Siderophores

DOI: 10.1002/anie.201300751

Interactions of a Periplasmic Binding Protein with a Tetradentate Siderophore Mimic**

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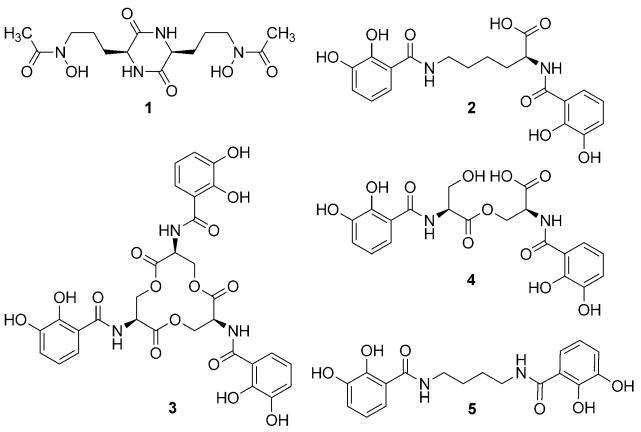
To ensure the uptake of essential Fe^{III} ions, microorganisms have developed iron chelators known as siderophores, of which over 500 examples have now been documented.[1–4]

Whilst the most powerful siderophores are hexadentate ligands and form coordinatively saturated octahedral 1:1 complexes with Fe^{III} ions, others are ligands of lower denticity but still bind Fe^{III} ions and aid their transport into the bacterial cell.[1,5–7] Examples include the bidentate 2,3-dihydroxybenzoylserine[8] and citrate, which is likely to act as a tridentate ligand.[9] In addition, tetradentate siderophores from a variety of microorganisms have been identified, including those of the bis(hydroxamate) type, such as rhodotorulic acid[10] (**1**), and the bis(catecholamide) type, such as azotochelin[5] (**2**) or the hydrolysis product of enterobactin (**3**), *N*,*N*′-bis(2,3-dihydroxybenzoyl)-*O*-seryl serine[6,11] (**4**; Scheme 1). Whilst **1** is structurally related to hexadentate hydroxamate siderophores derived from δ-*N*-hydroxy ornithine, such as ferrichrome and ferricrocin,[1,2,4] catecholate siderophores **2** and **4** are fragments of their hexadentate counterparts protochelin and enterobactin, respectively. It is still unclear if "secondary" siderophores, such as **2** and **4**, are produced as siderophores in their own right or whether they are biosynthetic precursors or degradation products of their hexadentate parent compounds.[12–14] However, the use of low denticity siderophores by pathogenic bacteria has been documented.[15] Further examples of bis(catecholamide) siderophores are summarized in the Supporting Information (Scheme S1). In contrast to the well characterized iron uptake mediated by hexadentate siderophores, the exact biological roles of tetradentate siderophores and their interactions with ferric siderophore transport proteins have yet to be established.



Scheme 1. Molecular formulas of H_2-rhodotorulic acid (**1**), H_5-azotochelin (**2**), H_6-enterobactin (**3**), H_6-*N*,*N*′-bis(2,3-dihydroxybenzoyl)-*O*-seryl serine (**4**), and H_4-4-LICAM (**5**).

Owing to the reduced number of chelating groups, the Fe^{III} complexes of tetradentate siderophores have a lower thermodynamic and kinetic stability than those of their hexadentate counterparts,[1,2] a disadvantage in environments with low Fe^{III} ion concentrations. However, under less challenging conditions low-denticity siderophores, which are likely to have a lower biosynthetic cost, may confer a competitive advantage to the bacteria that are able use them. In addition to faster Fe^{III} dissociation kinetics, the Fe^{III} complexes of tetradentate siderophores possess more positive redox potentials,[1,2] which can be beneficial to Fe^{III} ion uptake mechanisms that rely on reductive iron release.

Along with the evolution of diverse siderophore structures, microorganisms have had to adapt their receptor and transport proteins to accommodate the uptake of the resulting Fe^{III} complexes.[16] Interestingly, most bacteria do not rely solely on their own siderophores for iron acquisition but also acquire siderophores from competing species by producing suitable receptor proteins. The Gram-negative, food-borne pathogen *Campylobacter jejuni* relies on such exogenous siderophores (xenosiderophores) for iron uptake, in particular the hexadentate enterobactin and possibly its hydrolysis products also.[17] Enterobactin is produced by commensal intestinal bacteria. In *C. jejuni*, ferric enterobactin is recognized by the outer membrane receptors CfrA and CfrB and transported into the periplasm, where it is captured by the periplasmic binding protein (PBP) CeuE. The resulting complex interacts with the inner membrane transporter to enter the cytoplasm of the cell.[18,19]

Previously, we co-crystallized CeuE with the Fe^{III} complex of the enterobactin mimic $MECAM^{6-}$ (Figure 1).[20] This structure revealed that the CeuE binding pocket contains three positively charged arginine residues, which balance the threefold negative charge of the $[Fe(catecholate)_3]^{3-}$ unit and donate hydrogen bonds to oxygen donors that are coordinated to the iron center. Two additional hydrogen bonds are

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[**] We thank the UK Biotechnology and Biological Sciences Research Council (BBSRC) for financial support, the Diamond Light Source for access to beamlines i03 and i24, Dr. J. Turkenburg, S. Hart for assistance with data collection, and Dr. A. Leech for help with circular dichroism spectroscopy.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201300751.

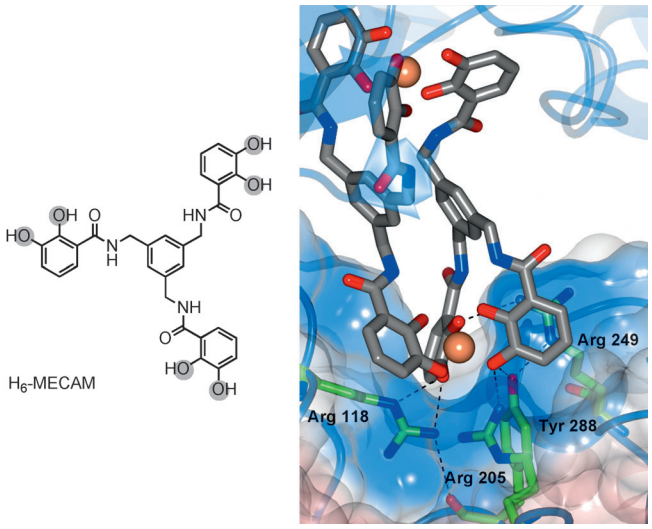
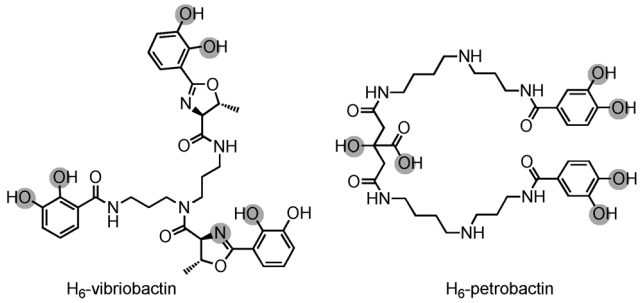


Figure 1. Molecular formula of H_6-MECAM with Fe^{III} coordinating donor atoms highlighted in gray (left) and crystal structure of $[\{Fe^{III}(MECAM)\}_2]^{6-}$ in complex with two CeuE molecules (right; PDB code: 2chu; figure produced using CCP4mg[26]). Ligand atoms: C gray, O red, N blue, Fe orange, electrostatic surface representation of the binding pocket with green cylinders for carbon atoms of the binding amino acid residues.

formed, one between a tyrosine residue and a coordinated catecholate oxygen and one between a lysine residue and the carbonyl oxygen of one of the three catecholamide units. Unexpectedly, the $MECAM^{6-}$ was found to bridge between two Fe^{III} centers and two CeuE monomers.

Co-crystal structures of two related ferric siderophore PBPs have since been reported, FeuA from *Bacillus subtilis* in complex with ferric bacillibactin,[21] enterobactin and MECAM,[22] and ViuP from *Vibrio cholerae* in complex with ferric vibriobactin (Scheme 2).[23] These proteins bind mono-



Scheme 2. Molecular formulas of H_6-vibriobactin and H_6-petrobactin with Fe^{III} coordinating donor atoms highlighted in gray.

nuclear, coordinatively saturated Fe^{III} complexes of hexadentate siderophores. In addition, CeuE shares structural similarities with the *B. subtilis* protein YclQ, which binds the mixed citrate-bis(3,4-catecholamide) siderophore, petrobactin (Scheme 2),[24] and the second periplasmic catecholate-siderophore binding protein of *V. cholerae*, VctP.[25]

It has been suggested that one of the outer-membrane siderophore receptors of *C. jejuni*, CfrA, may have evolved to bind a variety of siderophores.[27] We therefore investigated if the next component of the relevant iron import system, the PBP CeuE, shows similar ligand promiscuity. Because *C. jejuni* needs to acquire its essential iron from a wide variety of sources, ranging from animal hosts, poultry, milk, and drinking water to the colonized human host, this pathogen would benefit greatly from a flexible iron uptake system. Given that *C. jejuni* depends on xenosiderophores and considering that enterobactin is prone to hydrolysis,[6] we focused our attention on H_4-4-LICAM (**5**),[28] a mimic of the tetradentate enterobactin fragment *N*,*N*′-bis(2,3-dihydroxybenzoyl)-*O*-seryl serine (**4**; Scheme 1).

Herein we report the structures of both apo-CeuE and of the CeuE-$[Fe(4\text{-LICAM})]^-$ complex (PDB codes: 3zkw and 3zk3, experimental details provided in the Supporting Information). The fold of apo-CeuE is that of a typical type III PBP (cluster A-II), with two domains linked by a long α-helix.[29,30] In the 4-LICAM complex, the ligand is bound in a shallow binding pocket formed by the two domains. Superposition of the apo and the complex structure shows that the binding of the ferric complex of 4-LICAM triggers only minor structural changes (Supporting Information, Figure S1).

CeuE-$[Fe(4\text{-LICAM})]^-$ is the first co-crystal structure that provides insights into the interactions of a PBP with a tetradentate siderophore (Figure 2). The 4-LICAM^{4-} ligand chelates a single iron center in a tetradentate fashion with the catecholate oxygens interacting with the positively charged side chains of Arg118, Arg205, and Arg249. The two

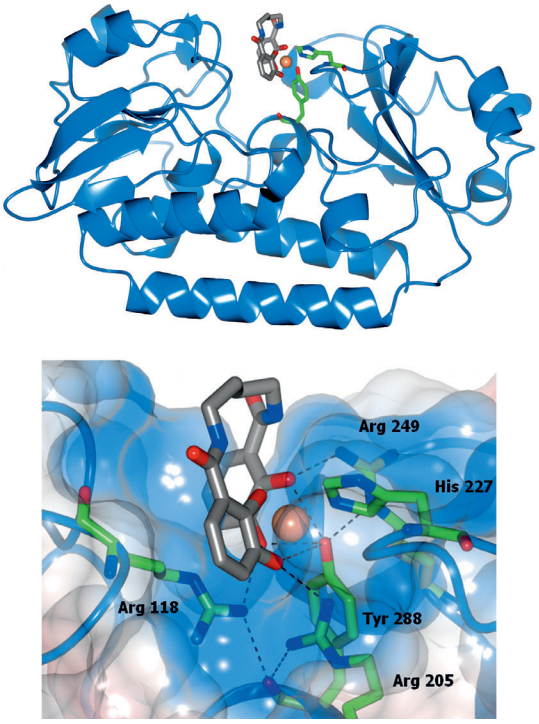


Figure 2. Top) Crystal structure of the CeuE-$[Fe^{III}(4\text{-LICAM})]^-$ complex. CeuE = blue ribbon, 4-LICAM^{4-} chelating a single Fe^{III} ion (orange). Bottom) Binding pocket (blue ribbon backbone with green cylinders for carbon atoms of the binding amino acid residues), 4-LICAM^{4-}, and Fe^{III} (orange). Images created using CCP4mg.

remaining coordination sites of the octahedral Fe^{III} center are occupied by a nitrogen atom and an oxygen donor provided by nearby His227 and Tyr288, respectively. The Fe–O bond lengths involving the catecholate groups range from 1.9 to 2.3 Å and are consistent with those previously found in both the crystal structures of ferric bis(2,3-catecholamide) complexes[31] and co-crystal structures of ferric tris(2,3-catecholamides) with their respective binding proteins.[20–23] The Fe–O and Fe–N bond distances for the tyrosine and histidine interactions are 1.9 Å and 2.3 Å, respectively and agree well with those in the crystal structure of the inorganic ion transporter cFbpA from *C. jejuni*, in which an Fe^{III} ion is coordinated by four tyrosines and one histidine residue.[32]

4-LICAM^{4-} binds with a 1:1:1 Fe/siderophore/protein stoichiometry, in contrast to the unusual bridged dimer seen with MECAM^{6-}, in part reflecting the linker between the iron-binding groups being too short for bridging. Similar mononuclear binding is observed in the structures of FeuA in complex with ferric bacillibactin, enterobactin, and MECAM,[22] and ViuP with ferric vibriobactin.[23] The oxygen donor of Tyr288, directly coordinated to the Fe^{III} center in CeuE-[Fe(4-LICAM)]$^-$, is positioned 3.5 Å away from the metal center in $(CeuE)_2$-[{Fe(MECAM)}$_2$]$^{6-}$, where it instead donates a hydrogen bond to one of the deprotonated catecholate oxygen donors of the ligand. His227, the second Fe protein ligand, is disordered, forming part of a flexible loop in both the apo-CeuE and $(CeuE)_2$-[{Fe(MECAM)}$_2$]$^{6-}$ structures.

Two of the other structurally characterized ferric siderophore PBPs, YclQ and VctP, have Tyr and His side chains located in positions similar to those found in CeuE-[Fe(4-LICAM)]$^-$ (Figure 3; Figure S3). YclQ binds the ferric

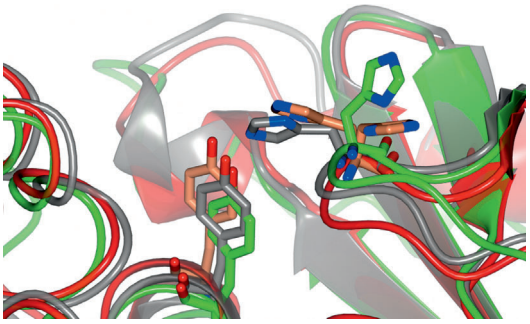


Figure 3. Overlay of the substrate binding pockets of CeuE (gray), YclQ (red; PDB code: 3gfv; His disordered over two sites) and VctP (green; PDB code: 3tef). Tyr and His side chains involved in Fe^{III} coordination in CeuE-[Fe(4-LICAM)]$^-$ are shown as cylinders with the carbon atoms colored the same as their ribbons.

bis(catecholate) complex formed by 3,4-dihydroxybenzoic acid,[24] whilst VctP is proposed to bind both [Fe(enterobactin)]$^{3-}$ and [Fe(H-vibriobactin)]$^{2-}$ by being able to accommodate different numbers of negative charges.[25] We propose that these two amino acid residues are part of a mechanism that enables certain PBPs to capture more than one type of ferric siderophore.

The structure of the CeuE-[Fe^{III}(4-LICAM)]$^-$ complex has the metal center bound in a Λ-configuration, as seen in the co-crystal structures of $(CeuE)_2$-[{Fe(MECAM)}$_2$]$^{6-}$,[20] FeuA-[Fe(MECAM)]$^{3-}$, FeuA-[Fe(enterobactin)]$^{3-}$, and FeuA-[Fe(bacillibactin)]$^{3-}$.[21,22] To confirm that the Λ-configuration is retained in solution, the circular dichroism (CD) spectrum of CeuE-[Fe(4-LICAM)]$^-$ was recorded in the wavelength range of the ligand-to-metal charge transfer (LMCT) band at around 500 nm (Figure 4). The negative

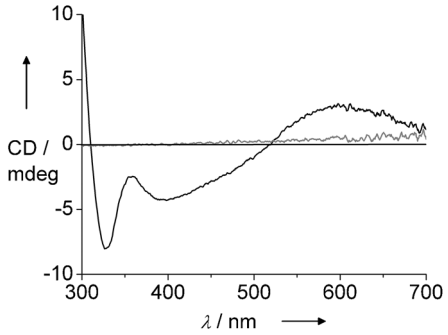


Figure 4. CD spectra of CeuE with ferric 4-LICAM bound (black) and ferric 4-LICAM in the absence of protein (gray); concentrations 68 μM in 20 mM TRIS buffer, 10 mM NaCl, 0.6% DMSO, pH 8.

band with a minimum at 400 nm and the positive band with a maximum at 595 nm are indicative of the Λ-configuration.[33] In the absence of CeuE, the ferric 4-LICAM complex shows no CD, confirming that the protein selectively binds the complex in the Λ-configuration from the racemic mixture.

In summary, the tetradentate siderophore mimic H_4-4-LICAM was synthesized, coordinated to Fe^{III} and co-crystallized with the periplasmic binding protein CeuE of *C. jejuni*. In addition to the expected electrostatic and hydrogen-bonding interactions between the binding pocket of CeuE and [Fe^{III}(4-LICAM)]$^-$, the structure revealed the direct coordination of two amino acid side chains to the Fe^{III} center. By displaying this previously unobserved binding mode, CeuE is the first siderophore binding protein to provide insights into the recognition and capture of both tetradentate and hexadentate siderophores, as exemplified by 4-LICAM^{4-} and MECAM^{6-}. It is remarkable that despite its flexibility with regard to ligand denticity, CeuE retains selectivity for the Λ-configuration at the Fe^{III} center. His and Tyr residues are conserved in a number of PBPs including YclQ and VctP. It would therefore be interesting to establish if these proteins can undergo similar structural changes to adapt to the binding of lower denticity siderophores. Such adaptability would be consistent with the observation that one PBP often serves a whole group of outer-membrane receptors, for example FepB of *E. coli* serves a range of ferric catecholamide receptors, including Fiu, Cir, and FepA.[1]

Received: January 28, 2013
Published online: ■■ ■■, ■■■■

Keywords: iron uptake · metalloproteins · periplasmic binding proteins · protein structures · tetradentate siderophores

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Communications

Siderophores

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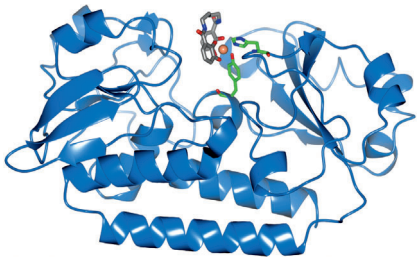
Interactions of a Periplasmic Binding Protein with a Tetradentate Siderophore Mimic



Iron-bound structure: The ferric complex of a tetradentate siderophore mimic was synthesized and co-crystallized with the periplasmic binding protein CeuE of *Campylobacter jejuni*. In addition to electrostatic and hydrogen-bonding interactions between the binding pocket and the substrate, the structure showed direct coordination of two amino acid side chains to the Fe^{III} center (orange, see figure).