino acids) with and without $[Fe^{III}(BB)]^{3-}$, and solved the phases by molecular replacement (see the Supporting Information).[23] Basically, FeuA is composed of two domains, which show a Rossmann-like fold and are connected by a 22 amino acid long α helix (Figure 1). These structural elements are indicative of siderophore binding proteins of the "helical-backbone" metal-receptor superfamily such as FhuD and CeuE.[10,11]

The binding of the substrate at the interface of the N- and C-terminal domains induces a movement towards the binding site. Superposition of the N-terminal domains of apo- and holo-FeuA results in a shift of 20.2° in the C-terminal domain (Figure 2). This bending is not as large as that observed for binding proteins with flexible antiparallel β-strand linkers, such as the maltose binding protein,[12] but larger than in other

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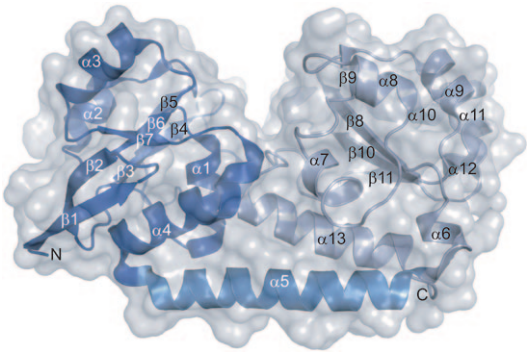

Figure 1. Overall structure of FeuA. The two asymmetric lobes and the connecting α helix are shown in different color shades (N-terminal domain: dark blue, connecting α helix: medium blue, C-terminal domain: light blue). The protein surface is shown in gray.

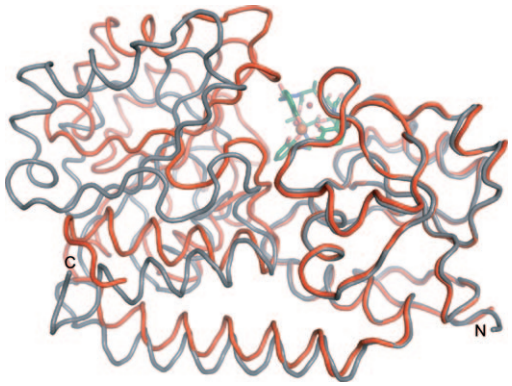

Figure 2. Movement of the FeuA domain upon binding of $[Fe^{III}(BB)]^{3-}$ (see Figure 3 for the color scheme). The N-terminal domains of native protein (gray) and its siderophore complex (red) are superpositioned. The Cα backbone is shown in a ribbon representation.

proteins of the "helical-backbone" superfamily (see the Supporting Information).[13–16] The siderophore-dependent domain connection leads to a stabilization of the protein towards heat denaturation, which was monitored by CD spectroscopy (Table 1).

The profile of the protein–ligand interactions revealed a basic triad made up of K84, K105 (both provided by the N-terminal domain), and R180 (from the C-terminal domain), which holds $[Fe^{III}(BB)]^{3-}$ by electrostatic interactions directed towards the deprotonated catecholate oxygen atoms of the ligand. Direct binding to the siderophore is accomplished only by two further residues: Q181 and Q215 form hydrogen bonds with parts of the ligand. Residues T104, R178, N183, Y185, Y187, E239, and Q277 contribute to the overall fold of the binding site, although they do not participate directly in ligand binding (see Figure 3 and the Supporting Information).

To examine the importance of the interacting residues for ligand binding and structural stabilization, the basic triad residues and the residues R178 inside the binding pocket as well as K213 on the surface of the C-terminal domain outside the binding pocket were each mutated to alanine and subjected to ligand-dependent melting-point analysis (Table 1). The native protein and K213A, which are able to

Table 1: Melting points of FeuA and its variants determined by CD spectroscopy.

FeuA variant	T_M [°C][a]	
		+ $[Fe^{III}(BB)]^{3-}$
native	58.15 ± 0.02	65.99 ± 0.04
K84A	63.12 ± 0.03	62.93 ± 0.06
K105A	59.64 ± 0.03	59.44 ± 0.05
R178A	56.96 ± 0.04	57.33 ± 0.06
R180A	58.82 ± 0.98	58.78 ± 1.04
K213A	50.84 ± 0.05	58.74 ± 0.07

[a] T_M: melting temperature, calculated with Spectra Analysis Software, JASCO Corporation.

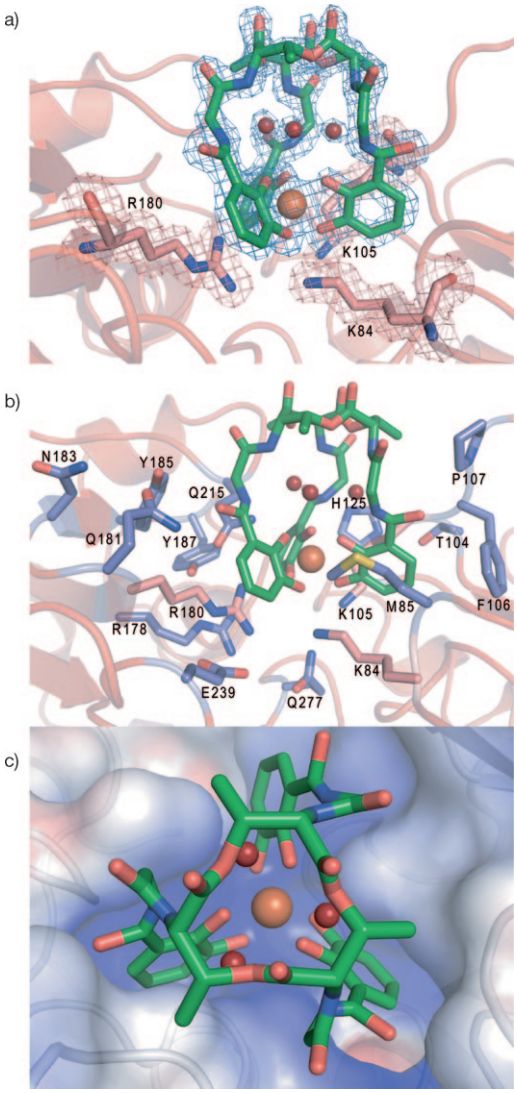

Figure 3. Detailed view of the siderophore binding pocket of FeuA; ligand atoms: green C, red O, blue N, orange sphere Fe. a) SIGMAA-weighted ($F_{obs}-F_{calc}$) electron-density difference (blue) calculated at 1.7 Å resolution (contour level 2.7σ) for $[Fe^{III}(BB)]^{3-}$ and the three water molecules (dark red spheres); $2F_{obs}-F_{calc}$ electron density (contour level 1σ) is shown for residues K84, K105, and R180 (C light red). b) The side chains of other residues which make up the binding site are also illustrated (C light blue). c) Electrostatic surface representation of the binding pocket (from −5 (red) to +5 (blue) k_BT/e_c).

bind $[Fe^{III}(BB)]^{3-}$, showed a shift in the melting temperatures by about 8 °C in the presence of the ligand. In contrast, the stabilities of the three single substitutions of the basic triad and also variant R178A were not affected by the presence of the siderophore. Although R178 does not participate directly in ligand binding, it seems to be indirectly involved by stabilizing residue R180 within the binding pocket. Additionally, R180 seems to have a significant influence on the overall folding stability of the protein, because, in contrast to all other variants, precipitate was formed during heating. The higher melting point of K84A compared to native FeuA is likely due to decreased electrostatic repulsion with other positively charged residues within the binding pocket. Additional fluorescence quenching studies supported the data by revealing a strong decrease in ligand binding affinity for the K84A, K105A, R180A, and R178A variants. The estimated binding affinities decreased by about three orders of magnitude relative to native FeuA (see the Supporting Information). It would thus appear that the basic triad is a widespread binding motif for triscatecholate siderophores and can be found in nonhomologous binding proteins from bacteria up to humans.[5,6,11]

The siderophore scavenger siderocalin binds $[Fe^{III}(Ent)]^{3-}$ and $[Fe^{III}(BB)]^{3-}$ very tightly by hybrid electrostatic/cation–π interactions with residues R81, K125, and K134.[5,6] Additionally, the crystal structure of the enterobactin binding protein CeuE, which was dimerized by a $[\{Fe^{III}(mecam)\}_2]^{6-}$ bridge (H_6-mecam; Scheme 1), revealed residues R117, R204, and R248 to be the corresponding basic triad.[11]

Although the trilactone backbone of $[Fe^{III}(Ent)]^{3-}$ bound to siderocalin was partly degraded, its components seemed to adopt a Λ configuration.[5] The unbound complex is known to exist as the Δ diastereomer in solution, which was also observed in a crystal structure of $[V^{IV}(Ent)]^{2-}$.[17,18] $[Fe^{III}(BB)]^{3-}$, on the other hand, is present in the Λ configuration in solution,[19] and is also bound as the Λ species in the present FeuA crystal structure. Furthermore, the racemic mixture of $[Fe^{III}(mecam)]^{3-}$ preferred the Λ configuration upon binding by CeuE.[11]

The observed binding pocket in the FeuA crystal structure seems to be specific for Λ substrates and would not allow binding of Δ-$[Fe^{III}(Ent)]^{3-}$. Since the configuration of ferrisiderophores is decisive for their biological function during uptake and further processing, it was examined which configuration is preferred by FeuA, and if FeuA might be able to trigger or change the absolute configurations of the ligand to provide its substrate promiscuity. Therefore, CD spectroscopic analysis of different iron–triscatecholate complexes in the presence of FeuA was carried out (Figure 4).

Negative bands around 450 nm and positive bands around 550 nm are characteristic of ferric–triscatecholate complexes with Λ chirality.[20] The measurements confirmed the Λ configuration of $[Fe^{III}(BB)]^{3-}$ when bound to FeuA in solution. Achiral $[Fe^{III}(mecam)]^{3-}$ was observed in the Λ configuration, and strikingly the same chirality was found for $[Fe^{III}(Ent)]^{3-}$ when FeuA was present. These results show that FeuA influences the configuration of these siderophores. The strict binding of the Λ-configured triscatecholate substrates may be one reason for the lower affinity of FeuA towards $[Fe^{III}$-

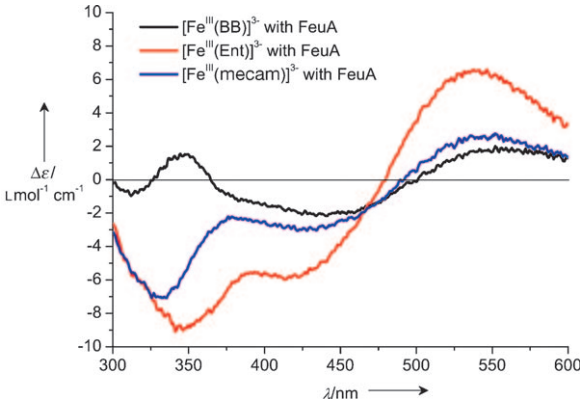

Figure 4. CD spectra of $[Fe^{III}(BB)]^{3-}$, $[Fe^{III}(Ent)]^{3-}$, and $[Fe^{III}(mecam)]^{3-}$ complexed with FeuA in buffered water (pH 7.0) at 22.5 °C.

(Ent)]$^{3-}$ compared to $[Fe^{III}(BB)]^{3-}$.[8] This was also observed for the binding of $[Fe^{III}(Ent)]^{3-}$ and its non-native Λ stereoisomer $[Fe^{III}$(enantio-Ent)]$^{3-}$ to the FepB protein from *E. coli*.[21]

Remarkably, although $[Fe^{III}(BB)]^{3-}$ binds in the favored Λ configuration, the trilactone ring does not adopt the conformation calculated to be more stable (although with a low energy difference of 2.3 kcal mol^{-1}).[19] Instead, our electron-density map reveals three highly ordered water molecules between the trilactone ring and the iron(III)–catecholate center (Figure 3). These water molecules stabilize the structure of the iron–siderophore complex through the formation of hydrogen-bonding networks, which lead to the unexpected trilactone conformation which resembles that of $[Fe^{III}(Ent)]^{3-}$. This may have implications for subsequent transport or trilactone hydrolase recognition processes.

In conclusion, we have reported the first crystal structure of a binding protein in a complex with an intact triscatecholate-trilactone siderophore and demonstrate that the mecam-dependent dimerization of binding proteins does not seem to be extended to trilactone derivatives. Detailed binding analysis determined the importance of a basic triad for recognition of the iron–triscatecholate ligand center, and this motif seems to be highly conserved in different species. Protein interactions with the ligand backbone were found to be insignificant and, thus, the trilactone scaffold may be used for cross-linking strategies to design siderophore–antibiotic conjugates, so called sideromycins, and siderophore import inhibitors.

The ability of FeuA to alter the configuration of triscatecholate substrates suggests that stereochemical recognition must play a key role in at least one step of siderophore import and subsequent intracellular processing. The adoption of another conformation to enable binding of Δ-triscatecholate siderophores seems to be unlikely, since the binding pocket of the related hydroxamate siderophore binding protein FhuD changes minimally when binding different but related substrates.[10,22]

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[1] S. C. Andrews, A. K. Robinson, F. Rodriguez-Quinones, *FEMS Microbiol. Rev.* **2003**, *27*, 215 – 237.
[2] M. Miethke, M. A. Marahiel, *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 413 – 451.
[3] E. A. Dertz, J. Xu, A. Stintzi, K. N. Raymond, *J. Am. Chem. Soc.* **2006**, *128*, 22 – 23.
[4] L. D. Loomis, K. N. Raymond, *Inorg. Chem.* **1991**, *30*, 906 – 911.
[5] D. H. Goetz, M. A. Holmes, N. Borregaard, M. E. Bluhm, K. N. Raymond, R. K. Strong, *Mol. Cell* **2002**, *10*, 1033 – 1043.
[6] R. J. Abergel, M. K. Wilson, J. E. Arceneaux, T. M. Hoette, R. K. Strong, B. R. Byers, K. N. Raymond, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18499 – 18503.
[7] J. J. May, T. M. Wendrich, M. A. Marahiel, *J. Biol. Chem.* **2001**, *276*, 7209 – 7217.
[8] M. Miethke, O. Klotz, U. Linne, J. J. May, C. L. Beckering, M. A. Marahiel, *Mol. Microbiol.* **2006**, *61*, 1413 – 1427.
[9] J. Ollinger, K. B. Song, H. Antelmann, M. Hecker, J. D. Helmann, *J. Bacteriol.* **2006**, *188*, 3664 – 3673.
[10] T. E. Clarke, S. Y. Ku, D. R. Dougan, H. J. Vogel, L. W. Tari, *Nat. Struct. Biol.* **2000**, *7*, 287 – 291.
[11] A. Müller, A. J. Wilkinson, K. S. Wilson, A. K. Duhme-Klair, *Angew. Chem.* **2006**, *118*, 5256 – 5260; *Angew. Chem. Int. Ed.* **2006**, *45*, 5132 – 5136.
[12] A. J. Sharff, L. E. Rodseth, J. C. Spurlino, F. A. Quiocho, *Biochemistry* **1992**, *31*, 10657 – 10663.
[13] Y. H. Lee, R. K. Deka, M. V. Norgard, J. D. Radolf, C. A. Hasemann, *Nat. Struct. Biol.* **1999**, *6*, 628 – 633.
[14] Y. H. Lee, M. R. Dorwart, K. R. Hazlett, R. K. Deka, M. V. Norgard, J. D. Radolf, C. A. Hasemann, *J. Bacteriol.* **2002**, *184*, 2300 – 2304.
[15] E. L. Borths, K. P. Locher, A. T. Lee, D. C. Rees, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16642 – 16647.
[16] N. K. Karpowich, H. H. Huang, P. C. Smith, J. F. Hunt, *J. Biol. Chem.* **2003**, *278*, 8429 – 8434.
[17] M. Llinas, D. M. Wilson, J. B. Neilands, *Biochemistry* **1973**, *12*, 3836 – 3843.
[18] T. B. Karpishin, K. N. Raymond, *Angew. Chem.* **1992**, *104*, 486 – 488; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 466 – 468.
[19] M. E. Bluhm, B. P. Hay, S. S. Kim, E. A. Dertz, K. N. Raymond, *Inorg. Chem.* **2002**, *41*, 5475 – 5478.
[20] T. B. Karpishin, T. D. P. Stack, K. N. Raymond, *J. Am. Chem. Soc.* **1993**, *115*, 6115 – 6125.
[21] C. Sprencel, Z. Cao, Z. Qi, D. C. Scott, M. A. Montague, N. Ivanoff, J. Xu, K. M. Raymond, S. M. Newton, P. E. Klebba, *J. Bacteriol.* **2000**, *182*, 5359 – 5364.
[22] T. E. Clarke, V. Braun, G. Winkelmann, L. W. Tari, H. J. Vogel, *J. Biol. Chem.* **2002**, *277*, 13966 – 13972.
[23] The structural data have been deposited in the Protein Data Bank under the accession codes 2WI8 (apo) and 2WHY (complex).

